Institut National de la Recherche Scientifique

EFFETS DES PROPRIÉTÉS RHÉOLOGIQUES SUR LA FERMENTATION DES EAUX USÉES ET DES BOUES D'ÉPURATION PAR *BACILLUS THURINGIENSIS* VAR. *KURSTAKI* ET SUR LE DÉVELOPPEMENT DE BIOPESTICIDES EN SUSPENSIONS AQUEUSES CONCENTRÉES

Par

SATINDER KAUR BRAR

Thèse présentée

pour l'obtention du Grade de Philosophiae Doctor (Ph.D.) en Sciences de l'Eau

Jury d'évaluation

Examinateurs externes	Dr. Peter J. Laughton, P.Eng. Consulting Engineer (Environmental Engineering Services) Dr. Jean-Charles Côté (Centre de recherche et développement en horticulture, AAFC) Prof. Guy Charpentier (UQTR)
Examinateur interne	Prof. Jean-Louis Sasseville (INRS-ETE)
Directeur de recherche	Prof. Rajeshwar D. Tyagi (INRS-ETE)
Co-directeur de recherche	Dr. José R. Valéro (INRS-ETE)

Janvier 2007

EAU, TERRE ET ENVIRONNEMENT INRS

Dedicated

To God, my grandparents, my parents and my brother who have lend me a moral support during this travail.

WAHEGURU JI KA KHALSA, WAHEGURU JI KI FATEH

ਵਾਹਿਗੁਰੂ ਜੀ ਕਾ ਖਾਲਸਾ ॥ ੴ ॥

ਵਾਹਿਗੁਰੂ ਜੀ ਕੀ ਫਤਹ ॥ ੴ ॥

ACKNOWLEDGEMENT (S)

This is, without any doubt, the more difficult part of my thesis to write. Many of you have helped me through this very difficult part of my life; my apologies if I have forgotten anyone.

I sincerely thank my **Ph.D. supervisor**, **Prof. Rajeshwar D. Tyagi** and **co-supervisor, Dr. José R. Valéro** for giving me valuable suggestions and guidance during the course of my doctoral tenure. I have learnt many scientific aspects and honed my research aptitude through their continuous encouragement and support. I owe my sincere thanks to **Dr. R.Y. Surampalli** who has been guiding me and encouraging me all through my Ph.D. I also take the opportunity to express my gratitude to **my examiners** (Dr. Peter Laughton, Dr. Jean-Charles Côté, Prof. Guy Charpentier and Prof. Jean-Louis Sasseville) who have played an important role in contributing to my doctoral project through their excellent suggestions. My heartfelt thanks to **my best friend and colleague**, **Mausam Verma** for his rigorous brainstorming sessions and moral support, in pursuing my doctoral studies.

I highly acknowledge the efforts of my internal examiner, **Prof. Jean-Louis Sasseville** in his consistent efforts to ameliorate the French content of my *synthèse* and also the modification of structural contents of the same. I equally acknowledge the translation efforts of Dr. Valéro and my friend, Kokou Adjalle in making this thesis writing a success. My gratitude to insect rearing centre at Sault-Sainte Marie, Ontario for being in thick and thin of my bioassay experiments. I would equally like to acknowledge the assistance provided by my colleagues, in particular, Simon Barnabé, Mathieu Drouin, Jean-Phillipe Chenel and laboratory personnel (Stéfane Prémont, Michelle Bordeleau, Marc Greendale, Pauline Fournier, René Rodrigue, Sébastien Duval and Lise Rancourt) in each venture. My warm thanks to my stagaires from France, Virginie Gueyne, Sébastien Durand, Campton Pierre, Nicolino Luc, Rémi Baranoff, Nathalie Siméon and Virginie Clet-Ortgea who have played a great role in my research project. My sincere thanks to National Sciences and Engineering Research Council of Canada (NSERC), Canadian Forestry Service and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing financial support during my research. Furthermore, my heartfelt thanks to Student's secretary, Suzanne Dussault who has been very patient and prompt in any kind of administrative help required despite her very busy schedule. Likewise, my thanks to Johanne Desrosiers for her ever glowing face and active aid in the various miscellaneous, yet very important activities related to research. My sincere gratitude to the informatics support at INRS-ETE and everybody at INRS-ETE who have encouraged me through the course of my doctoral studies.

Finally, I would like to thank my parents (Bahadur Singh Brar and Surjeet Kaur Brar), my brother Parminder Singh Brar; my uncle and aunt (Gurnam and Balwinder Sekhon); my friends (Tania, Sandeep, Vishakha, Selvia, Maushmi) and Monsieur Robert Richer for their beaming encouragement during the doctoral studies.

AVANT-PROPOS

PUBLICATIONS DANS CETTE THÈSE

1. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. and S. Banerji. 2004. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations.*Water, Science and Technology*, 50 (9), 229-236.
2. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. and Banerji, S. 2004. Comparative rheology and particle size analysis of various types of *Bacillus thuringiensis* fermented sludges. *Journal of Residuals Science and Technology,* 1(4), 231-237.
3. **Brar, S.K.,** Verma, M., Barnabé, S., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges. *Process Biochemistry*, 40, 2695–2705.
4. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005. Sludge based *Bacillus thuringiensis* biopesticides: Viscosity Impacts. *Water Research*, 39, 3001-3011.
5. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. *Process Biochemistry,* 41, 323-342.
6. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005. Starch industry wastewater based stable *Bacillus thuringiensis* liquid formulations. *Journal of Economic Entomology,* 98(6), 1890 - 1898.
7. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006. Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge. *Water Research* 40, 1310-1320.
8. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006. Screening of different adjuvants for wastewater/wastewater sludge based *Bacillus thuringiensis* formulations. *Journal of Economic Entomology* 99(4), 1065-1079.
9. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2007. *Bacillus thuringiensis* fermentation of hydrolyzed sludge - rheology and formulation studies. *Chemosphere* 67, 674–683.
10. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006. Value addition of sewage sludge- a clean management strategy. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management* (under review).
11. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006. Ramification of solids concentration on entomotoxicity and enzyme activity of *Bacillus thuringiensis* fermented wastewater sludge. *Bioresource Technology* (under review).
12. **Brar, S.K**., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006. *Bacillus thuringiensis* fermentation of primary and mixed sludge - rheology and process performance. *Journal of Environmental Engineering* (under review).
13. **Brar, S.K.**, Verma, M., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006. Particle size variations during production of wastewater sludge based *Bacillus*

thuringiensis biopesticides. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management* (under review).

14. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006. Presence and characterization of chitinases present in *Bacillus thuringiensis* fermented wastewater and wastewater sludge. *Environmental Technology* (under review).
15. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2007. *Bacillus thuringiensis* proteases: production, sporulation and synergism. *Process Biochemistry* doi:10.1016/j.procbio.2007.01.015.

PUBLICATIONS HORS THÈSE

1. **Brar, S.K.**, Verma, M., Surampalli, R.Y., Misra, Kshipra, Tyagi, R.D., Meunier, N. and Blais, J.F. 2006. Bioremediation of hazardous wastes – a review. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management – special issue on bioremediation,* 10 (2), 59-72.
2. Verma, M., **Brar, S.K.**, Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005. Production of antagonistic fungus *Trichoderma* sp. from wastewater sludge: effect of pre-treatment and solids concentration. *Water Research*, 39, 3587–3596.
3. Verma, M., **Brar, S.K.**, Blais, J.F., Tyagi, R.D. and Surampalli, R.Y. 2005. Aerobic biofiltration processes – advances in wastewater treatment. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management* 10(4), 1-13.
4. Surampalli, R.Y., **Brar, S.K.**, Verma, M., Tyagi, R.D. and Valéro, J.R. 2006. Wastewater Treatment. Encyclopedia of Chemical Processing. Marcel Dekker, Inc. Editor: Sunggyu (K.B.) Lee DOI: 10.1081/E-ECHP-120008009.
5. Verma, M., **Brar, S.K.**, Tyagi, R.D. Surampalli, R.Y. and Valéro, J.R. (2006). Dissolved oxygen as principal parameter for conidia production of biocontrol fungi *Trichoderma viride* in a non-Newtonian wastewater. *J. Industrial Microbiology and Biotechnology,* 33, 941–952.
6. Verma, M., **Brar, S.K.**, Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2005. Antagonistic fungi, *Trichoderma* spp.: panoply of biological control. *Biochemical Engineering Journal* (under review).
7. Verma, M., **Brar, S.K.**, Tyagi, R.D. Surampalli, R.Y. and Valéro, J.R. (2007). Starch industry wastewater as a substrate for antagonist, *Trichoderma viride* production: Evaluation of medium additives for conidiation. *Bioresource Technology*, 98, 2154–2162.
8. Verma, M., **Brar, S.K.**, Riopel, A.R., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. (2006). Pre-treatment of wastewater sludge – biodegradability and rheology study. *Environmental Technology* (In Press).
9. Verma, M., **Brar, S.K.**, Tyagi, R.D. Surampalli, R.Y. and Valéro, J.R. (2006). Wastewater and wastewater sludge: rich nutrient source for growth of biocontrol agent, *Trichoderma viride. World Journal of Microbiology and Biotechnology* (under review).
10. Adjalle, K.D., **Brar, S.K.**,Verma, M., Tyagi, R.D. Surampalli, R.Y. and Valéro, J.R. (2006). Recuperation of entomotoxicity from supernatant of *Bacillus thuringiensis* fermented wastewater and wastewater sludge. *Process Biochemistry* (under review).
11. Faout, N., **Brar, S.K.**,Verma, M., Tyagi, R.D. and Blais, J.F. (2006). Chromium recovery from tannery wastewater by adsorption on various types of wastewater sludge. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management* (under review).
12. Misra, K., S. S. Bala, **Brar, S. K.**, Tyagi R.D. and Surampalli, R.Y. 2005. Biological assimilation and degradation. In: Natural processes and systems for hazardous waste treatment. ASCE book series on environmental and water resources engineering (In Press).

13. Misra, K., S.S. Bala, **Brar, S.K.,** Tyagi R.D. and Surampalli, R.Y. 2005. Bioremediation of hazardous wastes. In: Remediation technologies for soil and groundwater contamination task committee. ASCE Book (In Press).
14. Blais J.F., **Brar, S. K.,** Meunier N., Mercier, G., Tyagi R.D. and Surampalli, R.Y. 2005. Decontamination of toxic metals from soil and ground water. In: Remediation technologies for soil and groundwater contamination task committee. ASCE Book (In Press).
15. Verma, M., **Brar, S.K.,** Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005. Saprotrophs and mycorrhizal fungi in bioremediation – ephemeral vs sustainable approach. ASCE book series on environmental and water resources engineering (In Press).
16. **Brar, S.K.,** Verma, M., Misra K., and Tyagi, R.D. 2006. Lindane – A Contaminant of Concern. *Pollution Control* (under review).

CONGRÈS ET CONFÉRENCES

1. **Brar, S.K.**, Verma, M., Tyagi, R.D. Valéro, J.R. and Surampalli, R.Y. Techno-economic analysis of *Bacillus thuringiensis* biopesticides production from wastewater sludge. **IWA Conference on Wastewater Biosolids Sustainability – Technical, Managerial and Public Synergy, Moncton, New Brunswick, Canada, June 24 - 27, 2007 (accepted).**
2. Verma, M., **Brar, S.K.**, Tyagi, R.D. and Surampalli, R.Y. Wastewater and wastewater sludge: rich nutrient source for growth of biocontrol agent, *Trichoderma viride*. **IWA Specialized Conference on Sustainable Sludge Management, ECWATECH-2006, Moscow, Russia, 30 May-02 June, 2006.**
3. **Brar, S.K.**, Verma, M., Tyagi, R.D. Valéro, J.R. and Surampalli, R.Y. Do wastewater and wastewater sludge possess natural features to sustain *Bacillus thuringiensis* biopesticide production and formulation? **41st Central conference of the CAWQ (Canadian Association on Water Quality), Canada Centre for Inland Waters, Burlington, Ontario, February 13-14, 2006.**
4. Verma, M., **Brar, S.K.**, Tyagi, R. D. Surampalli, R.Y and Valéro, J.R. Potential of fungi *Trichoderma* spp. grown on wastewater and wastewater sludge for simultaneous biocontrol and degradation of estrogenic compounds. **41st Central conference of the CAWQ (Canadian Association on Water Quality), Canada Centre for Inland Waters, Burlington, Ontario, February 13-14, 2006.**
5. **Brar, S. K.**, Verma, M., Tyagi, R.D. Valéro, J.R. and Surampalli, R.Y. Role of rheology in fermentation and formulation of biopesticides using wastewater and wastewater sludge as raw materials. **21st Eastern conference of the CAWQ (Canadian Association on Water Quality), INRS-ETE, Université du Québec, Québec, November 04, 2005.**
6. Verma, M., **Brar, S. K.**, Tyagi, R. D. Valéro, J.R. and Surampalli, R.Y. Enhanced conidiation of biocontrol fungi-*Trichoderma* spp. in wastewater and wastewater sludge. **21st Eastern conference of the CAWQ (Canadian Association on Water Quality), INRS-ETE, Université du Québec, Québec, November 04, 2005.**
7. **Brar, S. K.**, Verma, M., Tyagi, R. D. and Valéro, J. R. Rheological comparison of *Bacillus thuringiensis* fermented wastewater and wastewater sludge for biopesticide production. **40th Central Canadian symposium on Water Quality Research (CAWQ), National Water Research Institute, Burlington, Ontario, Februaury 14-15, 2005.**
8. Verma, M., **Brar, S.K.**, Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. Production of antagonistic fungus *Trichoderma* sp. conidia on wastewater sludge: effect of sludge pre-treatments and sludge solids concentration. **40th Central Canadian symposium on Water Quality Research (CAWQ), National Water Research Institute, Burlington, Ontario, February 14-15, 2005.**
9. **Brar, S. K.**, Verma, M., Tyagi, R. D. and Valéro, J. R. Ultraviolet degradation studies of wastewater sludge based *Bacillus thuringiensis* biopesticides. **20th Eastern conference of the CAWQ (Canadian Association on Water Quality), Department of Engineering, University of Ottawa, Ottawa, October 29, 2004.**

10. Verma, M., **Brar, S.K.**, Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. Enzyme production by antagonistic fungi *Trichoderma* spp.: municipal sludge as potential substrate. **20th Eastern conference of the CAWQ (Canadian Association on Water Quality), Department of Engineering, University of Ottawa, Ottawa, October 29, 2004.**
11. **Brar, S.**, Verma, M., Tyagi, R.D. and Valéro, J.R. Screening of various anti-microbial agents (AMAs) for stability of sludge based *Bacillus thuringiensis* biopesticides. **CCE Environmental Geochemistry Gananoqué Meeting, Gananoque, Ontario, February 6-8, 2004.**
12. Verma, M., **Brar, S.K.**, Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. Municipal and industrial wastes as raw material for production of potential antagonistic fungi *Trichoderma* spp. **CCE Environmental Geochemistry Gananoqué Meeting, Gananoque, Ontario, February 6-8, 2004.**
13. **Brar, S.**, Verma, M., Tyagi, R.D. and Valéro, J.R. Development of stable aqueous flowable formulations of sludge based *Bacillus thuringiensis* biopesticides. **19th Eastern conference of the CAWQ (Canadian Association on Water Quality), Department of Engineering, Université de Sherbrooke, October 24, 2003.**
14. Verma, M., **Brar, S.K.**, Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. A Novel Strategy for the Production of Potential Antagonistic Fungi *Trichoderma* spp. Using Wastes as Raw Material. **19th Eastern conference of the CAWQ (Canadian Association on Water Quality), Department of Engineering, Université de Sherbrooke, October 24, 2003.**
15. **Brar, S.K.**, Verma, M., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. and S.K. Banerji. Comparative rheology and particle size analysis of various types of *Bacillus thuringiensis* fermented sludges. **Resources from Sludge: Forging New Frontiers, IWA International Specialized Conference, Singapore, March 28-29, 2003.**

RÉSUMÉ

Ce projet de recherche vise à évaluer les possibilités de développer des procédés de production par fermentation de biopesticides à base de *Bacillus thuringiensis* var. *kurstaki* HD-1 (Bt) et ce, en utilisant des eaux usées et/ou des boues d'épuration comme milieux de culture. Ces substrats alternatifs sont de riches sources de nutriments qui peuvent être exploités pour cultiver Bt et produire des biopesticides à haute valeur ajoutée.

Les propriétés rhéologiques des bouillons constituent un élément clé devant être pris en considération lors de la fermentation des boues et de la formulation des biopesticides. Les études rhéologiques (viscosité et taille des particules) réalisées avec plusieurs types de substrats de fermentation (boues primaires, secondaires et mixtes, eaux usées de l'industrie de l'amidon, boues pré-traitées par hydrolyse ou par stérilisation) ont permis de mettre en évidence que des lois exponentielles et de puissance étaient préférentiellement suivies par les boues hydrolysées fermentées en comparaison avec les boues brutes fermentées, alors qu'une augmentation exponentielle de la viscosité a été observée avec la hausse de la concentration en solides. Les tests ont démontré que les boues secondaires contenant 25 g/L de solides et les eaux usées d'amidon sont rhéologiquement compatibles pour la fermentation et le développement de la formulation.

Les milieux alternatifs fermentés ont montré un comportement pseudoplastique (ayant une viscosité diminuant avec la vitesse de l'écoulement ou le taux de cisaillement) et un comportement thixotropique (présentant une viscosité diminuant avec la durée de l'écoulement ou la durée d'application de la contrainte de cisaillement). Parallèlement, les boues secondaires hydrolysées ont montré un profil rhéologique amélioré par rapport aux boues non hydrolysées, résultant en un bouillon de fermentation avec une forte entomotoxicité et une formulation stable. Quant aux boues non hydrolysées, suite à l'addition de Tween 80, elles ont aussi présenté des profils rhéologiques mieux adaptés pour les fermentations de substrats à des concentrations de 25 à 30 g solides totaux par litre. D'ailleurs, pour les boues hydrolysées, la diminution de la taille des particules présentait une bonne corrélation avec l'augmentation de l'entomotoxicité, alors que la distribution de la taille des particules se situait dans la région inférieure de 0,2 à 12 µm. En fait, la courbe de

distribution de la taille des particules des formulations faites à partir de la fermentation de boues hydrolysées était comparable à celle de la formulation commerciale.[0]

Des études complémentaires ont été réalisées sur les récoltes des bouillons fermentés de Bt, le traitement en aval et la formulation. Des valeurs de pH de 4 et 5 et une température de 20°C sont optimales pour la récolte de tous les bouillons : soya, eaux usées d'amidon, boues non hydrolysées et boues hydrolysées à pH alcalin et température élevée. Après centrifugation, une concentration de 10% en solides dans le bouillon produit une biomasse utilisable pour le développement de la formulation. Le pourcentage d'entomotoxicité décroît avec la vitesse de centrifugation, alors qu'une récupération maximale est obtenue à 48 000 x *g*. Une récupération de 70% de l'entomotoxicité est obtenue avec une centrifugeuse commerciale opérant à 9000 x *g*. Les calculs de vitesse de sédimentation pour les différents bouillons fermentés ont permis de calculer le facteur Σ pour la centrifugeuse commerciale qui était plus élevé pour le milieu à base de soya. De plus, la puissance requise pour la récupération d'entomotoxicité était plus élevée pour le milieu conventionnel à base de soya par rapport aux bouillons fermentés basés sur des eaux usées ou des boues d'épuration.

Différents adjuvants, à savoir, des agents de suspension (20% p/v), des phagostimulants (0,5% p/v), des agents adhésifs (0,2% p/v), des agents antimicrobiens (0,5% p/v) et des agents protecteurs contre les rayons UV (0,2% p/v) ont été testés afin de développer des suspensions aqueuses de biopesticides. Ces essais ont permis de définir une formulation comportant :du sorbitol, du monophosphate de sodium, du métabisulfite de sodium comme agents de suspension ; de la mélasse et de la farine de soya comme phagostimulants ; de la mélasse et du lait écrémé en poudre comme adhésifs ; de l'acide sorbique et de l'acide propionique comme agents antimicrobiens et, finalement, du lignosulfate de sodium, de la mélasse et du rouge Congo comme écrans protecteurs contre les rayons UV. Les formulations liquides pour les boues non hydrolysées ou des boues hydrolysées comprenant du sorbitol, du monophosphate de sodium et du métabisulfite de sodium donnent de meilleures caractéristiques physiques (viscosité, taille des particules et potentiel de suspension) et biologiques (entomotoxicité et comptes de spores viables) à des pH de 4 et 5 et à des températures entre 4 et 20°C. De la même façon, les formulations stables des eaux usées d'amidon et du soya comportaient du sorbitol, du monophosphate de sodium, et du métabisulfite de sodium en tant qu'agents de suspension. Un effet minime a été observé sur

l'entomoxicité et sur la concentration des spores après 120 jours à pH 6 et 6,5 et à des températures de 40 à 50^{0}C, alors que de faibles changements dans la viscosité pouvaient être observés. Les acides sorbique et propionique ont montré un effet anti-microbien plus important et cela est essentiel pour garantir la pureté de la formulation.

L'entomotoxicité de la formulation se manifeste par la présence d'enzymes (protéases et chitinases) mélangées à des protéines insecticides. Les chitinases de Bt issues de la fermentation des boues d'épuration ont été caractérisées : une stabilité optimale a été observée à un pH de 4 et à une température de 50^{0}C. Les poids moléculaires des chitinases variaient entre 35 et 45 kDa. Les tests d'exposition aux rayons UV ont permis de mettre en évidence des demi-vies plus longues pour les boues fermentées par rapport au milieu conventionnel : boues non-hydrolysées (11,14 jours) > boues hydrolysées (9,51 jours) > eaux usées de l'amidon (9,02 jours) > soya (2,8 jours). Ces résultats démontrent la faisabilité de produire une formulation stable aqueuse à partir d'eaux usées ou de boues d'épuration fermentées par Bt. Ces résultats serviront de référence pour le développement de formulations sous d'autres formes, comme de la poudre humide ou des capsules qui représentent un grand intérêt en agriculture.

Enfin, une étude technico-économique détaillée a été effectuée sur le procédé de production de biopesticides à base de Bt en tenant compte de sept scénarios pour quatre substrats de fermentation, c'est-à-dire : 1) les boues non hydrolysées, 2) les boues hydrolysées, 3) les eaux usées d'amidon et 4) le milieu à base de soya. Il s'agissait de la première étude technico-économique intégrant les résultats des études sur la rhéologie, le traitement en aval et la formulation du présent projet. Les sept scénarios étaient les suivants : 1) les formulations liquides (récolte du bouillon par centrifugation à 9000 x *g*) ; 2) les formulations liquides (récolte du bouillon par centrifugation à 9000 x *g* et ultrafiltration par membrane de taille 5 kDa) ; 3) les formulations sèches (récolte du bouillon par centrifugation à 9000 x *g*) ; 4) les formulations sèches (récolte du bouillon par centrifugation à 9000 x *g* et ultrafiltration par membrane de taille 5 kDa) ; 5) l'ajout de mélasse (phagostimulant à 0,5% p/v dans les formulations); 6) l'ajout de Tween-80 (comme modificateur de la rhéologie à 0,2% p/v pendant la fermentation, seulement pour les boues non hydrolysées) et; 7) procédé de croissance <<Fed-Batch>> (seulement pour les boues non hydrolysées). Les boues hydrolysées ont donné le plus faible coût de production, soit 0,228 $CAN par milliard

d'unités internationales (ou 0,228 $/MUI) pour une capacité en usine de 3 x 10^7 MIU (3 x 10^7 MUI/an). Le taux escompté du rendement sur l'investissement était jugé acceptable (supérieur à 40%) pour les matières premières alternatives. L'impact du niveau de production sur les coûts de production n'est pas très significatif ($Can/MUI) : à un niveau de production de 3 x 10^7 MUI/an, les coûts de production étaient 30 à 45% des coûts de production pour un niveau de production de 7,5 x 10^6 MUI/an. Les calculs élaborés dans cette étude pourraient éventuellement être appliqués à d'autres procédés de fermentation utilisant des eaux usées et des boues d'épuration comme substrat, tels la production de biopolymères ou de biofertilisants.

ABSTRACT

The present project deals with the feasibility of development of formulations of *Bacillus thuringiensis* ssp. *kurstaki* HD-1 (Bt) biopesticides ("value added product") by utilizing wastewater and wastewater sludge as a growth medium. The biopesticide development from alternative raw materials forms a part of the sustainable effort to manage wastewater sludge in an eco-friendly manner. To ascertain the possibility of development of formulations of Bt fermented sludge, detailed rheology studies (viscosity and particle size) of different types of media, namely, primary, secondary and mixed sludge and starch industry wastewater were carried out. Viscosity studies were also carried out on raw, pre-treated (sterilized and thermal alkaline hydrolyzed or both types of treatment) and Bt fermented sludges at different solids concentration (10–40 g/L). Exponential and power laws were preferentially followed by hydrolyzed fermented compared to raw fermented sludge as well as there was an exponential increase in viscosity with solids concentration. The results showed secondary sludge (25 g/L) and starch wastewater to be rheologically compatible for fermentation and formulation development. The alternative fermented media showed pseudoplastic and thixotropic behaviour. Similarly, hydrolyzed secondary sludge showed an improved rheological profile resulting in fermentation broth with higher entomotoxicity and stable formulation. Non-hydrolyzed sludge rheology was also improved by addition of Tween-80 due to improved physical properties of fermentation medium at 25 and higher (30 g/L) total solids concentration. Moreover, the decrease in particle size correlated well with the increase in entomotoxicity and volume distribution of particles was in the lower particle size region of 0.2-12 μm for hydrolyzed sludge. In fact, the particle size distribution pattern of hydrolyzed sludge formulation overlapped with that of commercial formulation.

Harvesting pH and temperature for all broths (soya, starch industry wastewater, non-hydrolyzed and thermal alkaline hydrolyzed sludge) were found to be 4-5 and 20°C, respectively. Concentration of broth to 10 % solids produced usable slurry for formulation development. Percent entomotoxicity losses decreased with centrifugal force with maximum Tx recovery at 48 000 g and specific recovery at commercial centrifugal force of 9000 g. The settling velocity calculations for different fermented broths led to the calculation of Σ factor for continuous commercial centrifuge which was higher for soya medium. Furthermore, power requirements for a given entomotoxicity recovery efficiency were highest for conventional medium (soya) in comparison to wastewater and wastewater sludge based fermented broths.

The screening of different adjuvants, namely, suspending agents (20% w/v), phagostimulants (0.5% w/v), stickers (0.2% w/v), anti-microbial agents (0.5% w/v) and UV screens (0.2% w/v) was carried out to develop aqueous biopesticidal suspensions. The screening yielded a formulation which will comprise sorbitol, sodium monophosphate, sodium metabisulphite (suspending agents); molasses, soya flour (phagostimulants); molasses and skimmed milk powder (rainfasteners); sorbic and propionic acids (anti-microbial agents) and sodium lignosulphate; molasses and Congo red (UV screens). Liquid formulations for non-hydrolyzed and hydrolyzed sludge containing sorbitol, sodium monophosphate and sodium metabisulphite yielded improved physical (viscosity, particle size and suspendibility) and biological (spore count and entomotoxicity) characteristics at pH 4-5 and temperatures 4-20°C. Likewise, stable formulations of starch industry wastewater and soya comprised sorbitol and sodium monophosphate as suspending agents. A small effect on entomotoxicity and spore concentration was observed after 120 days at pH 6, 6.5 and temperatures 40 and 50°C and likewise a small viscosity change was also reported. Sorbic and propionic acid provided better anti-microbial properties – mandatory adjuvant of formulation to retain its purity.

The entomotoxicity of formulations was a manifestation of presence of enzymes (proteases and chitinases) along with insecticidal proteins. Chitinases originating from Bt based sludge fermentation were characterized and were found to be optimally stable at pH 4.0 and temperature 50°C. The chitinases were found to lie in the 35-45 kDa weight range. UV studies reflected better half-life of formulations vis-à-vis fermented sludges. Half-life of formulations (in days) was found to be in the order: non-hydrolyzed sludge > Thermal alkaline hydrolyzed sludge > starch industry wastewater > soya as 11.14>9.51>9.02>2.8. The formulations were prone to washout during rainfastness studies and this warranted addition of stickers. These results show the probability of forming stable Bt based sludge formulations in the aqueous suspension form. This will lay foundation to develop formulations in other forms like powder and encapsulations to be used in agricultural sector.

Further, detailed techno-economic study was carried out on the Bt biopesticides production process taking into account seven scenarios for all the four raw materials, namely, non-hydrolyzed wastewater sludge, hydrolyzed wastewater sludge, starch industry wastewater and soya. The seven scenarios included: a) liquid formulations (harvesting by centrifugation); b) liquid formulations (harvesting by centrifugation and ultrafiltration); c) dry formulations (harvesting by centrifugation); d) dry formulations (harvesting by centrifugation and ultrafiltration); e) Molasses (phagostimulant in formulations); f) Tween-80 (rheology modifier during formulation, only NH sludge) and; g) Fed-batch process (only NH sludge). The hydrolyzed sludge gave the lowest product cost of $Can 0.228/BIU for a Bt plant

production capacity of 3 x 10^7 BIU/year. The discounted cash flow return rate (measure of profitability) was in the acceptable range (>> 40 %) for alternative raw materials. The production scale had a large impact on the total per-unit operating cost ($Can/BIU), which at 3 x 10^7 BIU/year was 30-45% of that at 7.5 x 10^6 BIU/year. Moreover, the capital and product cost calculations developed in this study can be further extended to the techno-economics of other fermentation technologies to develop value-added products from wastewater/wastewater sludge.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

4. Amélioration physique des bouillons fermenté par Bt

Partie I. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges

Partie II. Ramification of solids concentration on entomotoxicity and enzyme activity of *Bacillus thuringiensis* fermented wastewater sludge

5. Procédés en aval des bouillons fermentés par Bt

Partie I. Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge

Partie II. Presence and characterization of chitinases present in *Bacillus thuringiensis* fermented wastewater and wastewater sludge

6. Développement des formulations aqueuses

Partie I. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides- Review

7. Analyse technico-économique

Partie I. Techno-economic analysis of *Bacillus thuringiensis* biopesticides production from wastewater and wastewater sludge

8. Conclusions et Recommandations

LISTE DES FIGURES

Partie II. Sludge based *Bacillus thuringiensis* biopesticides: viscosity impacts

Partie III. Particle size variations during production of wastewater sludge based *Bacillus thuringiensis* biopesticides

Partie IV. Broth rheology and process performance of *Bacillus thuringiensis* fermented primary and mixed sludge in fermenter

4. Amélioration physique des bouillons fermentés par Bt

Partie I. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges

Partie II. Ramification of solids concentration on entomotoxicity and enzyme activity of *Bacillus thuringiensis* fermented wastewater sludge

5. Procédés en aval des bouillons fermentés par Bt

Partie I. Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge

Partie II. Presence and characterization of chitinases present in *Bacillus thuringiensis* fermented wastewater and wastewater sludge

6. Développement des formulations aqueuses

Partie I. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides- Review

Partie II. *Bacillus thuringiensis* proteases: production, sporulation and synergism – Review

<u>Partie III</u>. Screening of different adjuvants for wastewater/wastewater sludge based *Bacillus thuringiensis* formulations

<u>Partie V</u>. Starch industry wastewater based stable *Bacillus thuringiensis* liquid formulations

Partie VI. Rheology of hydrolyzed sludge *Bacillus thuringiensis* fermentation and development of stable liquid formulations

7. Analyse technico-économique

Partie I. Techno-economic analysis of *Bacillus thuringiensis* biopesticides production from wastewater and wastewater sludge

ABBREVIATIONS*

AAU	Aedes aegypti unit (1 ITU = 2.5 AAU)
a.i.	Active ingredient
$AM_1 \ldots AM_n$	Anti-microbial agents
AMA	Anti-microbial agent
ANOVA	Analysis of variance
Bt	*Bacillus thuringiensis*
BIU	Billion international units
BITU	Billion international toxic units
CA	Chitinase activity (U/ml)
CDA	Controlled droplet application
CFM	Cross-flow microfiltration
CMC	Critical micelle concentration
COD	Chemical oxygen demand (mg/L)
CFU	Colony forming units
CUQ	Communauté Urbaine de Québec
CUQP	Communauté Urbaine de Québec Primary sludge
CUQM	Communauté Urbaine de Québec Mixed sludge
CUQS	Communauté Urbaine de Québec Secondary sludge
$D_1 \ldots . D_n$	Suspending agent
DO	Dissolved oxygen
DS	Dissolved solids
D_{10}	Particle size; 10% or undersize (μm)
D_{50}	Particle size; 50% or undersize (μm)
D_{90}	Particle size; 90% or undersize (μm)
EDTA	Ethylene diamine tetraacetate
EPS	Extra-cellular polymeric substances
FGD	Flue gas desulfurization
ISPR	In-situ product removal
Tx	Entomotoxicity (SBU/μL)
H	Hydrolyzed/ Thermal alkaline hydrolyzed sludge
HDPE	High density polyethylene
HF	Hydrolyzed fermented sludge
HLB	Hydrophile-lipophile balance
IP	Insecticidal protein
ICP	Insecticidal crystal protein
IU	International Units
IUPAC	International Union of Pure and Applied Chemistry
ITU	International Toxic Units
M	Mixed sludge
MEUF	Micelllar enhanced ultrafiltration
NAG	N-acetyl glucosamine
NH	Non-hydrolyzed sludge
NHF	Non-hydrolyzed fermented sludge
NHM	Non-hydrolyzed mixed sludge
NHP	Non-hydrolyzed primary sludge
NHS	Non-hydrolyzed secondary sludge

NMD	Number mean diameter
NTU	Nephelometric turbidity units
OFS	Oil from sludge
OTR	Oxygen transfer rate (mmol/L)
OUR	Oxygen uptake rate (mmol/L)
$P_1...P_n$	Phagostimulant
PPC	Protein polysaccharide complex
PPG	Polypropylene glycol
P	Primary sludge
PA	Protease activity (IU/ml)
PMSF	Phenylmethane sulfone fluoride
PCR	Polymerase chain reaction
PGA	Polyglutamic acid
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyric acid
PVP	Polyvinlylpyrrolidone
$R_1...R_n$	Rainfasteners
RBD	Rational biopesticide development
RCF	Relative centrifugal force (g)
RQ	Respiratory quotient
S	Secondary sludge
SBU	Spruce budworm units
SCOD	Soluble chemical oxygen demand (mg/L)
SCP	Single cell protein
SIW	Starch industry wastewater
SRB	Sulphate reducing bacteria
SS	Suspended solids (g/L)
SSA	Sewage sludge ash
STF	Sludge to fuel
ST	Surface tension (mN/m)
TOP	Thermophilic oxic process
TS	Total solids (g/L)
TVS	Total volatile solids (g/L)
TH/TAH	Thermal alkaline hydrolyzed/hydrolyzed sludge
THM	Thermal alkaline hydrolyzed mixed sludge
THP	Thermal alkaline hydrolyzed primary sludge
THS	Thermal alkaline hydrolyzed secondary sludge
UF	Ultrafiltration
ULV	Ultra low volume
UV	Ultraviolet
Vip	Vegetative insecticidal protein
VS	Viable spores
VC	Viable cells
VSS	Volatile suspended solids (g/L)
VMD	Volume mean diameter (μm)
K_La	Volumetric mass transfer coefficient (h^{-1})
WADB	Waste anaerobic dead biomass
WW	Wastewater
WWS	Wastewater sludge

WWTP	Wastewater treatment plant
WP	Wettable powder
YE	Yeast extract

French Abbreviations

APG	Acide polyglutamique
CFM	Microfiltration de croisement de flux
DCO	Demande en chimique oxygène
ISPR	Récupération de produit in situ
MES	Matières en suspension
MIU	Milliard Unité Internationale
MST	Matières en suspension totale
Piv	Protéine insecticide végétative
PCI	Protéines cristal insecticide
RQ	Quotient respiratoire
SPE	Substances polymères extracellulaires
STEP	Stations d'épuration
Tx	Entomotoxicité
UFMA	Ultrafiltration micellaire augmentée

* Chaque article contient sa propre série d'abbréviations

CHAPITRE 1.

SYNTHÈSE

1. Introduction

Les biopesticides à base de *Bacillus thuringiensis* (Bt) ont retenu l'attention des chercheurs pendant plusieurs décennies, mais cette technologie écologique n'est pas encore parvenue à remplacer complètement l'usage de pesticides chimiques malgré ses avantages qui ont été démontrés par plusieurs études. L'impact envers l'organisme nuisible ciblé, la taille du marché, la performance sur le terrain, le rapport coût-efficacité, la satisfaction des utilisateurs et de nombreux défis technologiques relevés (production par fermentation ou autres procédés, traitement en aval, formulation, pulvérisation sur le terrain), sont des facteurs qui affectent la commercialisation des biopesticides.

Même s'ils occupent la majeure partie de ce marché, les insecticides bactériens à base de Bt ne représentent que 1 % du marché mondial des pesticides (Jarvis, 2001). En dépit des progrès dans le développement des procédés, leur utilisation demeure limitée par leur coût de production. Dans les procédés conventionnels de production de Bt, le coût des matières premières varie de 35 à 59 % du coût de production selon la capacité de l'usine (Lisansky *et al.,* 1993; Stanbury *et al.*, 1993). Diverses études ont évalué l'addition de certaines matières résiduelles et d'autres matières alternatives pour la production de biopesticides, mais le coût de l'ajout de ces suppléments nutritifs au substrat de fermentation affecte leur compétitivité sur les marchés (Sachdeva *et al.*, 1999). Plusieurs chercheurs ont développé des solutions alternatives plus rentables pour la fermentation de Bt, notamment par l'utilisation de sous-produits agro-alimentaires comme les pelures de citron, les résidus de germe de blé, la farine de maïs, les graines de dattes, le sang de boeuf, l'exuvie de ver à soie, les résidus de noix de coco, la mélasse de canne à sucre et les résidus de transformation de l'arachide, dans lesquels sont souvent ajoutés des micronutriments tels : Mn, Mg et Ca (Salama *et al.*, 1983; Mummigatti et Raghunathan, 1990; Liu *et al.*, 1994; Aveluizapa *et al.*, 1999; Vora et Shethna, 1999; Saskinchai *et al.*, 2001; Özkan *et al.*, 2003).

Ces recherches ont mis en évidence plusieurs carences nutritives, reliées aux sources d'azote organique et inorganique, aux besoins en acides aminés et à la répression métabolique par le carbone, qui influencent la production des dérivés microbiens insecticides de Bt (toxines insecticides, spores et autres facteurs de virulence). Ainsi, l'addition de plusieurs sources nutritives synthétiques est nécessaire pour combler les insuffisances alimentaires dans les milieux de culture non conventionnels (résidus agro-alimentaires). Ces additions augmentent considérablement les coûts de production.

En contrepartie, l'utilisation des eaux usées ou des boues d'épuration comme substrat de fermentation pourrait apporter une solution aux problèmes reliés à l'ajout de nutriments. Les eaux usées et les boues d'épuration sont, en effet, une source riche en nutriments et peuvent être utilisées avec succès pour cultiver Bt et obtenir des biopesticides à haute valeur ajoutée. Au Québec, approximativement 200 000 tonnes de boues sont produites chaque année et 33 % sont utilisées pour l'épandage agricole et la production de compost (Charbonneau *et al.*, 2000). Les boues restantes sont enfouies ou incinérées, ce qui constitue une ressource intéressante comme matière première de haute qualité, disponible en tout temps à un coût presque nul, sinon négatif.

Au cours de la dernière décennie, plusieurs études approfondies ont été effectuées à l'INRS-ETE en vue de mettre au point un tel procédé (procédé de Bt-INRS) à base de boues d'épuration. Les résultats ont révélé qu'il était possible de produire des biopesticides à base de Bt ayant une entomotoxicité équivalente et supérieure aux biopesticides conventionnels sur le marché et ce, à des coûts inférieurs (Tirado-Montiel *et al.*, 1999; Sachdeva *et al.*, 2000; Lachhab *et al.*, 2001; Tyagi *et al.*, 2001; Vidyarthi *et al.*, 2002; Tirado-Montiel *et al.*, 2001, 2003; Yezza *et al.*, 2004; 2005 a,b,c,d; Barnabe, 2004, Barnabé *et al.*, 2005). Le coût de production évalué dans ces études était de 0.35 $/L avec les boues, comparé à 0.70 $/L avec un milieu de culture conventionnel à base de soya.

Cette estimation n'incluait toutefois pas les étapes de formulation du produit. À l'étape de la formulation, en effet, les additifs auront un effet important sur le coût, ce qui influencera le choix du milieu de culture alternatif. De plus, d'autres enjeux, en ce qui concerne la formulation, entrent en ligne de compte lors du choix du milieu de culture alternatif car la viscosité et la taille des particules doivent être considérées.

Du point de vue de la rhéologie, les boues d'épuration sont généralement des fluides non Newtoniens, c'est à dire thixotropiques (diminution de viscosité avec le temps) ou pseudoplastiques (diminution de viscosité avec le taux de cisaillement). Les paramètres rhéologiques (viscosité et taille des particules) affectent fortement la plupart des opérations de traitement, de disposition et de réutilisation des boues tel que : le stockage, le pompage, le transport, la manipulation, l'épandage dans les champs agricoles, la déshydratation, le séchage et l'enfouissement (Lotito *et al.*, 1997; Honey et Pretorius, 2000). Dans cette optique, les propriétés rhéologiques influenceront la préparation des substrats, en particulier l'homogénéisation ainsi que les étapes de l'hydrolyse et de la stérilisation. Évidemment, ces propriétés affecteront aussi le mélange du milieu de fermentation et le transfert de masse entre les phases solides, liquides et gazeuses (Richard et Margaritis, 2003;

Vasconcelosa *et al.*, 2003). Par conséquent, ceci influencera le procédé de fermentation et donc l'entomotoxicité du bouillon fermenté.

Les boues d'épuration subissent des changements importants pendant leur fermentation par Bt (Tirado-Montiel *et al.*, 2001). Ces changements régissent également le comportement rhéologique de la formulation finale du biopesticide. De plus, l'intégration des connaissances sur la rhéologie de la boue au développement des procédés de production et de traitement en aval (centrifugation/homogénéisation), joue un rôle crucial dans la performance globale du biopesticide. À ce propos, le choix du meilleur équipement à utiliser pour leur épandage sur le terrain et de la stratégie de pulvérisation à employer est fortement influencé par la consistance physique du produit. C'est pourquoi l'évaluation des propriétés rhéologiques est essentielle. En somme, les études rhéologiques donnent des informations capitales permettant : (a) d'évaluer la qualité des matières premières ou des produits finaux; (b) d'élaborer les procédés; (c) d'optimiser les formulations en se basant sur les relations entre la microstructure des formulations et les propriétés physiques; et (d) de formuler les produits commerciaux de Bt.

Un des défis, dans la mise au point du procédé de production de Bt à partir de boues, réside dans le développement de formulations physiquement et biologiquement stables. Cette difficulté est accrue par le fait que les boues fermentées par Bt ont un comportement non Newtonien, et que ce comportement n'a pas été étudié antérieurement. Ainsi, il serait important de connaître les propriétés rhéologiques des boues lors des différentes étapes de la production (fermentation, récolte des bouillons fermentés, formulation) et de l'application du biopesticide sur le terrain.

Le présent chapitre présente la synthèse de la recherche de manière à couvrir certains aspects concernant les biopesticides à base de Bt dont la production; l'utilisation des boues d'épuration et le procédé Bt-INRS; la rhéologie de la fermentation; la récolte des produits désirés; et la formulation. Les objectifs de recherche, ainsi que l'originalité et les hypothèses de recherche, sont présentés à la fin de ce chapitre.

Ce premier chapitre constitue une synthèse suivie par une série d'articles scientifiques présentés dans les chapitres subséquents :

- le deuxième chapitre présente une revue de littérature complète sur la valorisation des boues d'épuration (Brar *et al.*, 2006a);

- le troisième concerne l'impact des propriétés rhéologiques sur la fermentation en vue de produire le Bt. Il concerne plus particulièrement l'effet de la taille et de la viscosité des particules lors des différentes étapes de la production, plus précisément les produits bruts non traités ou hydrolysés, la stérilisation et la fermentation, ainsi que le rôle de la taille des particules sur le potentiel entomotoxique (Brar *et al.*, 2004a, 2005a, 2006b,c);
- le quatrième chapitre 4 traite de l'influence de l'amélioration physique par addition de surfactants sur les propriétés rhéologiques, l'entomotoxicité et la production enzymatique du Bt obtenu (Brar *et al.*, 2005b, 2006d);
- le cinquième chapitre porte sur la récolte de Bt par centrifugation et ce, vis-à-vis la rhéologie, les procédés de mise à l'échelle et les possibilités de récupérer les chitinases produites par Bt et qui sont des facteurs importants de virulence de ce bacille (Brar *et al.*, 2006e,f);
- le sixième chapitre concerne le développement de suspensions aqueuses de Bt efficaces et stables et, plus particulièrement, une estimation de la validité des principaux adjuvants pouvant être ajoutés aux préparations de cet insecticide bactérien et leur mise au point en termes des propriétés physiques et biologiques, ainsi qu'une revue exhaustive de la chronologie du développement, des formulations de Bt et les recherches relatives aux protéases produites par Bt et qui peuvent avoir deux rôles opposés de synergisme et d'antagonisme sur le potentiel de ce bacille (Brar *et al.*, 2004b, 2005 c,d, 2006g,h,i);
- le septième chapitre traite des études technico-économiques du procédé Bt-INRS pour explorer la faisabilité du procédé (un extrait de rapport, Brar *et al.*, 2006j).

2. Description des procédés de fermentation, de formulation et du micro-organisme

2.1. Complexité du substrat – boues d'épuration – généralités

Les boues des usines de traitement des eaux usées sont des composés complexes dont les caractéristiques varient en fonction de leur origine, de la durée de leur vieillissement et des types de traitement qu'ils ont subis (Metcalf et Eddy, 2003). Même si elles sont hétérogènes et variables, les boues contiennent des produits énergétiques, des matières organiques, des sources de carbone et d'azote. Ces composés peuvent être recyclés en produits pouvant avoir une valeur intéressante sur les marchés.

Toutefois, le principal obstacle pour l'utilisation des biosolides est la présence de contaminants tels que : des métaux lourds, des résidus de pesticides, des composés organiques toxiques émergents (produits pharmaceutiques ou de soin personnel, additifs pour les plastiques...) et les pathogènes.

Certains de ces facteurs limitatifs peuvent être contrés en ajoutant une étape de prétraitement pour transformer les boues et les rendre plus aptes à leur bioconversion.

2.2. Production

L'efficacité de la production du Bt en termes de spores et de cristaux protéiques dépend principalement de la composition du milieu de culture et des conditions de fermentation (Dulmage, 1970; Dulmage *et al.*, 1971). Tel que mentionné précédemment, le coût des matières premières est l'un des facteurs limitatifs dans la production globale de Bt[1].

2.2.1. Sommaire du procédé Bt-INRS

Depuis 1992, les connaissances à la base du procédé « Bt-INRS », permettant d'optimiser le procédé de production et d'augmenter l'entomotoxicité des biopesticides à base de boues fermentées, ont grandement progressé. Plusieurs études ont démontré qu'il était possible de produire, à partir des boues d'épuration, un insecticide à base de Bt ayant un bon rendement en entomotoxicité pour le Bt cultivé dans le milieu conventionnel de soya (Sachdeva *et al.*, 2000; Tirado-Montiel *et al.*, 1998, 2001; Tyagi *et al.*, 2001). Les divers paramètres tels que : la concentration en solides des boues (25 g/L), le rapport de C/N (7.9-9.9), la concentration en oxygène et la stratégie de fermentation en « Fed-batch » ont été optimisés pour l'obtention de fortes entomotoxicités (Vidyarthi *et al.*, 2002; 2003 a,b,c; Yezza *et al.*, 2004c). Un volume de 2 % v/v s'est avéré le niveau optimal d'inoculum pour obtenir une entomotoxicité élevée (Lachhab *et al.*, 2001). D'autres études ont porté sur le contrôle de la mousse durant la fermentation, l'ajout d'un agent tensio-actif pour accroître l'entomotoxicité et la performance à l'échelle pilote (Vidyarthi *et al.*, 2000, 2001; Yezza *et al.*, 2004, 2005a,b,c,d). Ces études sont résumées au tableau 1.

En somme, ces études n'ont pas tenu compte du changement de la rhéologie pendant la fermentation et les procédés en aval, alors que les caractéristiques rhéologiques du milieu de fermentation pourraient avoir un impact déterminant sur les formulations des produits. Ainsi, les caractéristiques physiques et rhéologiques des boues et leurs effets sur la fermentation des boues, et les procédés en aval, doivent être appréhendés pour mieux comprendre le procédé de production de biopesticides et maximiser la valeur ajoutée aux boues (mentionné dans les chapitres suivants).

---

[1] Pour des études plus approfondies sur la production de Bt en utilisant les milieux synthétiques, les matières premières, semi-synthétiques et alternatives, voir Hameed, 1990; Lisansky, 1993; Liu *et al.*, 1994; Zouari *et al.*, 1998, 2002; Adams *et al.*, 1999; Aveluizapa *et al.*, 1999; Vora et Shethna, 1999; Saskinchai *et al.*, 2001; Özkan *et al.*, 2003; Devi *et al.*, 2005; Prabakaran et Balaraman, 2006.

Tableau 1. Synthèse des études antérieures portant sur le procédé Bt-INRS

Objectifs	Approche scientifique / Paramètres testés	Faits saillants	Conclusions	Commentaires/ Références
Faisabilité de croissance de Bt dans les boues d'épuration	a) Études en fioles pour tester la croissance de Bt dans différentes boues d'épuration (primaires, secondaires, boues de papetières et surnageants des boues). b) Hydrolyse acide pour améliorer l'assimilation des nutriments contenus dans les boues d'épuration. c) Optimisation des paramètres de fermentation en fioles. d) Effet de suppléments nutritifs. e) Études des gènes impliqués dans l'entomotoxicité pendant la sporulation.	a) Croissance de Bt avec spores viables = 1 x 10^7CFU/mL dans les boues secondaires. b) L'hydrolyse acide augmente l'entomotoxicité de 1300 à 3500 SBU/µL†. c) Conditions optimales : pH = 7,0 ±0,1; Temp. : 30,0±1 °C; agitation : 250 rpm d) Nutriments importants - source de carbone et d'azote. e) L'étude de PCR a démontré des gènes codant pour des protéines Cry contribuant à l'entomotoxicité.	Boues d'épuration = bons milieux de culture alternatifs pour la croissance de production des biopesticides Bt.	Comparaison avec le bioinsecticide Foray 48B Tirado-Montiel 1997; Tirado-Montiel *et al.*, 1998, 2001, 2003
Croissance de Bt dans les boues d'épuration	a) Optimisation du volume de l'inoculum et de la concentration en solides. b) Évaluation du coût de procédé pour la production de Bt à partir de boues.	a) Un volume d'inoculum de 1 % et une concentration en solides de 25 à 35 g/L sont les valeurs optimales pour la production et l'entomotoxicité. b) Épargne de 0,45$/L quand le Bt a été fermenté dans les boues.	Les boues donnent une entomotoxicité plus élevée par rapport au soya et sont un milieu économique pour la fermentation de Bt.	Comparaison avec bioinsecticide Foray 48B Sachdeva *et al.*, 1999, 2000
Production de Bt dans les boues –effet de l'inoculum et des solides totaux	a) Différents âges d'inoculum. b) Différents volumes d'inoculum – 1 %, 2 %, 3 %, 4 %, 5 %. c) Différentes concentrations en solides totaux – 10, 20, 30, 40 et 50 g/L.	a) Acclimatation de l'inoculum dans les boues donnent le plus fortes entomotoxicités. b) 2 % inoculum : spores viables =2,1x10^9 CFU/mL; Entomotoxicité =12900 SBU/µL†. c) 25 g/L – solides totaux optimaux	Inoculum acclimaté et 25 g/L de solides totaux optimaux pour la croissance de Bt.	Comparaison avec le bioinsecticide Foray 48B Lacchab *et al.*, 2000, Lacchab, 2001

Tableau 1. Synthèse des études antérieures portant sur le procédé Bt-INRS

Objectifs	Approche scientifique / Paramètres testés	Faits saillants	Conclusions	Commentaires/ Références
		avec spores viables = $5x10^9$ CFU/mL; entomotoxicité = 12970 SBU/µL†.		
Isolement des nouvelles souches de Bt à partir de boues d'épuration et criblage moléculaire	a) Croissance de 20 différentes souches de Bt isolées à partir de boues dans des flacons et bioréacteur - criblage moléculaire. b) Étude de la variation saisonnière des boues c) Test sur la présence de β-exotoxine dans les boues fermentées par Bt.	a) Les études de reproductibilité ont montré une excellente croissance des nouvelles souches de Bt dans les boues; spores viables= $1,6x10^7$ à 7 x 10^8 CFU/mL; l'entomotoxicité = 11000 à 15000 SBU/µL†. b) La variation saisonnière affecte l'entomotoxicité de 9 à 13 %. c) Absence de β-exotoxine dans les boues fermentées par Bt.	La présence des nouvelles souches de Bt dans les boues et leur croissance confirment la polyvalence et la durabilité du procédé; les boues fermentées de Bt sont sécuritaires.	Comparaison avec le bioinsecticide Foray 48B Mohammedi, 2004; Mohammedi *et al.*, 2006
Production simultanée de protéases et de biopesticides pendant la fermentation de Bt dans les boues d'épuration	a) Faisabilité de la production simultanée de protéases de biopesticides en fioles et en fermenteur (15 L). b) Caractérisation de protéases (pH, température et résistance aux inhibiteurs).	a) La production de protéases a augmenté de 1 IU/mL dans les fioles et à 4 IU/mL dans le fermenteur de 15 L avec une entomotoxicité = 8500 SBU/µL† et une entomotoxicité = 9200 SBU/µL†, respectivement. b) Température optimale = 60-70 °C pH optimal = 7 et 10-11 Sensible à EDTA, mais pas à PMSF (phenylmethane sulfone fluoride) Tolère des sels comme $CaCl_2$.	Les boues secondaires répondent bien à la production simultanée de protéases et de biopesticides : protéases neutres et alcalines et thermiquement stables.	Comparaison avec le bioinsecticide Foray 48B Tyagi *et al.*, 2001
Substitution des solutions d'acide-base et des chocs de	a) Remplacement de H_2SO_4 et NaOH par CH_3COOH et NH_4OH.	a) Augmentation des spores viables = 6,6 x 10^8 CFU/mL et une	Ces deux stratégies ont augmenté la	Comparaison avec le

Tableau 1. Synthèse des études antérieures portant sur le procédé Bt-INRS

Objectifs	Approche scientifique / Paramètres testés	Faits saillants	Conclusions	Commentaires/ Références
pH pour augmenter la sporulation de Bt dans les boues	b) Chocs de pH pour stimuler la sporulation.	entomotoxicité = 12200 SBU/µL[†] b) Chocs de pH stimulant les spores viables = 4,4 x 10^8 CFU/mL et une entomotoxicité = 14400 SBU/µL[†].	production de Bt var. *kurstaki* dans les boues.	bioinsecticide Foray 48B Barnabe *et al.*, 2000, 2001
Optimisation de divers paramètres de fermentation en fermenteur pour la production de Bt dans les boues et criblage d'agents tensio-actifs et anti-mousse en fioles	a) Essais d'agents d'anti-mousse pour contrôler la mousse pendant la fermentation de Bt dans les boues. b) Essais de différents agents tensio-actifs - ATLOX 847, ATLOX 848, ATMOS 300, ATPLUS 401, ATPLUS 522, SPAN 20, SPAN 85, Tween 85 pour augmenter l'entomotoxicité. c) Optimisation des solides en suspension et du ratio C:N. d) Optimisation de l'âge et de la concentration en solides de l'inoculum.	a) Les huiles naturelles comme l'huile d'arachide et l'huile d'olive étaient efficaces comme suppresseurs de mousse. b) ATPLUS 522, Tween 80 et Tween 85 ont augmenté Tx de 24 % comparé au contrôle. c) MES optimale = 25 g/L et rapport C:N = 7,9-9,9. d) L'âge de l'inoculum à 12 h a donné un plus haut compte de spores viables = 4,2 x 10^9 CFU/mL; entomotoxicité = 10800 SBU/µL[†]; 25 g/L MES a donné un plus haut OTR = 250 mmol/L/h et un taux de transfert de dioxyde de carbone = 120 mmol/L/h et RQ = 0,6 à 9 h de fermentation.	Agents d'anti-mousse essentiels pour supprimer la mousse pendant le croissance de Bt; augmentation de l'entomotoxicité par des agents tensio-actifs; le ratio de C:N est un paramètre important pour le métabolisme de Bt; la production de Bt dépend de l'âge de l'inoculum.	Comparaison avec le bioinsecticide Foray 48B Vidyarthi *et al.*, 2000, 2001, 2002, 2003a,b,c
Effet de différentes stratégies de prétraitement de fermentation sur la croissance, la sporulation et le Tx de Bt en fioles	a) Hydrolyse des boues. b) Addition des différents nutriments (glucose, sulfate d'ammonium et d'extrait de levure). c) Mélange des boues (déshydratées et résidus brassicoles de levures). d) Addition d'agents tensio-actifs (Tween-80).	a) L'entomotoxicité était plus élevée dans les boues hydrolysées thermo-alcaline à 16000 SBU/µL[†]. b) Addition de glucose (2 g/L), une entomotoxicité élevée dans les boues non-hydrolysées de 11000 à 12500 SBU/µL[†]. c) Le mélange des boues n'a pas donné	Les stratégies de prétraitement et d'addition d'agents tensio-actifs sont d'excellentes méthodes pour augmenter le Tx des boues fermentées de	Comparaison avec le bioinsecticide Foray 76B Leblanc, 2003

Tableau 1. Synthèse des études antérieures portant sur le procédé Bt-INRS

Objectifs	Approche scientifique / Paramètres testés	Faits saillants	Conclusions	Commentaires/ Références
		de bons résultats, cependant, addition de levures résiduels aux boues non-hydrolysées a augmenté Tx de 2000 SBU//μL†. d) L'addition de 0,4 % et 0,1 % de Tween-80 a augmenté Tx de 25 % et de 65 % en boues hydrolysées et non- hydrolysées, respectivement.	Bt, alors que l'addition de nutriments augmente l'entomotoxicité.	
Études approfondies sur la production simultanée de Bt et des protéases en utilisant des nouvelles souches de Bt isolées dans les boues	La croissance de différentes souches de Bt dans les boues secondaires.	INRS 4, INRS 14 et INRS 21 a donné une excellente entomotoxicité = 15000 à 16000 SBU/μL† et protéase = 3 to 4,5 IU/mL.	Les souches de Bt isolées des boues ont un bon potentiel pour la production de biopesticides et de protéases	Comparaison avec le bioinsecticide Foray 76B Lamontagne, 2004
Augmentation de l'entomotoxicité par l'hydrolyse et l'oxydation partielle des boues	a) Prétraitements chimiques (acide, alcalin, oxydant), thermiques (au pH neutre et alcalin) et combinaison de traitement thermique et d'oxydant. b) Évaluation de l'entomotoxicité des spores, des cristaux protéiques et des facteurs de virulence.	a) Le traitement thermo-alcalin et l'oxydation thermo-alcaline ont donné d'excellentes entomotoxicités de 19000 et 17896 SBU/μL respectivement alors que les boues contenaient 35 g/L MES. b) Les rapports Tx/spores et Tx/protéines solubles étaient plus élevés dans les boues préalablement traitées que dans le milieu de soya.	Le prétraitement des boues facilite l'utilisation de boues à fortes concentrations en solides; permettent de produire des spores et des cristaux plus toxiques, et une grande quantité de facteurs de virulence.	Comparaison avec le bioinsecticide Foray 76B Barnabe, 2005; Barnabe *et al.*, 2005
Stratégies de mise à l'échelle (fermenteurs de 15 et 150 L) pour la production	a) Mise en échelle de la fermentation de Bt dans les eaux usées et les boues à l'échelle pilote (150 L).	a) Fermentation à l'échelle pilote augmente l'entomotoxicité par 25 % alors que les spores viables ont peu	La mise à l'échelle de Bt augmente les cellules totales,	Comparaison avec le bioinsecticide

Tableau 1. Synthèse des études antérieures portant sur le procédé Bt-INRS

Objectifs	Approche scientifique / Paramètres testés	Faits saillants	Conclusions	Commentaires/ Références
de biopesticides de Bt dans les eaux usées et les boues d'épuration	b) Fermentation en « Batch » et « Fed-batch ». c) Influence des agents de contrôle de pH sur différents substrats de fermentation. d) Stratégies de prétraitement pour augmenter Tx. e) Corrélation entre l'entomotoxicité et activité protéolytique.	changé (5,2 à 5,5 x 10^8 CFU/mL); la fermentation des boues industrielles a donné une entomotoxicité élevée = 18000 SBU/µL et une teneur en protéases de 4 IU/mL dans 150 L fermenteur. b) La stratégie de « Fed-batch » en fermenteur de 15 L a augmenté les spores viables de 5,6 x 10^8 à 8,6 x 10^8 CFU/mL et une entomotoxicité de 13000 à 18000 SBU/µL. c) La stratégie de contrôle de pH (150 L fermenteur) de CH_3COOH/ NH_4OH a augmenté les spores viables de 28 % et l'entomotoxicité de 22 % dans les boues. d) Le traitement thermo-alcalin des boues a augmenté les spores viables par 46 % et l'entomotoxicité était 17000 SBU/µL. e) L'activité de protéase corrélée exponentiellement avec les cellules totales et linéairement avec l'entomotoxicité; l'activité des protéases a atteint la valeur maximum à 36 h.	l'entomotoxicité et la production de protéases et les améliore avec différents agents de contrôle de pH <<fed-batch>> et des stratégies de prétraitement. Il existe une relation linéaire entre l'entomotoxicité et l'activité protéolytique.	Foray 76B Yezza *et al.*, 2004, 2005 a,b,c, 2006 a,b

[†] *Les valeurs d'entomotoxicité étaient à l'origine rapportées comme IU/µL, mais elles sont maintenant présentées en SBU/µL.*

2.3. Techniques de formulation

2.3.1. Récolte – Centrifugation et autres

Les bouillons fermentés comprennent des cristaux protéiques, des spores, des débris cellulaires et des particules solides résiduelles avec d'autres facteurs de virulence (tel que mentionné précédemment) qui doivent être récoltés de façon économique pour l'étape subséquente de la formulation (Bernhard et Utz, 1993; Rowe et Margaritis, 2004). La plupart des produits commerciaux de Bt contiennent des cristaux protéiques insecticides (CPI) et des spores viables. Pendant la production à grande échelle de Bt, il pourrait y avoir une perte importante d'ingrédients bio-actifs. Celle-ci est souvent due à la méthode de récolte utilisée. Au début, le procédé de lactose-acétone a été utilisé comme technique de laboratoire pour récolter des spores de Bt (Dulmage *et al.*, 1970; Dulmage et Rhodes, 1971). Cependant, l'utilisation des méthodes avancées telles que : l'ultracentrifugation, la microfiltration et la filtration sous vide pour séparer les particules insolubles (ingrédients actifs) du liquide (fraction inerte) du bouillon fermenté, a donné des résultats probants lors de la récolte des composantes actives (des lipases et des protéines) (Gulati *et al.*, 2000; Boychyn *et al.*, 2000). Le séchage par pulvérisation a été communément utilisé pour produire des formulations à partir de grands volumes de bouillons fermentés. Le séchage peut être précédé d'une étape d'épaississement par centrifugation ou filtration du bouillon fermenté en présence d'additifs comme le celite (facilite l'épaississement) (Bonnefoi, 1963; Cords et Fisher, 1966; Tamez-Guerra *et al.*, 1996). Une récolte efficace du complexe actif spore-cristal de Bt a été documentée en utilisant soit une centrifugeuse à disques empilés ou un appareil de filtration à vide avec une efficacité de récolte de spores de plus de 99 % (Zamola *et al.*, 1981; Rojas *et al.*, 1996).[2]

La récolte des ingrédients actifs peut augmenter ou diminuer l'activité insecticide. Les techniques courantes basées sur la centrifugation – conventionnelle, différentielle gradient de densité – et le séchage par pulvérisation peuvent entraîner des pertes lors de la récolte des δ-endotoxines. Ces méthodes pourraient être adaptées avec succès aux biopesticides à base de boues. Par exemple, il n'est peut-être pas nécessaire d'ajouter des additifs pour favoriser l'épaississement. En effet, les

[2] Il y a d'autres procédés avancés de filtration comme l'ultrafiltration de type micellaire-amélioré (UFMA); la purification des produits in-situ (ISPR) et la microfiltration à flux croisée (CFM) qui sont utilisés pour récupérer des protéines en général (Agrawal et Burns, 1996; Tzeng *et al.*, 1999; Persson *et al.*, 2004; Lee *et al.*, 2004). Ces méthodes peuvent être explorées lors du procédé de récolte pour les biopesticides à base de boues fermentées par Bt, bien que l'on soupçonne que des problèmes de colmatage et d'épaississement de la membrane puissent survenir comme pour les milieux synthétiques.

boues fermentées contiennent des flocs qui peuvent se comporter comme des adsorbants pour les spores et les cristaux protéiques pendant la centrifugation et peuvent également agir comme protecteurs pendant les conditions défavorables de séchage suite à la pulvérisation.

2.3.2. Formulations des biopesticides

Le développement de la formulation est un procédé important dont le succès dépend des facteurs suivants : 1) la stabilisation des ingrédients actifs pendant la distribution et le stockage; 2) la facilitation de la manipulation et de l'application du produit; 3) la protection contre des facteurs environnementaux défavorables; et 4) l'amélioration de l'activité des ingrédients actifs sur le terrain. Principalement, une formulation comporte des ingrédients actifs (i.a.) (mycètes, bactéries, virus, nématodes...) et des adjuvants/additifs (agents de suspension et de protection contre les UV, agents anti-microbiens, phagostimulants, adhésifs) pour atteindre ces objectifs.

Le critère de sélection des adjuvants/additifs est régi par le type de formulation désiré et s'applique à toutes les formulations des bouillons fermentés par Bt.[3] Les formulations de Bt peuvent être globalement classées en deux groupes : les formulations solides (poussières, granules, poudres, briquettes) et les formulations liquides (suspensions ou émulsions) (Rhodes, 1993). Les formulations solides, incluant les poussières et les poudres humides, comportent principalement des adhérents et des déshydratants et sont très utilisées pour le contrôle de la pyrale du maïs (Sundara-Babu *et al.*, 1970; Lynch *et al.*, 1980; McGaughey, 1985; Hewitt, 1998). Les granules sont faits à partir de la farine de blé, de la fécule de maïs, de la gélatine et de plusieurs autres matériaux (Tamez-Guerra *et al.*, 1996; Navon *et al.*, 1997; Maldonado *et al.*, 2002). Les briquettes sont un type de formulation flottante faites avec des matériaux comme la farine de blé et sont couramment utilisées pour les formulations de Bti (Mittal, 2003). Les suspensions liquides comportent les concentrés liquides qui ne sédimentent pas à cause de l'agglomération réversible par les agents de dispersion. Elles comportent aussi des agents tensio-actifs qui agissent comme des agents mouillants favorisant la pulvérisation. Les agents tensio-actifs de nature non ioniques (dérivés de polyoxyéthylène et de polyoxypropylène) sont généralement utilisés en combinaison avec des agents de protection contre

[3] Les caractéristiques et la composition des formulations de biopesticides doivent être adaptées aux facteurs suivants : le type d'habitat (feuillage, sol, eau, superficie...), l'espèce d'insecte ciblé (habitude et lieu d'alimentation, cycle de vie...); le mode d'action (oral/contact); les interactions hôte-pathogène-environnement (changements de comportement, résistance, stabilité...); le mode d'application (aérien, terre); et le taux d'application (L/ha et kg/ha).

les rayons UV hydrosolubles (Burges, 1998). Il y a également des émulsions huile/eau et eau/huile dont l'usage domestique ou agricole est restreint (US EPA, 1989; Langley, 1998).

D'ailleurs, les progrès dans les formulations de bio-insecticides garantissent une très bonne protection envers des conditions environnementales défavorables (rayons UV, pluie...). La stabilité résiduelle améliorée permet une diffusion lente de la formulation dans le milieu. Les biopesticides comprennent des formulations encapsulées à base d'amidon et de lignine (Owaga *et al.*, 1998; Lechelte-Kunze *et al.*, 2000; Toreki *et al.*, 2004). Il existe également un type de formulation permettant d'augmenter l'entomotoxicité par stimulation ou par synergie avec des phagostimulants (Broderick *et al.*, 2000; Martin, 2005).

Néanmoins, les formulations de Bt ont une faible persistance sur le terrain à cause des facteurs environnementaux défavorables (rayons UV, pH du feuillage, température, rosée, pluies) qui doivent être pris en compte pour développer de nouvelles formulations. La lumière naturelle du soleil, en particulier, le rayonnement des spectres UV (UV-B, 280-310 nm et UV-A, 320-400 nm), est principalement responsable de l'inactivation des ingrédients actifs. Plusieurs études ont été effectuées avec l'incorporation de divers agents protecteurs contre les rayons UV tels : le rouge Congo, l'acide folique, la mélasse, la lignine, l'alginate, la cellulose, l'acide p-amino benzoïque, et les résultats étaient plus ou moins intéressants (Salama *et al.*, 1993; Hobbs *et al.*, 1999; Wirtz, 1999).

Les précipitations représentent un autre facteur naturel important qui affecte l'efficacité des biopesticides en les enlevant du feuillage par lessivage avant leur interaction avec l'insecte cible. Les microencapsulations à base d'amidon et l'utilisation d'adhésifs naturels et synthétiques aident à atténuer le lessivage (Ferro *et al.*, 1997; Paukner *et al.*, 2003; Hatfaludi *et al.*, 2004). Des composés organiques volatils, comme les aldéhydes, les cétones, les acides carboxyliques et leurs dérivés présents sur le feuillage, auraient un effet antibiotique sur les spores de Bt et causent parfois leur inactivation ou retardent leur activité (Ferry *et al.*, 2004). Par ailleurs, la manière la plus efficace de contrer l'effet de pH sur les feuillages est de bien planifier la séance de pulvérisation et d'ajouter des tampons dans la formulation ou dans la cuve à mélanger. Les effets du pH sont souvent observés lorsque les mélanges à pulvériser sont entreposés dans des contenants en aluminium ou en fer qui sont sensibles à la corrosion et qui nécessitent l'utilisation de tampons. Des problèmes de corrosion peuvent affecter l'efficacité des biopesticides sur le terrain (Burges, 1998). Il est aussi démontré que

le pH affecte la performance de la formulation aussi bien pendant le stockage (action des protéases) que pendant l'application (corrosion des réservoirs) et après l'application (action du feuillage discutée plus loin dans la chapitre 5). De plus, la température élevée de l'atmosphère, lors des applications, peut diminuer l'activité des produits à base de Bt, spécialement dans les régions tropicales où les températures excèdent fréquemment 30°C (Morris, 1983).

Pour développer avec succès des biopesticides et maximiser leur efficacité sur le terrain, il faut tenir compte de facteurs biotiques (concentration des spores et l'entomotoxicité) et abiotiques (rayonnement UV, température, pH, pluie, feuillage, etc.). De plus, différents paramètres et additifs pour les différentes formulations doivent également être considérés (par exemple, la formulation ne doit pas contenir de matériau hygroscopique pour éviter la formation d'agrégats dans les produits secs). La possibilité de contamination et la dégradation des cristaux protéiques pendant le stockage doivent aussi être réduites en diminuant le pH avec des solutions très acides. Quant aux différents types de formulations solides et liquides, ils ont plusieurs avantages et inconvénients, mais ils trouvent tous une place sur le marché et répondent très bien aux besoins des utilisateurs.

En raison de l'obligation de minimiser les coûts de production, le procédé de fermentation du biopesticide doit être configuré de manière à satisfaire aux exigences de la formulation qui forment un pont entre la fermentation de Bt et l'application sur le terrain.

2.4. Bacillus thuringiensis var. kurstaki (Btk)

Btk est une bactérie Gram positif et aérobie qui forme des spores.[4] Elle est caractérisée par des inclusions cristallines parasporales de nature protéique comme le montre la figure 1. Celles-ci apparaissent souvent sous le microscope photonique comme des cristaux distincts pouvant avoir des formes bipyramidales avec des propriétés pathogènes spécifiques contre les larves de lépidoptères. Les cristaux protéiques insecticides jouent un rôle majeur dans l'entomotoxicité alors que les spores

[4] Il y a au moins 34 sous-espèces de Bt (également appelés sérotypes ou variétés) et probablement plus de 800 souches isolées (Entwistle *et al.*, 1993; Schallmey *et al.*, 2004). Bt est une bactérie ubiquiste de l'environnement et elle est entre autres présente dans les boues activées des stations d'épuration des eaux usées municipales (Mizuki *et al.*, 2001; Mohammedi *et al.*, 2006).

peuvent y contribuer. Les produits commerciaux à base de Bt sont inoffensifs pour l'humain et l'environnement.[5]

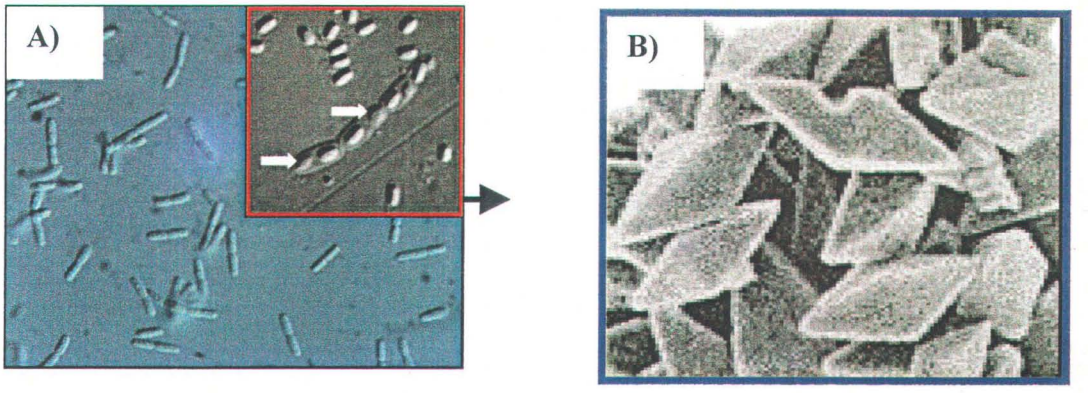


Figure 1. A) Spores et cristaux protéiques de *Bacillus thuringiensis* var. *kurstaki* (microscopie à contraste de phase interdifférentiel, 1600X) dans le milieu de boues d'épuration; B) forme bipyramidale des cristaux protéiques (microscopie électronique), (http://helios.bto.ed.ac.uk/bto/microbes/bt.htm).

2.4.1 Mode d'action (Btk)6

Quand les larves d'insecte ingèrent les cristaux protéiques, les spores et d'autres facteurs de virulence (qui agissent en synergie), les protoxines sont solubilisées et fragmentées par les protéases intestinales des larves, libérant alors des fragments dont certains sont entomotoxiques (δ-endotoxines) (figure 2). Le processus d'intoxication des larves d'insectes par Btk est divisé en six étapes : solubilisation, activation, liaison, formation des pores, septicémie et mort. Les protoxines ont approximativement un poids moléculaire de 130-135 kDa. La solubilisation des protoxines se produit à un pH alcalin (9-10) chez les larves d'insecte de l'ordre des lépidoptères et sont protéolytiquement convertis en toxines actives de 55-65 kDa. Les δ-endotoxines s'insèrent irréversiblement dans les membranes (Schwartz *et al.*, 1997). Finalement, l'association avec d'autres molécules toxiques par oligomérisation entraîne la formation des pores (Masson *et al.*, 1999). Le flux ionique à travers les

[5] Les études de laboratoire et les essais réalisés sur le terrain (dans le champ) ont démontré que les insecticides de Bt sont sécuritaires pour l'environnement et ne produisent aucun résidu toxique (Oshodi et MacNaughtan, 1990; Glare et O'Callaghan, 2000; Joung et Côté, 2000; Siegel, 2001).

[6] Pour plus de détails sur le mode d'action de Bt, voir Whalon et Wingred, 2003.

pores provoque la lyse cellulaire et éventuellement une septicémie (invasion du sang par les spores ou par la flore intestinale) (Bravo *et al.*, 2002) tel qu'illustré aux étapes 4 et 5 de la figure 2.

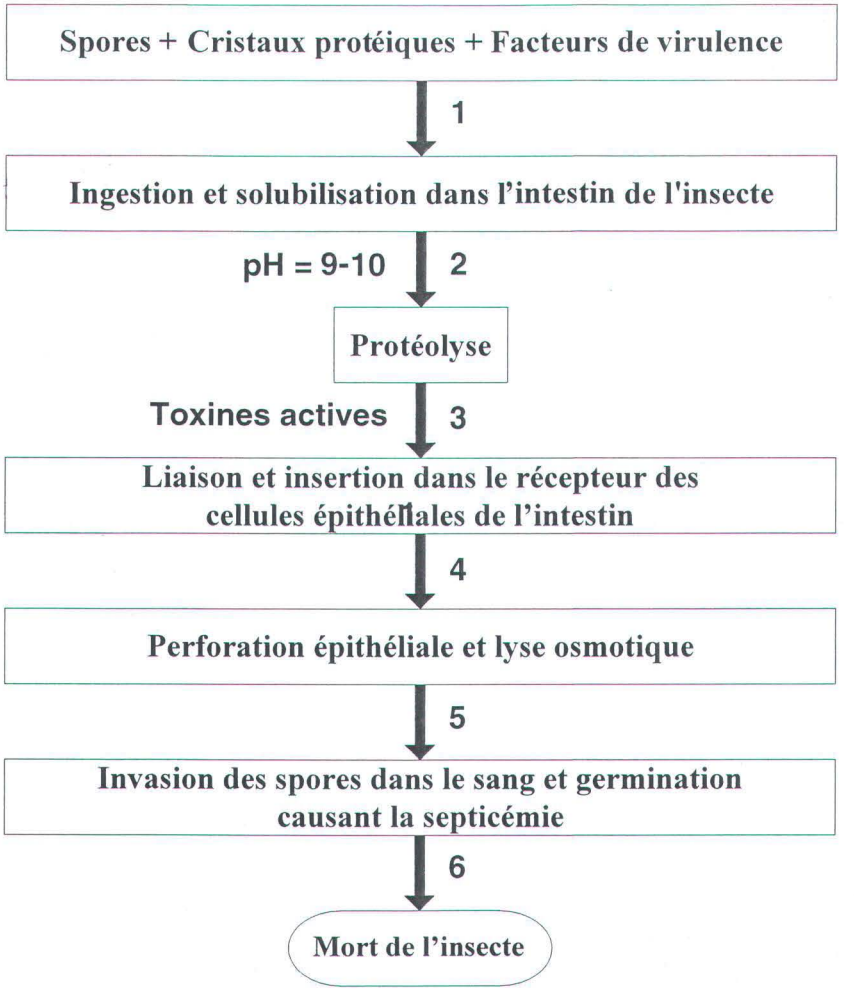


Figure 2. Mode d'action des spores de Btk, des cristaux protéiques et d'autres facteurs de virulence chez les larves de lépidoptères

Bt sécrète également d'autres facteurs de virulence comme des phospholipases, des chitinases, des protéases, des protéines insecticides végétatives (Piv) et des antibiotiques (bactériocines) qui, par synergie, permettent d'augmenter l'entomotoxicité des cristaux protéiques insecticides et/ou des spores de Bt (Johnson *et al.*, 1998; Schnepf *et al.*, 1998; Chang *et al.*, 2003). Cependant, aucune étude approfondie portant sur la synergie possible avec les protéases, n'a pas été répertoriée dans la littérature (Rukmini *et al.*, 2000).

3. Rhéologie

Les caractéristiques physiques des boues (taille de particule, viscosité) peuvent avoir un impact important sur l'efficacité des systèmes de traitement des eaux usées ainsi que la transformation ultérieure des différents produits (comme les biopesticides par exemple). Une de ces caractéristiques est la propriété d'écoulement qui peut être mesurée en utilisant des techniques rhéologiques.

3.1. La rhéologie des solutions aqueuses : principes généraux

Étymologiquement, la rhéologie est une science qui traite de l'écoulement, des déformations, et plus généralement, de la viscosité des matériaux sous l'action de contraintes. Deux grandeurs servent à caractériser quantitativement le cisaillement : la vitesse de cisaillement et la contrainte de cisaillement. La vitesse de cisaillement est la déformation qui correspond au vecteur déplacement de la particule fluide d'une couche sous l'effet du mouvement de cisaillement pendant une durée. Pour expliquer la contrainte de cisaillement, considérons deux couches au contact l'une de l'autre; elles se déplacent relativement l'une par rapport à l'autre. Il en résulte l'apparition de forces de frottement qui s'exercent tangentiellement à la surface de la couche : ce sont les forces de cisaillement.

De façon générale, il y a deux types de fluides : Newtoniens et non-Newtoniens. Pour les fluides Newtoniens, leur viscosité ne dépend pas du cisaillement appliqué (exemples : eau, la plupart des solvants, huiles minérales, certaines dispersions). Pour les fluides non Newtoniens (non linéaires), la viscosité n'est pas constante. À chaque valeur du couple vitesse de cisaillement, contrainte de cisaillement (τ) correspond une valeur de la viscosité, η. Il existe plusieurs types de fluides non Newtoniens - liquides rhéofluidifiants (parfois appelés pseudoplastiques) : la représentation passe par l'origine avec une décroissance de la dérivée, c'est-à-dire de la viscosité apparente, lorsque le gradient de vitesse augmente; liquides rhéoépaississants (à tord nommés épaississants) : il concerne des dispersions très concentrées, les solutions d'amidon, sables mouillés et compactés et certaines huiles polymériques. Certains de ces produits augmentent de volume sous la contrainte (épaississants); les liquides plastiques : indiquent qu'ils s'écoulent à partir d'une certaine valeur de contrainte; les liquides thixotropes : la viscosité diminue avec le temps (exemple : peintures) et; les liquides dilatants : la viscosité augmente avec le temps (exemple : les sables).

3.2. Rhéologie des boues d'épuration – viscosité et taille de particule

Les boues présentent des caractéristiques thixotropiques (Campbell et Crescuollo, 1982; Honey et Pretorius, 2000) qu'il est possible d'étudier en utilisant différents modèles rhéologiques comme le modèle de Bingham (Chilton *et al.*,1995; Lotito *et al.*,1997) et les modèles de Herschel-Bulkley (Baudez, 2001). Par exemple, la détermination de la contrainte de cisaillement (τ) en fonction du taux de cisaillement (γ•) permet de caractériser le comportement d'écoulement des boues, notamment en regard de ses propriétés non-Newtoniennes où une relation non linéaire est observée entre la contrainte de cisaillement (τ) et le taux de cisaillement (γ•) (Dentel, 1997). Le modèle généralement utilisé pour décrire le comportement rhéologique des boues est celui de Herschel–Bulkley (1) :

$$\tau = \tau_0 + K\,(\gamma\bullet)\,n \qquad (1)$$

où K et n sont des constantes et τ_0 est la contrainte d'écoulement.

Les boues d'épuration se présentent sous la forme d'une suspension biologique de flocs aux structures irrégulières et de diverses tailles qui évoluent dans le temps. À de très faibles concentrations en solides, les boues peuvent avoir un comportement se rapprochant de celui de l'eau (c'est-à-dire un comportement Newtonien). Cependant, avec l'augmentation de la concentration en solides, les boues montrent un caractère d'écoulement non-Newtonien. Ce comportement non-Newtonien est causé par les propriétés colloïdales des solides (Hiemenz et Rajagopalan, 1997). Les substances polymères extracellulaires (SPE) présentes dans les boues peuvent affecter plusieurs caractéristiques physiques et chimiques des boues, intervenant ainsi sur le taux de déshydratation, la charge et la structure des flocs, le taux de sédimentation et de floculation, ainsi que sur la biosorption des particules (Laspidou et Rittmann, 2002). La présence de SPE peut ainsi augmenter la résistance des solides des boues aux forces de cisaillement entraînant alors une augmentation de la viscosité qui affectera le comportement rhéologique. Les interactions de SPE entre les cellules permettent aux bactéries adjacentes de former des agrégats à la suite des attractions électrostatiques et physiques des surfaces cellulaires et, par conséquent, entraînent la formation des flocs changeant ainsi le comportement chimique des boues.

Les matières solides des boues activées ont fait l'objet d'études intensives et leur effet est bien documenté (Lotito *et. al.*, 1997; Sanin, 2002). La taille et la forme des particules des boues sont un

aspect très difficile à étudier. La distribution des tailles de particules des boues est large et varie en fonction du temps et des facteurs physiques et chimiques comme le cisaillement et la chimie de suspension. Les interactions « particule-particule » et « particule-milieu de dispersion » dépendent aussi bien des propriétés des particules que de la chimie physique de solution. Cependant, ce problème est surmonté avec l'arrivée de nouvelles techniques pour mesurer la taille des particules.

La mesure de la taille des particules a connu d'énormes progrès depuis l'usage des instruments comme le Coulter Counter ou de l'analyse des électrozones (Houghton *et al.*, 2002). À l'heure actuelle, la diffraction par LASER connaît une popularité croissante comme méthode d'analyse de la taille des particules pour des échantillons d'eaux usées (Neis et Tiehm, 1997; Biggs *et al.*, 2000), mais son application aux boues d'épuration demeure limitée (Houghton *et al.*, 2002).

Actuellement, les paramètres rhéologiques sont essentiellement utilisés pour le conditionnement des boues ou pour l'optimisation de leur consistance lors de leur stockage ou de leur épandage (Lotito *et al.*, 1997; Dentel *et al.*, 2000; Yen *et al.*, 2002). Malgré son rôle holistique pour la production de produits à valeur ajoutée (voir figure 2), il n'y a aucune étude effectuée sur l'utilisation des paramètres rhéologiques comme indice de qualité des boues pour cette option de valorisation.

3.3 Amélioration des propriétés rhéologiques entrant dans le procédé de fabrication de suspensions insecticides

Les propriétés rhéologiques, telles que la viscosité, et la taille des particules, peuvent affecter les différents aspects de la production des biopesticides, particulièrement la préparation du substrat de la fermentation, la fermentation, la récolte et la formulation, et l'application sur le terrain. Il est donc essentiel d'évaluer l'impact de ces propriétés rhéologiques sur les diverses étapes de la production de biopesticides, tel que décrit dans la figure 3.

3.3.1. Rhéologie et fermentation

Un bouillon de fermentation est un milieu semi-liquide comprenant, entre autres, la biomasse cellulaire et les produits microbiens sécrétés par les micro-organismes. La performance métabolique d'une culture microbienne en bioréacteur dépend fortement des interactions complexes entre les diverses conditions d'opération. Plusieurs conditions d'opération telles : la vitesse de l'agitation, l'espèce microbienne cultivée, les nutriments et les suppléments alimentaires, déterminent la

rhéologie du milieu et la morphologie cellulaire. La rhéologie affecte à son tour l'approvisionnement en nutriments, particulièrement l'oxygène, et le brassage du bouillon de fermentation. Les propriétés rhéologiques des bouillons de fermentation varient pendant une fermentation, ce qui influence, entre autres, le coefficient volumétrique du transfert de l'oxygène ($k_L a$) et le degré d'agitation (Aiba *et al.*, 1973). La croissance spécifique observée dans des conditions données dépend de plusieurs facteurs, dont : la souche microbienne, la méthode de culture de départ (par exemple spores, granules), l'aspect rhéologique du milieu de croissance, et le régime hydrodynamique dans le bioréacteur (Lopez *et al.*, 2005).

D'une manière importante, la contrainte de cisaillement du bouillon fermenté est normalement caractérisée par le modèle simple de la loi de puissance :

$$\tau = K.\dot{\gamma}^{n} \qquad (2)$$

où τ est la contrainte de cisaillement et $\dot{\gamma}$ est le taux de cisaillement.
Les constantes, K et n représentent respectivement les indices d'uniformité et de comportement d'écoulement. La viscosité apparente, η_a est alors donnée par :

$$\eta_a = K.\dot{\gamma}^{n-1} \qquad (3)$$

En prenant le logarithme des deux côtés de l'équation (3), l'équation (4) est obtenue :

$$\log(\eta_a) = \log K + (n-1)\log\dot{\gamma} \qquad (4)$$

Les valeurs de K et n sont évaluées à partir d'une courbe logarithmique de $\eta_a = f(\dot{\gamma})$.

Bt est commercialement produit par fermentation en mode « Batch » ou « Fed-batch » et les conditions de culture changent tout au long de la fermentation. Une caractéristique importante d'un procédé de fermentation en « Batch » est le changement de la rhéologie pendant la fermentation à cause de la variation de la composition du substrat (causée par la production ou la consommation des SPEs), de la concentration de la biomasse cellulaire, des conditions de fermentation et de la morphologie des micro-organismes (Berovic *et al.*, 1993). La fermentation de Bt est limitée par le transfert de l'oxygène. Celui-ci est grandement influencé par la rhéologie du milieu alors qu'une augmentation du taux du transfert d'oxygène s'accompagne d'une augmentation de l'entomotoxicité du biopesticide (Vidyarthi *et al.*, 2002; Rowe *et al.*, 2003). Tel que mentionné précédemment,

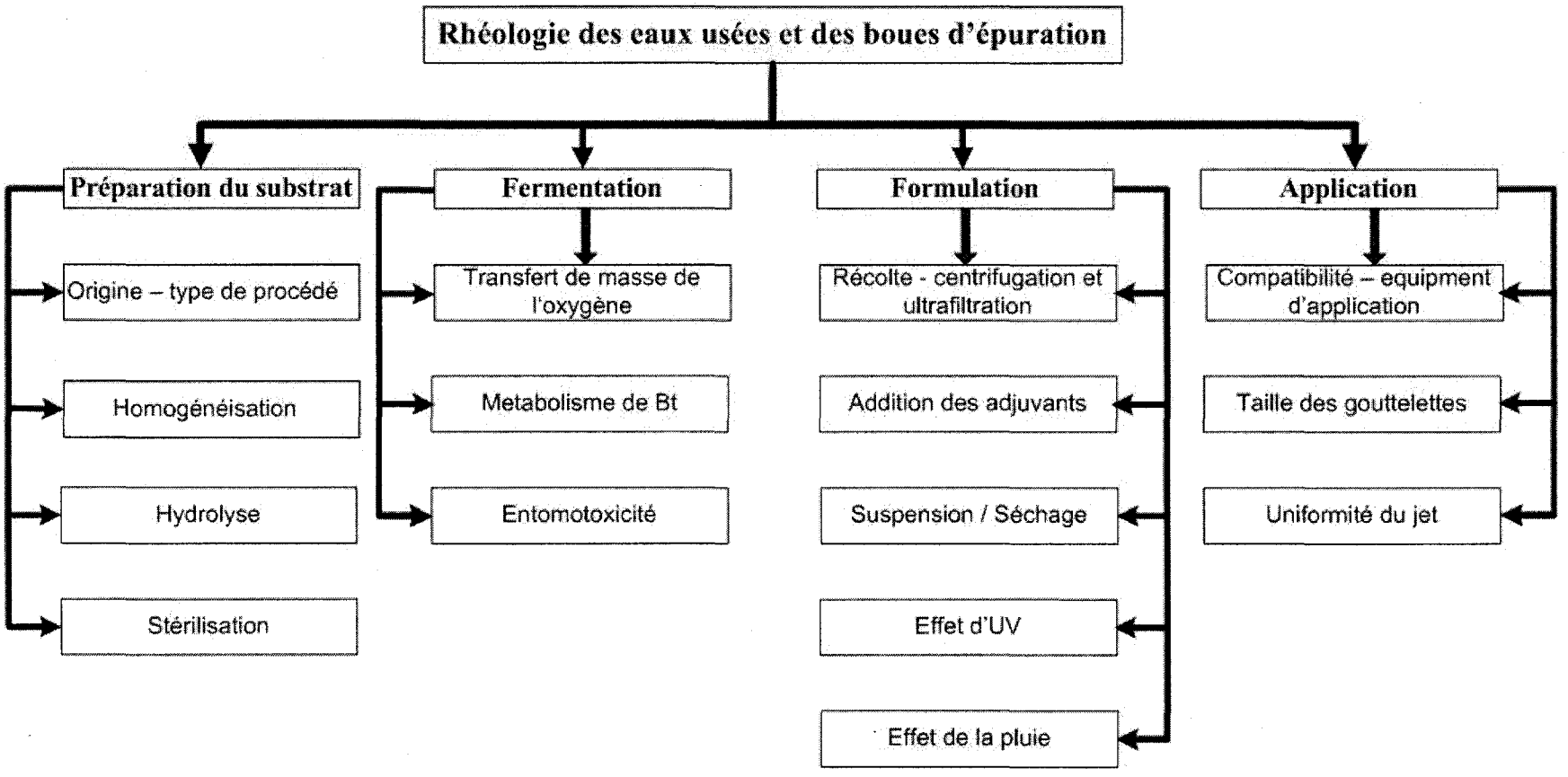


Figure 3. Le rôle holistique de la rhéologie des boues d'épuration dans la production de biopesticide.

la rhéologie de boues est non Newtonienne et, par conséquent, la production des biopesticides à base de boue suivra un procédé différent (désintégration des flocs, réduction de viscosité) de celui des milieux de culture conventionnels.

Plusieurs études ont mentionné l'influence de différentes conditions environnementales sur les caractéristiques rhéologiques du milieu de fermentation comme : la contrainte de cisaillement qui affecte la production de la β-exotoxine de Bt dans le fermenteur (Wu *et al.*, 2002); les variations morphologiques (Jong *et al.*, 1994); la production d'acide polyglutamique (APG) par *Bacillus subtilis* (Richard et Margaritis, 2003) et *B. licheniformis* (Yoon *et al.*, 2000) et la conception de bioréacteur (Kilonzo et Margaritis, 2004). À INRS-ETE, les divers paramètres comme l'addition d'agents tensio-actifs (Vidyarthi *et al.*, 2001) et d'agents anti-mousse (Vidyarthi *et al.*, 2000) pour la fermentation des boues par Btk ont déjà été étudiés, mais leur effet sur la rhéologie n'a pas été déterminé, surtout en ce qui a trait à l'entomotoxicité des préparations de Bt obtenues, potentiel devant être nécessairement affecté par l'impact important de la rhéologie sur le transfert de masse.

Considérant les connaissances sur le mode d'action du Btk (section 2.4.1), il est impératif d'explorer les effets des propriétés rhéologiques sur différents aspects de ce mode d'action. Lorsque les cristaux, les spores et les divers facteurs de virulence de Btk sont absorbés par l'insecte, il est possible que les particules présentes dans les préparations de ce biopesticide, particulièrement celles à base de matières recyclées comme les eaux usées et les boues d'épuration, peuvent réduire l'hydrolyse des protoxins en toxins actifs par des protéases et donc l'action subséquente de ces protéines (étapes 2 et 3, figure 2). Ainsi, toute modification, en ce qui concerne la rhéologie, peut altérer le mode d'action du Btk, et donc son pouvoir entomotoxique.

3.3.2. Propriétés rhéologiques, formulation et application

Tel que montré dans la figure 3, les propriétés rhéologiques affectent de plusieurs façons la mise en suspension de Btk. En premier lieu, la récolte d'un milieu fermenté d'une faible viscosité serait affectée par une centrifugation à basse vitesse par rapport à un bouillon qui aurait une viscosité élevée, ceci en accord avec la loi de Stoke (5) :

$$v_{sed} = \frac{g}{18}\frac{\left[(\rho_s - \rho_L)d^2\right]}{\eta} \qquad (5)$$

Dans laquelle :

v_{sed} = vitesse de sédimentation; (m s^{-1});

g = accélération due à la gravité; (m s^{-2});

ρ_s = densité des solides des boues, assumant que ces boues sont des sphères; (kg L^{-1}); ρ_L= densité des fluides (kg L^{-1});

et, η = viscosité de la suspension (mPa s^{-1}).

D'autres études doivent être effectuées pour établir une véritable corrélation entre la fermentation et la rhéologie (viscosité et taille de particule) de la formulation en considérant l'étape de la récolte. L'étude de la rhéologie de la fermentation (viscosité, taille des particules et densité) facilitera le choix des paramètres opérationnels pour la récolte (force centrifuge, débit, temps, pH, la température, temps de séchage, utilisation des agents de protection).

Ainsi, le développement de la formulation est fonction des propriétés de la suspension qui dépendent elles-mêmes de la rhéologie, en particulier de la viscosité et de la taille des particules. Les propriétés rhéologiques ont aussi un impact sur l'addition de différents adjuvants (agents mouillants, agents collants et autres agents de dispersion) pour la formulation et surtout, des techniques d'homogénéisation pour les différentes formulations.

Hwan Do *et al.* (2001) ont constaté qu'une bonne connaissance des changements rhéologiques pendant la fermentation facilite la récupération de l'acide polyglutamique (APG) du bouillon fermenté. De plus, Burges et Daoust (1998) ont rapporté que les conditions de formulation doivent dépendre des procédés de fermentation. Ceci est important, car les changements de la taille de particules et de la viscosité influencent la taille des gouttelettes dans les formulations (Chapple *et al.*, 2000) ainsi que la rétention et l'adhérence sur le feuillage (Lisansky *et al.*, 1993) tel que montré à la figure 3. Ainsi, la réduction de la taille des particules observée pendant la fermentation des boues par Bt (dans les fermenteurs) permettrait possiblement d'avoir la taille de particules désirée pour les formulations. Ceci permettra de trouver un compromis à l'étape de fermentation entre le rendement et la consistance physique de produit, pour alors atténuer les coûts de l'homogénéisation. Par conséquent, les données sur la rhéologie de fermentation pourraient servir de base pour la conception des bioréacteurs (transfert de masse de l'oxygène), la mise en échelle et la conception des procédés en aval de séparation et de purification, et pour prendre des décisions quant aux choix des additifs (agents tensio-actifs, dispersants, adhérents, épaississants et d'autres) lors du développement de la

formulation. De plus, des difficultés peuvent survenir lors de l'application sur le terrain en relation avec la rhéologie (ex. : la décantation dans les réservoirs est influencée par la taille des particules) d'où la nécessité d'améliorer les caractéristiques rhéologiques pour éviter le problème de mélange ou d'obstruction (qui dépend de la viscosité) tel que discuté par Mor et Matthews (2003). Dans ce contexte, Bateman (1998) a clairement rapporté que l'uniformité de la taille de gouttelettes (qui dépend de la rhéologie de la formulation) joue un rôle critique dans les applications des produits de Bt.

3.4. Stratégies pour l'amélioration des propriétés rhéologiques (préparation du substrat)

Le niveau de viscosité des boues peut être ajusté par des moyens physiques ou chimiques. Par exemple, l'addition d'agents tensio-actifs, qui ont été utilisés pour améliorer l'assimilation du substrat dans les milieux complexes (gruaux, farines de poisson...), et la croissance bactérienne (Zouari et Jaoua, 1999; Zouari *et al.*, 2002) permettent de réduire la viscosité. Il en est de même pour l'hydrolyse thermique des boues d'épuration (165–180 °C) qui, en brisant les molécules de glucides et de protéines (Haug *et al.*, 1983; Hiraoka *et al.*, 1985), réduit la viscosité. De même, le prétraitement thermo-chimique (à pH acide et alcalin) a été utilisé pour la solubilisation de la partie volatile des matières en suspension (MES) et la demande chimique en oxygène (DCO insoluble) (Neyens *et al.*, 2003a; Zhu et Chen, 2005). En fait, l'ajout de Tween 80 et l'hydrolyse préalable des substrats modifient la rhéologie, ce qui influence éventuellement tous les autres aspects de la production de biopesticides à base de Btk, à savoir : la fermentation, la récolte, la formulation et l'application, étapes qui sont toutes interdépendantes (figure 3).

Le traitement alcalin est une méthode rude où les valeurs du pH du milieu sont très élevées. Les concentrations alcalines élevées provoquent, entre autres, une rupture des cellules et une dégradation des débris cellulaires dans les boues d'épuration, ce qui entraîne la libération du matériel intracellulaire et des polymères (protéines, ARN, ADN, glucides) dans le milieu (Erdincler et Vesilind, 2000; Neyens *et al.*, 2003b). L'hydrolyse est souvent utilisée pour traiter la biomasse lignocellulosique (Pandey et Soccol, 2000; Mantzavinos et Psillakis, 2004) ou pour améliorer les procédés biologiques comme la digestion anaérobie des boues d'épuration (Chiu *et al.*, 1997; Mata-Alvarez et Llabrés, 2000). En fait, les boues hydrolysées n'ont jamais été explorées du point de vue

de la valorisation[7] sauf dans les études de Barnabé (2005). Cependant, dans cette recherche les études rhéologiques sont absentes.

Des détails sur les différents prétraitements des boues d'épuration sont donnés dans le tableau 2. La plupart de ces méthodes peuvent avoir un impact important sur la rhéologie des boues. Cependant, il n'y a pas d'études approfondies sur le changement de la rhéologie (viscosité et taille des particules) des boues ayant subies une hydrolyse, spécialement dans un contexte de production à valeur ajoutée.

4. Présentation du corps de la thèse

4.1. Objectifs et hypothèses scientifiques

4.1.1. Objectifs poursuivis dans les travaux présentés dans le corps de la thèse

En se basant sur les références scientifiques publiées à ce sujet, nous avons établi les objectifs suivants pour nos travaux de recherche et développement :

1. L'étude de la rhéologie des eaux usées et des boues d'épuration des usines de traitement des eaux, un paramètre vital qui n'a jamais fait l'objet d'une étude scientifique exhaustive dans une perspective de recyclage de ces résidus en produits à haute valeur ajoutée. Cette étude a été réalisée pour différentes étapes de l'obtention de Btk, en particulier, la préparation du substrat, la récolte et la fermentation, le tout ayant ultimement des répercussions sur l'épandage du biopesticide.
2. Le type de récolte du Btk est une étape très importante entre la fermentation et la formulation, l'optimisation des divers paramètres de centrifugation faisant donc l'objet d'une étude approfondie. Par ailleurs, la présence de chitinases produites par Btk, des enzymes ayant un rôle synergique élevé sur le potentiel insecticide du bacille, a aussi été évaluée dans les eaux usées et les boues d'épuration fermentées par la bactérie en question, tout ceci dans le but d'accroître l'entomotoxicité des préparations obtenues.
3. Des formulations de Btk à base d'eaux usées et de boues d'épuration fermentées ont été développées en tenant compte de la nécessité des expériences d'évaluations de divers paramètres, du développement de préparations stables, en tenant compte d'études préliminaires sur les

[7] Odegaard *et al.* (2002) rapportent une étude qui suggère la possibilité d'intégrer l'hydrolyse des boues dans la chaîne de traitement des eaux usées municipales pour produire des biofertilisants, de l'énergie et des sources d'éléments nutritifs (carbone, azote et phosphore).

Tableau 2. Différents prétraitements des boues d'épuration

Type de prétraitement	Agents utilisés	Mécanisme	Objectif (s)	Problèmes	Références
Acide	HCl, H_2SO_4	Dégradation des glucides, de l'acide carboxylique et des groupements ester; solubilisation du phosphore, Fe, Zn et d'autres métaux.	Digestion anaérobie des boues d'épuration et production de biofertilisants.	Corrosion des équipements; habituellement couplé à d'autres prétraitements .	Solomons, 1997; Aravinthan *et al.*, 2001; Ben Rebah; 2001
Alcalin	NaOH; KOH; $Mg(OH)_2$	Dégradation de matériel lignocellulosique, des protéines et polyamines; solubilisation des métaux comme le Mo et le Cu; favorise le procédé de désorption.	Digestion anaérobie des boues d'épuration et production de biogaz; bioconversion des boues en biofertilisants; dénitrification des boues d'épuration.	Corrosion des équipements.	McBride, 1998; Penaud *et al.*, 1999;

Tableau 2. Différents prétraitements des boues d'épuration

Type de prétraitement	Agents utilisés	Mécanisme	Objectif (s)	Problèmes	Références
Thermique	Différentes températures s'étendant de 121-200 °C dans des fours à micro-ondes ou des cuves pressurisées.	Hydrolyse et oxydation partielle des molécules; rupture prononcée des cellules mortes ou vivantes; augmente la surface des particules et améliore la biodégradabilité; la biodisponibilité du Mn et du Cu est augmentée et plusieurs des métaux sont oxydés.	Déshydratation des boues d'épuration; digestion anaérobie des boues et production de biogaz; solubilisation des boues d'épuration.	Les températures près de 200 °C peuvent causer des réactions de Maillard (les formes réduites des glucides réagissent avec des acides aminés pour générer mélanoïdes, qui sont difficiles à dégrader et parfois inhibiteur).	Hasagawa, 2000; Kepp *et al.*, 2000; Delgènes *et al.*, 2000; Schieder *et al.*, 2000; Müller, 2000; Shanableh et Jomaa, 2001; Zorpas *et al.* 2001; Obrador *et al.*, 2001; Neyens et Baeyens, 2003
Thermique-alcalin	NaOH; KOH; $Mg(OH)_2$; $Ca(OH)_2$ en combination avec une température élevée de 50 à 170 °C	Combinaison des effets des prétraitements thermiques et alcalins; spécialement efficace pour dégrader le matériel lignocellulosique.	Solubilisation des boues d'épuration; digestion anaérobie des boues et production de biogaz.	Problèmes de corrosion, mais moins importants qu'un prétraitement thermique à pH acide.	Novelli *et al.*, 1995; Tanaka *et al.*, 1997, 2002; Penaud *et al.*, 1999; Delgènes *et al.*, 2000; Neyens et Baeyens 2003; Neyens *et al.*, 2003b; Vlyssides et Karlis, 2004
Thermique-acide	H_2SO_4 et températures élevées de 121-160 °C	Combinaison des effets des prétraitements thermiques et acides	Dénitrification et déshydratation des boues d'épuration; digestion aérobie des boues; conditionnement.	Problèmes de corrosion.	Karlsson et Göransson,1993; Neyens *et al.*, 2003a
Oxydation partielle ou	Fe^{2+}/ H_2O_2; Fe^{3+}/ H_2O_2;	Dégradation des composés organiques	Digestion anaérobie des boues d'épuration et	Technologie coûteuse; certains produits organiques facilement	Kim et Huh, 1997; Jeworski et Heinzle,

Tableau 2. Différents prétraitements des boues d'épuration

Type de prétraitement	Agents utilisés	Mécanisme	Objectif (s)	Problèmes	Références
totale	O_2; O_3	réfractaires divers; oxydation complète ou partielle selon les conditions de réaction.	production des biogaz; augmentation de biodégradabilité des boues pour d'autres traitements; déshydratation des boues.	biodégradables peuvent également être dégradés en composés réfractaires.	2000; Kim *et al.*, 2000; Weemaes *et al.*, 2000; Ahn *et al.*,2002; Anderson *et al.*,2002; Camacho *et al.*, 2002; Neyens *et al.*, 2003c;

effets des UV, ce qui reflétera leur stabilité sur le terrain. Toutefois, étant donné leur durée dans le temps, ces études n'ont pas été aussi exhaustives que prévu.

4. L'obtention de Btk, en utilisant des eaux usées et des boues d'épuration comme substrat a été évaluée du point de vue économique. Dans ce contexte, pour définir clairement la rentabilité d'employer ces substrats, une analyze technico-économique a été réalisée en se basant sur les diverses étapes de la production de Btk, soit : l'accès aux substrats, la fermentation, la récolte et la formulation.

En somme, si les objectifs sont atteints, cette recherche permettra au procédé Bt-INRS de se déployer dans sa totalité – procédés en amont (procuration du substrat/prétraitement et fermentation) et procédés en aval (récolte et développement de formulation). De plus, les formulations stables pourront être testées pour évaluer la faisabilité de leur application. Ce test permettra ensuite de développer une stratégie pour le développement de formulation qui pourra être utilisée comme modèle pour la formulation d'autres produits biologiques. Aucune étude n'a été rapportée dans la littérature où le bouillon entier, obtenu à partir de la fermentation de Bt, est traité jusqu'à l'étape de formulation. Finalement, ce travail fera la lumière sur la faisabilité technique et économique du développement de biopesticides de Bt basés sur des eaux usées et des boues d'épuration qui n'ont pas les mêmes caractéristiques que les biopesticides issus de milieux conventionnels.

4.1.2. Hypothèses scientifiques à la base des recherches

En considérant la littérature disponible et pour compléter les objectifs scientifiques et techniques en rapport avec le procédé Bt-INRS, les hypothèses suivantes sont posées :

1. L'étude des paramètres rhéologiques pendant la fermentation, pouvant affecter le transfert de masse de l'oxygène et donc, l'entomotoxicité de Bt, facilitera le traitement en aval du biopesticide pour le développement de formulations stables. À ce titre, la rhéologie joue un rôle critique dans la relation entre les formulations de Bt et les équipements de pulvérisation. C'est pourquoi, toute amélioration physique et/ou chimique des milieux fermentés par Btk peut améliorer la rhéologie, et donc la fermentation des eaux usées et des boues d'épuration.
2. Les procédé conventionnels de récolte, comme la centrifugation sans additif favorisant la filtration, peuvent être utilisés avec succès pour la récolte des bouillons fermentés de

Bt, en raison de la présence de flocs qui peuvent agir comme des adsorbants naturels et aider à la filtration.

3. Les principales formulations de Bt sont des suspensions aqueuses sans danger pour l'environnement et possédant une viscosité qui augmentent l'efficacité de leur application (d'autres formulations, par exemple à base d'huiles, sont connues pour présenter quelques problèmes pour la santé humaine). Il est donc souhaitable de produire des formulations aqueuses à partir de boues d'épuration fermentées de Bt. Divers adjuvants ou ingrédients inertes ont été largement explorés pour le développement de formulations de biopesticides à base de Bt obtenus à partir de milieux synthétiques. Il est possible d'extrapoler les mêmes types d'adjuvants pour la stabilisation des formulations à base de boues fermentées par Bt.
4. Finalement, les biopesticides à base de boues fermentées peuvent posséder des propriétés inhérentes leur conférant un avantage sur la formulation par rapport aux milieux conventionnels. Une étude technico-économique approfondie peut donc contribuer à évaluer le coût de la production de Btk en utilisant comme substrats alternatifs, des eaux usées et des boues d'épuration.

4.2. Résumé des études présentées dans le corps de la thèse

4.2.1. Rhéologie des eaux usées et des boues d'épuration

4.2.1.1. Étude rhéologique (viscosité; la taille des particules) de la fermentation des eaux usées et des différentes boues d'épuration et de la corrélation entre le changement de la rhéologie et la croissance de Bt ayant un effet de cascade sur les procédés en aval : a) petite échelle (fioles); b) échelle de bioréacteur (15L).

La gestion des boues d'épuration a subi une série de changements à la suite de réglementations sévères. Ces changements ont fait passer la gestion des boues de l'élimination vers la production à valeur ajoutée qui gagne en popularité. Cette transformation a été présentée en détails dans la revue de littérature présentée au chapitre 2.

Bien que des études détaillées sur l'optimisation du procédé Bt-INRS aient été effectuées, la rhéologie n'a pas été étudiée pendant la fermentation. C'est pourquoi des études approfondies de viscosité et de taille des particules ont été réalisées sur les boues brutes, les boues pré-traitées (stérilisation et hydrolyse thermo-alcaline) et les boues fermentées de Bt à différentes concentrations en solides (10–40 g/L). Les boues, indépendamment du type,

étaient pseudoplastiques et thixotropiques. Ce comportement non Newtonien est un effet combiné de la viscosité et de la taille des particules, lesquelles sont ensuite modifiées par les procédés de traitement (stérilisation, hydrolyse et fermentation) (chapitre 3, partie I). Les boues hydrolysées fermentées suivent des lois exponentielles et de puissance en comparaison avec les boues brutes fermentées (chapitre 3, partie II). La distribution volumétrique de la taille des particules a montré un plus grand nombre de particules pour les boues hydrolysées fermentées dans la région de 0,2-12 µm par rapport aux boues non-hydrolysées fermentées de Bt où les particules sont concentrées dans la région de 0,5 à 10 µm, indépendamment de la concentration en solides (chapitre 3, partie III). En fait, une corrélation directe entre la taille des particules et l'entomotoxicité a été observée (chapitre 3, partie III). La plus faible taille de particules dans le cas des boues hydrolysées et fermentées a permis un meilleur transfert de l'oxygène et a donné une formulation adéquate pour les équipements de pulvérisation (figures 1,2,3; chapitre 3, partie III). D'ailleurs, la fermentation des boues primaires, mélangées et secondaires (brutes et hydrolysées) et des eaux usées de l'industrie de l'amidon ont également confirmé le modèle pseudoplastique respectant la loi de puissance ou un plus grand cisaillement pour les boues primaires (brutes et hydrolysées). La rhéologie a également présenté une excellente corrélation avec la morphologie (chapitre 3, partie IV). Les études en fermenteur ont montré que les boues secondaires et les eaux usées de l'industrie de l'amidon peuvent être utilisées comme milieux de culture alternatifs pour la production de biopesticides à base de Bt.

4.2.1.2. Addition d'agent tensio-actif (Tween-80) pendant la fermentation de Bt comme un modificateur physique de la rhéologie des boues d'épuration et son effet probable sur les formulations.

Tel que déjà mentionné, la rhéologie des milieux complexes comme les boues d'épuration peut être améliorée par l'addition de surfactants comme le Tween-80. Ainsi, le Tween 80 (0.2 % v/v) a été ajouté lors de la fermentation par Bt des boues non hydrolysées et hydrolysées à une concentration en solides totaux de 25 g/L. Avec les boues non hydrolysées, l'usage de Tween 80 a entraîné une augmentation de l'entomotoxicité (Tx) de 26,6 %. (chapitre 4, partie I). Cependant, il n'y a aucune augmentation apparente de Tx après la fermentation de boues hydrolysées contenant du Tween 80 alors qu'une formation d'intense de mousse a été notée. Ainsi, l'amendement des boues non hydrolysées avec du Tween 80 a permis d'atteindre trois objectifs : a) améliorer la rhéologie et augmenter le taux de

consommation de l'oxygène pendant la fermentation pour augmenter Tx; b) faciliter la détermination de Tx pendant la centrifugation (récolte) des boues; et c) augmenter la mouillabilité dans les formulations sans avoir ajouté des adjuvants, réduisant ainsi les coûts de formulation.

D'ailleurs, l'addition du Tween 80 dans les boues non-hydrolysées avec une concentration élevée en solides (30 g/L) entraîne également une consommation élevée de l'oxygène, ce qui résulte en une augmentation de Tx de 29,7 % (chapitre 4, partie II). Cependant, les différentes enzymes (protéase et chitinase) ont suivi les profils différents en présence du Tween 80.

4.2.2. Optimisation des procédés de récupération d'entomotoxicité

4.2.2.1. Optimisation de pH, la température et les conditions de centrifugation pour la récolte des boues fermentées par Bt.

L'étape de la récolte constitue un lien important entre les formulations et la fermentation et a aussi un impact sur l'application. Ainsi, l'étude de la centrifugation a été effectuée sur des bouillons fermentés des eaux usées de l'industrie de l'amidon, des boues d'épuration (brutes et hydrolysées) et du milieu semi-synthétique à base de soya pour augmenter l'entomotoxicité (Tx) (chapitre 5, partie I). Divers facteurs influençant Tx ont été étudiés : la concentration en solides, le pH, la température et la force centrifuge. L'efficacité de la centrifugation (récupération de Tx) est plus élevée à pH 4 et à une température de 20°C pour tous les bouillons. La récupération de Tx est également plus élevée pour les eaux usées de l'industrie de l'amidon et les boues d'épuration avec une force centrifuge typique de 9000 x g utilisée en industrie. La vélocité de la sédimentation, calculée avec les données, est un paramètre important pour les calculs du facteur Sigma lors de la mise en échelle de la centrifugation. De plus, pour une récupération de Tx donnée, les courbes de la simulation de puissance ont montré que les exigences de puissance étaient plus élevées pour le milieu semi-synthétique à base de soya que pour les milieux alternatifs.

4.2.2.2. La présence de l'enzyme chitinase dans les eaux usées et les boues fermentées par Bt et la caractérisation.

L'entomotoxicité est considérée comme la somme des cristaux protéiques, des spores et divers facteurs de virulence tels que les protéases, les chitinases, les phospholipases, les

protéines insecticides végétatives, et probablement les autres facteurs inconnus qui agissent en synergie. La chitinase est un facteur de virulence important qui agit en synergie avec des spores et des cristaux protéiques en clivant la chitine qui est une composante de la membrane péritrophique présente dans l'intestin. De plus, il est possible que la chitinase soit produite de façon induite et/ou constitutive dans les milieux alternatifs à base d'eaux usées ou de boues, ce qui ne nécessiterait pas d'ajout externe de cette enzyme dans les formulations. L'étude effectuée dans ce contexte a montré la présence des chitinases dans les boues (brutes et hydrolysées) et les eaux usées de l'industrie de l'amidon, mais pas dans le milieu semi-synthétique à base de soya (contrôle) (chapitre 5, partie II). Des bioessais réalisées avec l'ajout de chitinases ont montré une augmentation de l'entomotoxicité, confirmant ainsi le rôle de synergie des chitinases de Bt. En somme, cette étude a démontré la production in situ de chitinases durant la fermentation de Bt et la possibilité de ne pas ajouter cette enzyme dans les formulations.

4.2.3. Le développement des formulations liquides stables d'eaux usées et de boues fermentées par Bt.

La commercialisation et l'usage des biopesticides à base de Bt sont tributaires des coûts de production et de formulation. De plus, l'efficacité de la récolte détermine les traitements en aval et les besoins pour la formulation. La littérature abonde sur ce sujet et elle a été passée en revue au chapitre 6, partie I. De même, les protéases de Bt, comme facteurs de virulence, retiennent l'attention des chercheurs sur Bt alors que leur synergisme ou leur antagonisme ne sont toujours pas élucidés. Ainsi, une revue complète, sur le rôle probable des protéases de Bt dans l'augmentation ou de la diminution de l'entomotoxicité, a été effectuée (chapitre 6, partie II).

4.2.3.1. Essais des différents adjuvants/ingrédients inertes – agents de suspension, phagostimulants, agents anti-microbiens, agents de protection contre les rayons UV et adhérents (l'effet de la pluie) pour le développement de formulation.

Avant de commencer l'étape importante du développement de formulations stables de Bt, il est nécessaire d'étudier les différents adjuvants utilisés comme ingrédients inertes. Par conséquent, l'essai de divers adjuvants, à savoir : les agents de suspension (20 % p/v, meilleures suspensions pendant le stockage et application par pulvérisation), les phagostimulants (0.5 % p/v, stimulant de l'alimentation); les adhésifs (0,2 % p/v, pour

contrer l'effet de la pluie), les agents anti-microbiens (0,5 % p/v, inhibiteurs de la contamination microbienne) et les agents protecteurs contre des rayons UV (0,2 % p/v), a été effectué (chapitre 6, partie III). La meilleure combinaison d'adjuvants pour chacune des caractéristiques de la formulation est : le sorbitol, le monophosphate de sodium et le métabisulfite de sodium pour les agents de suspension; la mélasse et la farine de soya pour les phagostimulants; la mélasse et la poudre de lait écrémé pour les agents adhésifs pour les adhérents; les acides sorbiques et propioniques pour les agents anti-microbiens; et le lignosulphate de sodium, la mélasse et le rouge Congo pour les agents protecteurs contre les rayons UV.

4.2.3.2. Évaluation de la performance de divers types de formulations à différents pH et températures pour les boues secondaires (non-hydrolysées et hydrolysées), les eaux usées de l'industrie de l'amidon et le milieu à base de soya.

Des formulations liquides stables ont été développées pour différents milieux fermentés par Bt. Le potentiel de suspension a été analysé, car c'est un paramètre important pour les formulations liquides. Différents agents de suspension, à savoir : le monophosphate de sodium, le metabisulfite de sodium et le sorbitol, ont été étudiés en combinaison avec des adjuvants de base. Le mélange contenant 9 % de sorbitol, 7 % de monophosphate de sodium et 5 % de metabisulfite de sodium, a donné les meilleures caractéristiques physiques et biologiques pour la stabilité de la formulation des boues non hydrolysées (chapitre 6, partie IV). De même, le mélange de sorbitol, de monophosphate de sodium et de metabisulfite de sodium dans un rapport de 2,2:1:1 a donné la meilleure combinaison pour la formulation des boues hydrolysées fermentées (chapitre 6, partie V). Finalement, le mélange de monophosphate de sodium et de sorbitol dans un rapport de 3:1 a donné la meilleure combinaison pour les formulations des eaux usées d'amidon et du milieu à base de soya fermentés (chapitre 6, partie VI). Cependant, indépendamment du type de milieux fermentés, les formulations (stables et autres) se détérioraient à pH 6, 6,5 et entre 40 et 50 °C. Les formulations étaient stables à pH 4 –5 et entre 4 –30 °C. Les formulations à base de boues hydrolysées fermentées avaient une durée de vie plus élevée (2 ans), ce qui démontre l'intérêt d'utiliser des matières premières alternatives pour la fermentation de Bt et les formulations subséquentes.

4.2.3.3. Évaluation de l'effet d'UV sur les milieux fermentés et formulés – détermination de la demi-vie.

Les formulations de Bt subissent une perte d'entomotoxicité résiduelle au contact des rayons UV sur le terrain (Cohen et al. 1991). La littérature est abondante au sujet de l'ajout d'agents protecteurs contre les rayons UV. Cependant, l'addition de ces agents et/ou la modification de la matrice de formulation sont des méthodes très coûteuses. Ce coût pourrait être diminué si le milieu de fermentation possédait des caractéristiques inhérentes capables d'offrir une résistance contre les rayons UV. Les milieux fermentés par Bt (eaux usées d'amidon, boues non hydrolysées et hydrolysées et milieu à base de soya) ainsi que les formulations de Bt (avec et sans protecteurs contre les rayons UV) ont été donc étudiés (chapitre 6, partie III). Les observations faites révèlent que les formulations de Bt à base de boues fermentées, une fois exposées au rayonnement UV (avec et sans les protecteurs d'UV), présentent une demi-vie plus élevée que le milieu semi-synthétique à base de soya fermenté et la formulation commerciale de Bt. Les valeurs de demi-vie (en jours) pour les milieux étudiés se présentent dans l'ordre suivant : boues non hydrolysées (11,14) > boues hydrolysées (9,51) > des eaux usées de l'industrie de l'amidon (9,02) > soya (2,8).

4.2.4 Analyse technico-économique du procédé Bt-INRS

La production de Bt à base des eaux usées et de boues d'épuration permet à la fois d'obtenir des biopesticides économiques et de gérer les déchets de façon durable et économique. Ainsi, l'analyse technico-économique préliminaire du procédé de production de Bt est nécessaire et implique les étapes principales : fermentation; récolte (centrifugation et ultrafiltration) et formulation (chapitre 7). L'étude réalisée dans le présent projet comporte cinq principaux scénarios pour différents substrats de croissance : la formulation liquide à la suite de la récupération du bouillon fermenté par centrifugation; la formulation liquide après la récupération du bouillon fermenté par centrifugation et ultrafiltration; les formulations sèches à la suite de la récupération du bouillon fermenté par centrifugation; les formulations sèches après récupération du bouillon fermenté par centrifugation et ultrafiltration et les formulations liquides où la mélasse a été ajoutée à l'étape de la formulation en tant que phagostimulant (pour augmenter le potentiel entomotoxique de 13 %). Ces scénarios sont présentés au tableau 2 (chapitre 7). Les estimations des coûts du produit ont été calculées sur la base de l'entomotoxicité nette obtenue après que le développement de formulation ait été évalué en effectuant le bilan de masse pour tous les scénarios de procédé. Les boues

hydrolysées ont donné le plus bas coût de production de 0,228 $ Can/milliard unité internationale (MUI) avec l'ajout de mélasse par rapport à d'autres scénarios de procédé. Pour les matières premières, les coûts de production varient de 0,256 à 0,407 $ Can /MUI. La capacité de production de l'usine de Bt a eu un grand impact sur les frais totaux d'opération ($ Can/MUI), et c'est pourquoi une capacité de 3 x 10^7 MUI/an présentait un coût de 30-45 % de celui d'une usine de 7,5 x 10^6 MUI/an.

5. Discussion

5.1. L'effet des propriétés rhéologiques sur la performance du procédé de production de Btk est peu étudié dans la littérature

Le développement des biopesticides à base de Btk nécessite des études très élaborées sur la préparation du substrat, la fermentation, la récolte et la formulation. À cette fin, des études complètes ont été effectuées à l'INRS sur le contenu en nutriments, la concentration de solides, l'isolement et l'identification de nouvelles souches de Bt, l'augmentation de l'entomotoxicité des boues par des prétraitements physico-chimiques et la mise en échelle.

Cependant, peu ou pas d'études ont été réalisées sur la récolte et la formulation. De plus, la littérature scientifique ne contient que peu d'informations relatives à la récolte de Btk et au développement des formulations. Comme il fallait s'y attendre, ces informations sont intéressantes commercialement et sont ainsi à caractère confidentiel.

Pour que le procédé Bt-INRS puisse s'imposer sur le marché, la formulation doit être explorée en relation avec l'application sur le terrain et la commercialisation. Par exemple, les boues d'épuration sont généralement, des fluides non Newtoniens et les propriétés rhéologiques influencent les étapes principales de l'obtention de biopesticides, soit : la préparation du substrat, la fermentation, la récolte, la formulation et l'application sur le terrain. Les propriétés rhéologiques affectent aussi le mélange du milieu de fermentation et le transfert de masse entre les phases solides, liquides et gazeuses. De plus, l'étude de l'effet de la rhéologie des boues sur la formulation et sur l'application de ces formulations, peut jouer un rôle important dans la performance globale des biopesticides à base de Bt.

La revue de la littérature fait ressortir plusieurs aspects touchant la rhéologie des suspensions de biopesticides où il existe des déficits de connaissances :

1. L'origine du substrat (par exemple, boues primaires, secondaires ou mélangées) pourrait également affecter la rhéologie du milieu de fermentation. D'ailleurs, les étapes de prétraitements – la stérilisation et l'hydrolyse - peuvent changer de manière significative les propriétés rhéologiques avec des répercussions sur les étapes suivantes de production de Bt. Donc, les propriétés rhéologiques du substrat brut doivent être étudiées pour faciliter la production efficace du Bt.

2. Le procédé Bt-INRS cumule plus de 10 ans d'études sur divers aspects dont : l'isolement et l'identification des nouvelles souches de Bt issues des boues d'épuration, l'optimisation de procédé (inoculum, concentration en solides, étude de transfert de l'oxygène), le prétraitement pour améliorer l'assimilation des nutriments par Bt, des essais en fermenteur et la mise en échelle avec différentes stratégies de fermentation (ex. : culture en mode « batch » et « fed-batch »). Cependant, il existe un manque d'informations sur la rhéologie de la fermentation, laquelle peut limiter le transfert de masse et la mise à l'échelle du procédé Bt-INRS.

3. Il a déjà été démontré dans les recherches antérieures que l'addition de Tween-80 (études en fioles) et l'hydrolyse thermo-chimique (études en fioles et en bioréacteurs) augmentent l'assimilation des nutriments et améliorent subséquemment l'entomotoxicité des biopesticides. Cependant, les deux procédés (hydrolyse et ajout de Tween-80) n'ont pas toujours été combinés pour améliorer la rhéologie des boues et augmenter la production de Bt en termes de spores et d'entomotoxicité. Les boues d'épuration constituent une source riche de nutriments présents sous forme de matières en suspension. Afin d'utiliser au maximum le plein potentiel des boues, il est nécessaire d'accroître la biodisponibilité de ces nutriments en utilisant différents procédés de prétraitement physiques, thermiques ou chimiques, individuellement ou en combinaison, avec une attention particulière sur les propriétés rhéologiques.

4. L'entomotoxicité à partir des milieux fermentés est sensible à la taille des particules et à la viscosité des bouillons fermentés. Ainsi, les méthodes utilisées pour récolter les produits microbiens de Bt dans les eaux usées ou les boues fermentées peuvent entraîner des pertes en entomotoxicité.

5. Enfin, des études de différents adjuvants et des tests sur la durée de conservation doivent être réalisés pour développer des formulations stables qui sont nécessaires lors de l'application des biopesticides de Bt sur le terrain. Ces formulations dépendent en bonne

partie des propriétés rhéologiques des suspensions aqueuses d'insecticides. À ce sujet, la littérature contient de nombreuses informations sur des formulations à base de concentrés primaires et non à base de bouillons fermentés, ce qui augmente le défi d'optimiser toutes les étapes décrites précédemment.

5.2. Importance des études rhéologiques pour la production de suspensions biopesticides

Les études rhéologiques réalisées dans le cadre de l'amélioration du procédé Bt-INRS ont permis une meilleure intégration de l'ensemble des étapes de la production des suspensions entomotoxiques.

De plus, les résultats obtenus ont des répercussions notables sur les recherches qui seront réalisées afin d'amener le procédé dans une configuration acceptable pour la production commerciale de Btk :

a) Il a été confirmé que la production de spores et des cristaux protéiques de Btk varient selon les types d'eaux usées et de boues en raison non seulement de leur teneur en nutriments, mais aussi à cause de l'état physique du substrat. Ceci montre qu'il est essentiel de bien comprendre les propriétés rhéologiques des bouillons de fermentation. Ainsi, par exemple, l'amélioration du comportement rhéologique des boues hydrolysées par rapport aux boues non hydrolysées, à l'étape de la formulation finale, a démontré l'intérêt de bien connaître le rôle des propriétés rhéologiques sur l'ensemble du procédé de production.

b) La récolte de l'entomotoxicité de Btk par centrifugation et l'optimisation des divers paramètres contribuent notablement à la mise à l'échelle du procédé de production de Btk, et ainsi à son éventuelle commercialisation. Par exemple, la récolte des bouillons fermentés de Bt devant se faire de façon efficace et économique, il est possible d'éviter l'étape de la filtration.

c) Le développement de formulations aqueuses stables suscitera l'intérêt des producteurs et des utilisateurs de biopesticides, alors que cette stabilité est en partie affectée par leurs propriétés rhéologiques. Il faut dire que la demi-vie des suspensions de Btk, obtenues en utilisant les milieux alternatifs, est de une et demie à deux années, ce qui augure bien de leur compétitivité aussi bien que de leur stabilité, en dépit de leurs

caractéristiques hautement non-Newtonien observées à toutes les étapes du procédé de production. Ces études permettent aussi de prévoir les concentrations des adjuvants multifonctionnels pouvant être utilisés pour stabiliser les suspensions entomotoxiques et leur conférer les propriétés requises pour en faire un produit compétitif sur le marché.

d) L'analyse technico-économique du procédé de production de Bt, intégrant un traitement amélioré en aval et de nouvelles connaissances sur la formulation, améliore considérablement la fiabilité des études de faisabilité sur la production à l'échelle industrielle et la commercialisation. L'analyse contribuera à établir la stratégie de production de Btk la plus appropriée en se basant sur l'entomotoxicité finale après formulation et le potentiel insecticide des milieux fermentés par Btk. L'addition d'eaux usées et de boues d'épuration aux milieux de production de Btk apparaît clairement comme une option rentable. En effet, le coût d'investissement et d'opération pour produire des biopesticides à base de Btk, à partir des boues d'épuration, se compare avantageusement à l'utilisation de farine de soya.

5.3. Amélioration de la stabilité des suspensions biopesticides

Le développement des biopesticides de Bt, à partir d'eaux usées et de boues d'épuration (procédé Bt-INRS), a fait l'objet de plusieurs recherches sur : l'isolement et l'identification de nouvelles souches de Bt, l'optimisation des procédés, l'augmentation de l'entomotoxicité par des prétraitements et la mise à l'échelle. Toutefois, il reste encore beaucoup à faire pour augmenter l'entomotoxicité et obtenir finalement une technologie de production qui sera complète sous tous ses aspects.

L'étude rhéologique des milieux, entrant dans la fermentation ou produits par cette dernière, permet de mieux comprendre comment développer des suspensions aqueuses d'insecticides biologiques stables et efficaces. La complexité des boues et leur comportement non-Newtonien constituent cependant un défi de taille pour le développement de formulations stables. L'originalité du présent travail réside dans le développement de connaissances permettant la production de formulations stables de biopesticides en mesure de répondre aux

exigences du marché. Il permettra une meilleure intégration des étapes de production, de récolte et de formulation.

6. Principales conclusions et recommandations

6.1. État actuel du procédé et orientation future

Malgré que les pesticides chimiques soient considérés comme des solutions efficaces pour pallier aux pertes agricoles, les biopesticides à base de Bt ont attiré l'attention de la communauté scientifique, des autorités environnementales, de l'industrie et de certains utilisateurs du secteur agricole. Les coûts de production des biopesticides sur le marché demeurent élevés et nuisent à leur diffusion commerciale. Cependant, ces coûts peuvent être considérablement réduits en remplaçant les matières premières semi-synthétiques coûteuses par les eaux usées et les boues d'épuration qui sont des milieux de culture économiques, écologiques et durables. Dans le contexte du procédé Bt-INRS, diverses études ont été réalisées concernant l'isolement de nouvelles souches de Bt, l'optimisation de différents paramètres de fermentation en Erlenmeyers, ainsi que l'accroissement du rendement par l'addition de nutriments après un prétraitement. Cependant, pour que le procédé Bt-INRS soit une technologie intégrée et performante, la formulation doit être explorée en relation avec l'application sur le terrain et les contraintes de commercialisation. Les propriétés rhéologiques des bouillons de fermentation et des produits de la fermentation jouent un rôle important dans la formulation : elles affectent, en effet, toutes les étapes de la production dont, notamment, la préparation du substrat de fermentation, la fermentation elle-même, la récolte des composantes entomotoxiques, la formulation des suspensions des biopesticides commerciales en vue d'en maximiser la stabilité et les propriétés des suspensions requises pour leur application sur le terrain.

6.2. Principales conclusions

Les conclusions spécifiques suivantes sont émises en prenant en compte les résultats obtenus pendant les différentes études dans le cadre de ce projet de recherche (les détails sont présentés au chapitre 8) :

1. Les boues fermentées (primaires, secondaires et mélangées) présentaient des caractéristiques pseudoplastiques et thixotropiques. Les résultats en fioles ont permis de

prévoir le comportement de la viscosité en bioréacteur. La distribution de la taille des particules, de type log-normale bimodale, a été réduite de presque que 50 % à la suite d'une hydrolyse thermo-alcaline. En plus, les boues hydrolysées fermentées ont donné de meilleures suspensions liquides (augmentation de la dispersion) mieux adaptées aux équipements de pulvérisation sur le terrain.

2. Les eaux usées d'amidon et les boues secondaires fermentées ont permis la production de chitinases et l'augmentation de l'entomotoxicité des différents bouillons fermentés (boues non hydrolysées > boues hydrolysées > eaux usées d'amidon) et montré leur effet synergique.

3. La récupération de l'entomotoxicité (Tx) par centrifugation était plus élevée pour les milieux à base d'eaux usées et de boues que pour le milieu semi-synthétique à base de soya et ce, en réduisant l'ajout d'additifs, contribuant ainsi aux réductions des coûts de fabrication.

4. La combinaison recommandée, à la suite de l'essai des adjuvants, est présentée au tableau 3. Les demi-vies, pour les formulations de Bt basées sur des eaux usées/boues d'épuration en contact avec les rayons d'UV, étaient plus élevées que les demi-vies des formulations à base du milieu semi-synthétique. Ceci suggère que les suspensions biopesticides, produites à partir des boues/eaux usées, auront une plus grande efficacité entomotoxique sur le terrain que les suspensions commerciales.

5. Les études technico-économiques ont mis en évidence que les formulations à base de boues hydrolysées offraient la meilleure rentabilité pour les investisseurs.

Tableau 3. Concentration optimale des différents adjuvants pour les diverses formulations.

Type de formulation	Agents de suspension	Phagostimulants† (0,5 % p/v)	Agents collants / Adhérents† (0,2 % p/v)	Agents anti-microbiens† (0,5 % p/v)	Protecteurs UV † (0,2 % p/v)
Boues non hydrolysées	Sorbitol- 9 %; monophosphate de sodium – 7 %; metabisulfite de sodium -5 %	Mélasses; farine de soya; farine de maïs	Mélasses; poudre de lait écrémé	Acide sorbique; acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo
Boues hydrolysées	Sorbitol- 11 %; monophosphate de sodium – 5 %; metabisulfite de sodium -5 %	Mélasses	Mélasses	Acide sorbique; acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo
Eaux usées d'amidon	Sorbitol- 15 %; monophosphate de sodium – 5 %	Farine de soya	Mélasses	Acide sorbique; acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo; acide p-Amino benzoïque
Soya (milieu semi-synthétique)	Sorbitol- 15 %; monophosphate de sodium – 5 %	Avoine; farine de soya	Poudre de lait écrémé	Acide sorbique; methyl para benzoate; acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo

† Ces adjuvants ont été seulement testés à des concentrations données.

6.3. Principales recommandations

Compte tenu des résultats obtenus au cours de ces travaux de recherche et développement au niveau du doctorat, il est possible de faire les recommandations suivantes (les détails sont présentés au chapitre 8) :

1. L'addition de Tween-80 aux eaux usées d'amidon permettrait d'augmenter la récupération des spores pendant la centrifugation. De plus, la fermentation en mode <<fed-batch>> et l'addition de chitine pourraient être testées pour évaluer leur contribution à l'augmentation de l'entomotoxicité aux préparations de Btk obtenues en employant des eaux usées d'amidon comme matières premières.
2. Des études doivent être effectuées d'une part, pour vérifier la présence d'autres facteurs de virulence, à savoir : les phospholipases, les cytolysines et les protéines insecticides

végétatives et, d'autre part, pour comprendre l'action synergique de ces facteurs de virulence afin de maximiser l'entomotoxicité des Btk obtenus lors de la fermentation.

3. Des études sur le terrain, avec les formulations stables développées au cours des présentes recherches, devraient être effectuées pour en établir l'efficacité réelle sous différentes conditions environnementales de pluie, de rosée, de vent, de rayonnement UV et de feuillage.

References

ADAMS, T.T., M.A. Eiteman et M.J. Adang. 1999. *Bacillus thuringiensis* subsp, *kurstaki* spore production in batch culture using broiler litter extracts as complex media. Bioresource Technology, 67, 83-87.

AHN, K.-H., K.Y. Park, S.K. Maeng, J.H. Hwang, J.W. Lee, K.-G. Song et S. Choi. 2002. Ozonation of wastewater sludge for reduction and recycling. Water Science and Technology, 46(10), p. 71-77.

AIBA, S., A.E. Humphrey et N. Millis. 1973. Biochemical Engineering. Academic Press, London.

ANDERSON, N.J., D.R. Dixon, P.J. Habour et P.J. Scales. 2002. Complete characterisation of thermally treated sludges. Water Science and Technology, 46 (10), p. 51-54.

ARAVINTHAN, V., T. Mino, S. Takizawa, H. Satoh et T. Matsuo. 2001. Sludge hydrolysate as a carbon source for denitrification. Water Science and Technology, 43 (1), p. 191-199.

AVELUIZAPA, L.I.R., R.C. Camarilo, M.I. Guerrero, R.R. Vazquez et J.E. Ibarra. 1999. Selection and characterization of a proteo-chitinolytic strain of *Bacillus thuringiensis* able to grow in shrimp waste media. World Journal of Microbiology and Biotechnology, 15, p. 261-268.

BARNABÉ, S. 2000. Utilisation des boues d'épuration comme substrat pour la production de biopesticides : induction abiotique de la sporulation chez *Bacillus thuringiensis*. Mémoire de maîtrise, Université du Québec, INRS-Eau, Québec, Canada, 157 p.

BARNABÉ, S., A.S. Vidyarthi, R.D. Tyagi et J.R. Valéro. 2001. Methods to improve entomotoxicity of biopesticides based *Bacillus thuringiensis*. Déclaration de savoir-faire soumise le 15-10-01, INRS-Eau, Université du Québec, Québec, Canada, 13 p.

BARNABÉ, S. 2005. Hydrolyse et oxydation partielle des boues d'épuration comme substrat pour produire *Bacillus thuringiensis* HD-1. Ph.D thesis, Institut National de la Recherche Scientifique, Eau, Terre et Environnement, Université du Québec, Québec, Canada, 270 p.

BARNABÉ, S., M. Verma, R.D. Tyagi et J.R. Valéro. 2005. Culture media for increasing biopesticide producing microorganism's entomotoxicity, methods of producing same, biopesticide producing microorganisms so produced and method of using same. US patent International application PCT/CA2005/000235.

BATEMAN, R.P. 1998. Delivery systems and Protocols for Biopesticides. In *Biopesticides: Use and Delivery,* Eds. F.R. Hall & J.J. Menn, Ch. 27., p. 509-528. Humana Press: Totowa, NJ, USA.

BAUDEZ, J.C. et P. Coussot. 2001. Rheology of aging, concentrated, polymeric suspensions: application to pasty sewage sludges. Journal of Rheology, 45, p. 5.

BEN REBAH, F. 2001. Utilisation des boues d'épuration comme milieu de culture pour la production d'inoculants à base de Rhizobium. Thèse de doctorat, INRS-Eau, Université du Québec, Québec, Canada, 239 p.

BERNHARD, K. et R. Utz. 1993. Production of *Bacillus thuringiensis* for industrial and commercial uses. In: Entwistle, P.F., Cory, J.S., Baily, M.J., Higgs, S., editors, *Bacillus thuringiensis*: an environmental biopesticide: theory and practice, p. 255-267. Great Britain, Wiley publications.

BEROVIC, M., T. Koloini, E.S. Olsvik et B. Kristiansen. 1993. Rheological and morphological properties of submerged citric acid fermentation broth in stirred-tank and bubble column reactors. Chemical Engineering Journal, 53, p. B35–B40.

BIGGS, C.A. et P.A. Lant. 2000. Activated sludge flocculation: on-line determination of floc size and the effect of shear. Water Research, 34(9), p. 2542–2550.

BOYCHYN, M., W. Doyle, M. Bulmer, J. More et M. Hoare. 2000. Laboratory scaledown of protein purification processes involving fractional precipitation and centrifugal recovery. Biotechnology Bioengineering, 69(1), p. 1-10.

BRAVO, A., J. Sánchez, T. Kouskoura et N. Crickmore. 2002. N-terminal activation is an essential early step in the mechanism of action of the *Bacillus thuringiensis* Cry1Ac insecticidal toxin. Journal of Biological Chemistry, 277(27), p. 23985-23987.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. and Banerji, S. 2004a. Comparative rheology and particle size analysis of various types of *Bacillus thuringiensis* fermented sludges. Journal of Residuals Science and Technology, 1(4), 231-237.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. and S. Banerji. 2004b. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations. Water, Science and Technology, 50 (9), 229-236.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005a. Sludge based *Bacillus thuringiensis* biopesticides: Viscosity Impacts. Water Research, 39, 3001-3011.

BRAR, S.K.,Verma, M., Barnabé, S., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005b. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges. Process Biochemistry, 40, 2695–2705.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005c. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. Process Biochemistry, 41, 323-342.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005d. Starch industry wastewater based stable *Bacillus thuringiensis* liquid formulations. Journal of Economic Entomology, 98(6), 1890 - 1898.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006a. Value addition of sewage sludge- a clean management strategy. Practice periodical of Hazardous, Toxic and Radioactive Waste Management (submitted).

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006b. *Bacillus thuringiensis* fermentation of primary and mixed sludge - rheology and process performance. ASCE Journal of Environmental Engineering (submitted).

BRAR, S.K., Verma, M., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006c. Particle size variations during production of wastewater sludge based *Bacillus thuringiensis* biopesticides. Practice Periodical of Hazardous, Toxic & Radioactive Waste Management (submitted).

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006d. Tween-80 fortification of *Bacillus thuringiensis* fermented wastewater sludge at higher solids influencing entomotoxicity and enzyme activity. World Journal of Microbiology and Biotechnology (submitted).

BRAR, S.K.,Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006e. Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge. Water Research, 40, 1310-1320.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006f. Presence and characterization of chitinases present in *Bacillus thuringiensis* fermented wastewater and wastewater sludge. Environmental Technology (submitted).

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006g. Screening of different adjuvants for wastewater/wastewater sludge based *Bacillus thuringiensis* formulations. Journal of Economic Entomology, 99(4), 1065-1079.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006h. Rheology of hydrolyzed sludge *Bacillus thuringiensis* fermentation and development of stable liquid formulations. Chemosphere 67, 674-683.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006i.*Bacillus thuringiensis* proteases: production, sporulation and synergism. Process Biochemistry doi:10.1016/j.procbio.2007.01.015.

BRAR, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006j. Analyze technico-économique de procédé de production de *Bacillus thuringiensis*. Report submitted to INRS-ETE, University of Quebec, Quebec, Canada, Research report No. R-892, October 2006.

BURGES, H.D. et R.A. Daoust. 1986. Current status of the use of bacteria as biocontrol agents. In: Fundamental and applied aspects of invertebrate pathology (Edited by R.A. Samson, J.M. Vlak and D. Peters). Society of Invertebrate Pathology, Wageningen. p. 514-517.

BURGES, H.D. (ed.) 1998. *Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments*, Kluwer Academic Publishers, Dordrecht, The Netherlands.

CAMACHO, P., S. Deleris, V. Geaugey, P. Ginestet et E. Paul. 2002. A comparative study between mechanical, thermal and oxidative disintegration techniques of waste activated sludge. Water Science and Technology, 46 (10), p. 79-87.

CAMPBELL, H.W. et P.J. Crescuollo. 1982. The use of rheology for sludge characterization. Water Science and Technology, 14, p. 475-489.

CHANG, L., R. Grant et A. Aronson. 2003. Regulation of the packaging of *Bacillus thuringiensis* δ-endotoxins into inclusions. Applied Environmental Microbiology, 67(11), p. 5032-5036.

CHAPPLE, A.C., R.A. Downer et R.P. Bateman. 2000. Theory and practice of microbial insecticide application. In: *Field Manual of Techniques in Invertebrate Pathology*. Lacey, L.A., Kaya, H.A. (eds.), p. 5-37. Kluwer, Dordrecht.

CHARBONNEAU, H., M. Hébert et A. Jaouich. 2000. Portrait de la valorisation agricole des matières résiduelles fertilisantes au Québec – partie 1 : Aspects Quantitatifs, Vecteur Environnement, 33(6), p. 30-32, 49-51.

CHILTON, R., R. Stainsby et S. Thompson. 1995. The design of sewage sludge pumping systems. Journal of Hydraulic Research, 34, p. 3.

CHIU, Y.-C., C.-N. Chang, J.-G. Lin et S.-J. Huang. 1997. Alkaline and ultrasonic pretreatment of sludge before anaerobic digestion. Water Science and Technology, 36 (11), p. 155-162.

CORDS, H. et R.A. Fisher. 1966. Stable concentrated bacterial insecticide suspensions. US Patent 3,271,243.

DELGÈNES, J.P., V. Penaud, M. Torrijos et R. Moletta. 2000. Investigations on the changes in anaerobic biodegradability and biotoxicity of an industrial microbial biomass induced by a thermochemical pretreatment. Water Science and Technology, 41 (3), p. 137-144.

DENTEL, S.K. 1997. Evaluation and role of rheological properties in sludge management. Water Science and Technology, 36, p. 1–8.

DENTEL, S.K., M.M. Abu-Orf et C.A. Walker. 2000. Optimization of slurry flocculation and dewatering based on electrokinetic and rheological phenomena. Chemical Engineering Journal 80, p. 65–72.

DEVI, V., P.S., T. Ravinder et C. Jaidev. 2005. Barley based medium for the cost-effective production of Bacillus thuringiensis. World Journal of Microbiology and Biotechnology, 21, p. 173-178.

DULMAGE, H.T. 1970. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis var. alesti*. Journal of Invertebrate Pathology, 15, p. 232-239.

DULMAGE, H.T., O.P. Boening, C.S. Rehnborg, et G.D. Hansen. 1971. A proposed standardized bioassay for formulation of *Bacillus thuringiensis* based on the international unit. Journal of Invertebrate Pathology, 18, p. 240-245.

ERDINCLER, A.U. et P.A. Vesilind. 2000. Effect of sludge cell disruption on compactibility of biological sludges. Water Science and Technology, 42 (9), p. 119–126.

HASAGAWA, S., N. Shiota, K. Katsura et A. Akashi. 2000. Solubilization of organic sludge by thermophilic bacteria as a pretreatment for anaerobic digestion. Water Science and Technology, 41 (3), p. 163-169.

HAUG, R.T., T.J. LeBrun et L.D. Tortorici. 1983. Thermal pre-treatment of sludges, a field demonstration. Journal of Water Pollution Control Federation, 55, 23–34.

HIEMENZ PC and RAJAGOPALAN R. (1997) *Principles of Colloid and Surface Chemistry* (3rd edn.) Marcel Dekker, Inc., New York. 650 p.

HIRAOKA, M., N. Takeda, S. Sakai, A. Yasuda. 1985. Highly efficient anaerobic digestion with thermal pre-treatment. Water Science and Technology, 17, p. 529–553.

HONEY, H.C. et W.A. Pretorius. 2000. Laminar flow pipe hydraulics of pseudoplastic-thixotropic sewage sludges. Water SA, 26 (1), p. 19-26.

HWAN DO, J., H. Chang et S. Lee. 2001. Efficient recovery of poly (glutamic acid) from highly viscous culture broth. Biotechnology and Bioengineering, 76, p. 219–223.

JARVIS, P. 2001. Biopesticides: trends and opportunities, Agrow Reports, PJB Publications, 18/20 Hill Rise, Richmond, Surrey, TW10 6UA, UK, August, 98pp.

JEWORSKI, M. et E. Heinzle. 2000. Combined chemical-biological treatment of wastewater containing refractory pollutants. Biotechnological Annual Reviews, 6, p. 163-196.

JOHNSON, D.E., B. Oppert et W.H. McGaughey. 1998. Spore coat protein synergizes *Bacillus thuringiensis* crystal toxicity for the Indian meal moth (*Plodia interpunctella*). Current Microbiology, 36, p. 278–282.

JONG, J.Z., W.T. Wu, Y.H. Young et Y.M. Tzeng. 1994. Morphological variation on cultivation of *Bacillus thuringiensis* for thuringiensin production. In: Teo WK, Yap MGS, Oh S.K.W. (eds). Proceedings of the 3rd Asia-Pacific Biochemical Engineering Conference, National University of Singapore, Singapore, p. 390–392.

JOUNG, K.B. et J.C. Côté. 2000. A review of the environmental impacts of the microbial insecticide *Bacillus thuringiensis,* http://www.agr.gc.ca/science/, (cited 15 August, 2003).

KARLSSON, I. et J. Goransson. 1993. Thermic sludge treatment. Water Science and Technology, 27 (5-6), p. 449-456.

KILONZO, P. M. et A. Margaritis. 2004. The effects of non-Newtonian fermentation broth viscosity and small bubble segregation on oxygen mass transfer in gas-lift bioreactors: a critical review. Biochemical Engineering Journal, 17, p. 27–40.

KEPP, U., I. Machenbach, N. Weisz et O.E. Solheim. 2000. Enhanced stabilization of sewage sludge through thermal hydrolysis – three years of experience at full scale plant. Water Science and Technology, 42 (9), p. 86-89.

KIM, Y.-K. et I.-R. Huh. 1997. Enhancing biological treatability of landfill leachate by chemical oxidation. Environmental Engineering and Science, 14 (1), p. 73-79.

KIM, J.S., Y.Y. Lee et S.C. Park. 2000. Pretreatment of wastepaper and pulp mill sludge by aqueous ammonia and hydrogen peroxide. Applied Biochemistry and Biotechnology, 84-86, p. 129-139.

LACHHAB, K., R.D. Tyagi et J.R. Valéro. 2001. Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids concentration. Process Biochemistry, 37 (2), p. 197-208.

LAMONTAGNE, E. 2004. Caractérisation de nouvelles souches de *Bacillus thuringiensis* d'intérêt pour la production de biopesticides et d'enzymes par fermentation de boues d'épuration municipales. Master's thesis, Institut Nationale de la Recherche Scientifique-Eau, Terre et Environnement, Université du Québec, Canada, 112 p.

LASPIDOU, C.S. et B.E. Rittmann. 2002. A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. Water Research, 36, p. 2711–2720.

LEBLANC, M.-E. Effets des différentes stratégies et prétraitements des biosolides municipaux sur la croissance, la sporulation, l'entomotoxicité de *Bacillus thuringiensis* var. *kurstaki*. Master's thesis, Institut Nationale de la Recherche Scientifique-Eau, Terre et Environnement, Université du Québec, Canada, 2003, 153 p.

LEE, C.T., G. Morreale et A.P.J. Middelberg. 2004. Combined in-fermenter extraction and cross-flow microfiltration for improved inclusion body processing. Biotechnology and Bioengineering, 85(1), p. 103-113.

LISANSKY, S.G., R.J. Quinlan et G. Tassoni. 1993. The *Bacillus thuringiensis* Production Handbook, CPL Press, Newbury, p.124.

LIU, W.M., R.K. Bajpai et V. Bihari. 1994. High cultivation of sporeformers. Annals of New York Academic Sciences, 721, p. 310-325.

LOPEZ, C.J.L., S.J.A. Perez, F.J.M. Sevilla, R.E.M. Porcel et Y. Chisti. 2005. Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*, Journal of Biotechnology, 16, p. 61–77.

LOTITO, V., L. Spinosa, G. Mininni et R. Antonacci. 1997. The rheology of sewage sludge at different steps of treatment. Water Science and Technology, 36(11), p. 79-85.

MANTZAVINOS, D. et E. Psillakis. 2004. Enhancement of biodegradability of industrial wastewaters by chemical oxidation pre-treatment. Journal of Chemical Technology Biotechnology, 79, p. 431–454.

MASSON, L., B.E. Tabashnik, Y.B. Liu et J.L. Schwartz. 1999. Helix 4 of the *Bacillus thuringiensis* Cry1Aa toxin lines the lumen of the ion channel. Journal of Biological Chemistry, 274, p. 31996-32000.

MATA-ALVAREZ, J., S. Macé et P. Llabrès. 2000. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresource Technology, 74, p. 3-16.

McBRIDE, M.B. 1998. Soluble trace metals in alkaline stabilized sludge products. Journal of Environmental Quality, 27, p. 578-584.

METCALF AND EDDY. 2003. Wastewater Engineering – Treatment and reuse, 4th Edition, Eds. Tchobanoglous, G., Burton, F.L. and Stensel, H.D., Mc Graw Hill Inc., New York, p. 888 – 893.

MIZUKI, E., M. Maeda, R. Tanaka, D.W. Lee, M. Hara, T. Akao, S. Yamashita, H.S. Kim, T. Ichimatsu et M. Ohba. 2001. A common member of microflora in activated sludges of a sewage treatment plant. Current Microbiology, 42(6), p. 422 – 425.

MOHAMMEDI, S. 2004. *Isolement et caractérisation de souches de Bacillus thuringiensis dans les boues d'épuration.* Thèse de doctorat déposée, Université du Québec, INRS-Eau, Québec, Canada.

MOHAMMEDI, S., B. Subramanian, Y. Song, R.D. Tyagi et J.R. Valéro. 2006. Molecular screening of *Bacillus thuringiensis* strains from wastewater sludge for biopesticide production. Process Biochemistry, 41(4), p. 829-835.

MOR, S.G. et G.A. Matthews. 2003. Recent developments in sprayers for application of biopesticides-an overview. Biosystems Engineering, 84(2), p. 119–125.

MÜLLER, J.A. (2000). Pretreatment processes for the recycling and reuse of sewage sludge. Water Science and Technology, 42 (9), p. 167-174.

MUMMIGATTI, S.G. et A.N. Raghunathan. 1990. Influence of media composition on the production of d-endotoxin by *Bacillus thuringiensis* var. *thuringiensis.* Journal of Invertebrate Pathology, 55, p. 47–151.

NEIS, U. et A. Tiehm. 1997. Particle size analysis in primary and secondary waste water effluents. Water Science and Technology, 36(4), p. 151–158.

NEYENS, E. et J. Baeyens (2003). A review of thermal sludge pre-treatment processes to improve dewaterability. Journal of Hazardous Materials, B98, p. 51-57.

NEYENS, E., J. Baeyens, M. Weemaes et B. De Heyder. 2003a. Hot acid hydrolysis as a potential treatment of thickened sewage sludge. Journal of Hazardous Materials, B98, p. 275–293.

NEYENS, E., J. Baeyens et C. Creemers. 2003b. Alkaline thermal sludge hydrolysis. Journal of Hazardous Materials, B97, p. 295–314.

NEYENS, E., J. Baeyens, M. Weemaes et B. De heyder (2003c). Pilot-scale peroxidation (H_2O_2) of sewage sludge. Journal of Hazardous Materials, B98, p. 91-106.

NOVELLI, A., F. Ottonello, A. Converti, A. Lodi, M. Rovatti et M. Del Borghi. 1995. Alkaline hydrolysis for the treatment of the organic fraction of municipal solid wastes and sludges. Chemical Biochemical Engineering, 9(4), p. 195-199.

OBRADOR, A., M. I. Rico, J. M. Alvarez et J. Novillo. 2001. Influence of thermal treatment on sequential extraction and leaching behaviour of trace metals in contaminated sewage sludge. Bioresource Technology, 76, p. 259-264.

OSHODI, R.O. et R. MacNaughtan. 1990. Btk preparation: Acute inhalation toxicity study in rats. Volume 6. Danbury, CT: Novo Nordisk. (April 20).

ÖZKAN, M., F.B. Dilek, U. Yetis et G. Özcengiz. 2003. Nutritional and cultural parameters influencing antidipteran delta-endotoxin production. Research in Microbiology, 1, p. 1-5.

PANDEY, A. et C.R. Soccol. 2000. Economic utilization of crop residues for value addition: a futuristic approach. Journal of Scientific Industrial Research, 59, p. 12-22.

PENAUD, V., J.P. Delgènes et R. Moletta. 1999. Thermo-chemical pretreatment of a microbial biomass: influence of sodium hydroxide addition on solubilization and anaerobic biodegradability. Enzyme Microbial Technology, 25, p. 258-263.

RICHARD, A. et A. Margaritis. 2003. Rheology, oxygen transfer, and molecular weight characteristics of Poly (glutamic acid) fermentation by *Bacillus subtilis.* Biotechnology and Bioengineering, 82(3), p. 299-305.

ROJAS, J.V., E. Gutierrez et M. De la Torre. 1996. Primary separation of the entomopathogenic products of *Bacillus thuringiensis.* Biotechnological Progress, 12, p. 564-566.

ROWE, G.E., A. Margaritis et N. Wei. 2003. Specific oxygen uptake rate variations during batch fermentation of *Bacillus thuringiensis* subspecies *kurstaki* HD-1. Biotechnological Progress, 19, p. 1439-1443.

ROWE, G.E., A. Margaritis. 2004. Bioprocess design and economic analysis for the commercial production of environmentally friendly bioinsecticides from *Bacillus thuringiensis HD-1 kurstaki.* Biotechnology and Bioengineering, 86 (4), p. 377-388.

SACHDEVA V, R.D. Tyagi et J.R. Valero. 1999. Factors affecting the production of *Bacillus thuringiensis* biopesticides. Developments in Microbiology, 3, p. 363-365.

SACHDEVA, V., R.D. Tyagi et J.R. Valero. 2000. Production of biopesticides as a novel method of wastewater sludge utilization/disposal. Water Science and Technology, 42, p. 211-216.

SALAMA, H.S., Foda, M.S., Dulmage, H.T. and El-Sharaby, A. (1983). Novel fermentation media for production of d-endotoxins from *Bacillus thuringiensis*. Journal of Invertebrate Pathology, 41, p. 8–19.

SANIN, F.D. 2002. Effect of solution physical chemistry on the rheological properties of activated sludge. Water SA, 28(2), p. 207-212.

SASKINCHAI, S., M. Suphantharika et C. Verduyn. 2001. Application of a simple yeast extract from spent brewer's yeast for growth and sporulation of *Bacillus thuringiensis* subsp. *kurstaki*: a physiological study. World Journal of Microbiology and Biotechnology, 17, p. 307-316.

SCHALLMEY, M., A. Singh et O.P. Ward. 2004. Developments in the use of *Bacillus* species for industrial production. Canadian Journal of Microbiology, 50, p. 1–17.

SCHIEDER, D., R. Schneider et F. Bischof. 2000. Thermal hydrolysis (TDH) as a pretreatment method for the digestion of organic waste. Water Science and Technology, 41 (3), p. 181-187.

SCHNEPF, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler et D.H. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews, 62(3), p. 775–806

SCHWARTZ, J.L., M. Juteau, P. Grochulski, M. Cygler, G. Prefontaine, R. Brousseau et L. Masson. 1997. Restriction of intramolecular movements within the Cry1Aa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering. FEBS Letters, 410, p. 397-402.

SHANABLEH, A. et S. Jomaa. 2001. Production and transformation of volatile fatty acids from sludge subjected to hydrothermal treatment. Water Science and Technology, 44 (10), p. 129-135.

SOLOMONS, T.W.G. 1997. Fundamentals of organic chemistry, fifth edition. John Wiley & Sons Inc., New-York, É.-U., 1068 pages.

STANBURY, P.F., A. Whitaker et S.J. Hall. 1993. Principles of Fermentation Technology. 2nd ed. New York – Elsevier Science Ltd.

TAMEZ-GUERRA, P., M.R. McGuire, H. Medrano-Roldan et L.J. Galan-Wong. 1996. Sprayable granule formulations of *Bacillus thuringiensis*. Journal of Economic Entomology, 89, p. 1424-1430.

TANAKA, S., T. Kobayashi, K. Kamiyama et N.S. Biladan. 1997. Effects of thermochemical pretreatment on the anaerobic digestion of waste activated sludge. Water Science and Technology, 35 (8), p. 209-215.

TANAKA, S. et K. Kamiyama. 2002. Thermochemical pretreatment in the anerobic digestion of waste activated sludge. Water Science and Technology, 46 (10), p. 173-179.

TIRADO-MONTIEL, M.L. 1997. *Utilisation des boues des usines de traitement comme moyen alternatif pour la production de l'insecticide microbien Bacillus thuringiensis*. Thèse de doctorat, INRS-Eau, Université du Québec, Quebec, Canada, 223 pages.

TIRADO-MONTIEL, M.L., R.D. Tyagi et J.R. Valéro. 1998. Production of *Bacillus thuringiensis* using waste materials: In Bioconversion of waste materials into industrial.A.M. Martin (ed.). Blackie Academic and Professional, London, p. 480-516.

TIRADO-MONTIEL, M.L., R.D. Tyagi et J.R. Valéro. 2001. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. Water Research, 35 (16), p. 3807–3816.

TIRADO-MONTIEL, M.L., R.D. Tyagi, J.R. Valéro et R.Y. Surampalli. 2003. Production biopesticides using wastewater sludge as a raw material – Effect of process parameters. Water Science and Technology, 48 (8), p. 239-246.

TYAGI, R.D., V.S. Foko, S. Barnabé, A.S. Vidyarthi et J.R. Valéro. 2001. Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using sewage sludge as raw material. Water Science and Technology, 46 (10), p. 247-254.

TZENG, Y.M., H.Y. Tsun et Y.N. Chang. 1999. Recovery of thuringiensin with cetylpyridinium chloride using micellar-enhanced ultrafiltration process. Biotechnological Progress, 15, p. 580-586.

U.S. EPA 1989. Lists of Other (Inert) Pesticide Ingredients. 52 FR 13305 and 54 FR 48314.

VASCONCELOSA, J.M.T., J.M.L. Rodriguesa, S.C.P. Orvalhoa, S.S. Alvesa, R.L. Mendes et A. Reis. 2003. Effect of contaminants on mass transfer coefficients in bubble column and airlift contactors. Chemical Engineering Science, 58, p. 1431 – 1440.

VIDYARTHI, A.S., M. Desrosiers, R.D. Tyagi et J.R. Valéro 2000. Foam control in biopesticide production from sewage sludge. Journal of Industrial Microbiology and Biotechnology, 25, p. 86–92.

VIDYARTHI, A.S., 2001. Effect of surface active agents on the production of biopesticides using wastewater sludge as a raw material. Water Science and Technology, 44 (10), p. 253–259.

VIDYARTHI, A.S., R.D. Tyagi, J.R. Valéro et R.Y. Surampalli. 2002. Studies on the production of *B-thuringiensis* based biopesticides using wastewater sludge as a raw material. Water Research, 36 (19), p. 4850-4860.

VIDYARTHI, A.S., M. Desrosiers, R.D. Tyagi, J.R. Valéro. 2003a. Influence of oxygen concentration on biopesticide production from sewage sludge. Unpublished.

VLYSSIDES, A.G. et P.K. Karlis. 2004. Thermal-alkaline solubilization of waste activated sludge as a pre-treatment stage of anaerobic digestion. Bioresource Technology, 91, p. 201-206.

VORA, D. et Y.I. Shethna. 1999. Enhanced growth, sporulation and toxin production by *Bacillus thuringiensis kurstaki* in oil seed meal extract media containing cystine. World Journal of Microbiology and Biotechnology, 15, p. 747-749.

WHALON, M. E. et Wingerd, B. A. 2003. Bt: Mode of Action and Use. Archives of Insect Biochemistry and Physiology, 54, p. 200–211.

WU, W.T., Y.L. Hsu, Y.F. Ko et L.L. Yao. 2002. Effect of shear stress on cultivation *of Bacillus thuringiensis* for thuringiensin production. Applied Microbiology and Biotechnology, 58, p. 175–177.

YEN, P.S., L.C. Chen, C.Y. Chien, R.M. Wu et D.J. Lee. 2002. Network strength and dewaterability of flocculated activated sludge. Water Research, 36, p. 539–550.

YEZZA, A., R.D. Tyagi, J.R. Valero, R.Y. Surampalli et J. Smith. 2004. Scale-up of biopesticide production process using wastewater sludge as a raw material. Journal of Industrial Microbiology and Biotechnology, 31, p. 545-552.

YEZZA, A., R.D. Tyagi, J.R. Valero et R.Y. Surampalli. 2005a. Production of *Bacillus thuringiensis* based biopesticides in batch and fed cultures using wastewater sludge as a raw material. Journal of Chemical Technology and Biotechnology, 80, p. 502-510.

YEZZA, A., R.D. Tyagi, J.R. Valero et R.Y. Surampalli. 2005b. Wastewater sludge pre-treatment for enhancing entomotoxicity produced by *Bacillus thuringiensis* var. *kurstaki*. World Journal of Microbiology and Biotechnology, 21, p. 1165–1174.

YEZZA, A., R.D. Tyagi, J.R. Valero et R.Y. Surampalli. 2005c. Influence of pH control agents on entomotoxicity potency of *Bacillus thuringiensis* using different raw materials. World Journal of Microbiology and Biotechnology, 00, p. 1–10.

YEZZA, A., R.D. Tyagi, J.R. Valero et R.Y. Surampalli. 2006a. Correlation between entomotoxicity potency and protease activity produced by *Bacillus thuringiensis var. kurstaki* grown in wastewater sludge. Process Biochemistry, 41, p. 794-780.

YEZZA, A., R.D. Tyagi, J.R. Valero et R.Y. Surampalli. 2006b. Bioconversion of industrial wastewater and wastewater sludge into *Bacillus thuringiensis* based biopesticides in pilot fermentor. Bioresource Technology, 26, p. 1850-1857.

YOON, S., J. Do, S. Lee et H. Chang. 2000. Production of poly-γ-glutamic acid by fed-batch culture of *Bacillus licheniformis*. Biotechnology Letters, 22, p. 585–588.

ZAMOLA, B., P. Valles, G. Meli, P. Miccoli et F. Kajfez. 1981. Use of the centrifugal separation technique in manufacturing a bioinsecticide based on *Bacillus thuringiensis*. Biotechnology and Bioengineering, XXIII, p. 1079-1086.

ZORPAS, A.A., A.G. Vlyssides, G.A. Zorpas, P.K. Karlis et D. Arapoglou. 2001. Impact of thermal treatment on metal in sewage sludge from the Psittalias wastewater treatment plant, Athens, Greece. Journal of Hazardous Materials, B82, p. 291-298.

ZOUARI, N. et S. Jaoua. 1999. The effect of complex carbon and nitrogen, salt Tween 80 and acetate on delta-endotoxin production by a *Bacillus thuringiensis* subsp. *kurstaki*. Journal of Industrial Microbiology and Biotechnology, 23, p. 497–502.

ZOUARI, N., S.B.S. Ali et S. Jaoua. 2002. Production of delta-endotoxins by *Bacillus thuringiensis* strains exhibiting various insecticidal activities towards lepidoptera and diptera in gruel and fish meal media. Enzyme and Microbial Technology, 31, p. 411–418.

ZHU, H. et Ji-Hua Chen. 2005. Study of hydrolysis and acidification process to minimize excess biomass production. Journal of Hazardous Materials, B127, p. 221–227.

CHAPITRE 2.

VALORISATION DES BOUES D'ÉPURATION

Brar, S.K., Verma, M., Tyagi, R.D. et Surampalli, R.Y. (2009). Value addition of wastewater sludge: Future course in sludge reutilization. *Pract. Period. Hazard. Toxicol. Radioact. Waste Manage.* 13(1): 59-74.

http://dx.doi.org/10.1061/(ASCE)1090-025X(2009)13:1(59)

Partie I

Value Addition of Wastewater Sludge- Future Course in Sludge Re-Utilization

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*] and R.Y. Surampalli[2]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Practice Periodical of Hazardous, Toxic and Radioactive Waste Management - American Society of Civil Engineers (Under Revision)

Valorisation des boues d'épuration

Résumé

L'épuration des eaux usées urbaines et industrielles, fait maintenant l'objet d'une réglementation stricte, produit des grandes quantités de boues dont il faut disposer de manière efficace. Il existe aujourd'hui trois approches permettant d'améliorer l'efficacité des méthodes de disposition : (1) le contrôle de la qualité et la quantité des boues produites par une amélioration des procédés d'épuration, ceci permet la faciliter la valorisation comme fertilisant; (2) l'amélioration des méthodes de manipulation, de traitement et de disposition des boues; et (3) leur valorisation par transformation en produits à valeur ajoutée. Différentes options de gestion de boues ont fait l'objet de revues scientifiques et techniques et, généralement, les auteurs s'accordent pour dire que la clef d'une gestion efficace des boues réside dans leur valorisation. C'est cette perspective qui est explorée dans cette revue de littérature sur la valorisation des boues. Cette revue identifie les différents produits à valeur ajoutée pouvant être obtenus par la transformation des boues, notamment la production de fertilisants, de matériaux de construction, de carburants, de dérivés biologiques et d'adsorbants. Cette revue discute aussi des questions clés vis-à-vis les avantages et les limites des procédés de valorisation ainsi que les domaines d'application.

Mots-clés: Produits biotechnologiques ; biosorbants; matériaux de construction; carburants; amendements pour le sol; valorisation; boues d'épuration.

Abstract

Sludges are inevitable products of wastewater treatment processes which have to be managed efficiently to meet stringent laws and regulations being enforced day by day. Currently, there exist three major options for complete sludge management – control at source by process modification; improvement in handling, processing and disposal and reuse to produce value-added products. The source control and improvement in handling and disposal practices have been reviewed by various authors and it is actually sludge reprocessing to value-added products which holds the future key to sustainable management. Thus, the primary focus of this review is value-addition of sludge comprising recovery of different components and development of commercial products. The production of various value-added products, namely, soil amendments; construction aggregates; fuels; biotechnology products; biosorbents and miscellaneous have been examined addressing the key issues of production method limitations, benefits and possible field application.

Keywords: Biotechnology products; Biosorbents; Construction aggregates; Fuels; Soil amendments; Value-addition; Wastewater sludge.

Introduction

Increased global urbanization with concomitant growth in wastewater treatment plants have led to a very large increase in the production of wastewater sludge. In North America, several million tonnes of sludge is produced annually with the treatment cost being 50% of the annual operating costs of wastewater treatment (Metcalf and Eddy 2003). Sludge treatment and disposal has been investigated and reviewed extensively by different authors pointing to the challenges involved and future possibilities of reuse (Low and Chase 1999; Werther and Ogada 1999; Neyens and Baeyens 2003; Liu, 2003). The wastewater sludge is generally disposed by conventional means - landfills, incineration, and land application and other lesser means like advanced treatment and other uses as shown in Fig. 1. Although land filling is currently the most widely used disposal practice, yet this mode will not be sustainable at current or projected levels far into the future due to increasing competition for landfill space, higher cost, more stringent environmental standards and implementation of policies to promote recycling (Metcalf and Eddy 2003). Likewise, despite the reduction in sludge volume by incineration, it is known to cause secondary environmental pollution and is also cost intensive. On the contrary, land application had gained lot of popularity in 1990s as 60% of the sludge produced in France, 54% in Denmark, 50% in Spain, 44% in UK and 26% in USA was used in land spreading (McGhee 1991; Hall and Dalimier 1994). Although land spreading causes soil fertilization, yet some of the pollutants may be transferred to the soil and groundwater resulting in the subsequent introduction into the food chain restricting their future uses (Sort and Alcaniz, 1996; Andre 1999).

Thus, adverse environmental and health impacts of the sludge disposal options has paved way to sludge value-addition which is a sustainable option in the cradle-to-cradle journey of wastes as represented in Fig. 2. Normally, a life cycle approach is adopted for sludge management strategies which include: compliance with the best possible practice with respect to environment and health protection; energy use; greenhouse gas emissions; odour control; volume reduction and public acceptance (Bridle and Mantele 2000; Gaskin et al. 2003; Dichtl 2003; Spinosa 2004).

Nonetheless, these future trends and strategies will evolve rather than appear overnight. In this context, this review is an effort to understand the production of value-added products (Fig. 3.) and their feasibility and viability as a part of the entire sludge management plan.

Soil Amendments

Organic Matter- Soil Conditioner and Compost

The organic fraction of wastewater sludge is highly suitable for soil fertility improvement, increasing microbial activity in the soil, contributing to the gradual decomposition of organic matter and assimilation of specific nutrients depending on type, origin and characteristics of sludge (Sastre et al. 1996; Petersen et al. 2003; Smith and Tibbett 2004). Recycling of sewage sludge is already a common practice in several countries with 30% in Canada and 40% in the UK (Webber et al. 1996). In Western Europe, current trends in waste management policies favor land application as opposed to land fill deposition or incineration (Council Directive 1999/31/EC). Conventionally, dewatered sludge has been applied to agricultural land as a soil conditioner.

Roe et al. (1997) researched the effects of different compost amendments, including sludge and yard trimmings compost on seed emergence, seedling growth, plant growth yield, and fruit quality of vegetables. They found encouraging results and the same was reflected in the studies of Pinamonti et al. (1997) who found yields of cucumber, tomato and strawberry higher than the controls. The increase in the yield was associated with improvements in the physical properties of the soil, increases in soil pH, organic matter content and available phosphate and magnesium in the soil (Weir and Allen 1997). Despite the soil fertilization ability of sludge, presence of toxic heavy metals, organic pollutants and pathogens have been a cause of concern on long term basis and even improvement in application methods has not been of major benefit. In this context, Snyman et al. (1998) found that even the time gap between seedling planting and sludge application caused inherent problems of heavy metal contamination. Likewise, sludge when directly utilized in reed beds increased heavy metals content and the number of *Ascaris lumbricoides* per mass of soil (Zwara and Pempkowiak 2000). Elsewhere, it was found that the rye roots absorbed higher quantities of trace elements and formed a barrier to their transfer to above ground parts of the rye grass, with no significant increase in the soil metal distribution over one crop cycle (Maisonnave et al. 2001). Though, the metal concentrations were localised in roots, yet their disposal should be of major concern.

Combining agricultural use with degraded area recovery has been also known to accentuate the use of sludge. When aerobic lime stabilized sludge was applied to the fields, the production of dry matter of oats and maize was higher in the treatments and there was a significant increase in calcium and magnesium contents further supplemented by phosphorus and carbon (Andreoli et al. 2001; Gutierrez et al. 2001).

Likewise, composted wastewater sludges, retained highly stable organic materials that decomposed at a slow rate, therefore, releasing nutrients at a slower rate (Huang 1986; Wei et al. 2000; Nissen et al. 2000). Compost also contributed to water conservation by reducing water loss from percolation, evaporation, and runoff (Bart et al. 2002). In addition, compost could be used to bioremediate many toxic contaminants in soil (Garland, et al. 1995; U.S. EPA 1997; U.S. EPA 1998).

Lately, low temperature carbonization of sludge has been utilized to form products which can be used as soil amendments to improve permeability and water holding capacity. Some useful minerals such as nitrogen, phosphorus and potassium are retained in each type of carbon product (Shinogi et al. 2003). Although this process will lower the cost of sludge management, yet, presence of some heavy metals is questionable and the technology is yet to take off completely.

Nowadays, the research focus is also on integrated systems composed of sludge drying facility and a landfill for residual waste (Brautlecht et al. 1998). The system is based on the fact that thermal energy (biogas) produced in landfill operation is further used for sewage sludge drying. This strategy reduces drying costs and the existing resources can be used in an ecologically acceptable manner throughout the year.

The field application of sludge as a source of organic matter has been carried out with excellent outcomes. At Indiana, U.S., it was found that the actual crop yields after sludge application without use of any additional fertilizers increased. Hence, the prudent use of sludge provided the producer with the lowest cost per unit of production (Schreeg and Jarrett 1996). Some studies have been carried out where metalliferous mine wastes sites were improved by growing trees with dewatered sludge solids amendments (Black and Veatch, 1995; Whitbread-Abrutat 1997). In general, all doses of sewage sludge treatments as soil conditioner are known to influence the enzyme activity and contents of nutrients and organic matter of earthworm casts and surrounding soil (Kizilkaya 2004).

In another attempt to rehabilitate mined soils by using annual ryegrass (*Lolium rigidum*) and subterranean clover (*Trifolium subterraneum*), biosolids (digested sewage sludge) resulted in good organic matter supply, (Rate et al. 2004) which was similar to the observations made by Su et al. (2004). Meanwhile, ash amendment of sewage sludge has been found to significantly reduce the availability of heavy metals by chemical modification (through chemical speciation) into less available forms when greenhouse experiments were conducted on corn and other plants (Sajwan et al. 2003; Su and Wong 2003).

Thus, soil conditioning option of sludge application has been considered to be practical and successively employed over years with high success rate and would continue as a feasible option in the future. This reuse route will utilize large volumes of sludge aiding in sustainable sludge management. Meanwhile, many uncertainties still remain to be addressed concerning the transfer of pollutants to the environmental compartments and the food chain and in particular, the role of sludge.

Sheet Cover

Sludge could also be employed as a valuable sheet cover to mitigate erosion in quarries. Sort et al. (1996) in an exhaustive study on vegetation found that plot treated with sludge represented less than 10 % of the erosion from the control plot. Even when the vegetation was well developed, the erosion was lower in the plots where sludge had been applied. Composted sludge when applied to a severely burned, previously forested site near Colorado enhanced the plant cover and growth and controlled soil erosion. The runoff constituents were found below drinking water levels except that for Pb (Meyer et al. 2001).

Risk assessment studies on infection and illness from enteric viruses after application of class B biosolids to land proved that direct exposure to mesophilic digested sludge would result in risk exceeding the US EPA's recommendation of 1:10 000 yearly risk of infection from a one time exposure (Gerba et al., 2001). Isolation of filamentous fungal strains from domestic wastewater sludge showed five genera; *Aspergillus, Penicillium, Trichoderma, Myriodontium* and *Pleurotus*. They were proposed to have the ability to convert domestic wastewater sludge into compost by biomass production and growth rate on sludge enriched media and hence production of compost for plant growth by conserving soil fertility (Molla et al., 2003).

Despite positive impacts of erosion control, sheet cover utility may lead to transfer of various sludge constituents into food chain resulting in possible biomagnification which still needs to be determined.

Wasteland Rejuvenation

Sludge has also been directly employed to enhance biomass production of desert grasses, in particular, blue grama and tobosagrass as it increased the plant tissue nitrogen aiding in greening of arid and semi-arid rangelands (Fresquez et al. 1990; Benton and Wester 1998; Mata Gonzalez et al. 2002). An investigation was conducted to examine aerobic digestion of

the phosphorus-laden wastewater sludge produced at the Regina wastewater treatment plant in feasibility of land use of this sludge combined with the dewatered anaerobically digested primary sludge. Results showed that mixing the two digested sludges met the heavy metal criteria set by various guidelines for agricultural use, presenting the advantage of an increased concentration of nutrients and a decreased concentration of heavy metals, and a longer useful life of the agricultural site compared to using dewatered anaerobically digested primary sludge alone (Viraraghavan and Ionescu 2002).

Construction Aggregates/Bricks

When the sludge was subjected to conventional incineration, low bulk density and potential heavy metal content of the by-products posed problems in its safe environmental disposal. This stimulated the use of incinerated sewage sludge ash (SSA) as a valuable additive in the production of bricks. The mortar was normally composed of: wet sludge, calcareous sand, Portland I 45/A cement and tap water, the quantity of which was determined with the flow table test and the constituents were mixed homogeneously in a mixer (Valls and Vasquez 2001). In this kind of production, heavy metal content of sludge is crucial to ascertain leaching potential into the environment. Despite most of the metals being within the limits, there was still leaching of some specific metals and chloride ions which would augment the environmental burden.

SSA can be subjected to many possible reuse options such as fine aggregates or as filler in cement and road construction and as pozzolanic material. However, due to porous and irregular morphology of SSA, it is difficult to adequately maintain water-to-cement ratio and workability of SSA mortar simultaneously (Pan et al. 2003). Meanwhile, SSA influences the finished brick product in many ways: ashes work as opening agents (because of their particle size = 60-100 μm); high water absorption potential and Ca content increases demand for gauging water; work as pore forming agents; as fluxing agent i.e. decreases the sintering temperature of the mixture and finally affects the compressive strength depending on Ca (softening agent) and Fe (hardening agent) content (Valls et al. 2001). Despite all the ardent efforts to improve the product, leachability still remains questionable, limiting the product use over a long period of time.

Although the bricks so developed were superior to the traditional ones on the basis of strength and mechanical properties, but had various secondary problems like moss growth, icing surface and whitening (Okuno et al. 1997; Weibusch and Seyfried 1997). There have been several modifications made in the existing method of production of ceramic material,

whereby flame melting has been considered to be a better option and the finished product can be used as an extender for bentonite, an admixture for high flow concrete and additive for synthetic lumber (Teratani et al. 2001). This manufacturing scheme has been further improved by the use of crystallization furnace technology which saves energy and also enhances the properties.

Sludge also has been put to use in the manufacture of slag, pumice and even Portland cement (Onaka 1999). Sludge aggregate made from 100% sludge can be used to replace granite in concrete without any compromise in compressive strength and cement-like materials made from sludge can also replace ordinary Portland cement for up to 20% by weight (Tay et al. 2002; Paya et al. 2002). Sludge-marine clay combinations containing pelletized aggregates have demonstrated significantly low leachate levels after 150 days when used in concrete, indicating insignificant environmental contamination (Tay et al. 2004).

Another option has been the use of glass aggregate appended sludge in the manufacturing of floor tiles, abrasives, roofing shingles granules, and asphalt paving (O'Conor et al. 2001). Furthermore, sludge enriched with heavy metals has been incorporated into the production of bio-bricks (as no conventional material was utilized as ingredient), with incinerator ash as an additive in place of clay and it was found that these bricks did not release heavy metals during weathering or firing (Anderson et al. 1996, Anderson 2002). However, these bricks had some drawbacks like increase in tempering water which may increase the firing cost and also the requirement of addition of anti-scum agents which would increase the production costs. Thus, further investigations are necessary to verify the feasibility and actual performance of SSA based products in field.

The wastewater sludge has also been mixed with dam sediments and used as raw materials for brick making through sintering process (Weibusch and Seyfried 1997; Huang et al. 2001). A study carried out in Japan for six years proved that the dewatered sludge cake could be reduced to 1/7 in weight and to 1/4 in volume by melting and solidification process and the air cooled slag could be used as a substitute for natural coarse aggregate, including concrete aggregate and back filling material. Low boiling point heavy metals volatilized, but were trapped in the fume gas treatment process and no leaching of heavy metals was observed (Okuno et al. 1997). Meanwhile, Cusido et al. (2003) reported the emissions of various volatile organic compounds viz. methyl mercaptan, dimethyl disulfide and acetic acid with probability of dioxins and furans from the firing of a ternary mixture of clays, sewage sludge and forest debris.

Conclusively, there are two principal problems of SSA which sometimes affects their widespread use- a) strength sensitivity index is lower than normal fly ash and; b) water requirements are higher when compared to ordinary cement clinker. Mechanical grinding of the SSA could aid in overcoming these drawbacks, but incur mechanical costs which need to be looked into. A great deal of effort would be required in market development of construction aggregates (similar to that required for composts manufactured using sewage sludge) due to fixation of metals, toxics leachability after a period of time which remains a pertinent problem. Further, the processes/methods utilized in the construction aggregates production may impose an energy requirement which necessitates an energy-material-product benefit analysis to estimate the final product costs. Moreover, the abundance of traditional materials in certain geographical regions would also limit the use of aggregates.

Fuels

Oils

In the past, little quantity of oil was recovered from incineration processes tagged with secondary pollution problems. This led to the evolution of higher efficiency, moderate temperature processes with low emissions of NO_x and SO_x and also lower operational costs when compared to incineration (Avenell et al. 1996). Normally, these processes involved pyrolysis of sewage sludges under moderate temperatures (300–600°C) and varying gas residence times (1.5–3.5 s) in fluidized-bed, fixed bed and rotary reactors.

Cassidy et al. (1998) reported the pyrolysis of dewatered and dried sludge, when heated to 400-700°C in an oxygen deficient environment, the inert part was converted to coke like material and organic fraction was gasified, when cooled. The oil so condensed could be further used for energy production. The organic fraction could also be used as a raw material for activated carbon. There have been studies where the sludge has been converted thermally to liquid and solid fuels, oil yields have ranged from a low of 13% for an anaerobically digested sludge to a high of 46% for a mixed raw sludge. Char yields have ranged from 40 to 73% at the optimum operating temperatures (Bridle and Campbell 1984). A well developed technique called "sludge to fuel" (STF) involved a process that converted sludge organic matter into an incinerable oil using a solvent, atmospheric pressure, and temperatures in the range of 200-300°C (Millot et al. 1989) or alternatively, high pressures in the range of 10 MPa combined with high temperatures (Itoh et al. 1994; Boon and Thomas 1996).

The widely known "Enersludge^{TM}" process uses dewatered sludge, containing 28% solids, further sent to a dryer. The organics were catalytically (*in-situ* presence in sludge) converted to

hydrocarbons in the second reactor and then condensed, separated, and cleaned ready for either further processing or use as fuel in power generation. Non-condensable hydrocarbons, along with a non-volatile carbon char passed from the conversion reactor to the hot gas generator where they were burned to produce heat. The heat was used to dry the incoming sludge cake, thus, completing the cycle and utilising all the heat of combustion in the process. Ash remaining after combustion was cooled and stored ready for sale (Mantele et al. 2000). Typical product and energy yields of this process are presented in Table 1 and schematic is illustrated in Fig. 4.

Other oil production processes employed activated alumina pyrolysis of digested, dried sludges or toluene extracted sludge lipids at low temperatures of 300 – 600°C (Abu-Orf and Jamrah 1995; Abu-Orf et al. 2001). Shen and Zhang (2003) studied the recovery of oil from sewage sludge in a fluidised bed reactor. They found that a maximum oil yield of 30% (weight % of dry activated form of sludge fed) was achieved at a bed temperature of 525°C and a gas residence time of 1.5s. The structure of sewage oils comprised a group of aromatic clusters with one to three aromatic rings connected by long straight chain hydrocarbons with hydroxyl groups.

The successful development of oil from sludge (OFS) process was started by the Environmental Canada Wastewater Technology Centre in Canada in 1982, based on the research originally carried out at Tubingen University in Germany involving two stages – pyrolysis and catalytic conversion in the presence of char to oil (Kyriakos 1990; Hudson and Lowe 1996). The Tubingen process was carried out at a low temperature of around 300°C and produced 20–30% oil per kg of dry sludge.

Many tests have successfully revealed that the oil obtained from sewage sludge pyrolysis could be used directly in diesel fuelled engines and was comparable to low-grade petroleum distillates from commercial refineries (Campbell 1990; Werther and Ogada 1999).

Although, oil production from sludge has been commercialised to some extent and the entire process is sustainable as nothing has to be disposed off finally and even the ash produced has commercial application in concrete aggregates which compensates for its cost, yet the efficiency (calorific value) in comparison to conventional oils requires further investigation. In addition, the liquid sludge has to be dewatered to ensure better performance of the production process which in itself is an energy intensive process.

Gas

Traditionally, lignocellulosic biomass has been employed for the production of eco-friendly bio-fuels with low yields. But, recently, a study enumerated that biohydrogen production from solid waste lignocelluloses could be stimulated with the use of raw sewage sludge as microbial seed to enhance recovery in the anaerobic process. The hydrogen composition of the biogas was greater than 50% with lower concentration of methane. Nevertheless, the heavy metal content was a matter of concern in the gases too (Lay 2000). Likewise, digested sludge was utilized as an inoculum for enhanced degradation of ortho-fruit waste in the ratio 9:1 to produce biogas (Lastella et al. 2002).

Noell conversion process developed in Germany is a novel venture to gas production whereby the sludge is thermally digested to produce synthesis gas, sulphur and vitrified slag with no problem of emission of mercury or dioxins and furans (Jaeger and Mayer 2000). Noell has acquired significant operation experience from their pilot-scale plant in Freiburg/Sachsen which has been operating since 1979. The experience enabled them to construct and commission a 130 MW large scale plant which has been operating near Berlin since 1988.

The combustion process parameters of the sludge-fuel conversion comprise water; mineral contents and the calorific value of sludge. These parameters, in turn, influence the combustion process in the boiler, such as excess air ratio, temperature in the combustion chamber and volume of the flue gases. The critical parameter from environmental point of view was the content of heavy metals, which were sometimes emitted to the atmosphere with the flue gases and dust particles.

Several wastewater sludges have been tested for their ability to anaerobically degrade methanethiol waste. This process recovered 80-90 % of elemental sulphur with simultaneous production of biogas. However, the process at large scale lost its efficiency after 100 days of start-up time and required additional and external carbon sources viz. sucrose and acetate increasing production costs (Sipma et al. 2003).

The resulting biogas product from all reactors normally comprised H_2, N_2, CO, CO_2 and CH_4 with a maximum average gross calorific value of 4 MJ /m^3. Around 10 –11% (v/v) of this product gas was hydrogen which could be utilized for fuel cells (Midilli et al. 2002). The literature is replete with hydrogen production studies using various reactor configurations with sewage sludge as proton donor (Wu et al. 2002; Lee et al. 2003). Sludge can also serve as a valuable energy source after removing the inorganic fraction and water and improving the carbon source. Sludge hydrolysis is one of the means to gaining an indirect energy source

where both raw and digested sludge can be treated for improving biogas production by 40% (Odeby et. al. 1996). Lately, valuable biogas from homogenized sludge has been harnessed in significant amounts coupled with mass reduction of sludge. There was 30% extra energy obtained from thickened and disrupted sludge (obtained by ulrasonication) than untreated samples and was higher than that invested during disruption and digestion processes. There was a concomitant 23% sludge reduction and this new process could produce extra energy for local electrification and heating the digester while sludge reduction provided economic benefits (Onyeche 2004). Thus, concentration of sludge to higher solids concentration would cause reduction in digester investment cost as well as reduction in operational time for sludge dewatering. Another recent study has brought about the importance of pre-treatment of wastewater sludge for production of methane/hydrogen by using *Clostridium* strain. Hydrogen yield followed freeze/thawed > acidified > sterilized > original sludge > sonicated; while methane yield followed sonicated > freeze/thawed > sterilized > acidified > original sludge (Ting et al. 2004). Thus, production of energy in the form of methane/hydrogen from wastewater sludge via pre-treatment by enhancing nutrient assimilation and increase in methanogenic bacterial growth holds another key to "eco-friendly fuel" and requires further systematic research in this area.

Although chemical processes (example, pyrolysis) produce a high calorific fuel in comparison to biological processes, yet the former suffer the problem of secondary pollutant production and generation of secondary sludge ash. Considerable non-combustible particles in the fuel derived from wastewater sludge may cause serious problems and variable moisture levels that could make storing the fuel difficult. But, to solve these technical snags with further technological advances, research is required, keeping in mind the biological origin of the wastewater sludge. At this juncture, a compromise could be reached between chemical and biological production processes by carrying out elaborate studies on specific parameters affecting fuel production.

Biotechnology Products

Bioplastics

Development of bioplastics from activated sludge is another route of sludge reutilization. Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates that accumulate as carbon/energy or reducing-power storage material in various microorganisms. Bacterial populations adopt a survival strategy when exposed to feast and famine conditions, whereby they store a large fraction of the soluble substrate, when available, as storage polymers such as poly-β-hydroxybutyric acid (PHB). This served as an NADH-overflow mechanism to control

the redox state of heterotrophic cells during unbalanced growth conditions (Senior and Dawes 1973; Van Niel et al. 1995). These biopolymers could be processed to bioplastics (Kessler et al. 2001, Nonato et al. 2001, Kim and Lenz 2001). The main reasons for reduced diffusion of PHAs into the plastic market are higher cost of the selection of microorganisms and substrate (Anderson and Dawes 1990). Indeed, most processes for PHA production are based on pure cultures of particular microorganisms (e.g., *Ralstonia eutropha*) grown on well-defined nutrient-deficient synthetic media (Doi et al. 1987; Lee 1996a).

However, the conventional approach of using synthetic media has now been replaced by alternate cheaper substitutes. The PHA content of activated sludge can be increased to 62% in a microaerophilic–aerobic sludge process (Satoh et al. 1998; Takabatake et al. 2002). This process utilized mixed culture in comparison to conventional pure systems and hence the process was more cost effective. There have been some other studies on the usage of industrial food wastewater as nutrient source for microbes. Bench experiments showed that microorganisms from the municipal activated sludges used the nutrients from malt and soya wastes to biosynthesize PHAs very efficiently (Yu et al. 1999). When compared with a pure culture (more than 88% of cell dry weight) (Lee 1996b), the merits of PHA production in mixed culture would be an enhanced economy, a simpler process control, no requirement of monoseptic processing, and an improved use of wastes (Satoh et al. 1998). Hence, a considerable effort has gone in producing PHA using mixed cultures (Ueno et al. 1993; Matsuo 1994; Saito et al. 1995; Hu et al. 1997; Brdjanovic et al. 1998; Tsunemasa 1998; Chau and Yu 1999; Takabatake et al. 2000, 2002; Tohyama et al. 2002; Beun et al. 2002; Van Loosdrecht and Heijinen 2002).

Kumar et al. (2004) carried out studies using food processing wastewater treatment plant activated sludge for the production of PHB with inoculum from sewage sludge and they found that variation of C: N ratio played a vital role. PHB can be accumulated in a large quantity concurrently with the growth of the bacterium, by making the growth rate very slow compared with the generation time of a bacterium in the case. Thus, enrichment of municipal sludge with agro-industry sludge stimulated the production of bioplastics. PHB can also serve as a valuable carbon source for wastewaters with low C: N ratio acting as a cheap carbon substrate for bioplastics manufacture (Third et al. 2003). Sawayama et al. (1999) reported the PHB production rate based on acetate media and PHB content based on dry biomass of about 6.6–14 mg/L/d and 15.1–25.3%, respectively, using photosynthetic bacteria in anaerobic activated sludge. The results have shown that PHB was stored in the presence of ethanol but not in presence of glutamic acid (Beccari et al. 2002). There has also been production of bioplastics from activated sludges by feeding simple organic acids- acetic, propionic and lactic acid in a

periodic mode (feast and famine regime) in a sequencing batch reactor (Dionisi et al. 2001). The immediate biomass response to substrate excess (as determined through short-term batch tests) was characterized by a storage rate and yield of 649 mg PHA (as COD) g biomass (as COD) $^{-1}$ h^{-1} and 0.45 mg PHA (as COD) mg removed substrates (as COD^{-1}), respectively. As for the PHA composition, the copolymer poly (β-hydroxybutyrate/β-hydroxyvalerate) with 31% of hydroxyvalerate monomer was produced from the substrate mixture (Dionisi et al. 2004). Although this technology looks promising from point of view of scale-up, yet production should be further increased by performing enrichment and production of biomass at higher organic load rates than tested in most of the studies to make the process economically attractive.

The major technical difficulty with bioplastics production processes from wastewater sludges was the optimization of low organic loading rates and also economically lower yields. This necessitates extensive experimentation on enhancement of organic loading rates and efficacy of utilizing mixed substrates (e.g., municipal sludge and agro-industry wastewater sludges) with mixed cultures. Overall cost of these processes will be sustainably reduced due to cheaper substrates and non-sterile reactors with little process control. Further, inherent culture stability and use of open fermentation eliminates the traditional bottlenecks of continuous monoseptic fermentations. Sludge to bioplastics could be a novel option with extended studies on applicability options and its potential beneficial effects and after effects, if any.

Biosurfactants

They are biologically synthesized surface-active agents (extracellular macromolecules) produced as metabolic by-products during microbial transformation of organic substrates. They are readily biodegradable and possess properties comparable to chemically produced surfactants and can be used for various applications such as in food, personal-care products and household/laundry detergents (Khaled et al. 1992; Kosaric et al. 1992). Wastewater sludge has been explored to produce biosurfactants by various routes by using *Bacillus licheniformis* and *Bacillus subtilis* bacteria as discussed by Gallert and Winter (2002).

Initial study on biosurfactants was limited to batch reactors and the substrates comprised molasses, used vegetable oil, and olive oil, representing food processing wastewaters as sole carbon source with the salt medium (Vipulanandan and Xen 1995). Subsequently, activated sludge was successfully used in producing biosurfactants from various organic substrates (carbon source) under non-aseptic conditions. Performance of the biosurfactants produced

from activated sludge using wastewater as carbon source was comparable to the biosurfactants produced using pure cultures under aseptic conditions with expensive pure organic substrates (Nitschke and Pastore 2003). Recently, a study on *Gordonia amarae*, a filamentous actinomycete found in foaming activated sludge wastewater treatment plants was investigated for its biosurfactant production capability. The maximum biosurfactant production was 3 folds the critical micelle concentration (CMC) with hexadecane as the sole carbon source, and 5 folds of CMC with the mixture of hexadecane and acetate (Pagilla et al. 2002).

However, the pathogenicity of certain microbial strains has been always off-putting the large scale production of biosurfactants. The toxicity and antigenic properties of mycobacterial glycolipids, produced by pathogenic mycobacteria such as *M. avium-intracellure, M. scrofulaceum,* and *M. fortulitum,* which are habitats of water polluted with industrial and domestic residues (Jardine et al. 1989) and methylrhamnolipids from *Pseudomonas aeroginosa* (Hirayama et al. 1982), are well known.

The biosurfactants production from wastewater sludge is in its infancy and more optimization of process parameters and selection of non-toxic strains would push this research area. They have great application in bioremediation of non-polar compounds which requires more studies on fate and transport of different compounds. Hence, production of biosurfactants mandates stricter scrutiny of the microbes as some bacterial taxa may be of public health concern. Furthermore, biosurfactants could be explored as a novel option for sludge management by use of high performing non-pathogenic strains like *Bacillus licheniformis*, *Bacillus subtilis* and others.

Bioflocculants

Bioflocculants production from low molecular fatty acids has also been developed as an innovative strategy for the utilization of waste sludge digestion liquor. A large number of bioflocculants were purified and reported to belong to functional proteins (Koizumi et al. 1991) or polysaccharides (Takagi et al. 1985; He et al. 2002, 2004). They have been widely used in chemical and mineral industrial fields such as wastewater treatment, dredging, downstream processing, fermentation and food industries. In particular, they are very useful in fermentation, brewing and food industries for harvesting microbial cells from broth as a substitute for centrifugation and filtration (Nakamura et al. 1976).

Several bioflocculants have been developed by using synthetic growth media which have efficient flocculating activities comparable to synthetic flocculants (Kurane et al. 1994; Lee et

al. 1995; Yokoi et al. 1998). However, the higher production cost owing to usage of expensive substrates viz. glucose and sucrose limits their wide use. This propelled studies on the screening of low molecular weight volatile fatty acid utilizing bioflocculating bacteria *Bacillus* sp. As-101 from wastewater sludge (Kim et al. 2000). The flocculating activity of these bacteria was stimulated by the addition of Al^{3+}, Fe^{3+} and Ca^{2+} in optimum concentrations of 0.2, 0.25 and 8 mM, respectively (Salehizadeh et al. 2000). *Citrobacter* sp. TKF04 was screened for producing a bioflocculant from acetic and propionic acids which was recovered by ethanol precipitation. The bioflocculant was capable of flocculating a kaolin suspension and many other suspended particles like diatomite, bentonite, activated carbon, soil and activated sludge, when added at a final concentration of 1-10 mg/L. The structure of the bioflocculant resembled chitosan like polymer (Fujita et al., 2001).

In spite of various studies carried out on production of bioflocculants, none of them have been practically applied in industry due to lower productivity and higher production costs. Utilizing sludge as a raw material for their growth and further optimizing the fermentation process (different process parameters) needs to be addressed seriously to tackle the scale-up issues. Additionally, each bioflocculant obtained from wastewater sludges must be characterized (ionic properties, structure) as this would serve as the basis for selection of process parameters to realize process optimization.

Biopesticides and Enzymes

Another encouraging step has been the utilization of biotechnology in the development of biopesticides from wastewater and wastewater sludge. Production cost of conventional biopesticides which is a major restriction in their commercial use (Lisansky et al. 1993) could be substantially reduced by using wastewater (sewage) sludge as a raw material. Production of *Bacillus thuringiensis* (Bt) based biopesticides has been very attractive (Tirado-Montiel et al. 1998; Sachdeva et al. 2000; Vidyarthi et al. 2002) option to replace chemical pesticides. In a batch culture, the optimization of different fermentation parameters viz. temperature, pH, agitation and aeration, volume and age of inoculum, sludge suspended solids concentration, C/N ratio and various pre-treatments methods of sludge indisputably allowed to achieve higher entomotoxicity (biopesticidal potential) in the final product (Lachhab et al. 2001; Tirado-Montiel et al. 2001; Vidyarthi et al. 2001; Vidyarthi et al. 2002; Tirado- Montiel et al. 2003). Pilot scale studies and formulation development have also reaped encouraging results (Yezza et al. 2004, 2005 a, b; Brar et al. 2004, 2005, 2006). In fact, comprehensive studies on different aspects of biopesticides production, namely, fermentation, downstream processing, formulation development and potential field application studies have shown positive results with immense potential for this alternative as presented in Fig. 5. Recently, Verma et al. (2005)

have also reported the use of wastewater (sewage) sludge as raw material for production of *Trichoderma* sp. based bioherbicides/biopesticides which have broad spectrum activity in comparison to Bt. This technology is giving encouraging results in bench scale fermenters too (data unreported). Two principal problems associated with the use of wastewater sludge as a sole raw material are the presence of toxic heavy metals and pathogens. As far as pathogens are concerned, the sludge is sterilized prior to biopesticide and enzymes fermentation where all types of pathogens are eliminated. The metals concentration in sludge is regulated by federal and provincial laws for agriculture, forestry and other use. Thus, sludge respecting the regulations is selected for fermentation. Another aspect is the actual quantity of sludge required for Bt growth and subsequently for application in forest or agriculture to control insects. The current application rate of the formulated Bt product to control spruce budworm in Canadian forests is about 30 BIU (billions of IU) per hectare (or 1.5 L of the product with potency 76B or 76BIU/gallon) (Valéro et al. 1999). The calculations based on research results which were based on relative units defined as spruce budworm units required 37.5 BSBU per hectare (or 1.9 L of the product with potency 76B or 76BIU/gallon). Thus, the amount of Bt fermented sludge to kill spruce budworm is approximately 65 g of Bt fermented sludge per hectare. This quantity is very low with sludge application permitted for agriculture land application (15-30 tons per hectare depending on N, P, K content) and hence metal contamination risk is almost nil.

Excess sludge obtained from municipal wastewater treatment plants has been studied for possibility of recovery of enzymes, namely, protease, amylase, glucosidase, lipase and dehydrogenase by dyno mill disruption (Jung et al. 2001). Protease exhibited a higher activity of 69% compared with other examined enzymes implying its potential application to enhance *in-situ* hydrolysis of protein in wastewater treatment (Jung et al. 2002). Despite the protease production, its economical separation remains a big challenge. Tyagi et al. (2001) and Lamontagne (2004) observed the possibility of production of enzymes like alkaline proteases in substantial concentration (4.58 IU/ml) simultaneously along with the Bt biopesticides. In fact, the production of proteases from wastewater sludge in pilot scale fermenters has also yielded positive results (Yezza et al., 2005 a, b, 2006). Knowledge of the types of enzymes – intracellular and extracellular and constitutive and/or induced will aid in the production of high yields substantiating the process engineering and economics. Molecular taxonomic studies using rRNA genes revealed that up to 80–90% of the microorganisms that exist in activated sludge cannot be grown using standard cultivation techniques (Amann et al., 1995; Gessesse et al., 2003). Because of this limitation, one cannot easily produce different enzymes in the laboratory for biochemical and molecular characterization.

Production of biopesticides from sludges warrants intensive investigation into development of alternative biopesticidal formulations (microencapsulations; dry powder and granules) for use in agriculture which are currently in progress in our laboratory at INRS-ETE. Simultaneous production of enzymes and biopesticides ropes in dual production resulting in further cost reduction. Enzyme production would be a novel venture as it would reduce the cost of detergents by usage of cheaper sludge derived products, at the same time, utilizing the entire fermented product in the deal.

Nutrient Source and Biofertilizers

The sludge can also be used in the recovery of nutrients i.e. phosphorus and nitrogen. Nitrogen is mainly present as ammonium and organic nitrogen. Unlike nitrogen, phosphorus is not an endless resource. The apatite mines known today will be empty within 150 years and we will have no reliable phosphorous sources left, stimulating phosphorus recovery from sludge.

Phosphorus is recovered from sludge by solubilization (more than 90% dissolves) which can serve as a fertilizer as an addendum with the conventional NPK fertilizer (Cassidy 1998; Hansen et. al. 2000). There is also possibility of producing calcium magnesium acetate by using residual biomass (Palasantzas and Wise 1994) from sewage sludge; this production mechanism would generate cost savings of 68% over conventional disposal costs. There have been some studies carried out in the late 80s on the possibility of extraction of fats, proteins and vitamins from sludge (Frost and Campbell 1986; Webber et al. 1986; Vriens et al. 1989; Webber 1990, 1991). All these possibilities sound interesting theoretically, but practically do not solve the problems of enormous quantities of sludge encountered in reality. No current studies have been reported on these issues.

Kim et al. (2002) studied the feasibility of sludge as micro-media with addition of clinoptilolite where the organic matter was removed with 95% efficiency. This also enhanced sludge settleability (clinoptilolite acted as floc seed) and nitrification by 90%, due to high concentration of nitrifiers attached to micro media. Likewise, dewatered and dried powdered sewage sludge when used as an alternative nutrient to yeast extract (YE) to promote the degradation of lipid materials by a thermophilic oxic process (TOP) gave degradation efficiency of 82.9% while that attained with YE was 68.3% (Nakano and Matsumora 2002). Traditionally, energy recovery and nutrient reuse from sewage sludge has been achieved via anaerobic digestion/power generation with land application of the biosolids. Nevertheless, pyrolysis can also accrue some benefits of nutrient recovery in the pyrolysis char along with the production of oil (energy) as reported by Bridle and Pritchard (2004). Laboratory soil

incubation studies using char in Australia were conducted over an eight-week period to confirm nutrient availability. Results showed that phosphorus in the char was plant available although the nitrogen was insoluble (Bridle and Pritchard 2004).

Research findings have also led to the utility of various types of wastewater sludges as raw materials for growth of *Rhizobium* (nitrogen fixers) inoculants (Ben Rebah et al. 2002a). The sludge based biofertilizers have been tested on plants such as soya and alfalfa at pilot plant levels and proven to be more efficient than the pre-existing ones with same symbiotic efficiency (nodulation and plant yield) as yeast mannitol broth based inoculants. The inoculation increased the nodulation indices (capacity to form nodules) from 4–6 to 8–12, and the rhizobial number from 10^3 (uninoculated soils) to 10^6–10^7 cells/g in inoculated soils. Salts and heavy metals content in sludges showed no deleterious effects on soil fertility (Ben Rebah et al. 2002b). Acid and alkaline treatment of sludges resulting in production of simpler substrates for the growth of bacteria enhanced the rhizobia production and utility of more sludges in terms of higher solids concentration (Rebah et al. 2001). Although wastewater sludge in itself was found to be a complete nutrient source, yet nutrient supplements like yeast extract and glycerol did increase the cell yield (Ben Rebah et al. 2002c). Additionally, wastewater sludge offered better protection for rhizobia survival during freezing and thawing at -20°C than the conventional medium as well as the suitability of sludge as a carrier which will aid in maintaining high numbers of rhizobia during storage conditions (Ben Rebah et al. 2002d). Hence, sludge could serve as a novel substrate as well as formulation adjuvant (in dry form) for production of biofertilizers nourishing the soil and plants.

However, unlike manufactured fertilizers, whose nutrient properties can be formulated for optimum application, treated sludges sprayed at agronomic rates (a rate equivalent to the amount of fertilizer nitrogen applied to the soil for the crop grown) to satisfy the requirement for one nutrient may cause the levels of other nutrients to be excessive or remain deficient. Also, the inorganic forms of nitrogen (nitrate and ammonium) are immediately available to the crop, whereas, organic forms are not available to the crop and must first be mineralized by microorganisms to inorganic forms. If organic nitrogen mineralization is not properly accounted for, overfertilization may occur that will subsequently lead to nitrogen leaching. Therefore, taking into account the possible hurdles to account for probable commercialization of nutrient sources, serious and concerted efforts are required. Long term studies need to be carried out on probable leachability of toxics and heavy metals from soils comprising these biofertilizers.

Biosorbents

Sewage sludge is carbonaceous in nature and rich in organic materials. Hence, it has the potential to be converted into activated carbon, on pyrolysis under controlled conditions or with some chemical treatment. This conversion could offer the combined benefits of reducing the volume of sludge and producing a valuable adsorbent with lower cost than commercial activated carbon (Jeyaseelan and Lu 1996, Martin et al. 1996).

Conventionally, adsorbent materials from sewage sludge were produced by chemical activation by H_2SO_4 impregnation followed by pyrolysis. The parent sludges were initially oven dried at 105°C to constant mass and then subjected to chemical activation by impregnating with H_2SO_4. The ensuing activated sludges were then pyrolysed under inert nitrogen, and subsequently washed with dilute HCl (10% by mass).These adsorbent particles were ground to desired particle sizes of greater porosity and higher surface area (Otero et al., 2003). Flow melting of sludge ash into glass like material due to presence of silicon oxide and aluminium oxide has been also used for development of adsorbents for heavy metal removal or as liner in landfills (Pan and Tseng 2001). In general, similar to fly ash and blast-furnace slag, the SSA can also be reused as an adsorbent for copper removal from wastewater with a removal efficiency of 98% (Pan et al. 2003).

Alternate method of activated carbon preparation involved activation of anaerobically digested sewage sludge with 5 M $ZnCl_2$ followed by pyrolysis at 500°C for 2 h under nitrogen atmosphere. It was found that this method improved the properties viz. surface area and pore size distribution, elemental composition and ash content, surface chemistry structure and surface physical morphology and it turned out to be a cost effective option in comparison to existing conventional methods (Chen et al. 2002).

Sewage sludge-derived fertilizer, Terrene, was also used as a precursor of adsorbents tested for removal of hydrogen sulfide from moist air. The highest H_2S removal capacity was obtained for the sample carbonized at 950°C (Bagreev and Bandosz 2002).

Trials have also shown that the specific surface of sludge based adsorbents was lower than the commercial activated carbon, but they acted in the similar manner (Hagstorm et al. 1997). Sewage sludge can be conveniently used in the development of adsorbents by pyrolysis and chemical activation, which can play an important role in the removal of many adsorbates like, dyes, organic pollutants and many others. Further studies need to be carried out to study the synergistic/antagonistic effect of pollutants and also to ascertain the probable adsorptive

behaviour at molecular level (Otero et al. 2001). There have been other means of producing adsorbents by mixing with organic polymers to yield stable products, termed as composite adsorbents. Composite adsorbents have also been considered as a viable option by mixing water plant sludge with phenolic resin having the ratio of 1:1, 1:2, and 1:3 respectively, curing from 100°C to 170°C under N_2 atmosphere, and then activating with N_2 at 700°C. These adsorbents presented very promising total organic carbon removal efficiency of 98% and 32% for NH_4^+ salts, which was identical to commercial activated carbon (Myung and Kim 2001).

A study in the same context utilized four steps of adsorbent generation from sewage sludge viz. drying at 105°C, drying and pyrolyzing, drying and chemical activation. Although surface area corresponding to pyrolyzed and chemically activated sludge was higher (80-390m^2/g), yet dried sludges showed better adsorption of the adsorbate, methylene blue (Calvo et al. 2001) and safranin (Rozada et al. 2003). The sludge biomass can also be used directly for adsorption of dyes and it was found that the activation energy for the same process was 1.45 KCal/mol and kinetics was controlled by intraparticle diffusion (Chu and Chen 2002). Also, it has been found that sludge derived activated carbon performed better when removing dyes with a higher presence of anionic solubilizing groups and heavy metals (Martin et al. 2002). It was reported in one of the studies that methylene blue adsorption occurred faster than that of safranin, and it was preferably adsorbed when treating binary solutions (Rozada et al. 2003). Other similar studies have reported faster removal of dyes than organics. Otera et al. (2003) found that crystal violet adsorption had been higher and faster than indigo carmine or phenol. Another investigation by Annadurai et al. (2003) examined the adsorption capacity of an adsorbent for synthetic dye, Rhodamine 6G, derived from microwave treated activated sludge. Energy cost analysis performed in this study demonstrated the feasibility of applying the proposed microwave process for production of sludge based adsorbents.

Wastewater sludges have also been used as a carbon source for odorous gas treatment via adsorption and for flue gas treatment via desulfurization, albeit both with limited application (Krogmann et al. 1997). Digested sludge has been used as an adsorbent in many of the studies to remove dyes from water, and it was found that the specific surface area of the adsorbent was quite good as much as 82-150 m^2/g (Weng et al. 2001; Weng et al. 2003). Similar to powdered activated carbon being conventionally used for adsorption of dyestuffs, Kargi and Ozmihci (2004) explored the probability of acid washed powdered activated sludge (PAS) in shake flask tests for the removal of six different dyestuffs by adsorption. Only one dye was adsorbed in superior amounts (Direct Yellow 12) and otherwise all adsorptions profiles followed Freundlich isotherm. It was found that that PAS had a good adsorption capacity for large

molecular weight compounds and limited removal efficiency of smaller molecules such as phenol (Martin et al. 2004).

A study was conducted using waste anaerobic dead biomass (WADB) for the uptake of Pb (II), Cr(VI), Cu(II), Ni(II) and Zn(II). Metal absorptive capacity tests were evaluated at 25°C and pH of 4 and Pb (II) was adsorbed with the highest capacity fitting well into the Langmuir adsorption isotherm (Haytoglu et al. 2001). Zhai et al. (2004) also reported that WADB offered several advantages like high surface-to-volume ratios and anionic cell walls; no requirement of nutrients and easy availability.

Thus, many studies have investigated the biosorption of heavy metals using activated sludge, fungi, algae and yeast as biosorbent, in either living or non-living forms (Crist et al. 1990, 1992; Gourdon et al. 1990; Aksu et al. 1990; Zhou and Kiff 1991; Battistoni et al. 1993; Cordor and Reeves 1994; Churchill et al. 1995; Imai and Gloyna 1996; Yang and Volesky 1996; Butter et al. 1998; Kapoor and Viraraghavan 1998; Matheickal et al. 1999; Zhai et al. 2004). The effect of metals and chemical pre-treatment on biosorption has also been well established (Mueller and Steiner 1992; Bhattacharya et al. 1995; Alkan et al. 1996; Fang 1997; Lin and Chen 1997; Leighton and Forsters 1997). Many biosorbents suffer from the problems of leaching of organic matter and metals, however, this can be overcome to a small extent if sodium and calcium alginate immobilized sludge is used for the same purpose (Chen et al. 2002). But, all these studies for metal removal ignore effects of competitive inhibition and complex antagonism and most pertain to laboratory batch tests.

It could be inferred that sewage sludge-based activated carbon may be promising for dye removal from aqueous streams, but it remains to be ascertained as to which type of adsorbent can treat all wastes/pollutants (e.g. waste gas streams). Despite abundance of literature on adsorbent production from sludges and the feasibility analysis, this mode of value addition produced mixed results with organics and other pollutants, especially, waste streams removal and most results were an outcome of batch studies raising questions on their practical application. Hence, extensive studies need to be carried out to understand the synergistic/antagonistic effect of each pollutant with the adsorbent and its repercussions on the environment (secondary disposal/reuse options). Meanwhile, composite adsorbents gave better sorption performance, but cost factor was high which could be decreased by considering biosorbents.

Miscellaneous

Animal feed

Many studies in the early 1980s proposed the possibility of utilization of sewage sludges as animal feed. Amino acid analyses indicated that activated and trickling-filter sludges contained adequate amounts of amino acids required for normal avian and mammalian nutrition, with the possible exceptions of sulphur containing acids, methionine and cystine (Kavanagh et al. 1982). Activated sludges compared well with soyabean meal and fish meal in terms of protein content (Tacon 1979a; Tacon et al. 1979b). Some studies indicated that sewage sludge water extracts could serve as excellent nutrient source for algal growth (Wong 1977; Yip and Wong 1978). This was prompted by the fact that extensive usage of algae in oxidation ponds resulted in efficient removal of nitrogenous and phosphorus compounds. But, this application was limited by the processing costs in terms of separation processes involved at final harvesting stage. There were some novel methods of self-filtration of algae by predators in the aquatic system, but then this imposed the problem of heavy metal flow across the food chain leading to biomagnification (Suffern et al. 1981).

Utilization of photosynthetic bacteria from agro-industrial waste as well as activated sewage sludge has been reported to produce SCP (single cell protein) (Noparatnaraporn et al. 1986; Shier and Purwono 1994) which can subsequently enrich feedstock with a supplementary protein source together with valuable vitamin sources. A study well documented in literature evaluated nutrition and use of various waste materials as substrates in biomass production suitable for animal feed and human consumption (Goldberg 1985; Vriens et al. 1989) with various points of interrogation on subsequent food chain transfer.

Hung et al. (1996 a,b) studied sludge grown algae for aquaculture utility in two stages – growth of algae in sludge extracts and as feed for aquatic organisms. They found that sludge grown algae contained substantial amount of heavy metals (Zn, Cu, Ni, Mn, Cr and Fe). On feeding these algae to aquatic organisms, the body weights of zooplanktons, next in the chain showed no considerable increase, however, when fed directly to fish and shrimp, body weight increased by 7 and 11%, respectively with an increase in heavy metal concentrations too. Nevertheless, heavy metal concentration in the higher trophic levels was less owing to different body elimination mechanisms. A similar study enumerating the effect of heavy metal contaminated domestic sewage sludge on young male Wistar rats was tested by supplementing dehydrated activated sludge in their diet at concentrations of 5, 10, 15 and 20% and enzyme activities noted in different organs. It was observed that metals had adverse impacts on different enzyme systems causing biomagnification (Bag et al. 1999).

There have also been attempts made on feeding activated sludge to pigs, sheep and steers, in addition to chickens (Vriens et al. 1989). But, there were no significant differences in the body weights between control and sludge based diet. This suggested that mixing sewage sludge with conventional diets was feasible. However, problems associated with pathogens and heavy metals restricted wide application of this mode of sludge reutilization. Micro-algae grown on sewage sludge extract have been investigated to feed freshwater shrimps in aquaria (Wong and Cheung 1985). Harvested algae have been also used as proteins and animal or fish or poultry food, and mechanical paddles and other different techniques have been used to improve algal production and pond performance in India, California and Singapore (Polprasert 1989).

In one of the studies, pig-farm wastes which normally find its way into the sewage sludge was managed at source through reutilization again in the swine farms. Recovered swine manure solids were combined with milled sorghum and fermented. The fermented product was mixed with a nutritious swine supplement and when fed directly to the pigs, gave 16·5% savings of grain in fattening pigs (Covarrubias et al. 1994; Polprasert et al. 1994).

Utilization of sewage sludge as an animal feed did not yield any long term positive results and hence the studies have been restricted only to certain regions of the world and as such, there is no recent literature on the same. This could be due to the fact that there are other cheap sources of nutrients like agro-industry residues (Montgomery 2004) which could serve as better nutrient supplements without any compromise on pathogenic and metal toxicity (bioamplification in food chain). Thus, animal feed use of sewage sludge may not be of direct interest due to its long lasting biomagnification effects, however, there is a possibility of research in the field of production of algae which can serve as a potential feedstock.

Electricity

Producing electricity from microbial degradation of wastewater sludges has been considered as another route for beneficial use of sludge. Many different bacterial species including *Escherichia, Shewanella, Clostridum,* and *Desulfovibrio* have been reported to reduce metallic ions (e.g. manganese, ferric, uranium, and cupric) while oxidizing the available carbon substrates by redox mechanism (Lovley 1993). Sewage sludge can actually serve as a better biocatalyst completely oxidizing organics to carbon dioxide. Improved redox efficiencies and current densities obtained provide enough power to run sensing or telecommunications equipment in remote locations (Park and Zeikus 2003). A recent study in this context was

carried out by Dentel et al. (2004) by using a reactor with graphite foil electrodes in an aerated aerobic and anaerobic sludge zone, electrical current was generated, and enhanced when an additional organic substrate (acetate) was added. Given the demonstration of electricity generation from sludge, the potential for similar applications, using other organic waste sources, is possible.

Secondary Elemental Metabolites

There has been some research carried out on the possibility of production of secondary metabolites whereby sulfate reducing bacteria (SRB) can utilize digested sewage sludges as their carbon source to reduce flue gas desulfurization (FGD) gypsum to hydrogen sulfide. The sulfide was eventually oxidized to elemental sulfur via reaction with ferric sulfate, and accumulated calcium ions were eventually precipitated to calcium carbonate by using carbon dioxide (two important marketable products). This could also be referred to as substantial value-addition of sludge by conversion of another waste by one i.e. form a secondary value added product enroute conversion of another waste (Kaufman et al. 1996).

Conclusions

Stricter environmental regulations and adverse health and environmental impacts of disposal practices, namely, incineration and landfilling has catalyzed the value-addition strategy of sludge management. Although odour, pathogens and heavy metal presence has been always halting the pace of sludge reuse, these factors have been reduced and/or confined to acceptable levels by improvement /or optimization of production process. The value-addition of sludge has resulted in development of various products with some being commercialized like engineered soil, ornamental horticultural fertilizer, turf grass, synthetic coal and activated carbon. Development of products like construction materials and fuels are showing promising trends with further improvement in technologies to control metal leaching and/or toxic gaseous emissions. Likewise, biotechnology products, in particular, biopesticides, have been produced and formulated with great potential for field application. Similar studies are in progress for enzymes and bioplastics production. However, few products, namely, biosurfactants and bioflocculants are still at natal stage and their productivity could be improved after a complete understanding of the production process parameters by isolating and characterizing the respective microbial strains. Thus, production of bioflocculants and biosurfactants from wastewater sludge represents grey area of research due to their possible application in environmental clean-up i.e. bioremediation. In this respect, value-addition of wastewater sludge is an environmentally and socially useful venture.

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. The views and opinions expressed in this article are those of authors. We are also thankful to Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Service and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing Ph.D. postgraduate scholarship to Satinder K. Brar.

Notations used in this text:	
ADB	Anaerobic dead biomass;
CMC	Critical micelle concentration;
COD	Chemical oxygen demand;
FGD	Flue gas desulfurization;
LPG	Liquified Petroleum Gas;
NPK	Nitrogen, phosphorus, potassium;
OFS	Oil from sludge;
PHA	Polyhydroxyalkanoate;
PHB	Poly-β-hydroxybutyric acid;
PAS	Powdered activated sludge;
SAI	Strength activity index;
SBU	Spruce budworm units
SCP	Single cell protein;
STF	Sludge to fuel;
SSA	Sewage sludge ash;
SRB	Sulfate reducing bacteria;
TOP	Thermophilic oxic process;
Tx	Entomotoxicity
WADB	Waste anaerobic dead biomass and;
YE	Yeast extract

References

Abu-Orf, M.M., and Jamrah, A.I. (1995). "Biosolids and sludge management". *Water Env. Res.*, 67(4), 481-487.

Abu-Orf, M.M., Griffin, P., and Dentel, S.K. (2001). "Chemical and Physical pretreatment of ATAD biosolids for dewatering". *Water Sci. Technol.*, 44(10), 308-314.

Aksu, Z., Sag, Y., and Kutsal, T. (1990). "A comparative study of the adsorption of Cr (VI) ions to *C. vulgaris* and *Z. ramigera*". *Env. Technol.*, 11, 33-40.

Alkan, U., Anderson, G.K., and Ince, O. (1996). "Toxicity of Cr (III) in the anaerobic digestion process". *Water Res.*, 30(3), 731-741.

Amann R.I, Ludwig, W., and Schleifer, K.H. (1995). "Phylogenetic identification and in situ detection of individual microbial cells without cultivation". *Microbiol Rev,* 59, 143–69.

André, P. (1999). "Ecological risks of the use of sewage sludge as fertilizer in soil restoration: effects on the soil microarthropod populations". *Land Degrad. Develop.*, 10, 67-77.

Anderson, M.(2002). "Encouraging prospects for recycling incinerated sewage sludge ash (ISSA) into clay based building products". *J. Chem. Technol. Biotechnol.*, 77, 352-360.

Anderson. M., Skeratt, R.G., Thomas, J.P., and Clay, S.D. (1996). "Case study involving fluidised bed incinerator sludge ash as a partial clay substitute in brick manufacture". *Water Sci. Technol.*, 34(3/4), 507-515.

Anderson A.J., and Dawes E.A. (1990). "Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates." *Microb Rev.,* 54(4), 450– 472.

Andreoli, C.V., Castro, L.A.R., Picinatto, A.G., and Pegorini, E.S. (2001). "Effect of the employment of biosolids as recovery agent for degraded areas". Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Mexico,Oct. 25-27, 410-417.

Annadurai,G., Juang, R.S., Yen, P.S., and Lee, D.J. (2003). "Use of thermally treated waste biological sludge as dye absorbent". *Adv. Environ.Res.*, 7(3), 739-744.

Avenell, C.S., Sainz-Diaz, C., and Griffiths, A. (1996). "Solid waste pyrolysis in a pilot-scale batch pyrolyser". *Fuel*, 75, 1767–74.

Bag, S. Vora, T. Ghatak, R. Nilufer, I. D'Mello, D. Pereira, L. Pereira, J., Cutinho, C., and Rao V. (1999). "A study of toxic effects of heavy metal contaminants from sludge-supplemented diets on male-Wistar rats". *Ecotoxicol. Environ. Safety*, 42, 163-170.

Bagreev, A., and Bandosz, T.J. (2002). "H_2S adsorption/oxidation on materials obtained using sulfuric acid activation of sewage sludge-derived fertilizer". *J. Colloid and Interface Science*, 252, 188–194.

Bartl, B., Hartl, W., and Horak, H. (2002). "Long term application of biocompost versus mineral fertilisation: effects of the nutrient and heavy metal contents of soil and plants". *J. Plant Nutr. Soil Sci.*, 165,161-165.

Battostoni, P., Fava, G., and Ruello, M.L. (1993). "Heavy metal shockload in activated sludge uptake and toxic effects". *Water Res.*, 27(5), 821-827.

Beccari, M., Dionisi, D., Giuliani, A., Majone, M., and Ramadori, R. (2002). "Effect of different carbon sources on aerobic storage by activated sludge". *Water Sci. Technol.,* 45, 157– 168.

Ben Rebah, F., Tyagi, R. D., and Prévost, D. (2001). "Acid and alkaline treatments for enhancing the growth of rhizobia in sludge". *Can. J. Microbiol.,* 47, 467–474.

Ben Rebah, F., Tyagi, R. D., Prévost, D., and Surampalli, R. Y. (2002a). "Wastewater Sludge as a new medium for rhizobial growth". *Water Qual. Res. J. Canada*, 37(2), 353-370.

Ben Rebah, F., Prévost, D., and Tyagi, R. D. (2002b). "Growth of Alfalfa in sludge-amended soils and inoculated with Rhizobia produced in sludge". *J. Environ. Qual.,* 31, 1339-1348.

Ben Rebah, F., Tyagi, R.D., and Prévost, D. (2002c). "Production of *S. Meliloti* using wastewater sludge as a raw material: effect of nutrient addition and pH control." *Environ. Technol.*, 23 (6), 623-629.

Ben Rebah, F., Tyagi, R.D., and Prévost, D. (2002d). "Wastewater sludge as a substrate for growth and carrier for rhizobia: the effect of storage conditions on survival of *Sinorhizobium meliloti*." *Bioresource Technol.*, 83 (2), 145-151.

Benton, and M.W., and Wester, D.B. (1998). "Biosolids effect on tobosagrass and alkali sacaton in Chihuahuan desert grassland". *J. Environ. Qual.*, 27, 199-208.

Beun, J.J., Dirks, K., Van Loosdrecht, M.C.M., and Heijnen, J.J. (2002). "Poly-(hydroxybutyrate) metabolism in dynamically fed mixed microbial cultures". *Water Res.*, 36, 167– 180.

Bhattacharya, S.K., Leslie, R.E., and Madura, R.L. (1995). "Effects of bioavailable cadmium on anaerobic systems". *Water Env. Res.*, 67(7), 1092-1094.

Biosolids Management Update (1998). *BioCycle,* January.

Black and Veatch (1995). Wastewater Biosolids and Water Residuals: Reference manual on Conditioning, Thickening, dewatering and drying. Electric Power Research Institute, St.Louis.

Boon, A., and Thomas, V. (1996). "Resource or rubbish"? *The Chemical Engineer*, 25–30.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., and Banerji, S. (2004). "Development of sludge based stable aqueous *Bacillus thuringiensis* formulations". *Water Sci. Technol.,* 50(9), 229-236.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2005). "Starch industry wastewater based stable *Bacillus thuringiensis* liquid formulations". *Journal of Economic Entomology*, 98(6), 1890 - 1898.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2006). "Screening of different adjuvants for Wastewater/Wastewater sludge based *Bacillus thuringiensis* formulations." *Journal of Economic Entomology,* 99 (4), 1065 - 1079.

Brautlecht, P., and Gredigk, S. (1998)."Concept for an interlinked system of a sludge drying facility and a landfill for residual waste". *Water Sci. Technol.*, 38(2), 119-125.

Brdjanovic, D,. Van Loosdrecht, M.C.M., Hooijmans, C.M., Mino, T., Alaerts, G.J., and Heijnen, J.J. (1998). "Effect of polyphosphate limitation on the anaerobic metabolism of phosphorus accumulating microorganisms". *Appl. Microbiol. Biotechnol.* , 50, 273– 6.

Bridle, T., and Skrypsi-Mantele, S. (2000). "Assessment of sludge reuse options: a life cycle approach". *Water Sci. Technol.*, 44(10), 41-46.

Bridle, T.R., and Campbell, H.W. (1984). Conversion of sewage sludge to liquid fuel. 7th Annual AQTE Conference, Montreal, Quebec.

Bridle, T.R., and Pritchard, D. (2004). "Energy and nutrient recovery from sewage sludge via pyrolysis". *Water Sci. Technol.*, 50 (9), 169–175.

Butter, T.J., Evison, L.M., Hancock, I.C., Holland, F.S., Matis, K.A., Philipson, A., Sheikh, A.I., and Zoubolis, A.I. (1998). "The removal and recovery of cadmium from dilute aqueous solutions by biosorption and electrolysis at laboratory scale". *Water Res.*, 32(2), 400-406.

Calvo, L.F., Otero, M., Moran, A., and Garcia, A.I. (2001). "Upgrading sewage sludges for adsorbent preparation by different treatments". *Biores. Technol.*, 80(2), 143-148.

Campbell, H.W. (1990). "Converting sludge to fuel—a status report." In: Hogan et al., editor. Biomass Thermal Processing, Proceedings of the 1st Canada/European Community R and D Constructors Meeting, Ottawa, Canada, 1990, 78–85.

Cassidy, S. (1998). Recovery of valuable products from municipal wastewater sludge. In: Chemical Water and Wastewater Treatment V (eds. H. Hahn, E. Hoffmann and H., Odegaard)

Chau, H., and Yu, P.H.F. (1999). "Production of biodegradable plastics from chemical wastewater: a treatment". *Water Sci. Technol.*, 39, 273– 280.

Chen, J.P., Lie, D., Wang, L., Wu, S., and Zhang, B. (2002). "Dried waste activated sludge as biosorbents for metal removal: adsorptive characterization and prevention of organic leaching". *J. Chem. Technol. Biotechnol*, 77, 657-662.

Chen, X., Jeyaseelan, S., and Graham, N. (2002). "Physical and chemical properties study of the activated carbon made from sewage sludge". *Waste Management*, 22, 755 –760.

Chu, H.C., and Chen, K.M. (2002). "Reuse of activated sludge biomass: The rate processes for the adsorption of basic dyes on biomass". *Process Biochem.*, 37(10), 1129-1134.

Churchill, S.A., Walters, J.V., and Churchill, P.F. (1995). "Sorption of heavy metals by prepared bacterial surfaces". *J. Env. Eng.,* 121(10), 706-711.

Corder, S.L., and Reeves, M. (1994). "Biosorption of nickel in complex aqueous waste streams by cyanobacteria". *Appl. Biochem. Biotech.*, 45-46, 847-860.

Covarrubias, G.I., Cabrera, A.R., and Gómez, M.D.J.F. (1994). "Continuous solid-substrate fermentation of swine waste recovered solids for pig feed". *Biores. Technol.* , 50 (2) , 139-147.

Council Directive, 1999/31/EC of 26 April 1999 on the landfill of waste. Official Journal L 182, 16/07/1999, 1–19.

Crist, R.H., Robert, M.J., Guptill, P.W., Eslinger, J.M. and Crist, DeLanson R. (1990). "Interaction of metals and protons with algae. 2. Ion exchange in adsorption and metal displacement by protons." *Environmental Science and Technology*, 24(3), 337-342.

Crist R.H., Oberholser, K., McGarrity, J., Crist, D.R., Johnson, J.K., Brittsan J.M. (1992). Interaction of metals and protons with algae. 3. Marine algae, with emphasis on lead and aluminum. *Environ. Sci. Technol.,* 26 (3), 496-502.

Cusido, J.A., Cremades, L.V., and Gonzalez M. (2003). "Gaseous emissions from ceramics manufactured with urban sewage sludge during firing processes". *Waste Management*, 23, 273–280.

Dentel, S.K., Strogen , B., and Chiu, P. (2004). "Direct generation of electricity from sludges and other liquid wastes". *Water Sci. Technol.,* 50(9), 161–168.

Dichtl, N. (2003). "New objectives of sludge treatment considering a safe sludge disposal". *Chem. Eng.*, 26(2), 223-228.

Dionisi, D., Majone, M., Tandoi, V., and Beccari, M. (2001). "Sequencing batch reactor: influence of periodic operation on physiological state, microbial composition and performance of activated sludge in biological wastewater treatment". *Ind. Eng. Chem. Res.*, 40, 5110– 5119.

Dionisi, D., Majone, M., Papa, V., and Beccari, M. (2004). "Biodegradable polymers from organic acids by using activated sludge enriched by aerobic periodic feeding". *Biotechnol. and Bioeng.*, 85(6), 569-579.

Doi, Y., Kunioka, M., Nakamura, Y., and Soga, K. (1987). "Biosynthesis of copolyesters in *Alcaligenes eutrophus* H16 from ^{13}C-labeled acetate and propionate". *Macromol.*, 20, 2988–2991.

Fang, H.H.P. (1997). "Inhibition of bioactivity of UASB biogranules by electroplating metals". *Pure Appl. Chem.*, 69(11), 2425-2429.

Fresquez, P.R., Francis, R.E., and Dennis, G.L. (1990). "Soil and vegetation responses to sewage sludge on a degraded semi arid broom snakeweed/blue grama plant community". *J. Range Manag.*, 43, 325-331.

Frost, R.C., and Campbell, H.W. (1986). Alternative uses of sewage sludge. In: Processing and use of organic sludge and liquid agricultural wastes. P.L. Hermite (Eds.), EUR 10361, D. Reidel Publishing Co., pp. 94-109.

Fujita, M., Ike, M., Jang, J.H., Kim, S.M., and Hirao, T. (2001). "Bioflocculation production from low molecular fatty acids as a novel strategy for utilization of sludge digestion liquor". *Water Sci. Technol.*, 44(2-3), 237-243.

Gallert, C., and Winter, J. (2002). "Solid and liquid residues as raw materials for biotechnology". *Naturwissenschaften*, 89(11), 483 – 496 (in English).

Garland, G., Grist, T., and Green, R. (1995). "The compost story: from soil enrichment to pollution remediation. *BioCycle*, 36, 53-56.

Gaskin, J.G., Brobst, R.B., Miller, W.P., and Tollner, E.W., (2003). "Long-term biosolids application effects on metal concentration in soil and bermudagrass forage." *J. Environ. Qual.*, 32, 146–152.

Gessessea, A., Dueholmb, T., Petersena, S.B., and Nielsenb, P.H. (2003). "Lipase and protease extraction from activated sludge". *Water Res.*, 37, 3652–3657

Gerba, C.P., Pepper, I.L., and Whitehead III, L.F. (2001). A risk assessment of emerging pathogens of concern in the soil and application of biosolids. Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Mexico, Oct. 25-27, 457-463.

Goldberg, I. (1985). Single Cell Protein. Springer Verlag, New York.

Gourdon, R., Pus, E., Bhende, S., and Sofer, S.S. (1990). "A comparative study of cadmium uptake by free and immobilised cells from activated sludge". *J.Env. Sci. Health*, A25(8), 1019-1036.

Gutierrez, E.C., Acosta, Y., Ramirez, E., Lopez, J., Moral, R., Fernandez, N., and Marmol, Z. (2001). Sewage sludge application to increase soil fertility. Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Mexico, Oct. 25-27, 434-439.

Hagstorm, L., Jonsson, L.E., Magnusson, P., Bouvman, R., Cassidy, S., Hanssen, B., Karlsson, I., and Patterson, L. (1997). *Fosfor och energi aur avloppsslam.* Rapport 4822, Swedish Environmental Protection Agency.

Hall, J.E., and Dalimier, F. (1994). Waste management—sewage sludge: survey of sludge production, treatment, quality and disposal in the EC. EC Reference No: B4-3040/014156/92, Report No: 3646.

Hansen, B., Karlsson, I., Cassidy, S., and Patterson, L. (2000). "Operational experiences from a sludge recovery plant". *Water Sci. Technol.*, 41(8), 23-30.

Haytoglu, B., Demirer, G.N., and Yetis, U. (2001). "Effectiveness of anaerobic biomass in adsorbing heavy metals". *Water Sci. Technol.*, 44(2-3), 245-252.

He , N., Li, Y., Chen, J., and Lun, S.Y. (2002). "Identification of a novel bioflocculant from a newly isolated *Corynebacterium glutamicum". Biochem. Eng. J.*, 11(2-3), 137-148.

He , N., Li, Y., and Chen, J. (2004). "Production of a novel polygalacturonic acid bioflocculant REA-11 by *Corynebacterium glutamicum". Biores. Technol.*, 94(1), 99-105.

Hirayama, T., and Kato, I. (1982). "Novel methyl rhamnolipids from *Pseudomonas aeruginosa*". *FEBS Lett.*, 139(1), 81–85.

http://www.environ.com.au/enersludge.shtml. Typical Enersludge process™ - Product and energy yields, cited 16 May, 2004.

Hu, W.F., Chua, H., and Yu, P.H.F.(1997). "Synthesis of poly-(hydroxy butyrate-co-hydroxyvalerate) from activated sludge". *Biotechnol. Lett.*, 19, 695– 698.

Huang, C., Pan, J.R., Sun, K.D., and Liaw, C.T. (2001). "Reuse of water treatment plant sludge and dam sediment in brick making". *Water Sci. Technol.*, 44(10), 273-277.

Huang, J.Y.C. (1986). "Market potential for sludge compost product". *J.Env. Eng.*, 112(3), 454-467.

Hudson , J.A., and Lowe, P. (1996). "Current technologies for sludge treatment and disposal". *J CIWEM*, **10**, 436–440.

Hung, K.M., Chiu, S.T., and Wong, M.H. (1996a). "Sludge grown algae for culturing aquatic organisms: Part I. Algal growth in sludge extracts". *Environ. Mgmt.*, 20(3), 361-374.

Hung, K.M., Chiu, S.T., and Wong, M.H. (1996b). "Sludge grown algae for culturing aquatic organisms: Part I. Sludge grown algae as feeds for aquatic organisms". *Environ. Mgmt.*, 20(3), 361-374.

Imai, A., and Gloyna, E.F. (1996). "Speciation of Cr(III) in activated sludge". *Water Env. Res.*, 68(3), 301-310.

Itoh, S., Suzuki, a., Nakamura, T., and Yokoyama, S. (1994). "Production of heavy oil from sludge by direct thermochemical liquefaction". *Desalination*, 98(1-3), 127-133.

Jardine, I., Scanlan, McNeil, M., and Bennan, P. J. (1989). "Plasma desorption mass spectrometric analysis of mycobacterial glycolipids". *Anal. Chem.*, 61(5), 416–422.

Jaeger, M., and Mayer, M. (2000). "The Noell Conversion process- a gasification process for the pollutant free disposal of sewage sludge and the recovery of energy and materials". *Water Sci. Technol.*, 41(8), 37-44.

Jeyaseelan, S., and Lu, G.Q. (1996). "Development of adsorbent/catalyst from municipal wastewater sludge". *Water Sci. Technol.*, 34, 499–505.

Jung, J., Xing, X.H., and Matsumoto, K. (2002). "Recoverability of protease released from disrupted excess sludge and its potential application to enhanced hydrolysis of proteins in wastewater". *Biochem. Eng. J.*, 10, 67–72.

Jung, J., Xing, X.H., and Matsumoto, K. (2001). "Kinetic analysis of disruption of excess activated sludge by Dyno mill and characteristics of protein release for recovery of useful materials". *Biochem. Eng. J.*, 8, 1–7.

Kapoor, A., and Viraraghavan, T. (1998). "Removal of heavy metals from aqueous solutions using immobilised fungal biomass in a continuous mode". *Water Res.*, 32(6), 1968-1977.

Kargi, F., and Ozmihci, S. (2004). "Biosorption performance of powdered activated sludge for removal of different dyestuffs". *Enzyme Microbial Technol.*, 35, 267–271.

Kaufman, E.N., Little, M.H., and Selvaraj, P.T. (1996). "Recycling of FGD gypsum to calcium carbonate and elemental sulfur using mixed sulfate reducing bacteria with sewage digest as carbon source". *J.Chem. Technol.Biotechnol.*, 66(4), 365-374.

Kavanagh, B. V., Herbert, L. S., and Moodie, S. P. (1982). "Biological sludges as animal feed supplements – – analysis and composition of sludges". *Agricultural Wastes* 4 (4), 305-315.

Kessler, B., Weusthuis, R., Witholt, B., and Eggink, G. (2001). "Production of microbial polysters: fermentation and downstream processes". *Adv. Biochem. Eng./Biotechnol.*, 71, 159-182.

Khaled, N., Montet, D., Farines, M., Pina, M., and Graille, J. (1992). "Synthèses des monoesters de sucre par biocatalyse". *Oleagineux*, 47(4), 181-189.

Kizilkaya, R. (2004). "Cu and Zn accumulation in earthworm *Lumbricus terrestris* L. in sewage sludge amended soil and fractions of Cu and Zn in casts and surrounding soil". *Ecol. Eng.*, 22, 141–151

Kim, S.M., Ike, M., Tachibana, S., Kitada, G., Hirao, T., and Fujita, M. (2000). "Screening bacteria capable of producing bioflocculants from acetic and propionic acids". *Japan J. Water Treat. Bio*l., 36, 183-192.

Kim, Y.B., and Lenz, R.W. (2001). "Polyesters from microorganisms". *Adv. Biochem. Eng./Biotechnol.*, 71, 51-79.

Kim, C.G., Lee, H.S., and Yoon, T.I. (2002). "Resource recovery of sludge as a micro-media in an activated sludge process". *Adv. in Environ. Res.*, 7(3), 629-633.

Koizumi, J.I., Takeda, M., Kurane, R., and Nakamura, J.(1991). "Synergetic flocculation of the bioflocculant FIX extracellularly produced by *Nocardia amarae*". *J. Gen. Appl. Microbiol.*, 37, 447–454.

Kosaric, N. (1992). Biosurfactants in industry. *Pure and Appl. Chem.*, 64(11), 1731-1737.

Krogmann, U., Boyles, L.S., Martel, C.J., and McKomas, C.A. (1997). "Biosolids and sludge management". *Water Environmental Research*, 69(4), 534-550.

Kumar, M.S., Mudliar, S.N., Reddy, K.M.K. and Chakrabarti, T. (2004). Production of biodegradable plastics from activated sludge generated from a food processing industrial wastewater treatment plant. *Biores. Technol.*, 95(3), 327-330.

Kurane, R., Hatamochi, K., Kutano, T., Kiyohara, M., Hirao, M., and Taniguchi, Y. (1994). "Production of a bioflocculant by *Rhodococcus erythropolis* S-1 grown on alcohols". *Biosci. Biotech. Biochem.*, 58, 428-429.

Kyriakos, M. (1990). Flash pyrolysis of wood and dried sewage sludge for liquid fuel production. In: Grass G, Gosse G, dos Santos G, editors. Biomass for energy and industry, 5th EC Conference, vol. 2. Conversion and utilisation of biomass. 2.611–2.615.

Lachhab, K., Tyagi, R.D., and Valéro, J.R. (2001). "Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids concentration". *Process Biochem.*, 37, 197-208.

Lamontagne, E. (2004). "Caractérisation de nouvelles souches de *Bacillus thuringiensis* d'intérêt pour la production de biopesticides et d'enzymes par fermentation de boues d'épuration municipales." Master's thesis, Institut Nationale de la Recherche Scientifique-Eau, Terre et Environnement, Université du Québec, Canada, 112 pages.

Lastella, G.., Testa, C., Cornnachia, G., Notornicola, M., Voltasio, F., and Sharma, V.K. (2002). "Anaerobic digestion of semi-solid organic waste: biogas production and its purification". *Energy Conversion Management,* 43, 63-75.

Lay, J.J. (2000). Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. *Biotechnol. Bioeng.* 68 (3), 269– 278.

Lee, S.H., Lee, S.O., Jang, K.L., and Lee, T.H. (1995). "Microbial flocculant from *Arcuadendron* sp. TS-49". *Biotechnol. Lett.*, 17, 95-199.

Lee, S.Y. (1996a). "Bacterial polyhydroxyalkanoates". *Biotechnol. Bioeng.*, 49(1), 1-14.

Lee, S.Y. (1996b). "Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria". *Trends Biotechnol.*, 14, 431– 438.

Lee, K.S., Lo, Y.S., Lo, Y.C., Lin, P.J., and Chang, J.S. (2003). "H_2 production with anaerobic sludge using activated-carbon supported packed-bed bioreactors". *Biotechnol. Letts,* 25,133–138.

Leighton, I.R., and Forster, C.F. (1997). "The adsorption of heavy metals in an acidogenic thermophillic anaerobic reactor". *Water Res.*, 31(12), 2969-2972.

Lin, C.Y., and Chen, C.C. (1997). "Toxicity resistance of sludge biogranules to heavy metals". *Biotech. Lett.*, 19(6), 557-560.

Lisansky, S.G., Quinlan, R.J., and Tassoni, G. (1993). *The Bacillus thuringiensis production* handbook. CPL press, Newbury, pp 124.

Liu, Y. (2003). "Chemically reduced excess sludge production in the activated sludge process." *Chemosphere* 50, 1–7.

Lovley, D.R. (1993). "Dissimilatory metal reduction". *Annual Rev. Microbiol.*, 47, 297-299.

Low, E.W., and Chase, H.A. (1999). "Reducing production of excess biomass during wastewater treatment". *Water Research*, 33(5), 1119-1132.

Maissonave, V., Vignoles, M.M., Bonnin, C., and Revel, J.C. (2001). Impact on crops, plants and soils of metal trace elements transfer and flux, after spreading of fertilisers and biosolids. Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Mexico, Oct. 25-27, 440-447.

Martin, M.J., Balaguer, M.D., and Rigola, M. (1996). "Feasibility of activated carbon production from biological sludge by chemical activation with $ZnCl_2$ and H_2SO_4". *Environ. Technol.,* 17, 667–672.

Martin, M.J., Serra, E., Ros, A., Balaguer, M.D., and Rigola, M. (2004). "Carbonaceous adsorbents from sewage sludge and their application in a combined activated sludge-powdered activated carbon (AS-PAC) treatment". *Carbon*, 42, 1389–1394.

Martin, M.J., Artola, A., Balaguer, M.D., and Rigola, M. (2002). "Towards waste minimisation in WWTP: activated carbon from biological sludge and its application in liquid phase adsorption". *J. Chem. Technol. Biotechnol*, 77, 825-833.

Mata Gonzalez, R., Sosebee, R.E., and Wan, C. (2002). "Physiological impacts of biosolids application in desert grasses". *Environ. Experimental Botany*, 48, 139-148.

Matheickal, J.T., Yu, Q., and Woodburn, G.M. (1999). "Biosorption of Cd(II) from aqueous solutions by pretreated biomass of marine alga *Durvillaea potatorum*". *Water Res.*, 33(2), 335-342.

Matsuo, T. (1994). "Effect of the anaerobic SRT on enhanced biological phosphorus removal". *Water Sci. Technol.*, 30, 193– 202.

McGhee, T.J. (1991). *Water supply and sewerage*. New York: McGraw-Hill.

Metcalf and Eddy. (2003). *Wastewater Engineering – Treatment and reuse*, 4th Edition, Eds. Tchobanoglous, G., Burton, F.L. and Stensel, H.D., Mc Graw Hill Inc., New York, 888 – 893.

Meyer, V.F., Redente, E.F., Barbarick, K.A., and Brobst, R. (2001). "Ecosystem restoration". *J. Environ. Qual.*, 30, 1528-1532.

Midilli,A., Dogru, M.,Akay, G., and Howarth, C.R. (2002). "Hydrogen production from sewage sludge via a fixed bed gasifier product gas". *Int. J. Hyd. Ener.*, 27,1035 –1041.

Millot, N., Huyard, A., Faup, G.M., and Michel, J.P. (1989). "Sludge liquefaction by conversion to fuels". *Water Sci. Technol.*, 21(8-9), 917-923.

Molla , A.H., Fakhru'l-Razi , A., Abd-Aziz, S., Hanafi, M.M., Roychoudhury, P.K., and Alam, M.Z. (2003). "A potential resource for bioconversion of domestic wastewater sludge". *Biores. Technol.*, 85, 263–272.

Montgomery, R. (2004). "Development of biobased products". *Biores. Technol.*, 91, 1-29.

Mueller, R.F., and Steiner, A. (1992). "Inhibition of anaerobic digestion caused by heavy metals". *Water Sci. Technol.*, 26(3-4), 835-846.

Myung, H.S., and Kim, D.P. (2001). "Preparation of composite adsorbents by activation of water plant sludge and phenolic resin mixtures". *Carbon Sci.*, 1(3 & 4), 154-157.

Nakano, K., and Matsumura, M. (2002). "Utilization of dehydrated sewage sludge as an alternative nutrient to stimulate lipid waste degradation by the thermophilic oxic process". *J. Biosci. Bioeng.*, 94(2), 113-118.

Nakamura, J., Miyashiro, S., and Hirose, Y. (1976). "Modes of flocculation of yeast cells with flocculant produced by *Aspergillus soja* AJ-7002". *J. Agr. Biol. Chem.*, 40(8), 1565-1571.

Neyens, E., and Baeyens, J. (2003). "A review of thermal sludge pre-treatment processes to improve dewaterability." *Journal of Hazardous Materials* B98, 51–67.

Nissen, L.R., Lepp, N.W., and Edwards, R. (2000). "Synthetic zeolites as amendments for sewage sludge based compost". *Chemosphere*, 41, 265-269.

Nitschke, M. and Pastore, G.M. (2003). Cassava flour wastewater as a substrate for biosurfactant production. *Applied Biochemistry and Biotechnology* 106(1-3), 295-302.

Nonato, R.V., Mantelatto, P.E., and Rossell, C.E.V. (2001). "Integrated production of biodegradable plastic, sugar and ethanol". *Appl. Microbiol. Biotechnol.*, 57, 1-5.

Noparatnaraporn, N., Sasaki, K., Nishizawa, Y., and Nagai, S. (1986). "Stimulation of vitamin B12 formation in aerobically grown *Rhodopseudomonas gelatinosa* under microaerobic condition". *Biotech. Letts.*, 8, 491–496.

O'Conor, R.E., Carroll, T.W., and Flom, A. (2001). *Minergy glass aggregate technology.* Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Mexico, Oct. 25-27, 272-279.

Odeby, T., Netteland, T., and Solheim, O.E. (1996). *Thermal sludge treatment to decrease sludge volume and recycle the sludge to new products.* In: Chemical Water and Wastewater Treatment IV (eds. H. Hahn, E. Hoffmana and H.Odegaard), Springer Verlag, Heidelberg, 401-409.

Okuna, N., Takahasi, S., and Asada, S. (1997). Full scale application of manufacturing brick from sewage. IAWQ Conference Papers on Wastewater sludge, waste or resource? Part 1, 244-256, Czestochowa, Poland.

Okuno, N., Uriu, M., Horii, T., and Miyagawa, K. (1997). "Evaluation of thermal sludge solidification". *Water Sci. Technol.*, 36(11), 227-233.

Onaka, T. (1999). Sewage can make Portland cement. IAWQ Conference Paper on Sludge management for the 21st century, Poster presentation, Freemantle, Western Australia.

Onyeche, T.I. (2004). "Sludge as source of energy and revenue". *Water Sci. Technol.*, 50(9), 197–204.

Otera, M., Rozada, F., Calvo, L.F., Garcia, A.I., and Moran, A. (2001). *Water pollutants elimination by means of adsorbents obtained from sewage sludge.* Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Mexico, Oct. 25-27, 280-287.

Otero, M.,Rozada,F.,Calvo,L.F.,Garcý, A.I., and Moran, A. (2003). "Elimination of organic water pollutants using adsorbents obtained from sewage sludge". *Dyes and Pigments*, 57, 55 –65.

Pagilla, K.R., Sood, A., and Kim, H. (2002). "*Gordonia* (nocardia) *amarae* foaming due to biosurfactant production". *Water Sci. Technol.*, 46(1-2), 519–524.

Palantzas, I.A., and Wise, D.L. (1994). "Preliminary economic analysis for production of calcium magnesium acetate from organic residues". *Res. Conserv. Recycling*, 11, 225-231.

Pan, S.C., and Tseng, D.H.(2001). "Sewage as characteristic and its potential applications". *Water Sci.* Technol., 44(10), 261-267.

Pan, S.S., Lin, C.C., and Tseng, D.H. (2003). "Reusing sewage sludge ash as adsorbent for copper removal from wastewater". *Res., Conserv. Recycl.*, 39, 79-90.

Park, D.H., and Zeikus, J.G. (2003). "Improved fuel cell and electrode designs for producing electricity from microbial degradation". *Biotechnol. Bioeng.,* 81(3), 348-350.

Paya, J.,Monzo, J.,Borrachero, M.V., Amahjour,F., Girbe, I., Velagsquez, and Ordognez, L.M. (2002). "Advantages in the use of fly ashes in cements containing pozzolanic combustion residues: silica fume, sewage sludge ash, spent fluidized bed catalyst and rice husk ash". *J. Chem. Technol. Biotechnol.,*77, 331-335.

Petersen, S.O., Petersen, J., and Rubæk G. H. (2003). "Dynamics and plant uptake of nitrogen and phosphorus in soil amended with sewage sludge". *Appl. Soil Ecol.*, 24, 187–195.

Pinamonti, F., Stringari, G., and Zorzi, G. (1997). "Use of compost in soilless cultivation". *Compost Sci. Util.*, 5(2), 38.

Polprasert, C. (1989). *Organic Waste Recycling.* John Wiley and Sons, Chichester, UK.

Polprasert, C., Yang, P. Y., Kongsricharoern, N., and Kanjanaprapin, W. (1994). "Productive utilization of pig farm wastes: a case study for developing countries". *Resources, Conservation and Recycling,* 11(1-4), 245-259.

Rate, A.W., Lee, K.M., and French P.A. (2004). "Application of biosolids in mineral sands mine rehabilitation: use of stockpiled topsoil decreases trace element uptake by plants". *Biores. Technol.*, 91, 223–231.

Roe, N.E., Stofella, P.J., and Graetz, D. (1997). "Composts from various municipal solid waste feedstock affect vegetable crops". *J. Am. Soc. Hort. Sci.*, 122, 427.

Rozada, F., Calvo, L.F., Garcia, A.Y., Martin-Villacorta, J., and Otero, M. (2003). "Dye adsorption by sewage sludge-based activated carbons in batch and fixed-bed systems". *Biores.Technol.*, 87,221 –230.

Sachdeva, V., Tyagi, R.D., and Valéro, J.R. (2000). "Production of biopesticides as a novel method of wastewater sludge utilization/disposal". *Water Sci. Technol.*, 42(9), 211-216.

Saito, Y., Soejima, T., Tomozawa, T., Doi, Y., and Kiya, F. (1995). "Production of biodegradable plastics from volatile acids using activated sludge". *Environ. Systems Eng.*, 52, 145– 54.

Sajwan, K.S., Paramasivam, S., Alva , A.K., Adriano, D.C., and Hooda P.S. (2003). "Assessing the feasibility of land application of fly ash, sewage sludge and their mixtures". *Adv. Environ. Res.*, 8, 77–91.

Salehizadeh,H., Vossoughi,M., and Alemzadeh,I. (2000). "Some investigations on bioflocculant producing bacteria". *Biochem. Eng. J.*, 5, 39-44.

Sastre, I., Vicente, M.A., and Lobo, M.C. (1996). "Influence of the application of sewage sludges on soil microbial activity". *Biores. Technol.,* 57,19-23.

Satoh, H., Iwamoto, Y., Mino, T., and Matsuo, T. (1998). "Activated sludge as a possible source of biodegradable plastic". *Water Sci. Technol.*, 38(2), 103-109.

Sawayama, S., Tsukahara, K., and Yagishita, T. (1999). "Wastewater treatment and polyhydroxybutyrate production using lighted upflow anaerobic sludge blanket method". *Biosci. Bioeng.*, 7, 683 – 689.

Schreeg, T.M., and Jarrett, D.L. (1996). Biosolids cut fertilizer costs by $200 an acre. Oct., 69-71.

Senior, P.J., and Dawes, E.A. (1973). "The regulation of poly-beta-hydroxybutyrate metabolism in *Azotobacter beijerinckii". Biochem. J.*, 134, 225-238.

Shen, L., and Zhang, D.K. (2003). "An experimental study of oil recovery from sewage sludge by low-temperature pyrolysis in a fluidised-bed". *Fuel*, 82, 465–472.

Shier, W.T., and Purwono, S.K. (1994). "Extraction of single-cell protein from activated sewage sludge: thermal solubilization of protein." *Bioresource Technol.,* 49, 157–162.

Shinogi, Y., Yoshida, H., Koizumi, T., Yamaoko, M., and Saito, T. (2003). "Basic characteristics of low temperature carbon products from waste sludge". *Adv. in Environ. Res.*,7(3), 661-665.

Sipma, J., Janssen, A. J. H., Pol, L.W. H., and Lettinga, G. (2003). "Development of a novel process for the biological conversion of H_2S and methanethiol to elemental sulfur". *Biotechnol. Bioeng,* 82, 1–11.

Smith, M.T.E., and Tibbett, M. (2004). "Nitrogen dynamics under *Lolium perenne* after a single application of three different sewage sludge types from the same treatment stream". *Biores. Technol.*, 91, 233–241.

Snyman, H.G., Jong, J.M., and Aveling, T.A.S. (1998). "The stabilisation of sewage sludge applied to agricultural land and the effects on maize seedlings". *Water Sci. Technol.,* 38(2), 87-95.

Sort, X., and Alcañiz, J.M. (1996). "Contribution of sewage sludge to erosion control in the rehabilitation of limestone quarries". *Land Degradation & Development,* 7(1), 69-76.

Spinosa, L. (2004). "From sludge to resources through biosolids". *Water Sci. Technol.,* 50(9), 1–9.

Su, D.C., and Wong J.W.C. (2003). "Chemical speciation and phytoavailability of Zn, Cu, Ni and Cd in soil amended with fly ash-stabilized sewage sludge". *Environ. International*, 29, 895– 900.

Su, D.C. Wong J.W.C., and Jagadeesan, H. (2004). "Implications of rhizospheric heavy metals and nutrients for the growth of alfalfa in sludge amended soil". *Chemosphere*, 56, 957–965

Suffern, J.S., Fitzgerald, C.M., and Szluha, A.T. (1981). "Trace metal concentration in oxidation ponds". *J. Water Pollut. Control Fed.*, 53, 1599-1608.

Tacon, A.G. J., and Ferns, P. N. (1979). "Activated sewage sludge, a potential animal foodstuff. II. Nutritional characteristics". *Agriculture and Environment*, 4, 271.

Takabatake, H., Satoh, H., Mino, T., and Matsuo, T. (2000). "Recovery of biodegradable plastic from activated sludge process".*Water Sci. Technol.*, 42, 351– 6.

Takabatake, H., Satoh, H., Mino, T., and Matsuo, T. (2002). "PHA production potential of activated sludge treating wastewater".*Water Sci. Technol.*, 45,119–126.

Takagi, H., and Kadowaki, K. (1985). "Flocculant production by *Paecilomyces* sp., Taxonomic studies and culture conditions for production". *Agric. Biol. Chem.*, 49, 3151–3157.

Tay, J.H., Show, K.Y., Hong, S.Y., Chien, C.Y., and Lee, D.J. (2002). "Potential reuse of wastewater sludge for innovative applications in construction industry". *Bulletin of the College of Engineering, N.T.U.,* 86, *October,* 103-112.

Tay, J.H., Show, K.Y., Lee, D.J., and Hong, S.Y. (2004). "Reuse of wastewater sludge with marine clay as a new resource of construction aggregates". *Water Sci. Technol.*, 50(9), 189-196.

Teratani, T., Okuno, N., and Kouno, K. (2001). *New technology to manufacture fine spherical ceramic from sewage sludge.* Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Mexico, Oct. 25-27, 296-303.

Ting, C.H., Lin, K.R., Lee, D.J., and Tay, J.H. (2004). "Production of hydrogen and methane from wastewater sludge using anaerobic fermentation". *Water Sci. Technol.*, 50 (9), 223–228

Tirado-Montiel, M.L., Tyagi, R.D., and Valéro, J.R. (1998) Production of *Bacillus thuringiensis* using waste materials. In: Bioconversion of waste materials to Industrial Products, A.M. Martin (ed.), Blackie Academic and Professional, London, 480-516.

Tirado-Montiel, M.L., Tyagi, R.D., and Valéro, J.R. (2001). "Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticide". *Water Res.*, 35(16), 3807–3816.

Tirado-Montiel, M.L., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2003). "Production biopesticides using wastewater sludge as a raw material – Effect of process parameters." *Water Sci. Technol.,* 48(8), 239-246.

Third, K.A., Newland, M., and Ruwish, R.C. (2003). "The effect of dissolved oxygen on PHB accumulation in activated sludge cultures". *Biotechnol. Bioeng.*, 82(2), 239-250.

Tohyama, M., Patarinska, T., Qiang, Z., and Shimizu, K. (2002). "Modeling of mixed culture and periodic control for PHB production". *Biotechnol. Eng.*, 10, 157– 173.

Tsunemasa, N. (1998). "Utilization of excess sludge for the production of biodegradable plastics". *Water Waste*, 40, 981–986 [in Japanese].

Tyagi, R.D., Foko, V.S., Barnabe, S., Vidyarthi, A.S., and Valéro, J.R. (2001). Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using sewage sludge as a raw material. Specialised Conference on Sludge Management: regulation, treatment, utilization and disposal, Oct. 25-27, 312-319.

Ueno, T., Satoh, H., Mino, T., and Matsuo, T. (1993). Production of biodegradable plastics. *Polym Preprint*, 42, 981– 986 [In Japanese].

U.S. EPA, (1997). Compost—New Applications for an Age-Old Technology.

U.S. EPA, 1998. An Analysis of Composting as an Environmental Remediation Technology. *EPA530-R-98-008*. April.Uncorrected.

Valéro, J.R., Mohammedi, S., Payne, N.J., and Tyagi, R.D. (1999). "Microbial control of defoliating forest insects." *Recent Res. Devel. Microbiology*, 3, 455-464.

Valls,S., and Vazquez, E. (2001). "Accelerated carbonation of sewage sludge–cement–sand mortars and its environmental impact". *Cement and Concrete Research*, 31, 1271–1276.

Van Niel, E.W.J., Robertson, L.A., and Kuenen, J.G. (1995). "Rapid short term poly-beta-hydroxy-butyrate production by *Thiosphaera pantotropha* in the presence of excess acetate". *Enzyme Microbiol. Technol.*, 17, 977-982.

Van Loosdrecht, M.C.M., and Heijnen, J.J. (2002). "Modeling of activated sludge processes with structured biomass". *Water Sci. Technol.,* 45, 13–23.

Verma, M., Brar, S.K., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2005). "Production of antagonistic fungus *Trichoderma* sp. from wastewater sludge: effect of pre-treatment and solids concentration." *Water Research,* 39, 3587–3596.

Vidyarthi, A.S., Tyagi, R.D., and Valéro, J.R. (2001). "Effect of surface-active agents on the production of biopesticides using wastewater sludge as a raw material". *Water Sci. Technol.*, 44(10), 253-259.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2002). "Studies on the production of *B.thuringiensis* based biopesticides using wastewater sludge as a raw material". *Water Res.*, 36 (19), 4850-4860.

Vipulanandan, C., and Ren, X. (1995). "Pollution prevention by conversion of industrial waste materials into biosurfactants". *Pollution Prevention*, 7-8.

Viraraghavan, T., and Ionescu, M. (2002). "Land application of phosphorus-laden sludge: a feasibility analysis." *J Environ. Management*, 64(2), 171-177.

Vriens, L., Nihoul, R., and Verachtert, H. (1989). "Activated sludges as animal feed: A review". *Biological Wastes,* 27(3), 161-207.

Webber, M.D. (1986). Epandage des boues d'epuration sur terres agricoles – une évaluation. Direction Générale de la Recherche, Agriculture Canada, 42 pages.

Webber, M.D. (1990). Resource recovery through unconventional uses of sludge. Le premier colloque sur québécois sur le valorisation des boues de stations d'epuration municipales. Centre Québécois de Valorisation de la Biomasse, Hull, Québec, Canada, Compte-rendu, pp 277-296.

Webber, M.D. (1991). Resource recovery through unconventional uses of sludge. J.E. Hall (Eds.). Pergamonn Press, Oxford, U.K., pp 263-276.

Webber, M.D., Rogers, H.R., Watts, C.D., Boxall, A.B.A., Davis, R.D., and Scoffin, R., (1996). "Monitoring and prioritisation of organic contaminants in sewage sludges using specific chemical analysis and predictive non-analytical methods". *Sci. Tot. Environ.*, 185, 27–44.

Wei, Y.S., Fan, Y.B., Wang, M.J., and Wang, J.S. (2000). "Composting and compost application in China". *Resour. Conserv. Recycl.*, 30, 277-300.

Weibusch, B., and Seyfried, C.F. (1997). "Utilisation of sewage sludge ashes in the brick and tile industry." *Water Sci. Technol.*, 36(11), 251-258.

Weir, C.C., and Allen, J.R. (1997) "Effects of using organic wastes as soil amendments in urban horticultural practices in the district of Columbia". *J. Environ. Health*, Part A, 32, 323.

Weng, C.H., Chang, E.E., and Chiang, P.C. (2001). "Characteristics of new coccine dye adsorption onto digested sludge particulates". *Water Sci. Technol.*, 44(10), 279-284.

Weng, C.H., Lin, D.F., and Chiang P.C. (2003). "Utilization of sludge as brick materials". *Adv. Environ. Res.*, 7, 679–685.

Werther, J., and Ogada, T. (1999). "Sewage sludge combustion". *Progress in Energy and Combustion Science*, 25, 55–116.

Whitbread-Abrutat, P.H. (1997). "The potential of some soil amendments to improve tree growth on metalliferous mine wastes". *Plant Soil*, 192, 199.

Wiebusch, B., and Seyfried, C.F. (1997). "Utilisation of sewage sludge ashes in the brick and tile industry". *Water Sci. Technol.*, 36(11), 251-258.

Wong, M.H., and Cheung, S.P. (1980). "Sewage sludges and carrot wastes as supplementary feed for the common carp. Cyprinus carpio". *Environ. Pollution,* 23, 29-39.

Wong, M.H. (1977a). "The comparison of activated and digested extracts in cultivating *Chlorella pyrenoidosa* and *Chlorella saline". Environ. Pollution*, 14, 207-211.

Wu,S.Y., Lin,C.N., Chang, J.S., Lee,K.S., and Lin, P.J.(2002). "Microbial hydrogen production with immobilized sewage sludge". *Biotechnol. Prog.*,18, 921-926.

Yang, J., and Volesky, B. (1996). "Intraparticle diffusivity of Cd ions in a biosorbent material". *J. Chem. Tech. Biotech.*, 66, 355-364.

Yezza, A., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2004a). "Scale-up of biopesticide production process using wastewater sludge as a raw material". *J. Ind. Microbiol. Biotechnol.* 31, 545-552.

Yezza, A. Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2005a). "Production of *Bacillus thuringiensis* based biopesticides in batch and fed cultures using wastewater sludge as a raw material". *J. Chem. Technol. Biotechnol.* 80, 502-510.

Yezza, A. Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2005b). "Bioconversion of industrial wastewater and wastewater sludge into *Bacillus thuringiensis* based biopesticides in pilot fermentor". *Bioresource Technology* 26, 1850-1857.

Yezza, A. Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2006). "Correlation between entomotoxicity potency and protease activity produced by *Bacillus thuringiensis var. kurstaki* grown in wastewater sludge." *Process Biochem.* 41, 794-800.

Yip, S.W., and Wong, M.H. (1978). "The comparison of sewage effluent and sludge extracts in the cultivation of *Chlorella pyrenoidosa". Archiv. fur Hydrobiologie*, 84, 368-380.

Yokoi, H., Shiraki, M., Hirose, J., Hiyashi, S., and Takasaki, Y. (1998). "Flocculation properties of xanthan produced by *Xanthomonas campestris". Biotechnol. Technol.*, 10, 789-792.

Yu, P. H. F., Chua, H., Huang, A. L., Lo, W. H. and Ho, K. P. (1999). Transformation of industrial food wastes into polyhydroxyalkanoates. *Water Sci. and Technol.,* 40(1), 365–370.

Zhai, Y., Wei, X., Zeng, G., Zhang, D., and Chu, K. (2004). "Study of adsorbent derived from sewage sludge for the removal of Cd^{2+}, Ni^{2+} in aqueous solutions". *Separation and Purification Technol.*, 38, 191–196.

Zhou, J.L., and Kiff, R.J. (1991). "The uptake of Cu from aqueous solution by immobilised fungal biomass". *J. Chem. Tech. Biotechnol.*, 52, 317-330.

Zwara, W., and Pempkowiak, O.H. (2000). "Polish experience with sewage sludge utilisation in reedbeds". *Water Sci. Technol.,* 41(1), 65-68.

Table 1. Typical product and energy yields of Enersludge^{TM} process

Product	Raw Sludge		Digested Sludge	
	Yield (%)	**% of Sludge Energy**	**Yield (%)**	**% of Sludge Energy**
Oil	30	60	20	50
Char	45	32	55	40
Non Condensable Gases	13	5	13	7
Reaction Water	12	3	12	3

(Source: http://www.environ.com.au/enersludge.shtml, cited 16 May, 2004)

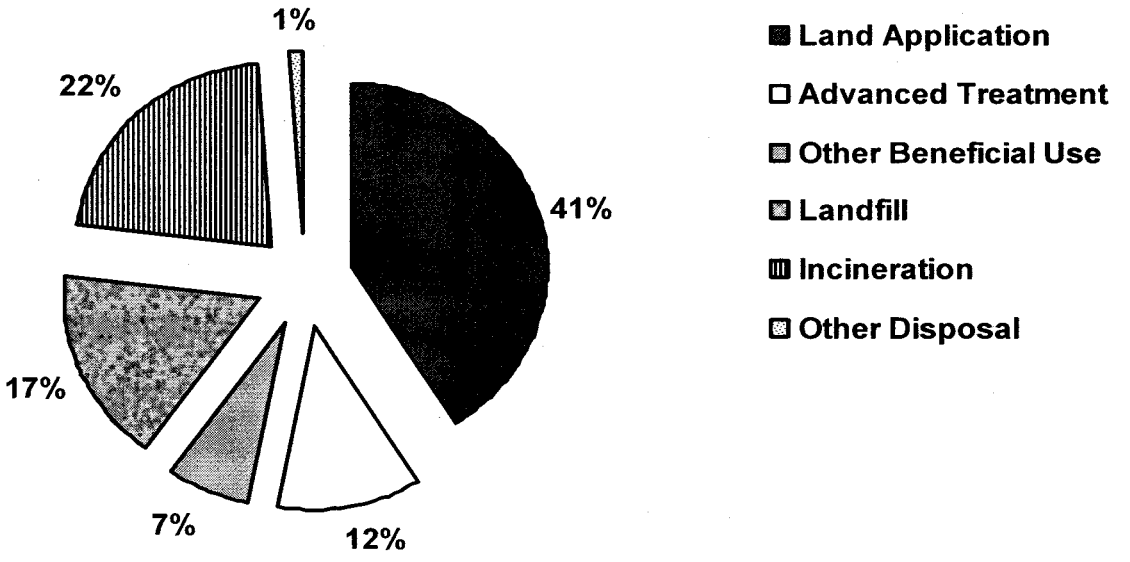


Figure 1. Average Biosolids Disposal and Reuse in United States (Source: Biosolids Management Update (1998). *BioCycle,* January); Note: Total annual production – 7 million tonnes dry solids/year; Combined beneficial use = 60% and combined disposal = 40%.

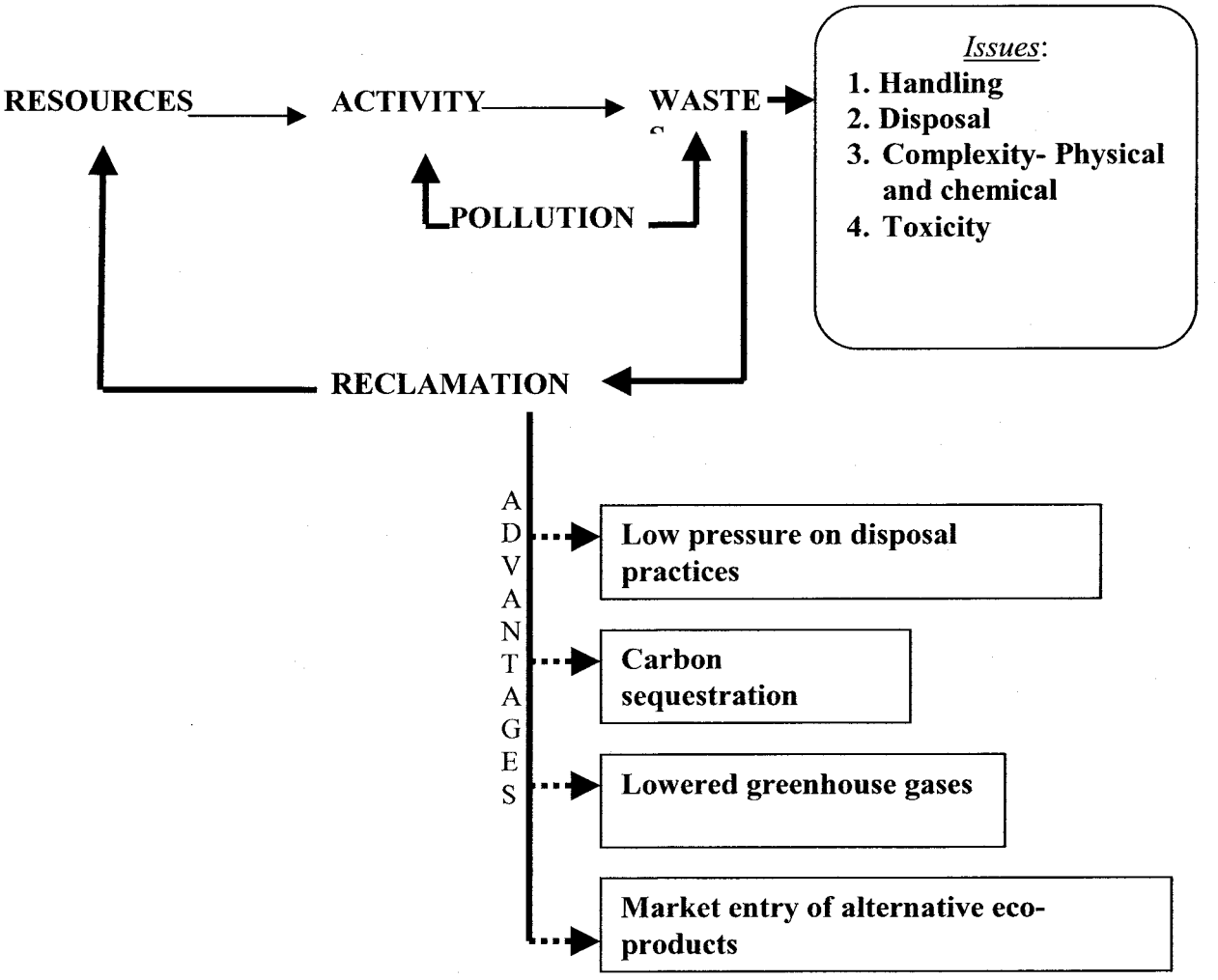


Figure 2. Simple representation of cradle-to-cradle journey of wastes

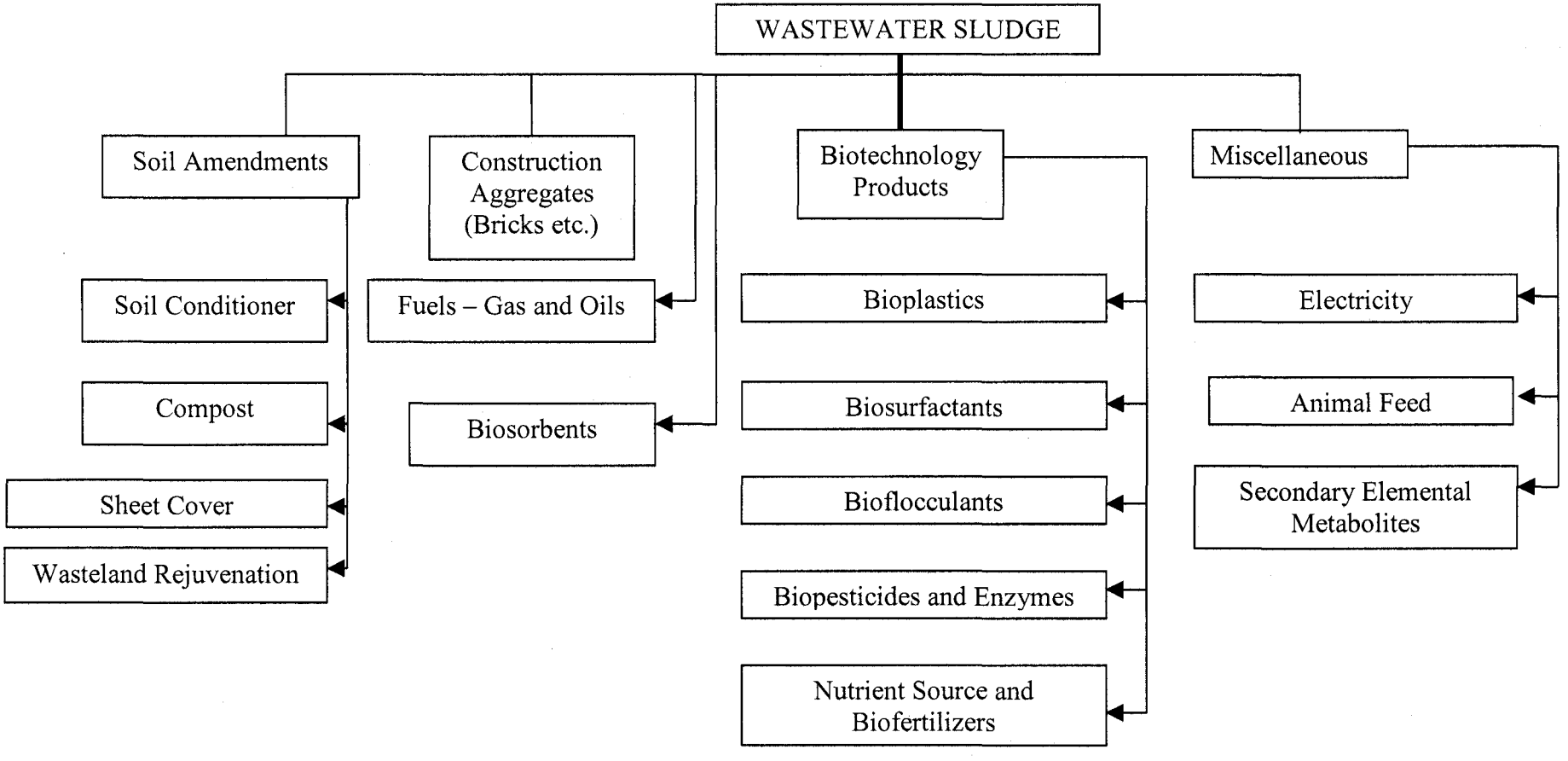


Figure 3. Schematic representation of value addition products of wastewater sludge

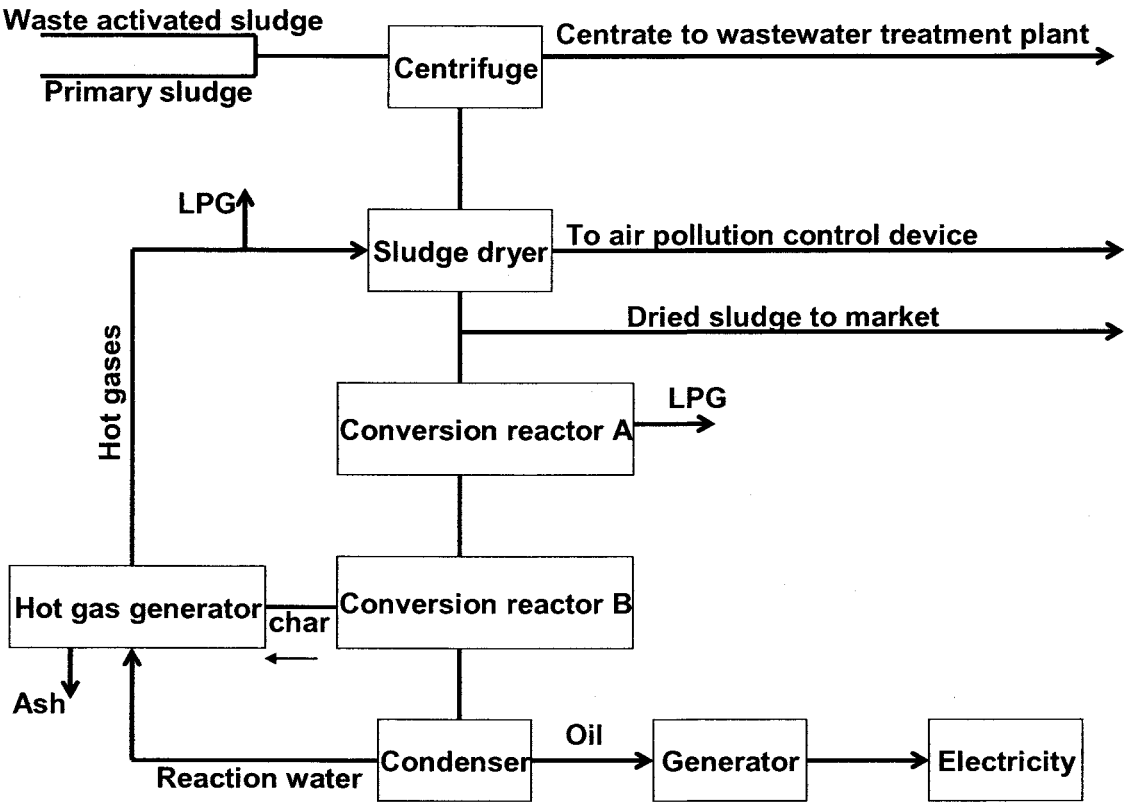


Figure 4. Typical Enersludge™ process (modified from http://www.environ.com.au/enersludge.shtml, cited 16 May, 2004)

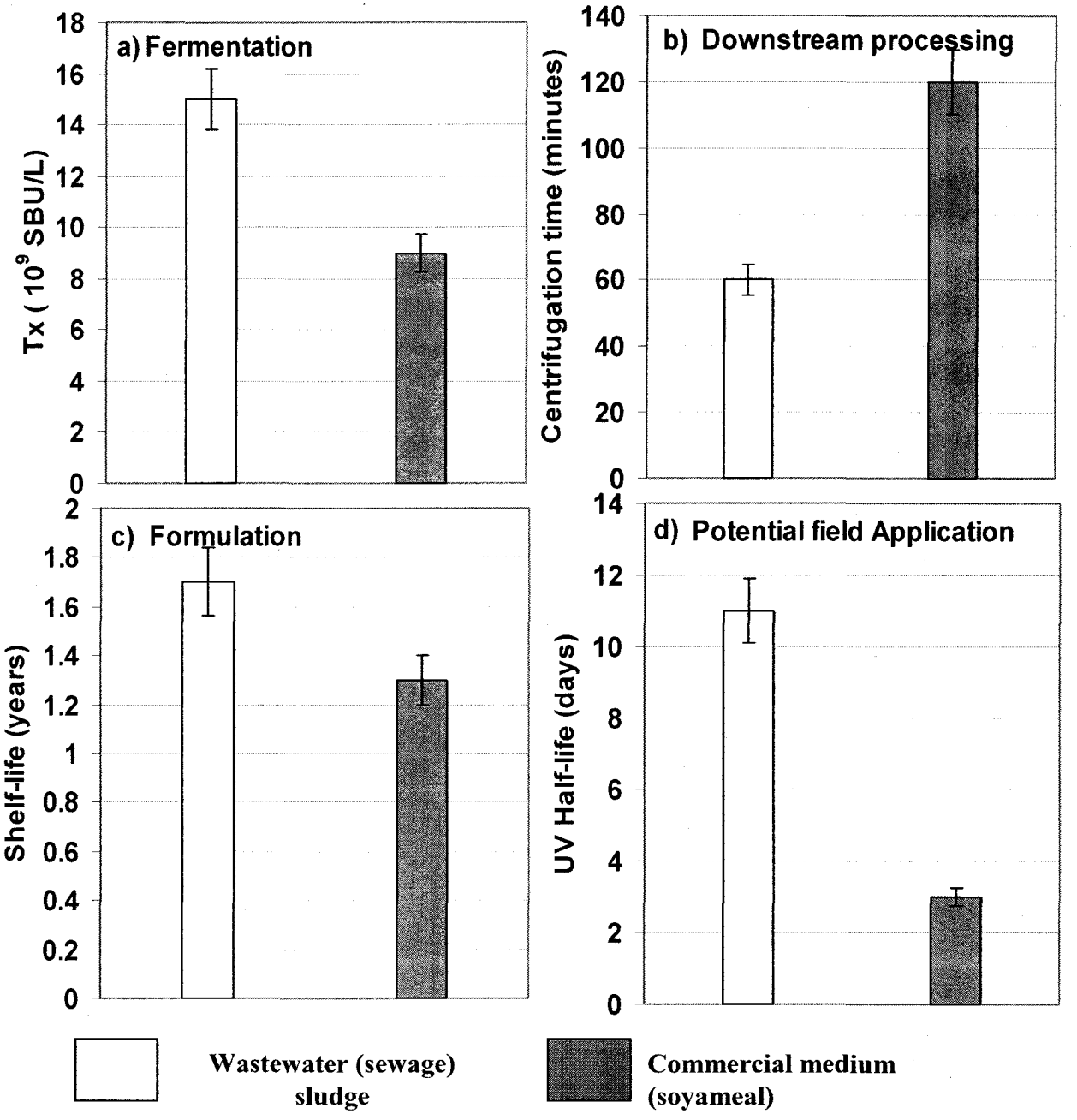


Figure 5. Biopesticide production potential of wastewater (sewage) sludge in comparison to semi synthetic conventional medium (soyameal) covering different aspects of production: a) Fermentation (reported as biopesticidal potential, entomotoxicity, Tx-spruce budworm units/L); b) Downstream processing-centrifugation (reported as time required for 70-80% Tx recovery, minutes); c) Formulation development (shelf-life storage, reported in years based on physical and biological stability) and; d) Prospective field application (laboratory UV studies, reported as half-life, days).

CHAPITRE 3.

ÉTUDES RHÉOLOGIQUES DES MILIEUX FERMENTÉS PAR Bt

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. et Banerji, S.K. (2004). Comparative rheology and particle size analysis of various types of *Bacillus thuringiensis* fermented sludges. *J. Residuals Sci. Technol.* 1(4): 231-237.

http://www.destechpub.com/pageview.asp?pageid=28024

Partie II

Sludge based *Bacillus thuringiensis* Biopesticides: Viscosity Impacts

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*], J.R. Valéro[1] and R.Y. Surampalli[2]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9
[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Water Research (2005)
39: 3001-3011

Biopesticides de *Bacillus thuringiensis* à base de boues d'épuration : effets de la viscosité

Résumé

Des études de viscosité ont été réalisées sur des boues brutes, pré-traitées (hydrolyse alcaline stérilisation thermique ou par ces deux types de traitement) et des boues fermentées par *Bacillus thuringiensis* (Bt) destinées à la production des biopesticides. Les concentrations de solides utilisées variaient entre 10 à 40 g/l. Des corrélations ont été établies pour la viscosité, pour la concentration en solides totaux et les solides dissous ainsi que pour l'entomotoxicité (Tx) des boues fermentées. Des lois exponentielles et de puissance ont été mises en évidence pour les boues hydrolysées fermentées comparativement aux boues brutes fermentées. La demande chimique en oxygène dissous (SDCO) variait avec l'augmentation de la concentration en solides dissous après prétraitement, alors que cette dernière contribuait aux changements de la viscosité. Par ailleurs, Tx était plus élevé pour bouillons fermentés obtenus avec les boues hydrolysées fermentées par rapport aux boues brutes fermentées. Le Tx élevé est apparu relié à une plus grande disponibilité des nutriments et à une basse viscosité qui a favorisé un meilleur transfert de l'oxygène. Les résultats obtenus en fioles étaient reproductibles en bioréacteur. Les résultats de cette étude permettent d'améliorer le choix des techniques de fermentation, des procédés en aval ainsi que de la formulation des bouillons fermentés par Bt.

Mots-clés: *Bacillus thuringiensis*, biopesticide, fermentation, hydrolyse, viscosité, boues d'épuration

Abstract

Viscosity studies were performed on raw, pre-treated (sterilized and thermal alkaline hydrolyzed (TAH) or both types of treatment) and *Bacillus thuringiensis* (Bt) fermented sludges at different solids concentration (10 - 40 g/L) for production of biopesticides. Correlations were established among rheological parameter (viscosity), solids (total and dissolved) concentration and entomotoxicity (Tx) of Bt fermented sludges. Exponential and power laws were preferentially followed by hydrolyzed fermented compared to raw fermented sludge. Soluble chemical oxygen demand (SCOD) variation corroborated with increase in dissolved solids concentration on pre-treatments, contributing to changes in viscosity. Moreover, Tx was higher for hydrolyzed fermented sludge in comparison to raw fermented sludge owing to increased availability of nutrients and lower viscosity that improved oxygen transfer. The shake flask results were reproducible in fermenter. This study will have major impact on selecting fermentation, harvesting and formulation techniques of Bt fermented sludges for biopesticide production.

***Keywords**: Bacillus thuringiensis,* biopesticide, fermentation, hydrolysis, viscosity, wastewater sludge

1. Introduction

Municipal wastewater treatment results in the production of large quantities of sludge that must be disposed off in an environmentally benign manner. Pre-existing modes of sludge disposal viz. landfilling, incineration and landspreading encompass multiple drawbacks and lack sustainable management. This stimulated a "re-thinking" to recycle or reuse these sludges. Henceforth, the sludges referred to as "biosolids" by US EPA have been utilized as a raw material to develop new processes for production of various value-added products including bricks, adsorbents, bioplastics, biosurfactants, enzymes and biopesticides etc. (Tay et al., 1992; Pan and Tseng, 2001; Tyagi et al., 2002). Therefore, it becomes essential to study physico-chemical properties of various types of wastewater sludges.

Literature is replete with various definitions of sludge based on physical constitution (Erdincler and Vesilind, 2000). According to some authors, sludge in "liquid state" was found to be thixotropic (Campbell and Crescuollo, 1982; Honey and Pretorius, 2000), and was characterized using different rheological models, including Bingham and Herschel-Bulkley models (Lotito et al., 1997; Baudez and Coussot, 2001). These rheological studies were primarily conducted to understand the flow properties of the sewage sludge, in order to optimize their transport processes (Battistoni et al., 1990 a,b).

The production of value-added products also requires extensive study of the physical, chemical and biological nature of the sludges. Viscosity is the simplest and most important process parameter to be explored. It can affect overall biopesticide production at different stages: fermentation, formulation and field application. The first important role of viscosity is in adjudging the feasibility of the sludge medium for fermentation by impacting oxygen mass transfer in the fermenter (Vidyarthi et al., 2002). It will equally affect formulation amendment of the fermented sludge (type and amount of adjuvants required to be added during formulation, ease of handling and improvement of final product properties) and its field application (compatibility with pre-existing equipment) (Mor and Matthews, 2003) and hence overall cost of the product and eventually its marketability. Earlier studies on production of biopesticide from sludge by *Bacillus thuringiensis* (Bt) investigated methods to enhance the entomotoxicity for economical reasons, by considering nutrient composition of sludges (Tirado-Montiel et al., 2001; Vidyarthi et al., 2002). However, there is no study that reports the rheological behaviour of sludges in relation to their bioconversion to value-added products.

This study is an effort to determine sludge viscosity at different process stages of Bt fermentation and to correlate it with other process parameters, thereby, aiding in adjudging better strategy for sludge as a raw material for biopesticide production.

NOMENCLATURE	
DS	Dissolved solids (g/L)
SCOD	Soluble chemical oxygen demand (mg/L)
SS	Suspended solids (g/L)
Sp. gr.	Specific gravity
TCOD	Total chemical oxygen demand (mg/L)
T	Temperature (°C)
TC	Total cell count (CFU/ml)
TS	Total solids (g/L)
ΔS	Change in entropy (kJ mol^{-1})
K	Consistency index ($Pa.s^n$)
ΔH	Enthalpy change (kJ mol^{-1})
n	Flow behaviour index
ΔG	Gibbs free energy change (kJ mol^{-1})
τ	Shear stress (Pa)
$\dot{\gamma}$	Shear rate (s^{-1})
η_a	Apparent viscosity (Pa.s)

2. Materials and methods

2.1 Sludge

Secondary sludge, utilized in experiments, was obtained from CUQ (Communauté urbaine de Québec) wastewater treatment plant, Ste-Foy with different sludge characteristics depicted in Table 1. The sludge was concentrated from 1.5 to about 4 % (w/v) solids gradually by gravity settling and centrifugation at 7650 g for 15 minutes in a Sorvall RC 5C plus Macrocentrifuge (rotor SA-600). The sludge supernatant was stored in the refrigerator at 4°C and used to dilute the sludge samples as per requirements. Concentrated sludge was homogenized using a blender for different solids concentration. The tests were conducted in the course of 5 to 6 d to prevent use of deteriorated sludge (there is possibility of suppressed and slow growth of microorganisms under storage conditions at 4°C).

To study the effects of solids concentration (10-40 g/L), each sludge sample was prepared in duplicates by diluting the concentrated sludge samples. In this study, sludge samples were defined for each set of total solids of 10, 15, 20, 25, 30 and 40 g/L as suffix 1, 1.5, 2, 2.5, 3 and 4 respectively as shown in Table 2. Thermal alkaline hydrolysis was carried out at pH 10.2 ± 0.1 and temperature 140 ± 1°C (microwave oven) as described in earlier studies (Barnabé et al., 2005).

2.2 Bacterial Strain

Bacillus thuringiensis var. *kurstaki* HD-1 (ATCC 33679) was used in this study.

2.3 Inoculum and Culture conditions

The culture conditions, maintenance, inoculum production, and shake flask fermentation (biopesticide production) details are described elsewhere (Vidyarthi et al., 2002). Standard deviation for total cell (TC) and viable spore (VS) count measurement was 6-8 %.

2.4 Viscosity

Viscosity of sludge during shake flask and fermenter experiments was determined at 25°C by using a rotational viscometer (Cole-Palmer Inc). The viscometer spindle diameter was 1.2 cm, with a gap size of about 2.5 mm between the spindle and the walls of the reservoir. Three different spindles were used in the entire study – L1, L2 and ultra-low centipoise adapter. Nature of sludge was ascertained at low shear rate (7.34 s^{-1}), preferable in most cases, as reported in viscometer manual. However, apparent viscosity (defined as the ratio of shear stress to shear rate at a fixed shear rate) for shake flask as well as fermenter samples was measured at higher shear rate of 36.71 s^{-1} (to reduce measurement lag time and uniformity of data to compare with the viscosities of formulations in downstream processing). In order to establish a best fit equation for viscosity variation with total solids concentration, viscosity was also measured at total solids concentration of 15 and 25 g/L. Viscosity was also determined at different shear rates of 0.36, 0.73, 1.83, 3.67, 7.34, 14.68, 36.71 and 73.42 s^{-1} to determine the shear rate behaviour.

The shear stress of the fermentation broth is normally characterized by the simple power law model:

$$\tau = K.\dot{\gamma}^{n} \quad (1)$$

where, τ is shear stress and $\dot{\gamma}$ is shear rate.

The constants, K and n represent consistency index and flow behavior index, respectively. The apparent viscosity, η_a is given by:

$$\eta_a = K.\dot{\gamma}^{n-1} \quad (2)$$

Taking logarithm on both sides of equation (2):

$$\log(\eta_a) = \log K + (n-1)\log\dot{\gamma} \quad (3)$$

The values of K and n were evaluated from logarithmic plot of η_a vs $\dot{\gamma}$.

These constants were estimated for fermented media at optimal solids concentration (maximum entomotoxicity value).

2.5 Bench-scale fermenter

Fermentation was carried out in a stirred tank 15L bioreactor (working volume = 10L, Biogenie, Quebec, Canada) equipped with accessories and programmable logic control (PLC) system for dissolved oxygen, pH, anti-foam, impeller speed, aeration rate and temperature as shown in Figure 1. The software (Fix 3.5, Intellution, USA) allowed automatic set-point control and integration of all parameters via PLC.

Initially, polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR-Canada). The oxygen probe was calibrated to zero (using N_2 degassed water) and 100 % (air saturated water). Subsequently, fermenter was charged with sludge and polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution as an anti-foam agent.

In-situ sterilization of non-hydrolyzed sludge was carried out at 121 ± 1°C for 30 minutes. When the fermenter cooled down to 30 ± 1°C, dissolved oxygen (DO) probe was recalibrated to zero by sparging N_2 gas and 100% saturation by sparging air at an agitation rate of 500 rpm. The fermenter was then inoculated (2 % v/v inoculum) aseptically with acclimated pre-culture of Bt in exponential phase. In order to keep the DO above 25 % saturation, air flow rate and agitation rate were varied between 0.133-0.233 vvm and 250–700 rpm, respectively. This ensured the critical DO level for Bt above 25% (Avignone-Rossa et al., 1992). The temperature was maintained at 30 ± 1°C by circulating water through the fermenter jacket. The pH was controlled at 7.0 ± 0.1 by using either 4N NaOH or 4N H_2SO_4 by peristaltic pumps. Foaming during fermentation was also simultaneously controlled by PPG injection and mechanical foam disruptor (Fundafoam™).

2.6 Analytical details

Total C and N, total solids (TS), total suspended solids (TSS), COD and dissolved solids (DS) concentrations for the sludge samples were analyzed according to Standard Methods

(APHA, 1995). The entomotoxicity (Tx) of the samples was measured by bioassay against spruce budworm (*Choristoneura fumiferana*) using diet incorporation method (Vidyarthi et al., 2002). The Tx data was reported as relative spruce budworm units (SBU/μL). Standard deviation for Tx measurement was 8-10 %. Volumetric oxygen transfer coefficient (k_La) was determined by conventional dynamic method.

3. Results and discussion

3.1 Thixotropic Behaviour

A representative viscosity behavior of unhydrolyzed sludge at TS of 20 g/L is illustrated in Figure 2. The apparent viscosity decreased appreciably from 210 to 110 mPa.s in the initial time frame of 40 minutes and later it became constant for the next 60 minutes conforming to thixotropic nature. This is in agreement with the basic definition of thixotropic fluids which are time dependent (Honey and Pretorius, 2000). Similar nature has been depicted by sludges at all process treatment stages. This nature of sludge can affect the stability of biopesticidal formulations in the downstream processing warranting addition of stabilizers and hence increasing the cost of final product.

3.2 Shear thinning behaviour

The apparent viscosity of fermented sludge suspensions decreased with increasing shear rate from 0.36 to 73.42 s^{-1} (data unreported) with corresponding K and *n* values given in Table 4. Detailed shear rate determination studies were carried out specifically for fermented sludges (Table 4) so as to ascertain the typical behaviour of such sludges which have been normally employed in biopesticide production studies (Vidyarthi et al., 2002).

When log-log viscosity-shear rate relationships were plotted, it yielded linear correlations for all fermented sludges, indicating that they followed power law in accordance with the literature (Lotito et al., 1997; Sanin, 2002). For NHF and HF sludge, K increased with solids concentration, showing non-Newtonian trend of the broth over entire solids range as observed from Table 4. This also explains stronger non-Newtonian behaviour (pseudoplastic nature) with increasing solids concentration in concordance with earlier studies (Sanin, 2002; Seyssiecq et al., 2003). It is known that at $n << 1$, more pronounced effects of pseudoplasticity on flow and transport phenomenon are observed (Aiba et al., 1973). The lower value of flow behaviour index (*n*) for NHF2.5 (0.67) as compared to HF2.5 (0.77) suggested greater pseudoplasticity or, more rapid decline in viscosity with increase in shear rate for NHF2.5 in contrast to HF2.5. This information will aid in designing heat and mass transfer factors for larger scale fermenters. In this context, NHF2.5 being more pseudoplastic than HF2.5 sludge could be advantageous at similar viscosities as HF2.5. However, higher

viscosity of NHF2.5 vis-a-vis HF2.5 might encompass more drawbacks in downstream processing. Moreover, distinguishing the best between the two sludges (HF2.5 and NHF2.5) in terms of viscosity still requires extensive study at fermenter scale. Similar trend of flow behavior indices was observed by Sinha et al. (2001) on varying glucose concentration during exo–biopolymer production by *Paecilomyces japonica* in a batch bioreactor.

Moreover, power law was obeyed until 20 and 30g/L for NHF and HF sludges respectively. This difference in power law obedience could be due to higher complexity of sludge medium in NHF when compared to HF (ameliorated by hydrolysis, discussed earlier). This behaviour was also evident from correlation coefficient (R^2) values given in Table 4.

Raw or NH sludge is known to obey power law only until 30g/l TS as per literature studies. There are no reported studies on rheology of sludge >30 g/l. However, when viscosity of raw sludge at solids concentration above 30 g/l was measured in our laboratory (data unreported), it was found that sludge at solids concentration >30 g/l did not obey the power law. Meanwhile, NHF did not obey power law beyond 20 g/l which could be probably due to change in morphology of medium caused by fermentation (>20 g/l), specifically, growth of Bt which might have resulted in enhanced solids interactions. Meanwhile, HF sludge obeyed power law up to 30g/l (against 20 g/l in NHF case). This was clearly due to dominant role of hydrolysis which caused thinning of sludge. Thus, hydrolysis of sludge shifted the power law disobedience from >20 g/l for NHF to >30g/l for HF sludge. Further, the disobedience of power law was not due to lack of precision of the instrument as 2.5 mm gap width (chosen in this study, as described earlier) was close to the gap width that Allen and Robinson (1990) showed to be insignificant for wall slip with pseudoplastic fermentation broths.

Alternatively, considering that sludge is a microbial mass slurry where particles constantly interact with each other, and after fermentation, more interactions will take place (due to growth of Bt, production of spores and crystal proteins) leading to a medium with entirely different physical regime. In this respect, it is possible that at higher solids concentration, there were intense particle interactions in NHF and HF sludges leading to different rheological behaviour and hence deviation from power law.

Trends in K and n of NHF and HF sludges will assist in design of downstream centrifugation criteria for different concentrations of fermented broths. For exemplification purpose, fermented broth has to be concentrated to increase spore concentration and overall entomotoxicity and as sedimentation rate is inversely related to viscosity, so NHF sludge will warrant higher centrifugal speed for product potency.

3.3 *Effect of solids concentration*

Overall variation of apparent viscosity values ranged from 2-156 mPa.s for raw/unhydrolyzed sludge and 3.67- 49 mPa.s for hydrolyzed sludge as seen in Figure 3. There was an increase in apparent viscosity with the increase in TS concentration from 10 to 40 g/L, irrespective of the type of sludge pre-treatment.

The increase in apparent viscosity with TS was due to resistance offered by increased inter and intra-particle interactions. As shown in Figure 3, there was not much change in the initial apparent viscosity until 20 g/L of TS. However, as particle concentration increased (either due to increase in solids concentration or disintegration of sludge solids due to treatment – TAH in this case; discussed later), the apparent viscosity changes were amplified. Additionally, formation of flocs or other cell aggregates is also mediated through extracellular polymeric substances (EPS), which form a highly hydrated gel and bind microorganisms together. Probably, low EPS concentration at lower TS could have decreased the floc strength resulting in lower viscosity values (Figure 3) as estimated in earlier studies (Sanin, 2002).

The increasing viscosity profiles were specific to process stages and followed exponential law as described by Equation 4:

$$y = a\, e^{bx} \qquad (4)$$

where; y = apparent viscosity (mPa.s) and x = solids concentration (g/L). Constants 'a' and 'b' are derivations of various process treatments and their respective values are shown in Figure 3. Constant 'b' will serve as a control parameter to adjudge the susceptibility of viscosity to change in solids concentration and if total solids concentration is too small, viscosity will yield to constant 'a'. The exponential law has also been found to be obeyed by raw and digested primary and secondary sludges (Dentel, 1997; Moeller and Torres, 1997). Exponential increase of viscosity with solids concentration implies that viscosity is a major governing factor at all process stages of biopesticide production.

3.4 *Effects of process treatment*

3.4.1 *Sterilization*

Sterilization is a mandatory step in biopesticide production, therefore, it will be pertinent to understand its effect on viscosity. The viscosity of ST sludge was lower than NH sludge (raw sludge) at all TS concentrations (Figure 3). Various physico-chemical changes like coagulation of sludge proteins and linking and de-linking of different bonds in sludge suspension would have contributed to the viscosity decrease. Additionally, decrease in

viscosity could be due to rupture of microbial cells (caused by thermal effect) and release of intracellular material and interstitial water into the medium.

At elevated pressure and temperature, the floc structure was disrupted and more free water was released from the aggregates (Muller et al., 1998). It was also possible that increased surface erosion of the flocs during heat treatment resulted in inter-particle cleavages and hence the decrease in viscosity (Figure 3). Even, EPS (bridges of inter-particle network of sludges flocs) may have also been destroyed during heat treatment. Thus, complete disorder and re-orientation of different sludge constituents would have contributed to viscosity decrease.

3.4.2 Thermal Alkaline Hydrolysis (TAH)

During the entire TAH process, many interesting changes occurred in terms of viscosity decrease (Figure 3). Firstly, on pH adjustment, there was partial hydrolysis of sludge particles and flocs. Chemically, there would be cleavage of many polar bonds and if NaOH was assumed to be equivalent to a catalyst (although not recoverable, but being used in smaller amounts). It will lower the energy of activation of different sludge constituents and cause partial cleavage of labile bonds which was further augmented by temperature effects (TAH).

Another explanation based on thermodynamics can be postulated for TAH phenomenon. If we assume thermal hydrolysis as disintegration in a pseudo-closed system (hydrolysis tubes) which increased due to the overall increase in rotational, translational and vibrational energies of the molecules, then this would further increase chaos in the system (partially reversible disintegration reaction). In this case, change in entropy (ΔS) would be positive, change in enthalpy (ΔH) would be negative as it would be an energy intensive process where inter and intra-particle bonds were cleaved and also some new intra-particle bonds generated. In the present case, ΔG would be negative ($\Delta G = \Delta H - T\Delta S$) and hence the reaction was favoured spontaneously towards disintegration.

Further, alkaline treatment disintegrated larger molecules into smaller and simpler ones. The disintegration thus increased effective cross-sectional area as the normal flocs were expanded, and hence there was more exposure to heat during consequent thermal hydrolysis (Kepp et al., 2000). Thus, combined effect of alkali and temperature resulted in lower viscosity of hydrolyzed sludge than ST at higher TS. Almost 50% of initial suspended solids (SS) were also dissolved during TAH process (data unreported). Lower viscosity so obtained was mainly due to solubilisation of SS (reflected in DS, caused by release of interstitial and

bound water from sludge flocs; lysis of cells; cleavage of bonds). This would be favourable for pumping of sludge, improving oxygen transfer during fermentation and enhance the productivity of wastewater sludge based Bt biopesticide process.

To sum up, there were two distinct phenomenon observed, during these studies; (1) increase in viscosity with total solids concentration, irrespective of type of treatment process (ST, TAH, HST); (2) decrease in viscosity of hydrolyzed sludge in comparison to NH and ST sludges, irrespective of total solids concentration. The former may be due to stronger inter-particle network structure at increased total solids concentration even after thermo-chemical treatment (Sozanski et al., 1997).

3.4.3 Hydrolyzed sterilized sludge (HST)

The increase in viscosity of hydrolyzed sludge on sterilization was possibly due to higher inter and intra-particle interactions leading to inter-particle bonds which offered resistance to flow. Contrary to ST, coagulation does not contribute much in HST as the proteinaceous matter has been already degraded to simpler compounds (refer to TAH). Therefore, it is advisable to use hydrolyzed (heat treatment) sludge directly for biopesticide production process so that initial viscosity during fermentation is low leading to better oxygen transfer and hence improved efficiency during the production process and reduction in sterilization costs.

3.4.4 Fermentation process

Results obtained on total cell concentration (TC), viable spore concentration (VS), change in pH and Tx during Bt fermentation of NHF and HF sludges are presented in Table 3. In general, TC,VS count and entomotoxicity values were higher in HF than NHF. After 48 hrs of fermentation, VS in NHF ($1.4 \times 10^5 - 1.6 \times 10^8$ CFU/ml at TS 10-40 g/L) was lower than that observed for HF sludge ($1.1 \times 10^5 - 2.4 \times 10^9$ CFU/ml at TS 10-40 g/L). High percent sporulation of 94 and 92 % was also observed for HF samples at TS of 25 and 30 g/L, respectively. Dissolved solids were reduced by almost two-third during fermentation due to their utilisation in the microbial metabolic processes. This decrease in DS contributed to higher Tx and TC and VS counts. During fermentation, DS decreased (Figure 5) with concomitant increase in TC. This would have increased viscosity at the end of fermentation which did not happen. This trend could be due to the fact that DS transformed into cells which eventually lysed releasing intracellular material (enzymes like proteases; chitinases; phospholipases and spores) resulting in a net decrease in viscosity. Various reports have also indicated that the accumulation of extracellular proteins secreted by the cells were

responsible for the shear-thinning behaviour and decrease in viscosity (Trejo-Tapia et al., 2001). Thus, various physico-chemical (pH, dissolved solids, soluble COD, suspended solids, particle size) as well as biological changes (enzymes, cell and spore concentrations, crystal protein formation) were contributory to the overall viscosity decrease.

Specifically, during sludge hydrolysis, solubilised complex matter, enhanced nutrient assimilation to produce specific products, biopesticide in this case, in the form of crystal proteins (δ-endotoxin) and also improved mass transfer due to amelioration in medium properties (Li et al., 1995; Berzins et al., 2001). Moreover, thermal pre-treatment has been considered as a viable option for many years to enhance the solubilisation of organic matter (Neyens et al., 2003). In toto, viscosity of sludge was decreased by TAH process (physico-chemical change) with further marked decrease due to fermentation process. Similar viscosity decrease was also obtained during fermentation of raw sludge (NH).

Viscosity of NHF sludge was higher with respect to ST due to biological changes (growth of Bt cells; formation of EPS) occurring during fermentation. On the other hand, a substantial decrease in viscosity of HF with respect to HST was attributed to more efficient fermentation of higher concentration of disintegrated particles with respect to NHF. Extracellular enzyme systems have been proved to be actively involved in formation of smaller entities during Bt fermentation (Vidyarthi et al., 2002). However, at this moment, difference in NHF and HF behaviour could be attributed to sludge complexity. Notably, lower viscosity will improve ease of handling for biopesticidal formulations (field application) and propitiate compatibility with the spraying equipment (Smirnoff, 1980).

Viscosity of HF sludge at low solids concentration (HF1, HF1.5, HF2) did not increase appreciably. This is in concordance with the reported literature (as explained before) that a very thin activated sludge (at TS 10-20 g/L) may behave very close to water ($n \rightarrow 1$; Newtonian behaviour) in terms of rheological properties (Campbell and Crescuollo, 1982). This is also confirmed in various other studies, where only thick activated sludge has been identified as either a plastic or a pseudoplastic non-Newtonian fluid (Baudez and Coussot, 2001).

3.5 Bench-scale fermenter studies

Fermentation results obtained in bench scale fermentation for NH2.5 sludge are presented in Figure 4. Total cell and viable spore concentration increased until 18h of fermentation and diauxic type of Bt growth was observed. Viscosity kept on decreasing all through the fermentation with a small hump at 12h. Interestingly, initial viscosity at 0h was higher than

ST sludge in shake flask. This could be due to two reasons – addition of anti-foam agent before sterilization in fermenter and sludge agglomerates (generated as a result of intense foaming during sterilization). Addition of anti-foam agent to sludge was essential to reduce foam during fermentation. However, viscosity decreased monotonically owing to continuous agitation and aeration to maintain DO above 25 % saturation (experimentally determined value). A small hump spanning over 9-12 h could be again due to anti-foam addition during exponential growth phase. Finally, at 48h of fermentation there was a four fold decrease in viscosity. A definite relationship between k_La and viscosity could not be established during fermentation owing to continuous variation of agitation and aeration rates and intermittent anti-foam addition. The k_La rose during first three hours of fermentation when the viscosity decreased. However, decrease in k_La from 3-6h despite a decrease in viscosity could be due to various reasons – changing complexity of sludge morphology; Bt growth (cell formation, spores and crystal protein formation) and addition of anti-foam to control the foam being continuously formed in the system. Further, increase in k_La from 6-12h despite increase in viscosity was probably influenced by increasing the speed of aeration and agitation to keep the dissolved oxygen above the limiting concentration of 25%. Viscosity at the end of fermentation was of the same order as in Erlenmeyer flasks. There was a 37.3 % increase in Tx (Tx = 12710 SBU/μL) as a consequence of more effective aeration and agitation in fermenter (monotonically decreasing viscosity) vis-à-vis shake flask (Tx = 9256 SBU/μL).

The requirement of shake flask studies was inevitable as a matter of fact that viscosity changes could be directly translated to bench scale bioreactor. This can drastically lower experimental load and as well provide an insight into probabilities in viscosity trends during scale-up and post-fermentation (harvesting and formulation development). Therefore, this study sets the ground for a compromise between biological performance (most suitable sludge type for Bt production) and physical efficacy (best sludge type for process operational conditions; agitation, aeration, viscosity modifiers) to be achieved. Further, similar studies are underway for hydrolyzed sludge, which needs optimization of hydrolysis conditions at bench-scale.

3.6 Dissolved solids

Profiles of dissolved solids (DS) for different sludges at various TS concentrations are presented in Figure 5. The increase of DS with TS in NH (raw) sludge could be due to the amount of dissolved matter coming into the solution after re-suspending solids which in turn is dependent on total amount of solids suspended in solution. DS increased with TS in the order: HST>TAH>ST>HF>NHF>NH. The amount of dissolved solids is a function of TS, pH, temperature and suspension medium (Neyens et al., 2003). In other words, increase in TS

probably increased the driving force of colloidal/ dissolved matter to re-dissolve into the suspension.

Among other factors, comparatively higher values of apparent viscosity for NH (3.36-156 mPa.s) could be contributed by lower DS. However, during TAH stage, the increase in DS led to thinning of the fluid and hence strongly contributed towards decreased viscosity. HST and TAH sludge showed similar DS values, however, viscosity for TAH was much lower than HST. Higher viscosity of HST sludge was due to inter-particle mechanisms (explained earlier). On the other hand, HF relative to HST sludge showed lower values of DS. Increase in DS during TAH resulted in decrease in viscosity due to physico-chemical phenomenon, whereas, during fermentation, DS decrease due to cell lysis and release of intracellular material caused net viscosity decrease. In particular, NHF sludge showed a relatively lower decrease in DS due to less efficient fermentation (lower TC, VS and Tx) in comparison to HF sludge, as explained in fermentation section. A noteworthy point was presence of bigger flocs in NHF vis-à-vis HF where the medium had smaller flocs (observed microscopically, results not reported). Thus, DS was not the sole contributing factor to change in viscosity due to complexity involved during fermentation (as discussed before and in fermentation section).

The specific gravity of sludge ranged from 0.979 to 1.018 for TS (10-40 g/L) and was almost near to water. The density does not change much and a small change was observed only with increase in TS and these values suggested that sludge was partially soluble in water (DS).

3.7 COD

Total COD varied from 11298-50628 mg/L at different solids concentration and did not change during pre-treatment processes. However, soluble COD (SCOD) followed a pattern similar to DS concentration (Figure 6). It increased from 2444-7369 mg/L for NH and 5993-10231 mg/L for TAH sludges as TS increased from 10 to 40 g/L. Evidently, the increase in SCOD in TAH sludge justified improvement in the Bt production process (growth, entomotoxicity, spore concentration) due to increase in biodegradable organic matter. There was a decrease in SCOD after fermentation (NHF, HF) as Bt utilized a part of SCOD as its carbon source.

In summary, at all process stages, sludges exhibited pronounced viscosity effects at 30 and 40 g/L. Thus, biopesticide production at high solids concentration needs critical evaluation of viscosity as it underwent various changes at different process stages, which is not true at lower solids concentration. Also, sterilization of raw and hydrolyzed sludge resulted in

different viscosity trends at all solids concentration which can aid in selection of process parameters (DO, agitation) in the fermenter (high viscosity and non-Newtonian behavior would cause decreased homogeneity in the fermenter and poor yield, e.g., NH). HF sludge showed higher Tx and lower viscosity. Hence, it could reduce cost due to high yield (Tx) and concomitant decrease in viscosity which may eliminate addition of viscosity modifiers. On the contrary, NHF showed an increase in viscosity and decrease in Tx questioning its utility for Bt production. Hence, these results will assist in making a smart choice of Bt fermented sludge for formulation purposes; estabilishing role of rheological parameters in production of value-added products from sludge.

4. Conclusions

The following conclusions can be drawn from this study:

1. Change in viscosity with total solids concentration was quantified by exponential law and sludges, in general, were thixotropic and pseudoplastic. Whilst, power law model was partially obeyed by fermented sludges.
2. Viscosity was strongly influenced by the type of process treatment. Viscosity was higher for non-hydrolyzed fermented vis-à-vis hydrolyzed fermented sludge.
3. Dissolved solids was one of the principal factors controlling viscosity which was also affected by soluble COD and fermentation efficacy.
4. Lower viscosity of hydrolyzed fermented sludge resulted in higher Tx as compared to non-hydrolyzed fermented sludge.
5. Shake flask results aided in predicting the viscosity trends in bench scale bioreactor.
6. Hydrolyzed sludge, preferentially, can be directly employed as a fermentation medium, omitting the sterilization step and further improving economy of the process.

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, and Canada Research Chair) for financial support. The views and opinions expressed in this article are those of authors. We are also thankful to Natural Sciences and Engineering Research Council of Canada and Canadian Forestry Service for providing Ph.D scholarship to Satinder K. Brar during the course of this research work.

References

Aiba, S., Humphrey, A.E. and Millis, N.F. (1973) Biochemical Engineering Academic Press, 2nd edition, New York.

Allen, D., Robinson, C.W. (1990) Measurement of rheological properties of filamentous fermentation broths. Chemical Engineering and Science, **45**, 37–48.

APHA-AWWA-WPCF (1995) Standard methods for the examination of water and wastewater (Ed. Clesceri, L.S., Greenberg, A.E., Trussell, R.R., and Franson, M.A.), 18th edition, APHA, Washington, DC 20005.

Avignone-Rossa, C., Arcas, J. and Mignone, C. (1992) *Bacillus thuringiensis*, sporulation and δ-endotoxin production in oxygen limited and non-limited cultures. World Journal of Microbiology and Biotechnology, **8**, 301-304.

Barnabé, S., Verma, M., Tyagi, R.D. Valéro, J.R. (2005) Culture media for increasing biopesticide producing microorganism's entomotoxicity, methods of producing same, biopesticide producing microorganisms so produced and method of using same. US patent International application PCT/CA2005/000235.

Battistoni, P., Fava, G. and Ruello, M.L. (1990a) Rheology of sewage sludge. Rheological parameters and sludge characteristics. Environmental Protection Engineering **16** (3- 4), 57-69.

Battistoni, P., Fava, G. and Ruello, M.L. (1990b) Rheology of sewage sludge (I) Development of a standardised methodology for rheological properties measurement. Ingegneria Sanitaria **2,** 10-19.

Baudez J.C. and Coussot P. (2001) Rheology of aging, concentrated, polymeric suspensions: application to pasty sewage sludges. Journal of Rheology **45**, 1123-1140.

Berzins, A., Toma, M., Rikmanis, M. and Viesteurs, M. (2001) Influence of micromixing on microorganisms and products. Acta Biotechnology **21**(2), 155 –170.

Campbell H.W. and Crescuollo, P.J. (1982) The use of rheology for sludge characterization. Water Science and Technology **14,** 475-489.

Dentel, S.K. (1997) Evaluation and role of rheological properties in sludge management. Water Science and Technology **36** (11), 1-8.

Erdincler, A. and Vesilind, P.A. (2000) Effect of sludge cell disruption on compactability of biological sludges. Water Science and Technology **42**(9), 119-126.

Honey, H.C. and Pretorius W.A. (2000) Laminar flow pipe hydraulics of pseudoplastic-thixotropic sewage sludges. Water SA **26**(1), 19-26.

Kepp,U., Machenbach, I., Weisz, N. and Solheim, O.E. (2000) Enhanced stabilization of sewage sludge through thermal hydrolysis – Three Years of experience with full scale plant. Water Science and Technology **42**(9), 89-96.

Li, G.Q., Qiu, H.W., Zheng, Z.M., Cai, Z.L. and Yang, S.Z. (1995) Effect of fluid properties on mass transfer in a bioreactor. Journal of Chemical Technology and Biotechnology **62**, 385-391.

Lotito, V., Spinosa, L., Mininni, G. and Antonacci, R. (1997) The rheology of sewage sludge at different steps of treatment. Water Science and Technology **36**(11), 79-85.

Moeller, G. and Torres, L.G. (1997) Rheological characterization of primary and secondary sludges treated by both aerobic and anaerobic digestion. Bioresource Technology **61**(8), 207-211.

Mor, S.G. and Matthews, G.A. (2003) Recent Developments in Sprayers for Application of Biopesticides : an Overview. Biosystems Engineering **84** (2), 19-125.

Muller, J., Lehne, G., Schwedes, J., Battenberg, S., Naveke, R., Kopp, J., Dichtl, N., Scheminski, A., Krull, R. and Hempel, D.C. (1998) Disintegration of sewage sludges and influence on anaerobic digestion. Water Science and Technology **38**(8-9), 425-433.

Neyens, E., Baeyens, J. and Creemers, C. (2003) Alkaline thermal sludge hydrolysis. Journal of Hazardous Materials, **B97,** 295–314.

Pan, S.C. and Tseng, D.H. (2001) Sewage sludge ash characteristics and its potential applications. Water Science and Technology **44,** 261-267.

Sanin, F.D. (2002) Effect of solution physical chemistry on the rheological properties of activated sludge. Water SA **28**(2), 207-211.

Seyssiecq, I. Ferrasse, J.H. and Roche, N. (2003) State of the art: rheological characterization of wastewater treatment sludge. Biochemical Engineering Journal **16**, 41 - 56.

Smirnoff, W.A. (1980) Deposit assessment of Bt formulation applied from an aircraft. Canadian Journal of Microbiology **26**, 1364-1366.

Sozanski, M.M., Jempa, E.S., Grocholski, K. and Bien, J. (1997) The rheological experiment in sludge properties research. Water Science and Technology **36**(11), 69-78.

Tay J.H. and Show, K.Y. (1992) Reuse of wastewater sludge in manufacturing non-conventional construction materials/an innovative approach to ultimate sludge disposal. Water Science and Technology **26**, 165-1174.

Tirado-Montiel M.L., Tyagi R.D. and Valéro J.R. (2001) Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. Water Research **35**(16), 3807 - 3816.

Trejo-Tapia, G., Jimenez-Aparicio, A., Villarrea, L. and Rodriguez-Monroy, M. (2001) Broth rheology and morphological analysis of *Solanum chrysotrichum* cultivated in a stirred tank. Biotechnology Letters **23**, 1943–1946.

Tyagi, R.D., Foko, V. S., Barnabe, S., Vidyarthi, A.S., Valéro, J.R. and Surampalli, R.Y. (2002) Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using sewage sludge as a raw material. Water Science and Technology **46** (10), 247–254.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2002) Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as a raw material. Water Research **36**, 4850-4860.

Table 1 Secondary sludge composition of Communauté Urbaine du Québec (Québec, Canada)

Parameter	Concentration
TS (g/L)	17
TVS (g/L)	13
SS (g/L)	15
VSS (g/L)	11
C_t (mg/kg)	261712
N_t (mg/kg)	37889
P_t (mg/kg)	7554
$N\text{-}NH_3$ (mg/kg)	824
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$ (mg/kg)	15
$P\text{-}PO_4^{3-}$ (mg/kg)	4328
Al (mg/kg)	4056
Ca (mg/kg)	12643
Cd (mg/kg)	0.37
Cr (mg/kg)	27
Cu (mg/kg)	179
Fe (mg/kg)	10568
K (mg/kg)	987
Pb (mg/kg)	28
S (mg/kg)	3598
Zn (mg/kg)	279
Na (mg/kg)	1259

Table 2 Designated nomenclature of different sludge samples

No.	Sludge type	Symbol	Solids categorisation
1.	Non-hydrolyzed/Raw/Fresh	NH	NH1 ;NH2; NH2.5;NH3; NH4
2.	Sterilized	ST	ST1; ST2; ST2.5; ST3; ST4
3.	Thermal alkaline Hydrolyzed	TAH	H1; H2; H2.5; H3; H4
4.	Thermal alkaline Hydrolyzed sterilized	HST	HST1; HST2; HST2.5; HST3; HST4
5.	Thermal alkaline Hydrolyzed fermented	HF	HF1; HF2; HF2.5; HF3; HF4
6.	Non-hydrolyzed fermented	NHF	NHF1; NHF2; NHF2.5; NHF3; NHF4

Table 3 Entomotoxicity vis-a-vis cell and spore concentrations

S.No.	Sample type	TC at 48h (CFU/mL)	VS at 48h (CFU/mL)	% sporulation	TC (0h)	VS (0h)	$pH_i \pm 0.1$	$pH_f \pm 0.1$	Tx (SBU/µL)
1.	HF1	2.00E+06	1.10E+05	5.5	1.6E +03	1E+02	7.02	8.1	6239
2.	NHF1	1.00E+06	1.40E+05	14	1.42E +03	1E+02	7.02	8.14	4221
3.	HF2	8.30E+07	8.4E+06	10.12	1.53E+03	1E+02	7.1	8.2	8956
4.	NHF2	4.8E+06	8.5E+05	17.71	1.47E+03	6E+02	6.8	8.15	7543
5.	HF2.5	1.80E+08	1.70E+08	94.4	1.4 E +03	11 E+02	6.98	8.2	14656
6.	NHF2.5	1.10E+08	1.00E+08	90.9	1.4 E +03	13 E+02	7.1	8.01	9256
7.	HF3	2.60E+09	2.40E+09	92.3	1.2 E +03	9 E+02	7.1	8.2	10223
8	NHF3	2.00E+08	1.60E+08	80	1.4 E +03	11 E+02	7.1	8.2	8562
9.	HF4	1.30E+07	1.00E+07	76.9	1.1 E +03	1E+02	7.2	8.23	13965
10.	NHF4	2.30E+06	2.00E+06	86.9	1.5 E +03	14 E+02	7.1	8.1	8556

pH_i = initial pH; pH_f = final pH

Table 4 Consistency and flow behaviour index values of fermented sludges

TS (g/L) **NHF**	***K*** ***(mPa.s^n)***	**n**	***R^2***
10	13.6	0.83	0.9872
15	13.9	0.78	0.9603
20	25.4	0.76	0.9134
25	30.9	0.67	0.7347
30	49.2	0.58	0.5212
40	51.3	0.35	0.3856
HF			
10	8.1	0.91	0.9847
15	9.1	0.89	0.9792
20	10.9	0.89	0.9802
25	13.4	0.77	0.8453
30	16.3	0.72	0.8037
40	21.1	0.56	0.4718

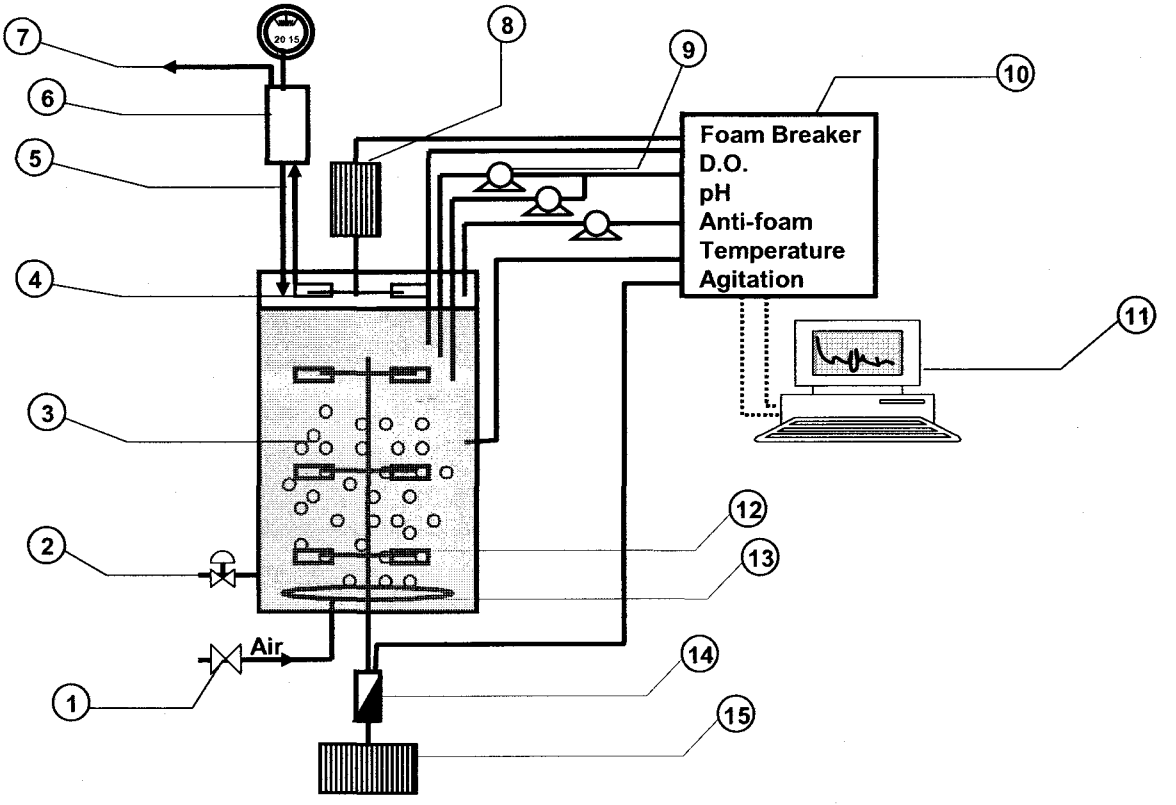


Fig. 1. Schematic diagram of 15 L bench scale fermenter: (1) Mass flow controller valve (for inlet air); (2) sampling port; (3) air bubbles; (4) foam breaker; (5) condensate; (6) condenser; (7) exit gas; (8) foam breaker motor; (9) peristaltic pump; (10) PLC panel; (11) data processing unit; (12) impeller; (13) air sparger; (14) tachometer; and (15) agitator motor

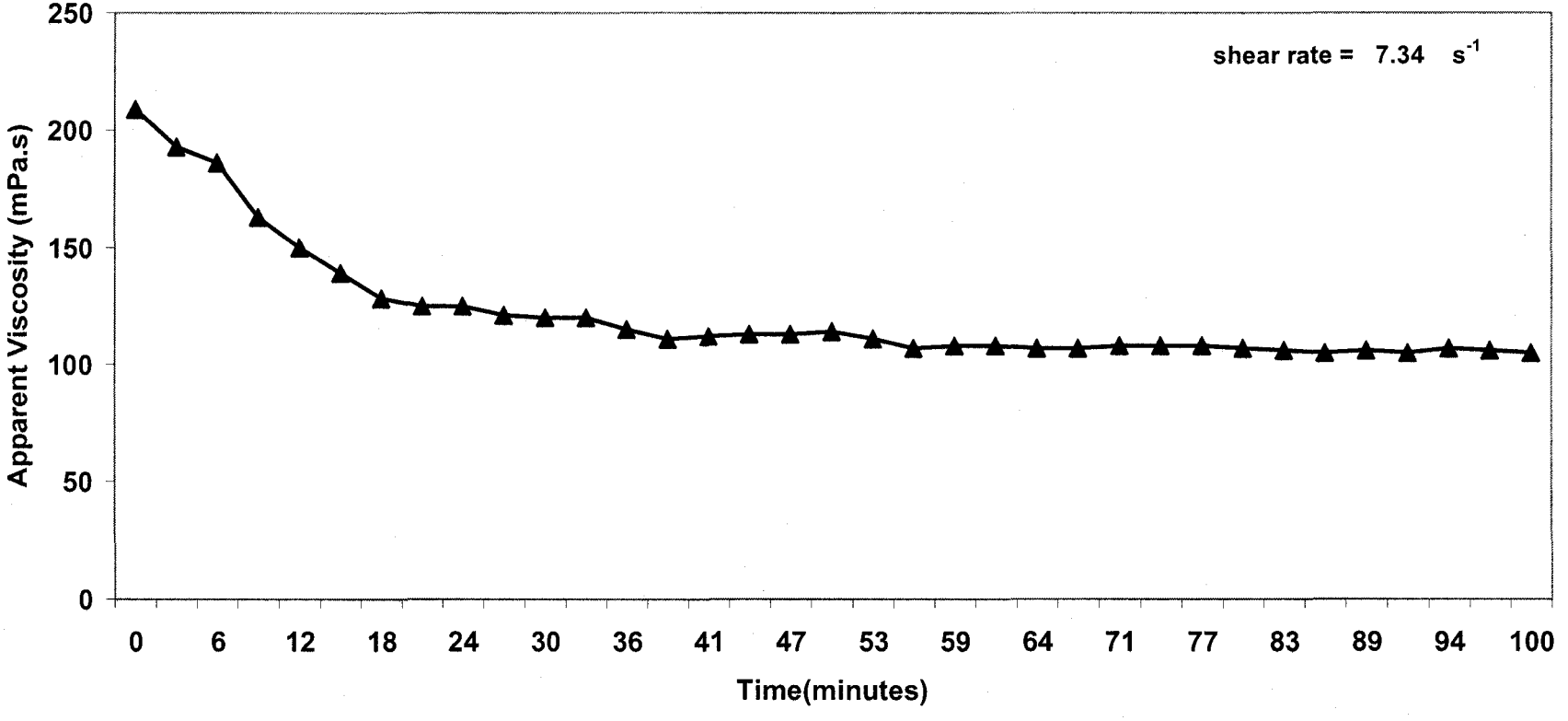


Fig. 2. Thixotropic behaviour of secondary sludge (TS = 20 g/L)

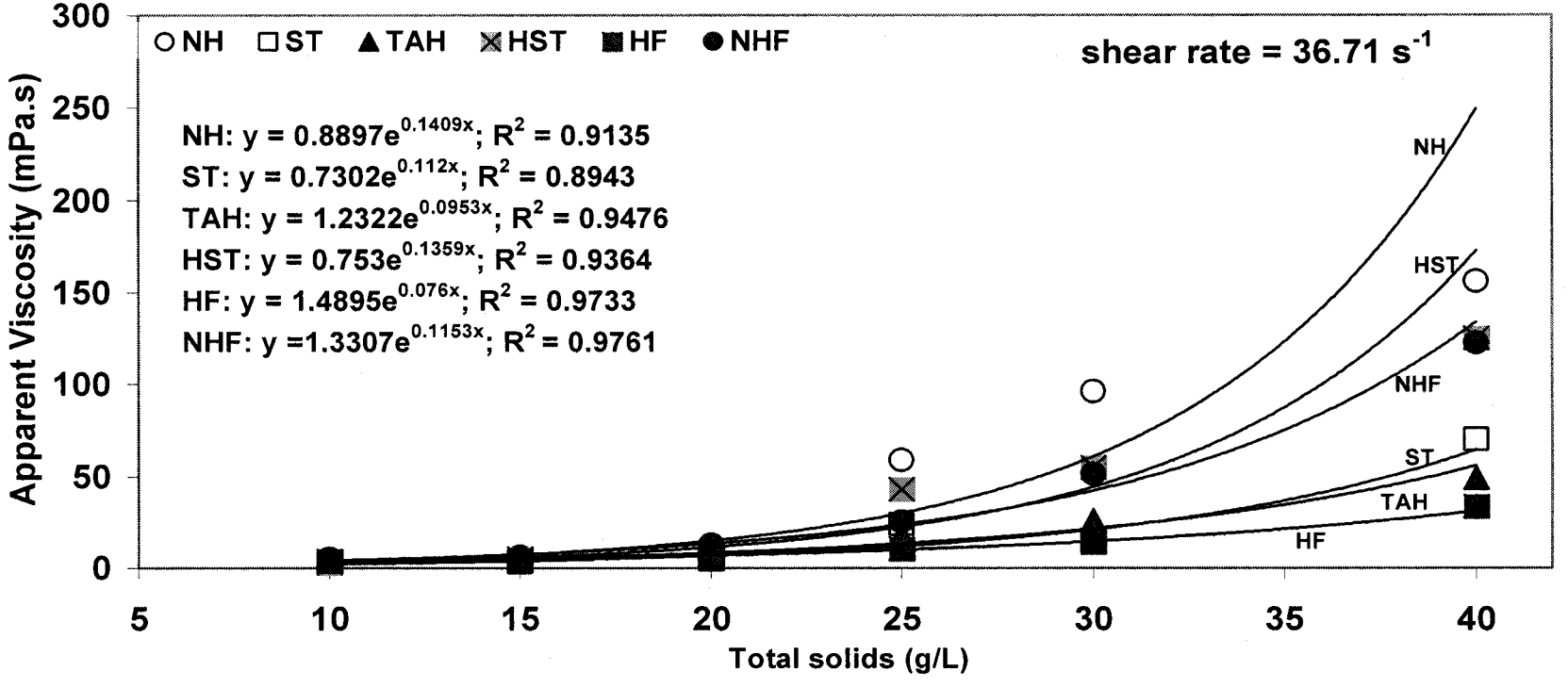


Fig. 3. Effect of process treatments and solids concentration

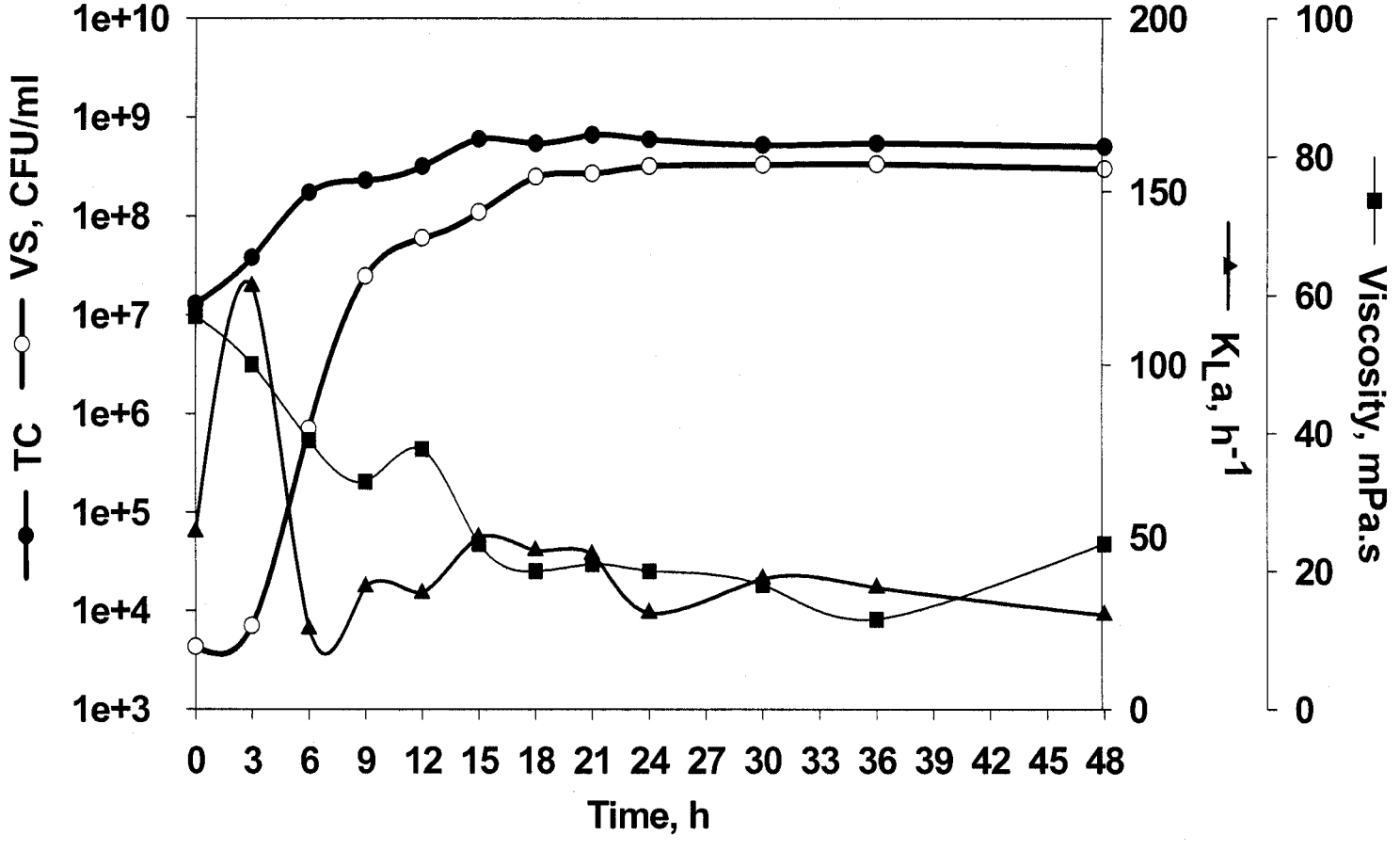


Fig. 4. Evolution of Bt growth profile with viscosity and k_La in a bench-scale fermenter (TS = 25g/L)

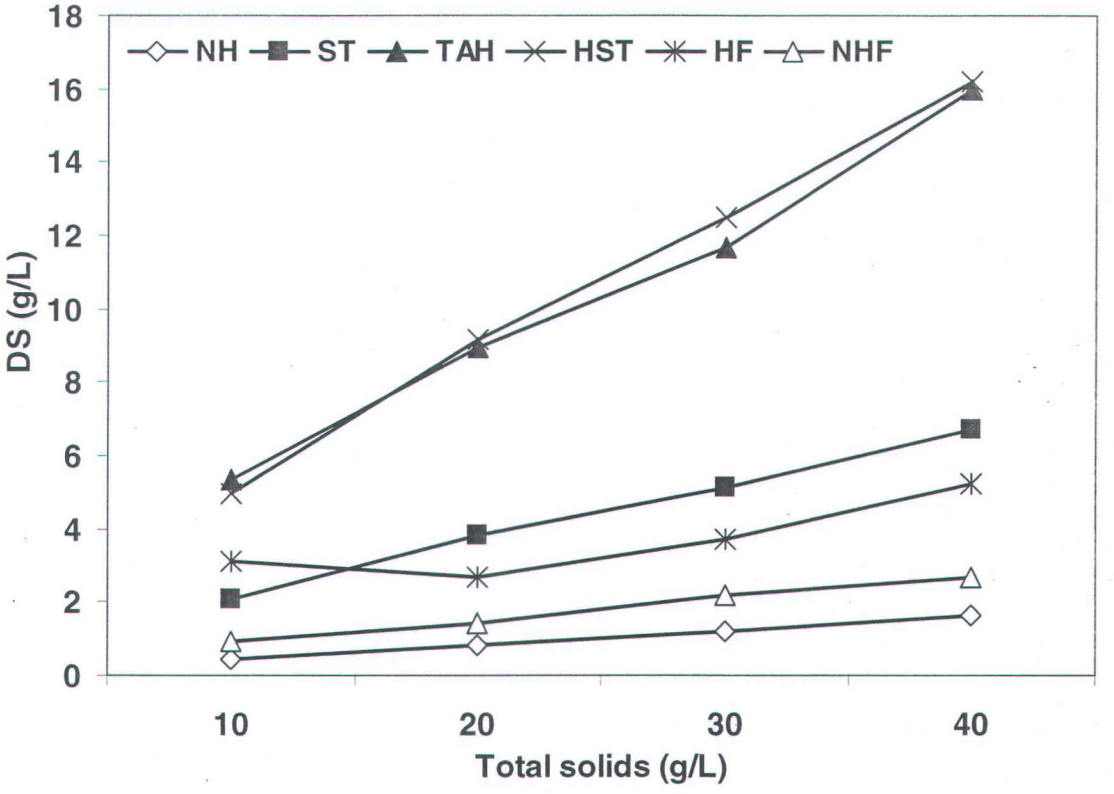


Fig. 5. Dissolved solids concentration profile at different TS for different treatments

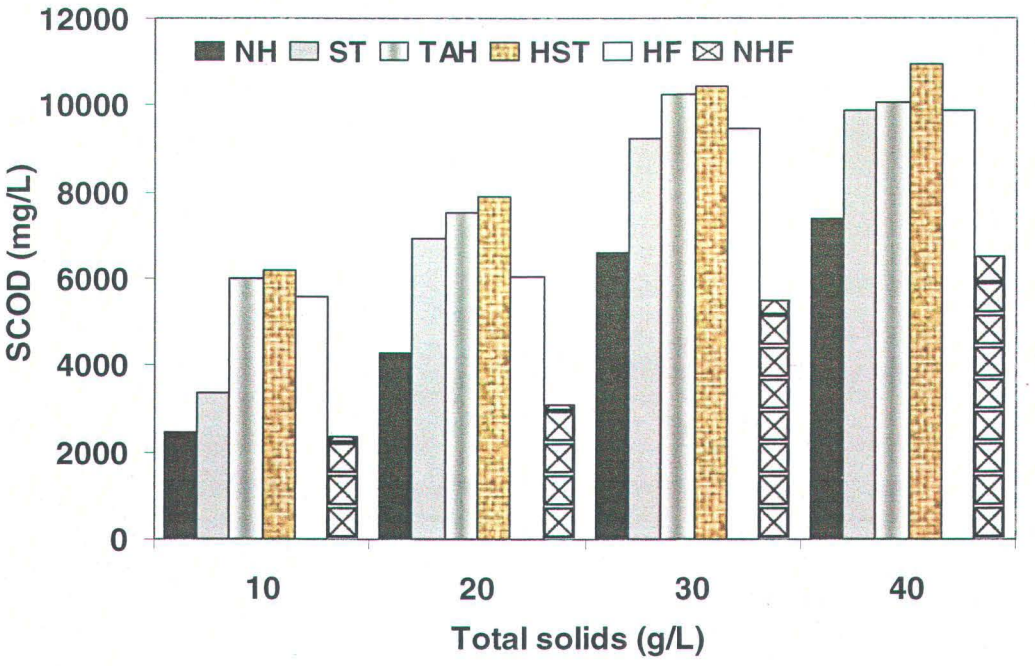


Fig. 6. SCOD profile for different sludges

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. et Surampalli, R.Y. (2008). Particle size variations during production of wastewater sludge-based *Bacillus Thuringiensis* Biopesticides. *Pract. Period. Hazard. Toxicol. Radioact. Waste Manage.* 12(1): 30-39.

http://dx.doi.org/10.1061/(ASCE)1090-025X(2008)12:1(30)

Partie III

Particle Size Variations during Production of Wastewater Sludge based *Bacillus thuringiensis* Biopesticides

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*], R.Y. Surampalli[2] and J.R. Valéro[1]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Practice Periodical of Hazardous, Toxic and Radioactive Waste Management - American Society of Civil Engineers (Under Revision)

Variation de la taille des particules pendant la fermentation des boues d'épuration par *Bacillus thuringiensis*

Résumé

Des études de la distribution de la taille des particules (PSD) ont été réalisées sur les biopesticides à base de *Bacillus thuringiensis* (Bt) pour tous les étapes de procédés tel que, bruts, stérilisation, hydrolyse et fermentation à différentes concentrations en solides (10 – 40 g L^{-1}). Des corrélations de la taille des particules (PRS) ont été mises en évidence avec différents paramètres comme l'indice du volume des boues (SVI), la vitesse de sédimentation ainsi que le nombre de spores viables et l'entomotoxicité de Bt. Le volume du PSD a mis en évidence qu'il y a plus de particules dont la PRS se situait entre 0,2-12 µm pour les boues hydrolysées fermentées par rapport aux boues non hydrolysées fermentées où la PSD variait entre 0,5 à 10 µm alors que le pourcentage cumulatif du volume était de 20 et 30 %, respectivement et indépendamment de la concentration en solides. Des tendances semblables ont été observées pour les profils de distribution de surface. Le potentiel insecticide diminuait et le SVI augmentait avec l'augmentation de la taille de particule. Le calcul de la vitesse de sédimentation a été réalisé avec la loi de Stoke et les valeurs étaient plus faibles pour les boues hydrolysées. Les faibles SVI et taille des particules pour les boues hydrolysées fermentées sont les caractéristiques requises pour les formulations de biopesticides. Ainsi, la PRS peut devenir un outil valable pour évaluer la qualité des boues utilisées comme matière première pour l'obtention de biopesticides à base de Bt, spécialement pour chacune des étapes de production (fermentation, centrifugation, mise en formulation et l'application sur le terrain).

Mots-clés: *Bacillus thuringiensis*; biopesticide; entomotoxicité; fermentation; hydrolyse; taille de particule; boues d'épuration.

Abstract

Particle size distribution (PSD) studies were performed on raw, hydrolyzed, sterilized and *Bacillus thuringiensis* (Bt) fermented wastewater sludge at different solids concentration (10 – 40 g L^{-1}). Correlations of particle size (PRS) were drawn with different parameters like sludge volume index (SVI), sedimentation velocity, viable spores and entomotoxicity. The volume distribution of particle size showed more number of particles for hydrolyzed fermented wastewater sludge in the 0.2-12 μm region in comparison to non-hydrolyzed Bt fermented sludge where the PRS was restrained to 0.5 to 10 μm region at a cumulative volume percent of 30 and 20 %, respectively, irrespective of solids concentration. Similar trends were observed for surface distribution profiles. The entomotoxicity decreased and SVI increased with increase in particle size. The sedimentation velocity was computed based on Stoke's law and was lower for hydrolyzed sludge. The lower SVI and particle size of hydrolyzed fermented sludge are the required characteristics suitable for biopesticidal formulations serving as an excellent Bt fermented medium for the purpose. Thus, PRS can act as an important tool in screening sludges as raw materials for Bt based biopesticide fermentation; centrifugation; formulation and field application.

Keywords: *Bacillus thuringiensis*; Biopesticide; Entomotoxicity; Fermentation; Hydrolysis; Particle size; Wastewater sludge

Introduction

As concerns for the wastewater sludge management increase, better understanding of various physical properties of sludge, namely, particle size, viscosity among others need to be determined to improve sludge disposal and reutilization options (Erikkson et al. 1992).

Sludge network is strengthened by inter-particle, intra-particle and particle-dispersion interactions, each playing a specific role in the multi-mode particle distribution (Wilén et al. 2003). These interactions influence the floc formation phenomenon which controls various physical, chemical and biological properties of sludge treatment systems (Jin et al. 2003). As sludge undergoes various stages of treatment, namely, digestion, dewatering, physical changes affect the PRS, which in turn, influences the sludge handling, utilization and disposal (Mikkelsen 2001). Furthermore, the particle size characterization gives better insight into the structure and inter-particle interactions inside bacterial flocs aiding in optimization of various sludge separation processes like filtration and centrifugation for dewatering (Mikkelsen and Keiding 2002). However, most of the reported studies discuss the role of particle size characterization in sludge treatment methods only for their ultimate disposal.

Currently, sludge is being considered as a raw material to develop sustainable value-added products. *Bacillus thuringiensis* (Bt) based biopesticides is one of the such value-added products leading to sustainable sludge management and enhanced penetration of biopesticides into the world pesticide market (Tirado-Montiel et al. 1998; Sachdeva et al. 2000). Production of biopesticides from wastewater sludge involves following steps: hydrolysis to augment nutrient availability; Bt fermentation of hydrolyzed sludge; centrifugation to concentrate Bt spores, and crystal proteins and formulation of the concentrated Bt broth (addition of different adjuvants to enhance field efficacy and ease of application). During fermentation, oxygen transfer is largely influenced by the physical nature of the fermentation medium (Vidyarthi et al. 2002). This also includes PSD of sludge undergoing biotransformation. The PSD will also influence the downstream processing of biopesticides during centrifugation step of Bt fermented sludge and amendment of various adjuvants during formulation development. It has been reported in literature that particle size of Bt based formulation controls synergy with the pre-existing application equipment and field efficacy (Mor and Matthews 2003). Furthermore, there is no reported study until date on the role of PSD in Bt fermentation. Thus, PSD study will aid in understanding the biopesticide production process from various points of view – fermentation, centrifugation (harvesting), formulation development and field application.

Therefore, the global objective of this research was to study the role of PSD during various steps of wastewater sludge based Bt biopesticide production process (treated and non-treated sludge, fermentation, centrifugation and formulation). The study was based on the hypothesis that different process stages at varying sludge solids concentration will influence the PSD having subsequent effect on Bt production.

Materials and Methods

Sludge

The secondary sludge used in this study was obtained from CUQ (Communauté Urbaine de Québec) wastewater treatment plant, Ste – Foy, Quebec, Canada with detailed characteristics given in Table 1. The sludge was concentrated from 1.7 % to about 4 % (w/v) suspended solids gradually by gravity settling and centrifugation at 7650 g for 15 minutes in a Sorvall RC 5C plus Macrocentrifuge (rotor SA-600). The sludge supernatant was stored in the refrigerator at 4°C and used to dilute the sludge samples as per requirements. The concentrated sludge was homogenized using a blender for different solids concentration. This was carried out to ensure well-mixed sample and this step has been adopted in all biopesticide studies (Tirado-Montiel et al. 2001; Vidyarthi et al. 2002). The tests were conducted in the course of 3 to 5 d to prevent deterioration of sludge (there is possibility of suppressed and slow growth of microorganisms under storage conditions at 4°C). However, insignificant change was observed in characteristics of sludge on cold storage over the specified period.

Each sludge sample was prepared in duplicate by diluting the concentrated sludge samples with sludge supernatant to obtain different solids concentration (10-40 g L^{-1}). The sludge samples were defined at different total solids of 10, 20, 30 and 40 g L^{-1} as suffix 1, 2, 3 and 4, respectively with distinct symbols and details of pre-treatment, if applicable, for each process stage shown in Table 2.

Bacterial strain

Bacillus thuringiensis var. *kurstaki* HD-1 (ATCC 33679) was used in this study.

Inoculum and culture conditions

The culture conditions, maintenance, inoculum production, and fermentation (biopesticide production) are described elsewhere (Vidyarthi et al. 2002).

Shake flask fermentation experiments

A 2.0 % (v/v) inoculum from the flask containing Bt culture acclimatized in sludge was used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of sterilized unhydrolyzed (ST) or thermal alkaline hydrolyzed (HST) sludge at pH = 7.0 ± 0.1. The flasks were incubated in an incubator-shaker (Sanyo, Japan) at 250 rpm for 48 hrs. Samples at definite intervals were drawn to determine total cell (TC) and viable spore (VS) count (Vidyarthi et al. 2002). Standard deviation for TC and VS measurements varied from 6-8 %.

Fermenter Experiments

A stirred tank 15L fermenter (Biogenie Inc., Quebec) equipped with accessories, computer coupled programmable logic controller (iFix 3.5, Intellution) for dissolved oxygen, pH, anti-foam, impeller speed, aeration rate, and temperature was utilized for fermentation as per the procedure detailed in Brar et al. (2005a). The fermentation for NH and TAH sludge was carried out at optimal suspended solids (SS) of 25 and 30 g L^{-1}, respectively. Similarly, the bench scale hydrolysis was carried out according to the method detailed by Brar et al. (2005a).

Viable spore (VS) count

The procedure specified in Vidyarthi et al. (2002) was utilized for VS count with a small modification of subjecting samples to heat treatment ("heat shock") in a silicone bath (Thermo-Lift, Buchler Instruments, USA) at 80 ±1°C for 10 min and then cooling in an ice bath for 5 min before plating on tryptic soya agar. The standard deviation for spore count was 8.0 %.

Particle Size Analysis

Particle size analysis was carried out by using Fritsch Laser particle sizer analyzette 22, which is based on LASER diffraction principle. During entire analysis, ultrasonic function was switched off to avoid breakage of sludge particles. The stirrer and recirculation pump speed were kept moderate at 250 rpm and 500, respectively to minimize the breakage/damage of sludge particles. To check the impact of ions present in tap water, in particular, calcium and carbonate, the analysis was repeated in deionized water, but there was no characteristic change in the PSD and this showed the reproducibility and consistency of data obtained as also observed by Houghton et al. (2002) for digested sludges.

For analysis, each sample was diluted approximately 400-fold in tap water; and each sample was analyzed 5 times to check the validity and reproducibility of results. This method is based on the principles of Fraunhoffer diffraction and Mie scattering. The results were then averaged to produce the PSD, and recorded as particle volume percent in 51 discrete particle ranges between 0.1 to 1000 μm. These conditions cause minimum floc disruption at the D_{10}, D_{50} and D_{90}, analogous to the PRS expressed as diameter 10%, 50% and 90% size distribution cut off points respectively. Results were reported as % v/v (volume of particles having a specific diameter out of the total volume of particles) and % s/s (specific area of particles having a specific diameter out of the total surface area of particles). The PRS refers to D_{50}, unless stated otherwise. In either case, the particles are assumed to have spherical shapes. Standard deviation of PRS measurement ranged from 10-12%.

Particle Density of Sludge

The particle density of sludge solids was estimated as follows: If V_{dw}, the volume of water displaced by the sludge solids, is equal to volume of sludge solids, V_s, then;

$$V_{dw} = \frac{M_{dw}}{\rho_{dw}} = V_s = \frac{M_s}{\rho_s} \tag{1}$$

Hence,

$$\rho_s \cong \rho_w \frac{M_s}{M_{dw}} \qquad (\rho_w \cong \rho_{dw}) \tag{2}$$

Where; M_{dw} is the mass of the displaced water and ρ_w is the water density. The values of M_{dw} and M_s were obtained by using a graduated volumetric flask and by taking the following measurements:

M_f = mass of the empty flask; M_{fs} = mass of the flask plus the dried sludge sample at 105 ± 0.1 °C; M_{fsw} = mass of the flask plus the sludge and filled with water up to a fixed volume, V_f; and M_{fw} = mass of the flask filled with pure water up to the fixed volume V_f.

The mass of the displaced water, M_{dw} and Mass of sludge solids, M_s, can be then calculated as:

$$M_{dw} = (M_{fsw} - M_{fw}) - (M_{fs} - M_f) \tag{3}$$

and;

$$M_s = M_{fs} - M_f \tag{4}$$

Substituting M_{dw} into the expression for sludge particle density, ρ_s, yields Equation 5 which was used to calculate the sludge particle density:

$$\rho_s = \rho_w \left[\frac{(M_{fs} - M_f)}{(M_{fsw} - M_{fw}) - (M_{fs} - M_f)} \right] \quad (5)$$

Storage temperature effect on particle size of different samples

The fermented broths, NHF and HF are normally freeze-dried so that they can be later used for formulation development. This necessitates study of effect of storage temperature on particle size of NHF and HF samples. Therefore, NHF and HF broths, pre-adjusted to pH 4.5±0.1, were stored at four different temperatures of –20, 4, 10 and 20°C overnight for 12 hours; the frozen samples were freeze thawed and subjected to further analysis namely, conductivity, viscosity and PRS. Viscosity ("apparent viscosity") was determined at 25°C and shear rate of 36.71 s^{-1} by using a rotational viscometer (Cole-Palmer Inc., Toronto, Canada). Conductivity was measured by using Bach-Simpson Conductivity meter (measuring range 0.5 µmho/cm to 500 mmho/cm).

Analytical details

Sludge volume index (SVI), total solids (TS), total suspended solids (TSS), dissolved solids concentrations, C, N, ammonia nitrogen, PO_4^{3-}-P for the sludge samples were analyzed according to Standard Methods (APHA 1998). Different metal (Al, Cd, Cr, Cu, Fe, Mn, Pb, Zn, Mg, and Ca) concentrations were determined by inductively coupled plasma – atomic emission spectrophotometer (IMPA.S-AES) Spectra AA-20 (Varian Techtron Pty. Ltd., Australia). There was a standard deviation of 10-15 % in metal analysis.

Bioassay for entomotoxicity measurements

Bioassays were conducted using the diet incorporation method (Beegle 1990). Sample preparation and diet protocol was followed as per method reported by Vidyarthi et al. (2002). The entomotoxicity (Tx, biopesticidal potential of Bt biopesticides) of sample preparations was obtained by comparing the final mortality (percentages) of eastern spruce budworm larvae (SB) (*Choristoneura fumiferana* Clemens), Lepidoptera: Tortricidae) with that of standard commercial product (Foray 76B, Abbott Industries, MI, USA) and expressed as relative spruce budworm units/µl (SBU/µl). Foray 76 B contained spores and crystals of Bt var. *kurstaki* at a potency of 20.1 x 10^9 IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni* Hübner). On comparison of Tx of Bt fermented sludge, it was found

that SBU in this study was 20-25% higher than IU. The standard deviation for Tx measurement was 8–10%.

Results and Discussion

Volume distribution of particle size

Volume distribution (frequency and cumulative frequency) semi-logarithmic PRS profiles of different process stages of Bt biopesticide production, namely, NH, ST, H, HST, HF and NHF at different solids concentration has been presented in Figs. 1 (a &b) and 2 (a&b). The PSD profile will be affected by the change in concentration of TS during different process treatment stages due to changes in inter-particle interactions as sludge floc is laden with such interactions (Wilen et al. 2003). The PSD varied from 0.2 to 500 μm for NH and ST sludge whereas for TAH sludge, PSD varied from 0.2 to 120 μm. Meanwhile, after fermentation, the PSD for NHF and HF sludge varied from 0.2 to 400 μm and 0.2 to 500 μm, respectively. However, irrespective of TS, it was observed that the volume % of particles for HF sludge was more (10% cumulative volume percent) towards smaller particle size region (Figs. 1 a & b and 2 a & b) in comparison to NHF sludge (5 % cumulative volume percent). Similarly, TAH and HST sludge had higher volume percent particles in the lower particle size region when compared to NH and ST sludge. There was 50% reduction in PRS on hydrolysis of ST sludge. The PSD on volume percent basis shifted to higher particle size region on moving from 10 to 40 g L^{-1}.

It was logical to use volume PSD in this study as during Bt biopesticide production, the interest lies in the production of entomotoxicity (Tx, biopesticidal potential) as a function of mass distribution of various contributing components. In the fermented broth, factors contributing to Tx include, spores and crystal protein, other virulent factors namely, vegetative insecticidal proteins (VIPs) and various enzymes like chitinases, proteases, phospholipases and some unknown antibiotics (Porcar and Juarez-Pérez 2003). Moreover, volume mean diameter is also used as a direct measure of droplet size (droplet is composed of several particles) for field application of Bt formulations in order to check the efficacy of formulations (Burges 1998). The droplet size is mainly a function of volume (volume application rate), as it relates to the dose (number of active particles i.e. Bt units per unit volume) applied per larva per unit area of the target surface.

Furthermore, if the settling velocity dependency was related only to particle size, then HF sludge particles would have more hindered (due to greater volume percent of particles towards smaller size) settling requiring higher centrifugal force to achieve higher Tx recovery

during centrifugation of the final fermented broth which has to be processed further to yield higher Tx values. A point of caution to be added is the consideration of Tx at the end of fermentation (higher Tx will lead to higher recovery efficiency) which is also a function of the solids concentration and nutrient requirements for Bt growth (Tirado-Montiel et al. 2001; Vidyarthi et al. 2002). Additionally, HF sludge formulations should have better suspendibility due to lower PRS and consequent dispersibility as also observed in blooming experimental results (Fig. 5). On the contrary, higher floc size/ higher range volume PSD as in case of NHF sludge has been already shown to protect the Tx components from harmful UV radiation and was reported to have a higher half-life of 11 days in comparison to 9.51 days for HF sludge formulations (Brar et al. 2005a, b).

Bimodal distribution of PRS

The particle size followed bimodal distribution as illustrated in Figs. 1 (a&b) and 2 (a&b). The distribution curve for NH was more flattened towards larger particle size; for ST sludge - higher percentage of particles towards higher particle size – two hump regions and for TAH – the curve moves towards lower particle region. On fermentation, the contrary was observed, for NHF sludge, the distribution shifted more towards relatively lower volume percent whereas for HF sludge, the shift was more towards high volume percent of particles. This suggested that in HF sludge due to better growth of Bt, more number of particles were formed (a function of increase in suspended solids) as seen in the net area under the curve in Figs. 1 (a&b) and 2 (a&b) when compared to NHF sludge. A bimodal distribution might result from a process involving multiple sources of particles (NH sludge), breakup of large particles (TAH system), or variable growth mechanisms (NHF and HF) in the system. In general, the PSD profile followed log-normal distribution which is commonly observed in slurries and typically sludges (Mikkelsen and Keding 2002; Jin et al. 2003).

Surface PSD

When surface PSD curves (Figures not shown due to similarity with volume PSD curves) were compared for different process stages, it was again observed that HST sludge (40% volume particles) in comparison to ST sludge (15% volume particles) had greater surface distribution. This will lead to more surface area per unit particle, which will improve the rheology and affect Bt growth during fermentation enhancing Tx of HF sludge (Barnabe 2004). This could also be correlated to increase in per unit surface area for enzymatic attack, enhanced diffusion of available nutrients into Bt cells and enhanced Bt production.

Henceforth, volume PSD will be considered as a means to measure the particle size of different Bt production stages, namely, fermentation, downstream processing, formulation and field application.

Shake flask fermentation and particle size

Fig. 3 represents the comparative profile of VS, Tx and average PRS during fermentation of hydrolyzed (TAH) and non-hydrolyzed sludges (NH) at different TS of 10 - 40 g L^{-1}. The VS concentration was always higher for HF when compared to NHF sludge, irrespective of TS (Fig. 3). The PRS decreased with solids concentration upto 25 g L^{-1} (NHF sludge) and 30 g L^{-1} (HF sludge) with a concomitant increase in VS and Tx values. From 30 to 40 g L^{-1} (HF sludge), increase in PRS was observed with decrease in both VS and Tx values, whereas for NHF sludge, PRS showed abnormal behaviour with an increase (from 25 to 30 g L^{-1} solids) followed by decrease from 30 to 40 g L^{-1} solids concentration with no relation with VS and Tx. These abnormal trends were unclear at this point. However, the PRS decrease with solids concentration could be due to increased inter-particle interactions during agitation in shaker, which might have resulted in net deflocculation in contrast to agglomeration (increase in PRS) normally observed with increase in TS as also reported by Erikkson et al. (1992). Moreover, the abnormal decrease in VS, despite decrease in PRS (30-40 g L^{-1} solids, NHF sludge) could be due to increase in viscosity (Brar et al. 2005b) which resulted in reduced mass transfer and nutrient assimilation by Bt.

In general, at TS of 10 to 30 g L^{-1} for HF and 10 to 25 g L^{-1} for NHF sludge, there were three important phenomenon contributing to the increased VS and Tx with solids concentration: a) increase in solids which will increase nutrient availability; b) decrease in PRS which will enhance the surface area, increase mass transfer and thus augment nutrient assimilation and; c) decrease in PRS which will improve ingestion of sludge particles by SB larvae during bioassay leading to higher Tx (explained in the next section).

Furthermore, irrespective of the type of fermented sludge, when PRS data were pooled at all TS, Tx and PRS fitted well into the logarithmic law equation with correlation coefficient (R^2) of 0.81 (Tx= -9539.1ln (PRS) + 47588) and 0.91 (Tx = -7728.3ln (PRS) + 38887) for HF at TS 10 to 30 g L^{-1} and NHF sludge at TS of 10-25 g L^{-1}, respectively. Thus, Tx will be affected by logarithmic order of PRS within the limited range of TS concentration. A point to be mentioned here is that these NHF and HF results in shake flask will be entirely different from that of fermenter results. Therefore, conclusions drawn from shake flask will not be applicable in a real world situation. Despite this indifference, studies need to be carried out in shake flasks in order to get an estimate of the possibilities in fermenter which may be

difficult to estimate on a larger scale. Further, process step changes may impact floc (particle) size, such as a decrease in PRS may result in greater sensitivity of the specific process to shear.

Tx-PRS correlation in fermenter

The time course of Bt fermentation (15L fermenter) at optimal TS of 25 g L^{-1} and 30 g L^{-1} for NH and H sludge, respectively is shown in Fig. 4. The VS concentration was higher for HF sludge in comparison to NHF sludge whereas viscosity was higher for NH sludge. The PRS decreased with fermentation time (until 48h) due to agitation effects with a small increase between 12-30 h. There was no concrete reason for this temporary increase in PRS, however it was highly probable that addition of anti-foam agent (to control foam in the fermenter) might have caused temporary floc formation. After 25-30h of fermentation time, addition of anti-foam agent is almost stopped (normally, foam formation is intense in the beginning of fermentation and it ceases after 24 h). Subsequently, temporary flocs formed were broken by continuous agitation leading to further decrease in PRS until the end of fermentation. The PRS of TAH sludge was lower than NH sludge from the beginning (sludge hydrolysis process broke down the flocs to low particle size) and stayed at a lower level throughout the fermentation and resulted in enhanced oxygen transfer and assimilation of nutrients by Bt leading to higher VS for TAH vis-à-vis NH sludge. The PRS for NH and TAH sludge at 48 h was 21.85 and 8.16 μm, respectively with corresponding Tx values of 12.8 and 18.5 x 10^9 SBU l^{-1}, respectively. The higher Tx value for TAH sludge in comparison to NH sludge at the end of fermentation could be due to enhanced nutrient availability by hydrolysis effect and lower particle size (increased surface area).

The effect of comparatively lower PRS on Tx could be provided on the basis that the peritrophic membrane in the insect ("target pest") midgut completely separates the food tract from the intestinal epithelium and is usually permeable to molecules around 30–40 kDa. To reach their target, digestive enzymes must cross it and digestion products (including Bt protoxins that are activated by trypsin-like proteases) must traverse this membrane to reach the absorptive epithelium (Beaty and Marquardt 1996). The peritrophic membrane has been reported to serve as a solid support to digestive enzymes: as food moves along the digestive tract, it is digested by these immobilized enzymes (Beaty and Marquardt 1996). If the PRS is large (NHF sludge), these ingested particles may form an additional layer of particles by physically covering the peritrophic membrane. This may further delay proteolytic processing of Bt protoxins and crossing of the activated toxins to reach the absorptive epithelium as necessary for binding to their specific receptors lowering Tx and effect will be vice versa for HF sludge.

Variation of SVI and sedimentation velocity

SVI and sedimentation velocity profiles at different TS are given in Fig. 5. SVI followed the order ST<HST<NH≈TAH<HF<NHF at different TS concentrations from 10 to 40 g L^{-1}. Lowest SVI of ST sludge indicated better settleability. Sterilization influenced the chemistry of proteins resulting in their coagulation and faster settling and also increase in PRS.

According to Stoke's law, sedimentation velocity of particles in quiescent liquid is given by:

$$v_{sed} = \frac{g}{18}\frac{[(\rho_s - \rho_L)d^2]}{\eta} \quad (6)$$

As sedimentation velocity (v_{sed}) is influenced by square of PRS, an increase in PRS will favour settling as also seen from Fig. 5. The v_{sed} in Fig. 5 was calculated using Equation 6, where, g = 9.81 m/s^2; η = viscosity for each process stage; (mPa.s); ρ_s = 1.015 kg L^{-1} (experimental value); ρ_L = 1.000 kg L^{-1} (experimental value) and d = particle size for each process stage (D_{50}, μm). Ideally, Stoke's equation is valid only for discrete particles in dilute suspension under laminar flow condition. However, concentration of sludge varying from 10 g/l to 40 g/l could be correlated with Stoke's equation as a conservative estimate for determining sedimentation velocity as established by earlier studies (Rector and Bunker 1995). Meanwhile, for agglomerated systems (flocs), in this case, sludge, sedimentation rates can be estimated using Stokes' law by replacing the particle diameter with the agglomerate size (approximated as particle size, D_{50}), correcting ρ_s to agglomerate (flocs) density (experimentally obtained value in this case), and by correcting ρ_L as net density of various types of water (interstitial, free, capillary and particle water) flowing through agglomerate structures of sludge. Further, agglomerates (flocs), in this case were assumed to be spherical as measured by the LASER diffraction based particle size analyzer.

Later, the experimental SVI and calculated v_{sed} were pooled together to draw correlation with TS. As seen in Fig. 5, SVI decreased proportionately with TS with for all process stages. The decrease was influenced by hindered settling towards higher TS. Similarly, v_{sed} decreased with TS proportional to PRS and viscosity and at higher TS, hindered settling dominated. The Stoke's law was valid in both D_{50} and D_{90} regions.

In the Bt production process, centrifugation of the fermented broth is required to concentrate cells, spores and toxins in order to achieve higher Tx and enhance handling (Rojas et al. 1996). The above interpretation can throw some light on the centrifugation process where lower centrifugal force may be required to settle larger particles (e.g. NHF,

higher SVI). These shake flask studies (v_{sed} for NHF and HF of 2.11 x 10^{-9} and 2.38 x 10^{-9} m s^{-1} at 25 and 30 g L^{-1} TS, respectively due to gravitational action) were in close concordance with centrifugation studies carried out in our laboratory (Brar et al. 2006) where v_{sed} for NHF and HF sludge was reported to be 3.93 x 10^{-9} and 5.24 x 10^{-9} m s^{-1} at TS 25 and 30 g L^{-1} , respectively (due to gravitational and centrifugal action) providing valuable data for downstream processing. Although, there were differences in the values of two studies, yet they explained a trend as v_{sed} of NHF sludge was practically higher than HF sludge with some anomaly as observed in Fig. 5 which could not be explained at this stage.

On the contrary, lower v_{sed} was desirable for liquid formulations i.e. higher suspendibility was required to have stable shelf-life and minimize the settleability of Bt fermented and formulated biopesticide product in the application tank and hence to enhance synergy with the application equipment (Mor and Matthews 2003). Thus, HF sludge having lower v_{sed} (due to lower PRS) should be preferred over NHF sludge to achieve high Tx (during fermentation) for formulation characteristics and field application.

The HF sludge should also require comparatively lower concentration of suspending agents than NHF sludge. However, systematic studies are required to determine optimum concentration of suspending agents in two types of sludge. In light of the above contention, studies carried out in our laboratory (Brar et al. 2004) showed that HF sludge possesses better dispersibility and forms stable formulation, as depicted in Fig. 6. A simple blooming experiment was conducted and it was observed that after 24h, the HF sludge formulation showed excellent dispersibility (90-96 %) in comparison to NHF sludge formulation (dispersibility – 20-30%). Additionally, HF based formulation will be better from application point of view as smaller PRS will aid in eliminating choking problem of spraying equipment's nozzles during Bt field sprays.

Furthermore, in general, PRS alone is not a determining factor for SVI as it is also governed by other factors like particle density and suspended solids concentration. Moreover, when particle density was higher, the particles contributed more to hindered rather than discrete settling (especially, SVI for TAH sludges).

Effect of sample storage temperatures and reproducibility

The results of PRS for NHF and HF sludges at TS of 25 and 30 g L^{-1}, respectively (fermenter samples) after 12h storage at temperatures –20, 4, 10 and 25°C are presented in Table 3. The standard deviation values given in parentheses demonstrated that the

instrumental conditions used for analysis caused minimum floc disruption at the D_{10} and D_{50}, the 10% and 50% size distribution cut-off points, respectively.

The NHF and HF samples showed no greater variability in the D_{10}, D_{50} and D_{90} values after 12 h storage at 25 and 10°C, yet storage was better at 10°C. Henceforth, 10°C will be adopted as the storage temperature for PRS analysis. The PRS difference between zero time and 12 h sample storage increased the PRS at 4 and –20°C for both NHF and HF sludge. The increase was more at –20°C owing to the agglomeration of microbial (Bt) colonies during freezing. During thawing and homogenization of sample, the floc structure broke but did not revert to original PRS during sample measurement. These changes in PRS effects are important as they may affect the rheological properties, flowability and final properties of formulation. Besides, for large particles, the ratio of the surface area to the volume is small and gravitational forces are large compared to colloidal forces (Tiller et al. 1987). Thus, these particles have a tendency to settle faster leading to good sedimentation, desired property for centrifugation.

Furthermore, when the samples were stored at 10°C, there was no substantial change in PRS. If these samples are used for formulation, PRS of liquid suspensions will not be affected as the samples were subjected to blending after mixing with various adjuvants which reduced the PRS than reported here (Brar et al. 2004). The stored samples after concentration and formulation also need to be spray dried for production of solid formulations. During spray drying operation, the PRS will dictate the flow of wet pre-formulated sludge slurry into the orifice which will affect nature and type of the formulation (bigger PRS may choke the nozzle and variable PRS will cause flaking or caking of the resulting powder/granules, Burges 1998).

This study demonstrated that different process stages had diverse impacts on the PRS, which was equally affected by the increase in TS. Furthermore, LASER particle size analyzer has not been prominently used in wastewater sludge studies; however, this study established the vital use of the instrument with reproducibility of data. It is inferred that storage of fermented broths at -20°C after fermentation may affect the PRS and thus PSD studies will play an important role in later formulation of these broths which needs to be looked into while 10°C can be a preferred choice of sample storage. A particular point in attention was the PRS profile after fermentation – HF had smaller PRS vis-à-vis NHF. This was interesting for developing liquid biopesticidal formulations where HF would perform better due to lower settling during storage and show synergy with application equipment. On the contrary, NHF sludge could be concentrated at lower centrifugal force/in lesser time, as PRS was higher,

lowering centrifugation costs. Hence, a logical approach needs to be adopted based on physical characteristics of different process stages of Bt fermentation to develop high performing biopesticides.

Conclusions

The following conclusions were drawn from the above stated study:

1. Volume distribution of particle size was the correct representation of variations as it would incorporate the mass component of fermentation.
2. Volume particle size distribution shifted to higher particle size zone as the TS increased from 10 to 40 g L^{-1}.
3. Volume distribution of particles showed higher volume percent particles of hydrolyzed fermented sludge towards smaller size range in comparison to non-hydrolyzed fermented sludge.
4. PSD was greatly influenced by the treatment process. Thermal alkaline hydrolysis reduced the PRS by almost 50%.
5. Decrease in particle size and higher availability of nutrients in hydrolyzed fermented sludge resulted in increase in entomotoxicity due to enhanced surface area.
6. Based on Stoke's law correlation between average PRS and SVI (sedimentation velocity), non-hydrolyzed Bt fermented sludge could be harvested at lower centrifugal force. Meanwhile, hydrolyzed fermented sludge would give better liquid suspensions (enhanced dispersibility) and higher synergy with application equipment.
7. PSD of sludges at all process stages behaved as bimodal log-normal particle distribution suspensions.
8. The sludge samples can be stored at 10°C for 12 hours with no change in particle size.

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. The views and opinions expressed in this article are those of authors. We are also thankful to Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Service and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing scholarship to Satinder K. Brar. The views or opinions expressed in this article are those of the authors and should not be construed as opinions of the U.S. Environmental Protection Agency.

List of symbols

Bt	*Bacillus thuringiensis*
D or d	Diameter of the spherical particle (μm)
D_{10}	10% of the particles undersize (μm)
D_{50}	50% of the particles undersize (μm)
D_{90}	90% of the particles undersize (μm)
g	Acceleration due to gravity (m s^{-2})
HF_n	Hydrolyzed fermented sludge (n = 10, 20, 25, 30 , 40 g/L TS represented as 1, 2, 2.5, 3 and 4, respectively)
HST_n	Thermal alkaline hydrolyzed sterilized sludge (n = 10, 20, 30 , 40 g/L TS represented as 1, 2, 3 and 4, respectively)
M_{dw}	Mass of displaced water (kg)
M_f	Mass of empty flask (kg)
M_{fs}	Mass of flask + dried sludge sample (kg)
M_{fsw}	Mass of flask + dried sludge + water (kg)
M_{fw}	Mass of flask filled with pure water upto fixed volume (kg), V_f
M_s	Mass of sludge solids (kg)
NH_n	Non-hydrolyzed sludge (n = 10, 20, 30 , 40 g/L TS represented as 1, 2, 3 and 4, respectively)
NHF_n	Non-hydrolyzed fermented sludge (n = 10, 20, 25, 30 , 40 g/L TS represented as 1, 2, 2.5, 3 and 4, respectively)
PRS	Particle size (μm)
PSD	Particle size distribution
SS	Suspended solids (g L^{-1})
ST_n	Sterilized sludge (n = 10, 20, 30 , 40 g/L TS represented as 1, 2, 3 and 4, respectively)
SVI	Sludge Volume Index
TAH_n	Thermal alkaline hydrolyzed sludge (n = 10, 20, 30 , 40 g/L TS represented as 1, 2, 3, 4 respectively)
TC	Total cell count (CFU ml^{-1})
TS	Total solids (g L^{-1})
Tx	Entomotoxicity (SBU μL^{-1})
V_{dw}	Volume of water displaced by sludge solids (L)

VS	Viable spore count (CFU ml^{-1})
V_s	Volume of sludge solids (L)
v_{sed}	Sedimentation velocity (m s^{-1})
η	Viscosity of suspension (mPa s^{-1})
ρ_L	Density of fluid (kg L^{-1})
ρ_s	Density of the sludge solids, assumed to be spheres (kg L^{-1})
ρ_w	Density of pure water (kg L^{-1})
ρ_{dw}	Density of displaced water from sludge (kg L^{-1})

References

APHA-AWWA-WPCF. (1998). *Standard methods for the examination of water and wastewater* .(Ed. Clesceri, L.S., Greenberg, A.E., Trussell, R.R., and Franson, M.A.), 20th edition, APHA, Washington, DC 20005.

Barnabé, S. (2004). *Bioconversion of wastewater sludge into biopesticide improving sludge nutritive potential by hydrolysis and partial oxidation for higher entomotoxicity.* Ph.D thesis, Institut National de la Recherche Scientifique, Eau, Terre et Environnement, Université du Québec, Québec, Canada, pp 337.

Beegle, C.C. (1990). *Bioassay methods for quantification of Bacillus thuringiensis δ-endotoxin, Analytical Chemistry of Bacillus thuringiensis*, in: Hickle L.A. and Fitch W.L. (eds.) Analytical Chemistry of *Bacillus thuringiensis*, American Chemical Society, pp.14-21.

Beaty, B.J., and Marquardt, W.C. 1996. *The Biology of Disease Vectors.* Niwot: University Press of Colorado.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli R.Y., and Banerji S. (2004). "Development of sludge based stable aqueous *Bacillus thuringiensis* formulations." *Water, Sci. Technol.,* 50(9), 229-236.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. (2005a). "Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges." *Process Biochem.*, 40, 2695–2705.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2005b). "Sludge based *Bacillus thuringiensis* biopesticides: Viscosity Impacts." *Water Res.*, 39, 3001-3011.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2006). "Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge." *Water Res.*, 40, 1310-1320.

Burges H.D. (ed.) (1998) *Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments*, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Erikkson, L., Steen, I., and Tendaj, M. (1992). "Evaluation of sludge properties at an activated sludge plant." *Water Sci. Technol.*, 25, 251–265.

Houghton, J.I., Burgess, J.E., and Stephenson, T. (2002). "Off-line particle size analysis of digested sludge." *Water Res.*, 36, 4643–4647.

Jin B., Wilén, B.M., and Lant, P.A. (2003). "Comprehensive insight into floc characteristics and their impact on compressibility and settleability of activated sludge." *Chem. Eng. J.*, 95, 221–234.

MENV (2004). *Guide sur la valorisation des matières résiduelles fertilisantes : Critères de références et normes réglementaires.* Direction du milieu rural, environnement Québec, Canada, 138 pages.

Mikkelsen, L.H. (2001). "The shear sensitivity of activated sludge. Relations to filterability, rheology and surface chemistry." *Coll. Surf. A: Physicochem. Eng. Aspects*, 182, 1-14.

Mikkelsen, L.H., and Keiding, K. (2002). "Physico-chemical characteristics of full-scale sewage sludges with implication to dewatering." *Water Res.*, 36, 2451–2462.

Mor, S.G., and Matthews, G.A. (2003). "Recent developments in sprayers for application of biopesticides : an overview." *Biosystems Eng.*, 84(2), 119-125.

Porcar, M., and Juarez-Pérez, V. (2003). "PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes." *FEMS Microbiol. Rev.*, 26, 419-432.

Rector, D.R., and Bunker, B.C. (1995). "*Effect of Colloidal Aggregation on the Sedimentation and Rheological Properties of Tank Waste*". PNL-10761, Pacific Northwest National Laboratory, Richland, Washington.

Rojas, J.V., Gutierrez, E., and De la Torre, M. (1996). "Primary separation of the entomopathogenic products of *Bacillus thuringiensis*." *Biotechnol. Prog.*, 12, 564-566.

Sachdeva, V., Tyagi, R.D., and Valéro, J.R. (2000). "Production of biopesticides as a novel method of wastewater sludge utilization/disposal." *Water Sci. Technol.*, 42(9), 211-216.

Tiller, F.M., Yeh, C.S., and Leu, W.F. (1987). "Compressibility of particulate structures in relation to thickening, filtration, and expression -a review." *Separation Sci. Technol.*, 22 (2 and 3), 1037.

Tirado-Montiel, M.L., Tyagi, R.D., and Valéro, J.R. (1998). *Production of Bacillus thuringiensis using waste materials,* In: Bioconversion of waste materials into industrial products. A.M. Martin (ed.). Blackie Academic and Professional, London, pp. 480-516.

Tirado-Montiel, M.L., Tyagi, R.D., and Valéro, J.R. (2001). "Wastewater sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticide." *Water Res.*, 35(16), 3807-3816.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2002). "Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as a raw material." *Water Res.*, 36, 4850-4860.

Wilén, B.M., Jin, B., and Lant, P. (2003). "The influence of key chemical constituents in activated sludge on surface and flocculating properties." *Water Res.*, 77 (9), 2127–2139.

Table 1. Secondary sludge composition of Communauté Urbaine du Québec (Quebec, Canada)

Parameters	**Concentration ± SE (mg / Kg , unless stated)**
Total solids (TS) ($g\ L^{-1}$)	21 ± 1.1
Total volatile solids (TVS) ($g\ L^{-1}$)	15 ± 0.3
Suspended solids (SS) ($g\ L^{-1}$)	17 ± 1.1
Volatile suspended solids (VSS)($g\ L^{-1}$)	14 ± 0.7
Total carbon (C_t)	253709 ± 3456
Total nitrogen (N_t)	35899 ± 4325
Total phosphorus (P_t)	6963 ± 324
$N\text{-}NH_3$	795 ± 143
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$	16 ± 3.1
$P\text{-}PO_4^{3-}$	5324 ± 231
Al	3987 ± 347
Ca	11978 ± 213
Cd	2.59 (3-10)* ± 0.2
Cr	28 (210)* ± 2.2
Cu	169 (400)* ± 21.1
Fe	9877 ± 345
K	895 ± 106
Pb	56 (150)* ± 31
S	3498 ± 236
Zn	481(700)* ± 154
Na	1301 ± 362

* Digits in parentheses represent metal concentrations prescribed by Ministry of Environment, Quebec (MENV, 2004) for agricultural application.

Table 2. Designated nomenclature of different sludge samples

Sludge type	Symbol	Solids categorization*	Pre-treatment Details
Non-hydrolyzed	NH	NH1; NH2; NH3; NH4	Raw
Sterilized	ST	ST1; ST2; ST3; ST4	Raw sludge subjected to 121°C for 30 min (autoclaving)
Thermal alkaline hydrolyzed**	TAH	H1; H2; H3; H4	Raw sludge pre-adjusted to pH 10.25±0.1 subjected to 140°C and 440.9 psi for 30 minutes
Thermal alkaline hydrolyzed sterilized	HST	HST1; HST2; HST3; HST4	TAH sludge further subjected to ST conditions
Thermal alkaline hydrolyzed fermented	HF	HF1; HF2; HF2.5; HF3; HF4	HST sludge subjected to Bt fermentation at 30°C, pH 7±0.1 for 48 hours
Non-hydrolyzed fermented	NHF	NHF1; NHF2; NHF2.5; NHF3; NHF4	ST sludge subjected to Bt fermentation at 30°C, pH 7±0.1 for 48 hours

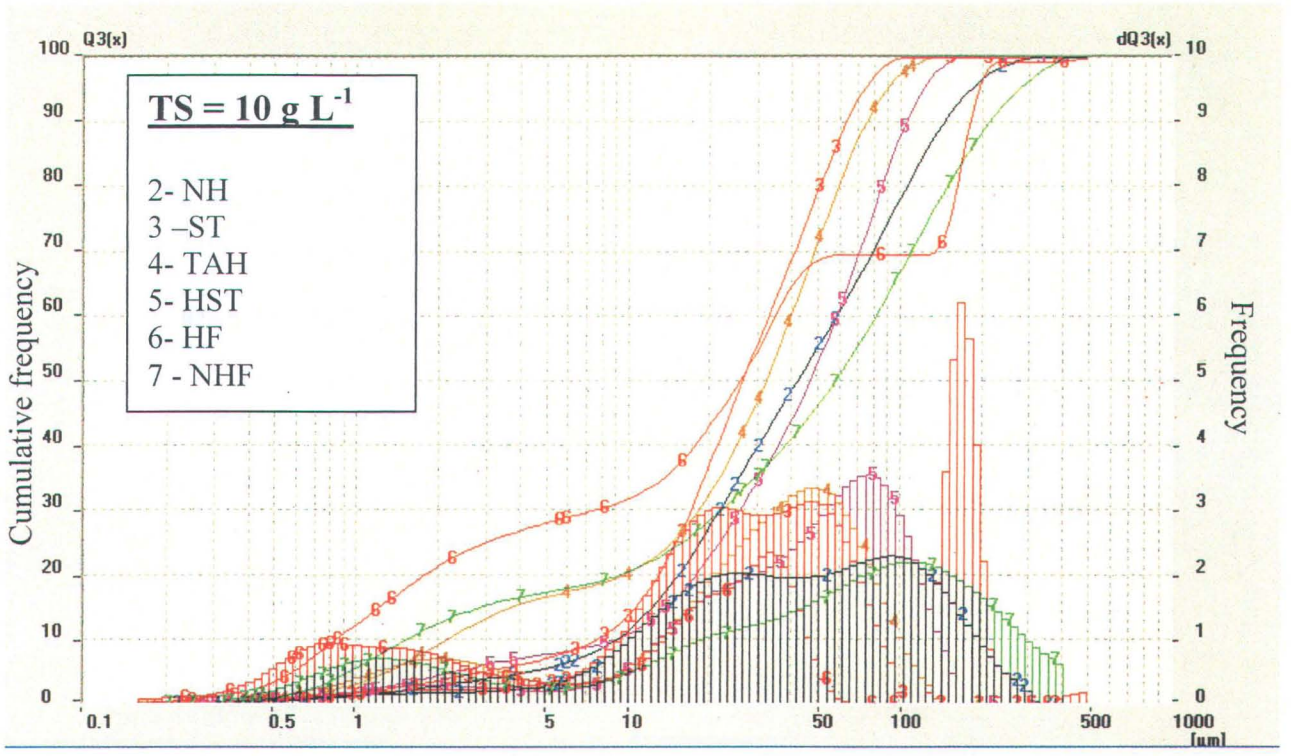
* - The numeral next to the sludge symbol code represents TS concentration (g L^{-1}), so 1, 2, 2.5, 3 and 4 refer to 10, 20, 25, 30 and 40 g L^{-1}, respectively.

* * - The process conditions were derived from studies carried out by Barnabé, 2004.

Table 3. Storage temperature effect on particle size of fermented sludges

Total solids (g L^{-1})	Temperature (°C)	Particle size (µm)		
		D_{10}	D_{50}	D_{90}
NHF (25)	Zero time	1.32 (1)	21.5 (1)	67.9 (21)
	25	1.31 (1)	28.5 (0)	74.6 (11)
	10	1.63 (1)	21.2 (0)	65.5 (21)
	4	3.5 (1)	24.12 (0)	78.9 (27)
	-20	14.5 (1)	56.7 (3)	89.6 (45)
HF (30)	Zero time	1.2 (1)	8.1 (1)	23.5 (17)
	25	1.83 (1)	7.1 (0)	26.9 (14)
	10	1.32 (1)	7.5 (0)	24.1 (16)
	4	1.82 (1)	9.39 (0)	54.9 (22)
	-20	6.7 (1)	11.7 (2)	73.8 (33)

Values in parentheses represent standard deviation obtained for 10 samples of each.



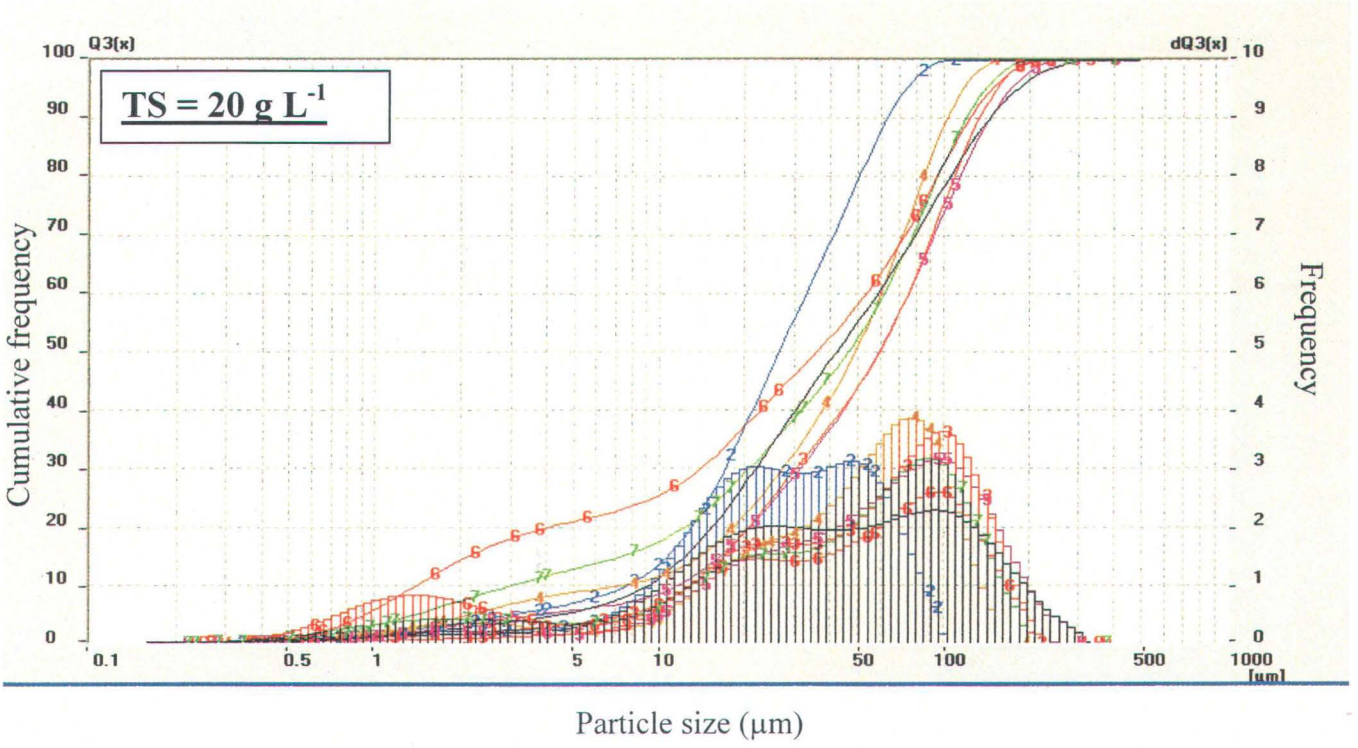
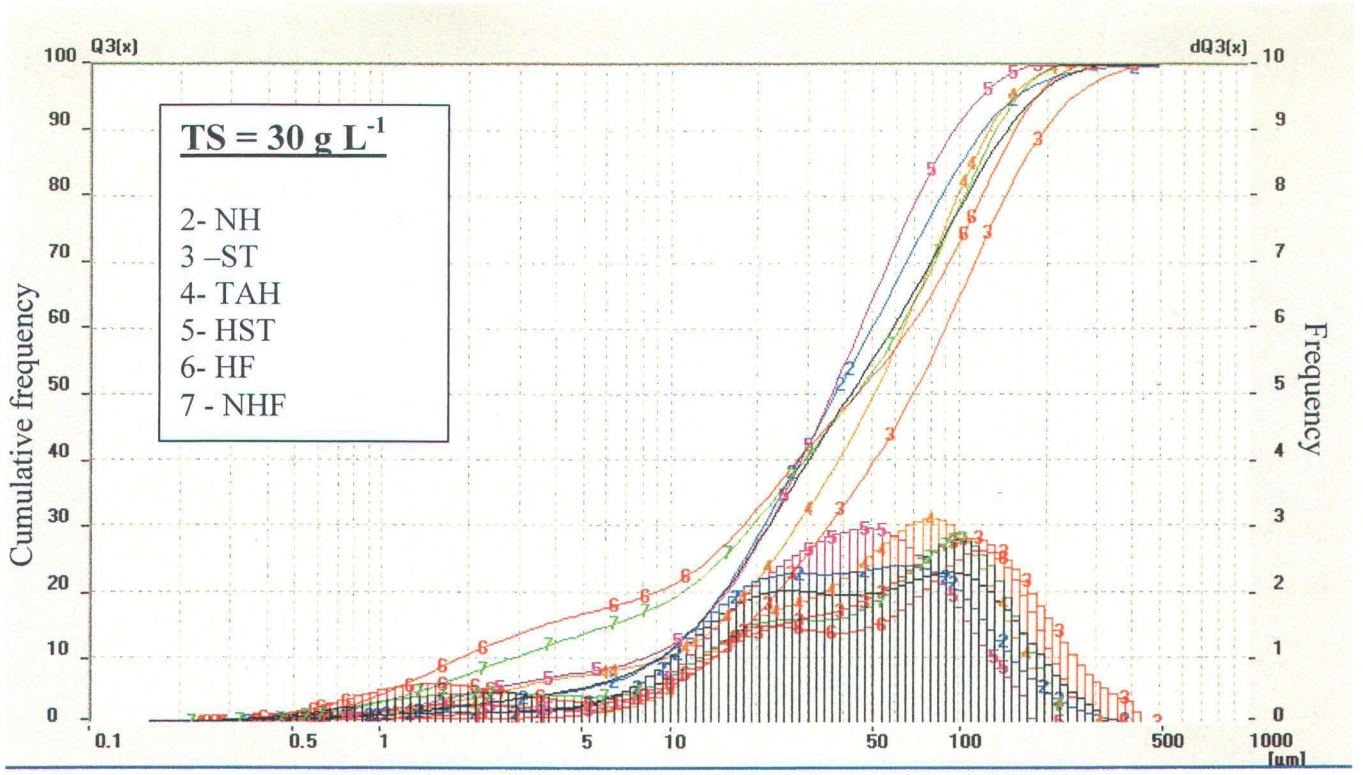


Fig. 1. Volume PSD of different process stages (NH-non-hydrolyzed sludge; ST-sterilized sludge; TAH-Thermal alkaline hydrolyzed sludge; HST-hydrolyzed sterilized sludge; NHF-non-hydrolyzed fermented sludge; HF-hydrolyzed fermented sludge).



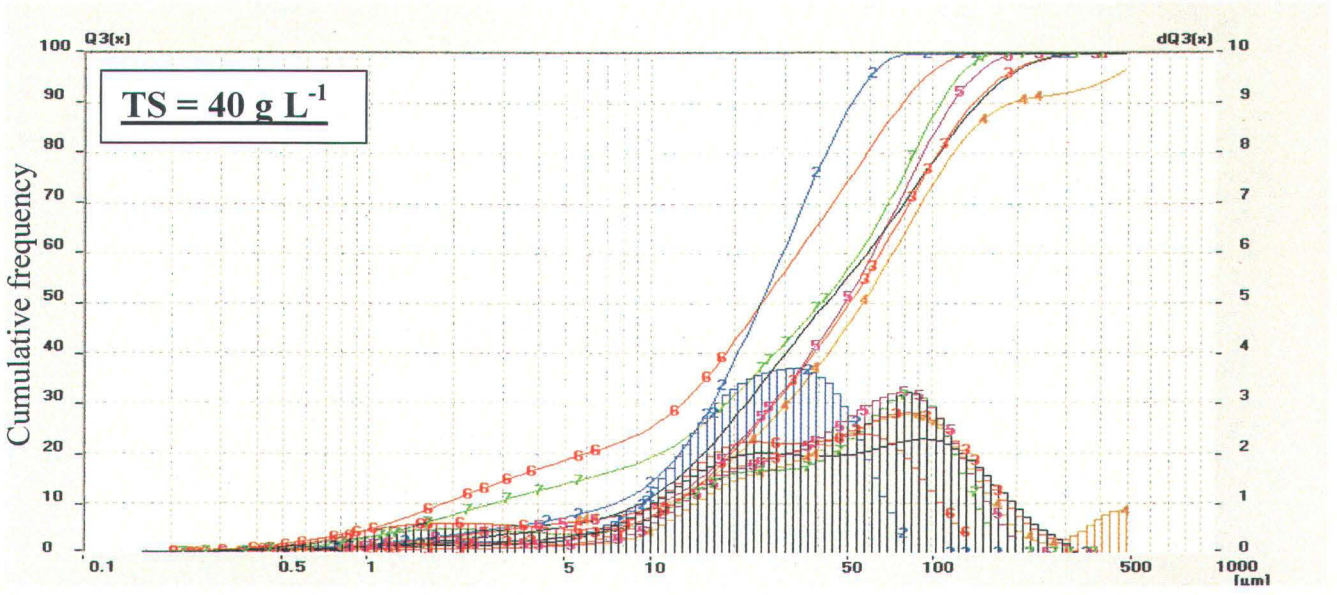


Fig. 2. Volume PSD of different process stages, (NH-non-hydrolyzed sludge; ST-sterilized sludge; TAH-Thermal alkaline hydrolyzed sludge; HST-hydrolyzed sterilized sludge; NHF-non-hydrolyzed fermented sludge; HF-hydrolyzed fermented sludge).

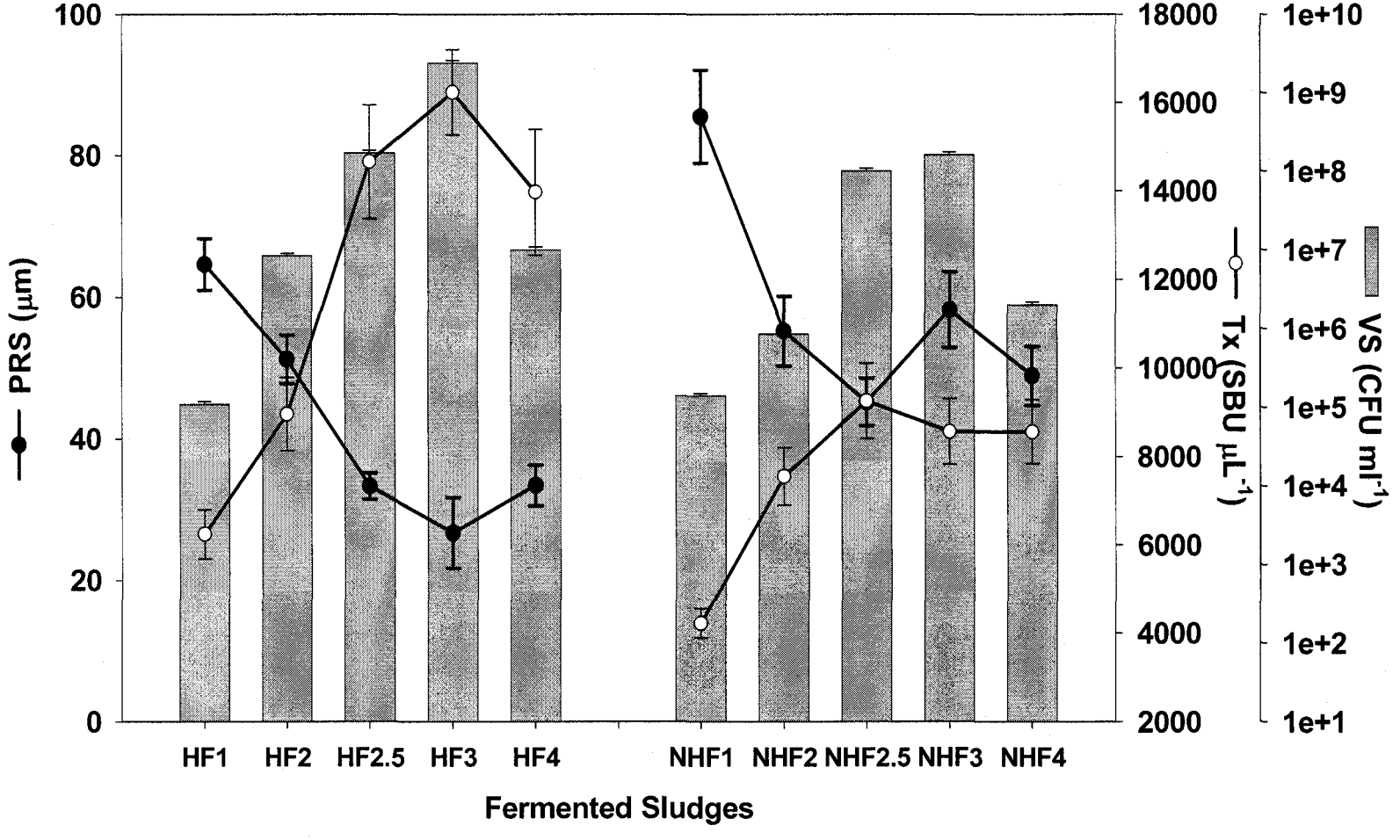


Fig. 3. Fermentation (shake flask) profile of fermented non-hydrolyzed and hydrolyzed sludges at different TS (PRS refers to D_{50}).

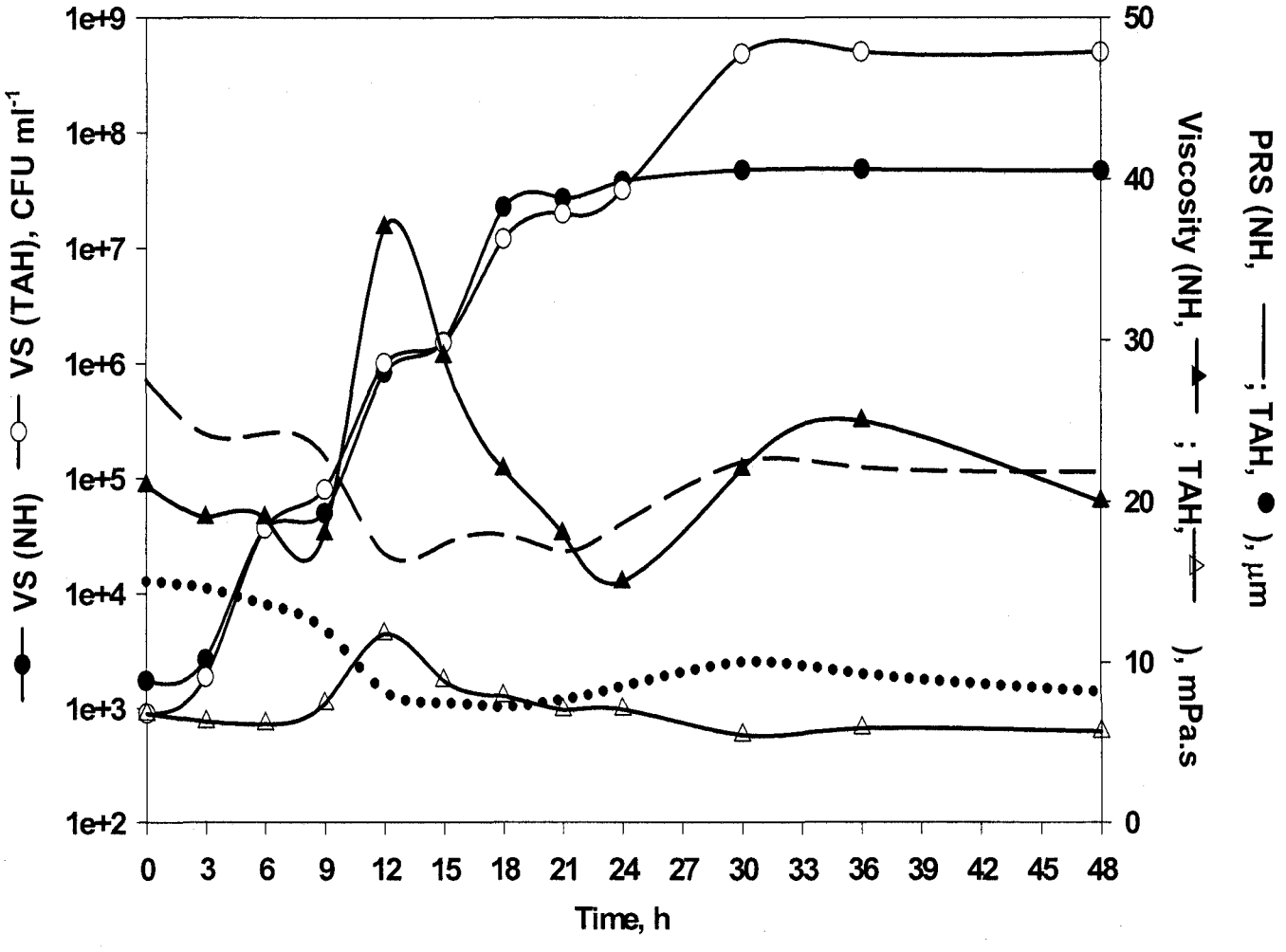


Fig. 4. Fermenter (15L) comparison of different growth and physico-chemical parameters.

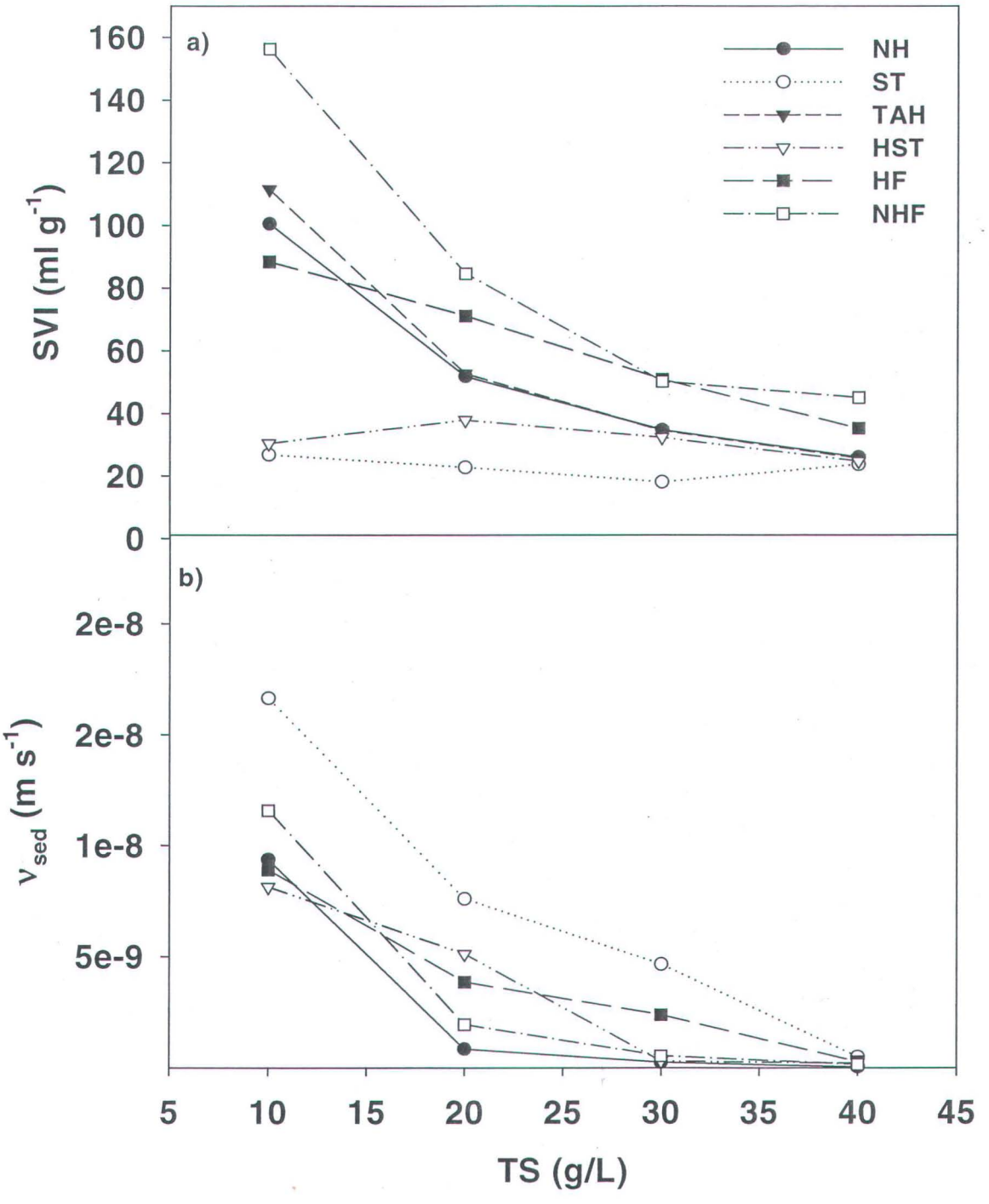


Fig. 5. Sedimentation velocity and SVI profile of different process stages with varying particle size.

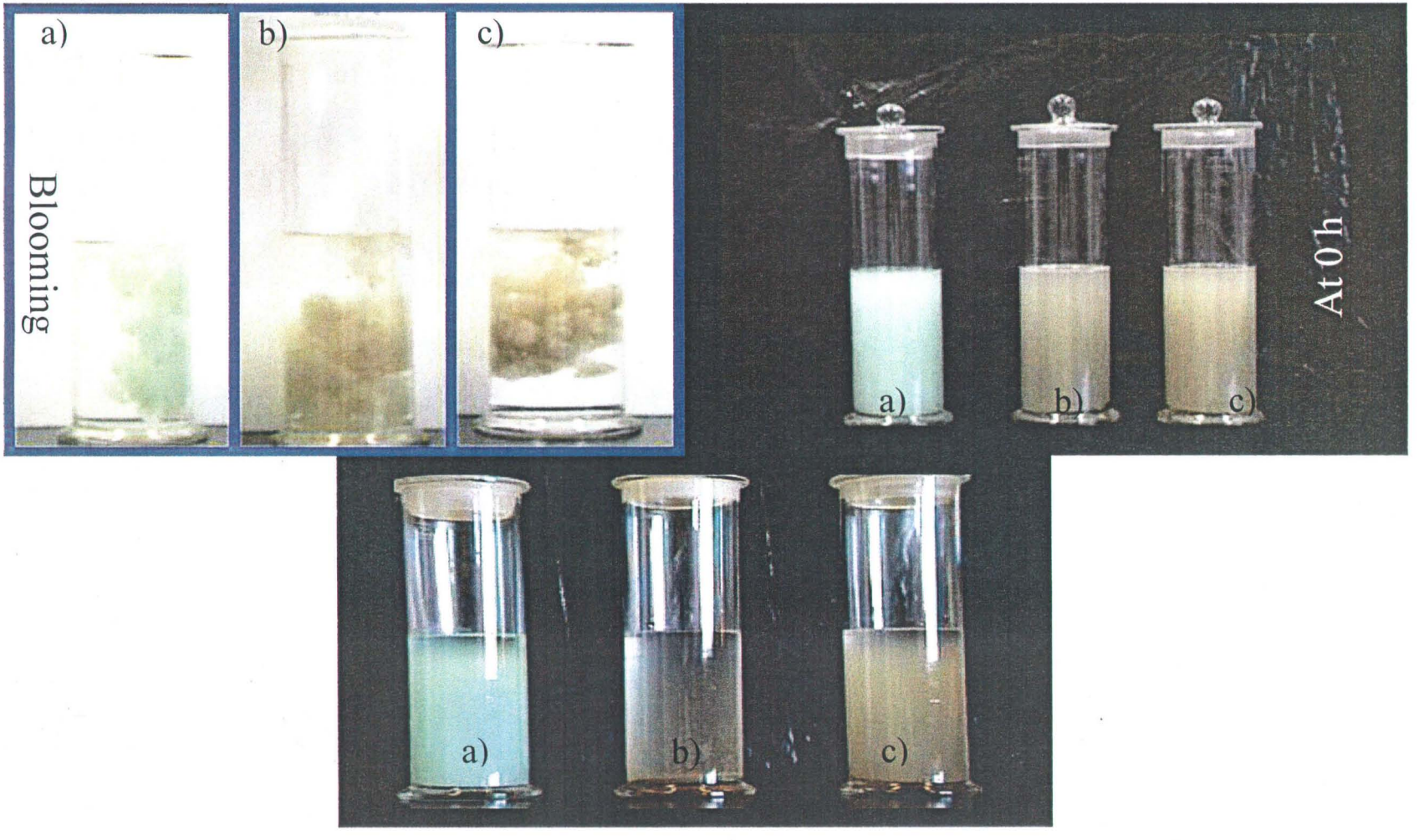


Fig. 6. Dispersibility of different secondary sludge based Bt formulations showing role of particle size; a) Commercial formulation (Foray 76 B); b) NHF sludge formulation; c) HF sludge formulation.

Partie IV

Bacillus thuringiensis Fermentation of Primary and Mixed Sludge - Broth Rheology and Process Performance

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*], J.R. Valéro[1] and R.Y. Surampalli[2]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Journal of Environmental Engineering - American Society of Civil Engineers (Under Revision)

Fermentation des boues primaires et des boues mélangées par *Bacillus thuringiensis* - Études rhéologiques des bouillons fermentés et de la performance du procédé

Résumé

Des boues d'épuration (brutes et hydrolysée) primaires, mélangées et secondaires et des eaux usées de l'industrie de l'amidon ont été employées pour la production de biopesticides à base de *Bacillus thuringiensis* var. *kurstaki* HD-1 (Bt) et pour l'évaluation subséquente de leurs possibilités d'utilisation comme substrats potentiels de fermentation. Cette évaluation a pris en considération les caractéristiques rhéologiques des bouillons fermentés en tenant compte de la rhéologie et des processus par rapport à ce qui est obtenu avec le milieu commercial de soya (semi-synthétique). Tous les milieux fermentés avaient un comportement de type pseudoplastique et suivait une loi de puissance pour la viscosité. Un plus grand cisaillement a été observé avec la boue primaire (brute et hydrolysée). Les nombre de cellules totales et de spores viables, la consommation de l'oxygène, le taux de croissance spécifique maximum (μ_{max}), et l'entomotoxicité étaient respectivement 2, 4, 1½ à 2, et 3 fois plus faibles dans les boues primaires contrairement aux boues mélangées. Cela indique que les boues primaires sont peu adéquates comme matière première pour la production de Bt et sa mise en formulation. De plus, les études de rhéologie des boues secondaires et des eaux usées d'industrie de l'amidon se sont avérées comme de bons substrats pour la production du Bt.

Keywords: *Bacillus thuringiensis*; fermentation; boues primaires; boues mélangées; rhéologie; soya

Abstract

Bench scale fermentation of primary, mixed and secondary (raw and hydrolyzed) sludge and starch industry wastewater was carried out using *Bacillus thuringiensis* var. *kurstaki* HD-1 to test their feasibility as potential growth substrates on the basis of rheology and process performance in comparison to soyameal (semi-synthetic) commercial medium. All fermented media exhibited pseudoplastic pattern, followed power law for viscosity with greater shear thinning for primary sludge (raw and hydrolyzed). Improved rheology correlated well with the fermented broth morphology. The total cell and viable spore counts, oxygen consumption, maximum specific growth rate (μ_{max}), and entomotoxicity were respectively, 2, 4, 1.5-2, and 3 folds lower in primary sludge in contrast to mixed sludge, rendering primary sludge unsuitable as a raw material for Bt fermentation and eventual formulation. Furthermore, the rheology studies of secondary sludge and starch industry wastewater proved them to be good Bt fermentation alternatives.

Keywords: *Bacillus thuringiensis*; Fermentation; Primary sludge; Mixed sludge; Rheology; Soyameal

INTRODUCTION

Wastewater sludge disposal via conventional routes like incineration, land application and landfills will be restricted in future due to stricter regulations. However, wastewater sludges are now being considered as valuable resources to produce numerous products, namely, cement aggregates, adsorbents, flocculants, enzymes, biofertilizer, biopesticides and many others, some of them successfully commercialized (Tirado-Montiel et al. 2003; Pan et al. 2003; Tay et al. 2004).

In a typical sewage treatment plant, each treatment step produces distinct sludge with specific characteristics which can serve as potential raw material for *Bacillus thuringiensis* (Bt) based biopesticides. This stems from comprehensive studies carried out on optimization of wastewater sludge based Bt biopesticides in shake flasks, pilot scale and even preliminary formulation studies (Vidyarthi et al., 2002;Tirado-Montiel et al. 2003; Brar et al. 2004; Barnabé et al. 2005; Yezza et al. 2005). Most of these studies pertained to secondary and dewatered sludges and studies on primary and mixed sludge were only carried out in shake flasks (Vidyarthi et al. 2002). Conditions during shake flask and fermenter are different due to continuous agitation and controlled environmental conditions in the fermenter so that sample is more uniform. On the other hand, shake flask presented various limitations: a) lack of pH control; b) different type of agitation (shaking) and; c) no control of aeration. Thus, these differences could change the rheological characteristics of the broth as well as the product concentration. Furthermore, scale-up involves testing of different raw materials in bench scale fermenters which will entail rheology study as an important step during fermentation. Moreover, hydrolysis (pre-treatment) of these sludges in order to increase nutrient availability, change in rheology (mass transfer) and, morphological changes during sludge Bt fermentation have not been tested.

The viscosity affects the rheology of the Bt fermented wastewater/wastewater sludge causing oxygen transfer problems, which limits the production of spores and crystal protein, important components of entomotoxicity (Tx) (Brar et al. 2005a). However, the viscosity of secondary sludge has been improved by addition of surfactant as well as by pre-treatment (hydrolysis) which increased nutrient availability and hence, the Tx (Brar et al. 2005 a,b). Viscosity is also an important factor influencing the separation performance of centrifugation and hence downstream processing. Thus, to sum up, rheology provides significant information in regard to: (a) quality control of either raw materials or final products; (b) process engineering; (c) downstream processing i.e. concentration and/or separation of products (entomotoxicity); d) optimization of formulations on the basis of the relationships

existing between microstructure and physical properties; (e) formulation development; and (f) field application of the product.

Therefore, effect of rheology during Bt fermentation of primary and mixed sludge (raw and pre-treated) in comparison with conventional soyameal medium and other media (starch industry wastewater and secondary sludge which are preferred substrates for Bt fermentation) was investigated.

MATERIALS AND METHODS

Wastewater and Wastewater sludge – Sources and Physico-Chemical Characterization

Sludges – primary, mixed (60% primary + 40% secondary sludge) and secondary were obtained from Ville de Québec wastewater treatment plant at Quebec (Canada). Starch industry wastewater (SIW) was obtained from ADM-Ogilvie at Candiac (Québec, Canada). Raw primary and mixed sludge and SIW were characterized according to Standard Methods (APHA et al. 1998) and different characteristics are provided in Table 1. The wastewater and wastewater sludge were immediately utilized (within 1 week) for fermentation as long term storage at even 4°C would lead to deterioration (slow endogenous respiration).

Solids amendment and pre-treatment procedure

The sludge was concentrated from about 3 to 5% w/v solids by gravity settling followed by centrifugation at 7650 g for 15 minutes at 20 ± 1°C in a Sorvall RC 5C plus Macrocentrifuge. The sludge supernatant was stored in the refrigerator at 4 ± 1°C and used to dilute the sludge samples as per requirements. The concentrated sludge was then homogenized in a blender to achieve suspended solids concentration of 25 kg/m^3 which was further used for all types of experiments. The SIW was used as such for fermentation.

The concentrated primary or mixed sludge was transferred into a custom made mechanical steam hydrolyzer (Stainless steel 316 L) with pure steam injection configuration; working volume: 10 liters with controlled agitation (EBR Quebec, Canada). Hydrolysis was carried out at optimal conditions: 140 ± 1°C for 30 minutes at a pressure of 276 kPa (Barnabe et al. 2005). Considering 1.67 times (experimental factor) dilution by steam condensation, the initial SS was 25 kg/m^3 x 1.67 so that after hydrolysis, the final SS would be 25 kg/m^3 (actually SS may be < 25 kg/m^3 due to solids dissolution). Raw primary and mixed sludge

will be henceforth referred to as NHP and NHM and hydrolyzed primary and mixed sludge as THP and THM, respectively.

Commercial medium and bacterial strain

The composition of semi-synthetic medium soybean meal medium is presented elsewhere (Vidyarthi et al. 2002). *Bacillus thuringiensis* var. *kurstaki* HD-1 (ATCC 33679) was used in this study. The culture conditions, maintenance and inoculum production are described elsewhere (Vidyarthi et al. 2002).

Fermenter set-up

A stirred tank fermenter (0.015 m^3) equipped with accessories and automatic control systems for dissolved oxygen, pH, antifoam, impeller speed, aeration rate and temperature was utilized for Bt fermentation of wastewater sludges. The software (iFix 3.5, Intellution, US) provided automatic set-point control and compilation of operational parameters.

Polarographic pH-electrode (Mettler Toledo, US) was calibrated using buffers of pH 4 and 7 (VWR-Canada). Subsequently, the oxygen probe was calibrated to zero (using N_2 degassed water) and 100 % (air saturated water). Later, the fermenter was filled with 0.010 m^3 individual media (sludge, SIW or semi-synthetic) and concentrated polypropylene glycol solution (PPG, Sigma-Canada) was added as an anti-foam agent so that its final concentration was 1% v/v. *In-situ* steam sterilization was carried out at 121°C for 30 minutes at a constant agitation of 300 rpm. When the fermenter cooled down to 30°C, the dissolved oxygen (DO) probe was recalibrated as stated earlier at agitation rate of 500 rpm. The fermenter was then inoculated (2 %v/v inoculum) with acclimated pre-culture of Bt in exponential phase. In order to keep the DO above 25 % saturation, air flow rate and agitation rate were varied between 0.13-0.23 vvm and 250–700 rpm, respectively. The temperature was maintained at 30 ± 1°C by circulating water through the fermenter jacket. The pH was controlled at 7.0±0.1 by using either 4N NaOH or 4N H_2SO_4 by peristaltic pumps. The foaming was controlled by PPG injection (1% v/v) and mechanical foam disruptor (Fundafoam^{TM}).

Sampling was carried out at regular intervals under aseptic conditions. Further, the samples were divided into four parts to analyze, namely, Tx (1ml, stored at -20°C until actual assay); TC and VS (5 ml, room temperature, done immediately); particle size and viscosity (50 ml, done immediately).

Volumetric oxygen transfer coefficient (k_La) and oxygen uptake rate (OUR) were measured at each sampling point by conventional dynamic method utilizing "air off and on" mechanism (Aiba et al. 1973).

Microscopy

Observation and image capture of sludge, SIW and soya, verification of Bt inoculum and pre-culture and contamination, if any, was carried out with an optical light microscope (Zeiss Axiolab, Germany) equipped with a video camera (Axiocam HRC Zeiss, Germany).

Total cell (TC) and viable spore (VS) count

Procedure specified in Vidyarthi et al (2002) was utilized for TC and VS count. The standard deviation for cell and spore count was 8.0 %.

Bioassay

Bioassays for entomotoxicity (Tx) determination were conducted using the diet incorporation method (Tirado-Montiel et al. 2003). Sample preparation and diet protocol was carried out as per method enumerated by Vidyarthi et al. (2002). Tx of sample preparations was obtained by comparing the final mortality (percentages) of eastern spruce budworm larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) with that of standard commercial product (Foray 76B, Abbott Laboratories, Chicago, IL) and expressed as relative spruce budworm units/µl (10^{-3} SBU/m^3). Foray 76B contained spores and crystals of Bt var. *kurstaki* at a potency of 20.1 x 10^{12} IU/m^3 (International Unit) measured against cabbage looper (*Trichoplusia ni*). On comparison of Tx of Bt fermented sludge and soyameal samples, it was found that specific SBU in this study was 20-25 % higher than IU. The standard deviation for Tx measurement was 8–10 %.

Viscosity and particle size

Rheological properties of Bt fermented sludges, especially, viscosity of SIW and soya were determined by using a rotational viscometer Brookefield DV II PRO+ (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) equipped with Rheocalc32 software. The spindle SC-34 with a sample cup volume of 18ml was used for all rheological measurements.

Samples for rheological study were drawn at 48h before harvesting the fermenter. Time dependent profile of fermented broths at 48 h was determined at low shear rate (7.34 s^{-1}) and the viscosity was measured at each sampling point at a shear rate of 36.71 s^{-1}. Viscosity was also determined at different shear rates ranging from 0.1 to 46.7 s^{-1} to characterize shear rate behaviour. All measurements were done at a constant temperature of 25±1°C and a time lag of measurement between consecutive shear rates was kept constant at 1 min. Different rheological models were drawn considering the viscosity input information. The viscosity always refers to "apparent viscosity", unless stated otherwise. The rheology (consistency index, K and flow behaviour index, n) of simpler fermented media for Bt fermentation, namely, secondary sludge (raw and pre-treated); SIW and soya (control) was carried out at different fermentation times. The detailed fermentation studies have been already reported in Brar et al., 2005 a, c.

Particle size analysis was measured by LASER diffraction particle sizer analyzette 22 (Fritsch GmbH, Germany) according to the method already specified (Brar et al. 2005a).

RESULTS AND DISCUSSION

Soyameal Fermentation

The fermentation profile of soyameal medium is illustrated in Figs 1a and b. It was observed that Bt followed exponential growth in the first 6h (TC increased from 1.8 x 10^6 to 1.78 x 10^8 CFU/ml) and later entered stationary phase (9-48h). However, spore formation still continued and reached a stationary phase at 15h (1.76 x 10^6 to 2.03 x 10^8 CFU/ml). Furthermore, lower VS resulted in lower Tx of 9.5 x 10^{12} SBU/m^3 (Table 2) inspite of higher μ_{max} (0.37 h^{-1}, Table 2) when compared to SIW, NHM and THM (discussed later). The TC and VS counts were in concordance with Yezza et al. (2005) who reported Btk fermentation of soyameal medium in pilot scale (0.100 m^3 fermenter), without assessing rheological properties which are very vital in any fermentation.

During the first stage of fermentation (0-6h), a decrease in DO was noticed due to higher oxygen uptake rate (OUR) caused by active Bt growth (exponential phase) and later, it increased until 36h and flattened until 48 h. The k_La increased during the exponential phase (51 to 283 h^{-1}) despite faster growth due to partial compensation by increments in the operating conditions (agitation rate and air flow rate), in order to maintain adequate DO.

Viscosity increased from 2.2 x 10^{-3} to 5.4 mPa.s in accordance with the TC increase (3 to 9h). The viscosity values remained high from 9 to 18h despite the beginning of stationary

phase at 12h. In spite of the increase in viscosity (3-9h), the k_La also increased which could be a manifestation of operating parameters (varying aeration and agitation conditions). On the contrary, particle size kept on decreasing continuously with a marginal increase from 13.5 to 27 μm (18 to 36h). Initial decrease in particle size was owing to shear stress induced by agitation with a small hump at 24h. The hump could be observed due to release of metabolites of fermentation and formation of extracellular polymeric substances (EPS) which interlinked the substrate particles and, TC and VS of Bt. Density remained practically constant throughout fermentation and rheology of fermentation was best translated in viscosity data.

SIW Fermentation

The fermentation profile of SIW has not been presented in details as it has been very comprehensively discussed in earlier publication (Brar et al. 2005c). However, for comparison, the fermentation parameters are provided in Table 2. The fermentation parameters revealed SIW to be a rheologically simple medium (power law Constants; K = 1.46; n = 1.04; Table 3) resulting in higher Tx (16.5 x 10^{12} SBU/m^3) and higher TC (1.67 x 10^9 CFU/ml) and VS (8.15 x 10^8 CFU/ml) counts when compared to primary, mixed (Table 2) or secondary sludge (Brar et al. 2005a). Further, the μ_{max} (0.34 h^{-1}) was comparable to soyameal medium.

Rheology and Morphology Correlation

The morphology (defined by floc characteristics –loose or compact and entangled; presence of extracellular polymeric substances and fibrous materials) of different fermented media is presented in Fig 2. The soyameal and SIW media before fermentation (0h – after inoculation) and after fermentation (48h) appeared to have simpler morphology (Fig 2 A&B, G&H) as also reflected in their K and n values. Contrarily, the primary sludge exhibited complexity marked by presence of fibrous network even in the fermented broth at 48h (Fig 2 C and D). The morphology did not change after hydrolysis (Figures not shown), as seen from power law constants, namely K and n (Table 3, discussed later). However, mixed sludge (Figs 2 E and F) complexity was reduced as it comprised 60% primary and 40% secondary sludge with further improvement on hydrolysis (K and n values, Table 3). These morphological characteristics can determine the rheological properties of the culture broth before and after fermentation by affecting the flow properties. Thus, the measurement of apparent viscosity of the medium during production (fermentation) phase could allow the predictions of probable Tx production potential.

Primary sludge (Raw)

Figs 3a and b present the Bt process and performance parameters, respectively of primary sludge (raw). Bt exhibited diauxic growth where the first growth phase lasted from 0 to 9h and second phase from 15 to 21h. In fact, Bt growth in mixed or complex medium has been shown to be diauxic as reported in earlier studies (Ribbons 1969). The maximum TC and VS concentration was 3.7 x 10^7 (24h) and 2.9 x 10^7 CFU/ml (36h), respectively. On the contrary, there was a slow increase in VS and also lower μ_{max} (0.13 h^{-1}, Table 2) when compared to soya or mixed sludge. It could be due to low availability of easily degradable carbon and lower nitrogen assimilation or availability. The slow increase in VS and lower μ_{max} resulted in lower k_La values (maximum, 98 h^{-1}) which were a consequence of poor rheological conditions (K = 583.3; n = 0.38) that limited mass transfer (oxygen and nutrients). The lower VS and poor rheology also resulted in reduced Tx value of 5.7 x 10^{12} SBU/m^3 (Table 2). Similar poor rheology observations were made in secondary sludge shake flask studies at higher solids (> 25 kg/m^3) concentration (Brar et al. 2005b). Poor Tx was due to combined effect of low VS as well as particle size which was higher (might inhibit ingestion by insects suppressing expression of Bt crystal proteins; VS and other virulence components). Moreover, the percent dissolved solids (DS) utilized during fermentation was 18.7% with an equivalent soluble chemical oxygen demand (SCOD) utilization of 22.6% (Table 2) resulting in lower TC (3.8 x 10^7) and VS (2.9 x 10^7) at the end of fermentation (48h) as compared to soya, SIW and mixed sludge. The percent DS and SCOD utilized could correspond to substrate consumption and hence fermentation. In literature, DS and SCOD utilization have been used as indicators of methanogenic fermentation for production of volatile fatty acids (Barajas et al. 2003).

Although air flow and agitation rates were maintained at 2.5-3.5 LPM and 350-500 rpm, respectively and initial DO was as high as 95%, yet the k_La values ebbed at lower levels in comparison to soyameal medium. This was due to complex medium rheology which showed irregularities in viscosity and particle size during fermentation (Fig. 3b) and also due to the presence of difficult to biodegrade carbon which resulted in low oxygen demand and hence lower k_La.

The density remained constant from 0.95 to 1.1 x 10^3 kg/m^3 all through the fermentation. The viscosity and particle size showed an ambiguous behaviour due to change in consistency index of the fermented broth (K = 583.3, Table 3 and also morphology; fibrous nature of the broth, Figure 2 B and C, discussed earlier).

Primary Sludge (Hydrolyzed)

The fermentation profiles (operating and performance parameters) of primary sludge (hydrolyzed) are illustrated in Figs 4a and 4b. The growth profile was nearly similar to NHP (diauxic phase; 1st from 0-9h and 2nd, 15-30h) with maximum TC of 1.2 x 10^7CFU/ml^3 (18h) and followed by a slow continuous growth until 30h of 3.9 x 10^7 CFU/ml as reflected in Fig. 4b. The μ_{max} (0.15 h^{-1}), DS (17.5 %) and SCOD (22.1 %) utilization values were similar to NHP (Table 2). VS also followed a similar profile with a maximum of 3.35 x 10^7 CFU/ml at 30h. Altogether, the Tx was also lower (5.6 x 10^{12} SBU/m^3) and equivalent to NHP (Table 2). Conventionally, hydrolysis of sludge would result in better assimilation of available nutrients by Bt due to hydrolysis of complex organic matter and floc break-up as reported by Barnabé et al. (2005) for secondary sludge. However, this was not the case in primary sludge where the morphology was more complex due to the presence of organic, inorganic and other extraneous matter.

Despite appreciable air flow rate (3 LPM, normally used in Bt fermentation) and agitation rate (300-450 rpm), k_La values remained lower with a maximum of 82 h^{-1} due to complex rheology (K = 515.3, Table 3 and also morphology in Figure 2) and low availability of degradable carbon. There was an initial increase in k_La until 18h, which was due to variable agitation and aeration rates and then it practically remained constant with small variations.

There were perturbations in viscosity and particle size similar to NHP sludge. Intense foam produced during the fermentation caused continuous addition of anti-foam agent, which might have caused inadvertent increase in viscosity. Thus, hydrolysis of primary sludge did not improve the rheological profile (Table 3) with minor changes in magnitude of values and highly variable behaviour.

Mixed sludge (raw)

Different fermenter operational and process performance parameters for mixed sludge (raw) are presented in Figs 5a and b, respectively. TC increased until 18h (maximum value of 3.9 x 10^8 CFU/ml) and likewise VS increased up to 18h (maximum value of 3.7 x 10^8 CFU/ml) with the TC count similar to THP and μ_{max} of 0.21 h^{-1}. There was a diauxic growth phase (1st from 0-9h with 9-12h of slow down of growth due to change of substrate and 2nd, 12-18h) similar to NHP and THP. The μ_{max} values obtained in this study were equivalent to secondary NH sludge at 25 kg/m^3 solids (Brar et al. 2005a). Moreover, 30.8% DS utilized during fermentation with an equivalent SCOD utilization of 38.5% (Table 2) resulted in higher TC and VS values at the end of fermentation (48h) compared to fermented NHP and THP.

Additionally, 1.7 fold increase in VS with reference to NH (secondary sludge, Brar et al. 2005a) sludge at 25 kg/m^3 solids resulted in higher Tx of 14.8 x 10^{12} SBU/m^3. The increase in Tx may also be due to abrasive nature of mixed sludge (siliceous material, sand) which may cause tearing of the insect midgut epithelium so that the action of spores and crystal protein was more intensified. In fact, abrasive materials have been added to Bt formulations to overcome the peritrophic barrier of chitin membrane and penetrate insect midgut (Burges 1998).

Although agitation rate was maintained at 400 rpm to compensate the falling DO, still k_La surfaced at lower values of 45 to 70 h^{-1} between 6 and 30h of fermentation. This irregular trend could be due to anomalous rheological and morphological conditions prevailing in the fermenter (note the ebbs and tides in viscosity and particle size in Fig 5b, fibrous network in Fig. 2B and C). Intermediate surges in viscosity were a manifestation of anti-foam addition due to intense foaming conditions normally observed in sludge fermentation (Vidyarthi et al. 2002). The ambiguous behaviour of mixed sludge was due to its physical constitution as it comprises 60% primary and 40% secondary sludge so that the presence of smaller silica particles escape primary treatment and thus affect the rheology and also the performance of fermenter (not recommended for bottom driven bioreactors as it may damage the mechanical seals).

Mixed sludge (Hydrolyzed)

Figs 6a and b represent the fermenter operational and process parameters of hydrolyzed mixed sludge, respectively. The exponential growth phase ranged from 0-6h with a subsequent small increase to reach maximum TC of 5.6 x 10^8 CFU/ml at 30h. VS kept on increasing until 18h (3.2 x 10^8 CFU/ml) followed by a small increase to 5.15 x 10^8 CFU/ml at 30h (Fig 6b). Due to better nutrient availability in comparison to NHM, the diauxic phase was not apparent, percent increase in sporulation was 28 % and similarly Tx increased by 18 % (16.6 x 10^{12} SBU/m^3). The μ_{max} was 0.30 h^{-1} which was equivalent to SIW but higher than NHM (Table 2). The Tx was higher than soyameal medium which was in concordance with earlier studies conducted on Bt fermentation of secondary sludge (Vidyarthi et al. 2002; Yezza et al. 2005; Barnabe et al. 2005). However, the percent DS (29.5%) and SCOD (39.9%) utilized during fermentation was similar to NHM (Table 2).

The DO decreased in the initial 6h and later increased until 12h and finally ceased to increase above 70%. Likewise, the k_La values remained relatively high at 94 h^{-1} at 9h and then started decreasing at 24h confirming better DO assimilation in the medium than NHM. Viscosity showed two humps at 6 and 21h (12h for 1st; 24-30h for 2nd hump in Fig. 6b) and

initial increase could be due to active cell growth and EPS formation and later the humps may be a manifestation of anti-foam addition and EPS degradation and possible cell lysis. As explained elsewhere (Brar et al. 2005b), increase in viscosity was difficult to correlate with Bt growth due to overriding factor of sludge solids. Similar to NHM, particle size distribution (D_{50}) showed an irregular yet smoother profile, and it was entirely an outcome of agitation (physical) conditions in the fermenter as well as inconsistent rheology and morphology of the medium.

OUR Profiles

Fig 7 represents OUR profiles of different fermented media with oxygen consumption values provided in Table 2. The SIW showed higher level of oxygen consumed (256.44 mol/m^3, Table 2) which was also reflected in the growth parameters, namely, TC, VS and finally Tx (Table 2). SIW was closely followed by THM sludge with OUR values ranging from 0.05 to 5.8 mol/m^3/h and the peak OUR of 2 to 3 mol/m^3/h (oxygen consumed – 156.13 mol/m^3) persisted from 18 until 36h. This increase in OUR, despite cessation of formation of TC and VS at 18h for THM sludge (Fig 6b) could be attributed to active metabolism related to spore maturation and formation of virulence factors, namely, crystal proteins and enzymes. The OUR of NHM was 1.8 to 6.3 mol/m^3/h and oxygen consumption was 67.68 mol/m^3 followed closely by soya with higher OUR from 6 to 12h (1.4 to 4.9 mol/m^3/h) with oxygen consumption of 52.38 mol/m^3. The differences in OUR were also due to higher TC and VS for NHM sludge (Figs 5 a and b) which also resulted in higher Tx when compared to soyameal medium.

The OUR profiles of primary sludge (NHP and THP with oxygen consumption of 34.92 and 43.92 mol/m^3, respectively) were also similar (1.3 to 3 mol/m^3/h), but lower than the OUR of NHM, THM, SIW and soyameal. The lower OUR values could be due to μ_{max} which was inferior for NHP and THP at 0.13 and 0.15 h^{-1} (Table 2), respectively. The final TC and VS values were also lower as reported in Table 2. In fact, slow growth of microorganisms has been found to lower the oxygen uptake (Aiba et al. 1973).

Rheology of Fermented broths

Fig 8 a represents rheograms of different fermented broths (primary and mixed sludge – raw and pre-treated; SIW and soyameal). All fermented broths indicated non-Newtonian behaviour. Non-linearity was higher for raw sludges followed by hydrolyzed sludges, whereas, soya and SIW showed the least non-linear behaviour. Similarly, the shear rate dependent profiles for different broths as represented in Fig 8b showed pseudoplastic

(decrease in viscosity with shear rate) behaviour. Additionally, all fermented broths, except soyameal and SIW, followed thixotropic (decrease in viscosity with time, data unreported) behaviour. The soyameal and SIW showed rheopectic behaviour (increase in viscosity with time, data unreported). This behaviour was in agreement with earlier study on Bt fermented wastewater sludge in shake flasks (Brar et al.2005b). There was difference in sampling conditions of shake flask and fermenter as continuous agitation and controlled environmental conditions in the fermenter assisted in taking a uniform sample. Thus, these differences might have altered the magnitude of the rheological characteristics (as expected), if not the profile.

Different rheological models were tested for data fits of various fermented broths (Table 3) and each broth showed a specific behaviour. The confidence of fit with a positive intercept for Bingham plastic model varied from 48-78% for all broths. On the other hand, the confidence of fit for the power law model varied from 82 to 95% indicating that the consistency behaviour of fermented sludges, SIW and soyameal medium could best be expressed by the pseudoplastic power law model as represented in Table 3. Other rheological models, viz. Casson, NCA-CMA (National Confectioners Association and the Chocolate Manufacturers Association) Casson over the whole shear rate range gave a poor confidence of fit (67 to 72%) for mixed sludge. But, the power law was universally followed by all fermented media with excellent confidence level (85-95%). Similarly, IPC (Institute for Interconnecting and Packaging Electronic Circuits) paste model, an extension of the power law also gave excellent fits for all fermented media.

The power law model was chosen over other potential models as it is the most commonly used for rheological analysis of microbial fermentation systems (Pollard et al. 2001). The consistency index (K) of 48h fermented broth followed the order: NHP>THP>NHM>THM>soyameal>SIW. The NHP fermented sludge showed maximum relative thickening (higher "K") at 48h. Likewise, n <1 for all fermented media types and hence more pronounced effects of pseudoplasticity would be felt on flow and transport phenomenon (Aiba et al. 1973). In any case, except soya and SIW, all fermented media exhibited a marked shear-thinning response ($n \leq 0.5$). The increase in n (example, soyameal and SIW) indicated the shift from pseudoplasticity towards a more Newtonian behavior at high shear rate. However, n is an exponent in the power law, hence, changes in n will have large effects on the broth apparent viscosity and vice versa. Nevertheless, the actual n value achieved could be medium specific. The flow behavior of the fermentation broth was essentially pseudoplastic for all wastewater sludge broths. The non-Newtonian behavior was relatively predominant at lower shear rate and higher medium complexity. These phenomena may be due to the higher energy dissipation rates to deform and break down the floc structure which could be negligible at very high shear rates.

The consistency index of fermented primary and mixed sludge was about 200 times higher than soyameal and SIW medium, which could be due to the presence of cellulosic fibres and other fibrous materials (which formed ramified structures) as seen in Fig 2. The K of primary sludge when compared to mixed sludge could also be influenced by the presence of relatively higher concentrations of metals, especially, higher valency forms (Ca, Fe, Mg, Zn, Al) which may have a tendency to link to EPS through more binding sites, thus enhancing viscosity of the medium. This could result in extended configuration of the sludge flocs which might otherwise be present as random coiled flocs. Bueno and Garcia-Cruz (2001) found that presence of bivalent ions intensified the viscosity during production of xanthan gum from *Xanthomonas campestris*. Thus, the increase in consistency index was an indicator of increasing medium complexity and also presence of metals with polyvalence capacity affecting fermentation as well as downstream processing steps. This study will assist in downstream processing pipe design so that higher non-Newtonian behaviour exhibited by primary sludge will cause greater non-linearity between pressure drop and flow rate when compared to mixed sludge and soyameal medium.

Power Law Behaviour of Different Media during Bt Fermentation

As discussed earlier, the primary and mixed sludge gave complex rheological profiles (Table 3, Figs. 3a, b; Figs. 4a, b and Fig. 2) which rendered them unfit for use as Bt fermentation raw materials. This stimulated the study of rheology of secondary sludge (non-hydrolyzed and hydrolyzed) since it is the most common and preferred medium used for Bt fermentation (Tirado-Montiel et al. 2003; Yezza et al. 2005; Barnabe et al. 2005; Brar et al. 2004, 2005a). As the Bt fermentation of secondary sludge has been already studied in great details (Brar et al. 2005a), this section only presents the rheological changes, particularly, "K" and "n" behaviour. Figs 9 a and b represent the representative "K" and "n" profiles, respectively of secondary sludge (non-hydrolyzed-NHS; hydrolyzed – THS); soya and SIW medium. The consistency index (K) increased until 12h (active exponential phase) which may be due to the possibility of continuous growth of Bt cells in the fermentation medium as suggested by TC trends (Brar et al. 2005a). Further, decrease in "K" after 12h may be due to release of spores and crystal proteins and various extracellular and intracellular enzymes or polymers into the surrounding fermented broth. The decrease in "K" could also be due to the agitation effects that predominated cell growth as the fermentation had reached stationary phase. The "K" continued to rise from the early fermentation period, and thereafter a significant decline was observed, as reported by Sinha et al., 2001. The *n* is also found to increase slightly towards the end of the fermentation as EPS of the sludge flocs would have degraded resulting in a more dispersed medium.

In summary, primary sludge (raw and hydrolyzed) should not be considered as an alternative raw material for Bt biopesticides production due to poor entomotoxicity; poor rheology; lower $\mu_{max;}$ lower k_La and lower OUR. All of these factors could negatively affect the total biopesticide process development. However, mixed (non-hydrolyzed as well as hydrolyzed) sludge could be considered as a feasible Bt growth alternative. Nevertheless, it would be expected to modify the fermenter configuration (example, in this case, replacing bottom mounted impeller by top mounted impeller) in order to protect the mechanical seal. Furthermore, its variable rheology and inherent nature (sand and fibers) could also affect downstream processing that remains to be investigated. This information provides valuable input for downstream processing where broth viscosity will impact the separation performance of the centrifugation. During downstream processing, mixed sludge may settle faster in shorter residence time due to presence of fibres sand and other debris. Further, the siliceous material (sand) may assist in development of dry powder formulations as it can act as a carrier. However, sand may be a drawback in suspensions due to the possibility of choking of spray equipment nozzles (particle size for formulations was 100 to 130 μm in comparison to desired field norm of $\leq$ 25 μm). Thus, mixed sludge may be used in dry product formulations as sand (silica) may also act as a natural protectant of crystal protein (biopesticidal component) from harmful effects of UV further enhancing field efficacy. Eventually, rheology of secondary sludge (non-hydrolyzed and hydrolyzed) and starch industry wastewater proved that they could serve as good fermentation substrates with ease of downstream processing and formulations as also established in earlier studies (Brar et al. 2004, 2005c).

Conclusions

The study on broth rheology and process performance of primary and mixed sludge led to following conclusions:

1. All fermented media showed pseudoplastic and shear thinning behaviour and for primary sludge it was more prominent than mixed sludge.
2. Power law and IPC Paste model was followed by all fermented media (soyameal; starch industry wastewater; raw and hydrolyzed primary and mixed sludge) with best confidence of fits from 82 to 95 %.
3. Rheology of fermented media agreed with the morphology which was less ramified (with fibrous networks) for mixed sludge when compared to primary sludge.
4. Primary sludge showed lower total cell and viable spore counts; lower oxygen consumption (35 to 45 mol/m^3) ; lower μ_{max} (0.13-0.15 h^{-1}) and lower percent of dissolved solids utilization (18%) resulting in lower entomotoxicity (6 x 10^{12} SBU/m^3) and thus may not be suitable as a future proponent for Bt fermentation and formulation.

5. Mixed sludge (raw and pre-treated) could be considered as feasible Bt growth alternatives with higher total cell and viable spore counts; higher μ_{max} (0.21-0.3 h^{-1}) and higher percent of dissolved solids utilization (30%) resulting in higher entomotoxicity.

Nomenclature	
K	Consistency index (mPa.s^n)
n	Flow behaviour index
μ_{max}	Maximum specific growth rate (h^{-1})
η	Plastic Viscosity (mPa.s)
R	Rotational speed, rpm
D	Shear rate (s^{-1})
τ	Shear stress (N/m^2)
n_s	Shear sensitivity factor
a	Spindle radius/inner cup radius
η_{10}	10 rpm viscosity
k_La	Volumetric oxygen transfer coefficient (h^{-1})
τ_o	Yield Stress (N/m^2)
List of abbreviations	
Bt	*Bacillus thuringiensis*
CFU	Colony forming units
D_{50}	Particle size (μm)
DO	Dissolved oxygen
DS_{0h}	Dissolved solids at 0 h, after inoculation (kg/m^3)
DS_{48h}	Dissolved solids at 48 h, after fermentation (kg/m^3)
NHS	Non-hydrolyzed secondary sludge
THS	Hydrolyzed secondary sludge
NHM	Non-hydrolyzed/raw mixed sludge
NHP	Non-hydrolyzed/raw primary sludge
OUR	Oxygen uptake rate ($2.8 \times 10^{-4} mol/m^3/s$)
$10^3 SBU/m^3$	Spruce budworm units/liter
$SCOD_{0h}$	Soluble chemical oxygen demand at 0h, after inoculation (kg/m^3)
$SCOD_{48h}$	Soluble chemical oxygen demand at 48h, after fermentation (kg/m^3)
SIW	Starch industry wastewater
SS	Suspended solids (kg/m^3)
TC	Total cells (CFU/ml)
THM	Hydrolyzed mixed sludge
THP	Hydrolyzed primary sludge
TKN	Total Kjeldahl nitrogen
TS	Total solids (kg/m^3)
TVS	Total volatile solids (kg/m^3)
Tx	Entomotoxicity (10^{12} SBU/m^3)
VS	Viable spores (CFU/ml)
VSS	Volatile suspended solids (kg/m^3)

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, and Canada Research Chair) for financial support. The views and opinions expressed in this article are those of authors. The authors thank Dr. Simon Barnabé for reading the manuscript and providing valuable suggestions in the preparation of manuscript. We are also thankful to Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Service and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing fellowship to Satinder K. Brar.

References

Aiba, S., Humphrey, A.E., and Millis, N.F. (1973). *Biochemical Engineering*, 2nd edition, Academic Press, New York.

APHA, AWWA, WPCF (1998). *Standard Methods for Examination of Water and Wastewaters.* 20th edn. (Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. eds.), American Public Health Association, Washington, DC, USA.

Barajas, M.G., Knobelsdorf, J., Escalas, A., and Mujeriego, R. (2003). "Solubilization and fermentation in a modified VFA-potential method." *Environmental Engineering Science*, 20 (4), 329-336.

Barnabé, S., Verma, M., Tyagi, R.D., and Valéro, J.R. (2005). "Culture media for increasing biopesticide producing microorganism's entomotoxicity, methods of producing same, biopesticide producing microorganisms so produced and method of using same." US patent International application PCT/CA2005/000235.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., and Banerji, S., (2004). "Development of sludge based stable aqueous *Bacillus thuringiensis* formulations." *Water Science and Technology*, 50 (9), 229-236.

Brar, S.K., Verma, M., Barnabé, S., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2005a). "Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges." *Process Biochemistry*, 40, 2695–2705.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2005b). "Sludge based *Bacillus thuringiensis* biopesticides: viscosity impacts." *Water Research* 39, 3001-3011.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2005c). "Starch industry wastewater based stable *Bacillus thuringiensis* liquid formulations." *Journal of Economic Entomology*, 98(6), 1890 – 1898.

Bueno, S.M., and Garcia-Cruz, C.H. (2001). "The influence of fermentation time and the presence of salts in the rheology of the fermentation broth as a polysaccharide-producing bacteria free of soil." *Journal of Food Engineering*, 50, 41-46.

Burges, H.D., (ed.) (1998). *Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments*, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Pan, S.S., Lin, C.C., and Tseng, D.H. (2003). "Reusing sewage sludge ash as adsorbent for copper removal from wastewater." *Resource Conservation Recycling*, 39, 79-90.

Pollard, D.J., Hunt, G., Kirschner, T.K., and Salmon, P.M. (2002). "Rheological characterization of a fungal fermentation for the production of pneumocandins." *Bioprocess Biosystems Engineering*, 24, 373–383.

Ribbons, D.W. (1969). "Automatic assessment of respiration during growth in stirred fermentors." *Applied Microbiology*, 438-443.

Sinha, J., Bae, J.T., Park, J.P., Kim, K.H., Song, C.H., Yun, J.W. (2001). "Changes in morphology of *Paecilomyces japonica* and their effect on broth rheology during production of exo-biopolymers." *Applied Microbiology and Biotechnology*, 56, 88–92.

Tay, J.H., Show, K.Y., Lee, D.J., and Hong, S.Y. (2004). "Reuse of wastewater sludge with marine clay as a new resource of construction aggregates." *Water Science and Technology*, 50(9), 189-196.

Tirado-Montiel, M.L., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2003). "Production biopesticides using wastewater sludge as a raw material – Effect of process parameters." *Water Science and Technology*, 48 (8), 239-246.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2002). "Studies on the production of *B-thuringiensis* based biopesticides using wastewater sludge as a raw material." *Water Research*, 36 (19), 4850-4860.

Yezza, A., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. (2005). "Production of *Bacillus thuringiensis* based biopesticides in batch and fed-batch cultures using wastewater sludge as a raw material." *Journal of Chemical Technology Biotechnology*, 80, 502-510.

Table 1. Raw Wastewater and wastewater sludge composition

Parameter (s)	**Primary**	**Mixed**	**Starch industry wastewater**
TS (kg/m^3)	46.2 ± 1.2	39.5 ± 3.2	17.1 ± 0.1
TVS (kg/m^3)	31.2 ± 1.8	21.4 ± 2.2	14.1 ± 0.1
SS (kg/m^3)	40.8 ± 2.2	30.6 ± 4.2	2.1 ± 0.11
VSS (kg/m^3)	28.2 ± 2.2	16.9 ± 1.7	2.1 ± 0.4
pH	5.3 ± 0.1	6.1 ± 0.1	3.2 ± 0.1
Concentration (x 10^{-3} g/kg of TS)			
Total carbon	504000 ± 764	412800 ± 896	587552 ± 1324
Total nitrogen	22325 ± 1346	34308 ± 1237.4	44231 ± 2011
Total phosphorus	12549 ± 3768	12140 ± 1132.4	28756 ± 1987
$N\text{-}NH_3$	615± 264	580 ± 379.2	110 ± 56.7
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$	15 ± 3.2	8 ± 0.8	4 ± 1.3
$P\text{-}PO_4^{3-}$	4200 ± 245.6	6194 ± 219	15001 ± 2345
Al	27229 ± 3456.7	21237 ± 1389.3	55987 ± 2234
Ca	34971± 4879	19826 ± 4367	11987 ± 198
Cd	6.7 ± 1.2	2.0 ± 0.2	-
Cr	54 ± 9.2	147 ± 77.1	1 ± 0.05
Cu	346 ± 34.2	310 ± 112.2	375 ± 143.6
Fe	16020 ± 1025.4	18717 ± 1456.2	8239 ±1001
K	5632 ± 238.9	7486 ± 1098.2	23021 ± 3566
Pb	55 ± 21.2	50 ± 11.5	2.7 ± 1.7
S	6170 ± 1257.4	5820 ± 1308.2	2307 ± 87.9
Zn	520 ± 124.1	390 ± 138.4	302 ± 97.3
Na	13817 ± 47.9	5487 ± 39.7	2305 ± 248
Mg	823 ± 40.5	422 ± 19.8	62 ± 32.1
Ni	22 ± 5.2	16 ± 3.1	-

± refers to standard error.

Table 2. Summary of fermentation parameters for different fermented broths

Time (h)	Parameter(s)	Soya	SIW	NHP	THP	NHM	THM
36	Viscosity (mPa.s)	2.7	2.21	194	128	35	11
	D_{50} (μm)	17.53	2.02	76.8	47.8	9.8	4.36
	Tx (10^{12} SBU/m^3)	8.78 (36.6)	15.87 (19.8)	4.7 (16.2)	5 (15.4)	12.6 (35)	14.8 (28.9)
	TC (CFU/ml)	2.60E+08	1.65E+09	3.70E+07	3.90E+07	3.95E+08	5.65E+08
	VS (CFU/ml)	2.40E+08	8.0 E+08	2.90E+07	3.25E+07	3.60E+08	5.10E+08
48	Viscosity (mPa.s)	2.8	2.4	156	62	37	9
	D_{50} (μm)	34.8	2.53	47.9	27.8	42.2	9.2
	Tx (10^{12} SBU/m^3)	9.54 (39.3)	16.5 (20.24)	5.7 (19.6)	5.6 (16.7)	14.8 (40)	16.6 (32.2)
	TC (CFU/ml)	2.70E+08	1.67E+09	3.80E+07	4.00E+07	3.90E+08	5.60E+08
	VS (CFU/ml)	2.43E+08	8.15E+08	2.90E+07	3.35E+07	3.70E+08	5.15E+08
Oxygen consumed (mol/m^3) †		52.34	256.4	34.92	43.92	67.68	156.13
Maximum specific growth rate†† (μ_{max},h^{-1})		0.37	0.34	0.13	0.15	0.21	0.30
DS_{0h} (kg/m^3)		-	-	23.9	10.2	2.5	10.7
$SCOD_{0h}$ (kg/m^3)		-	-	10.14	13.6	5.75	7.6
% DS utilized †††		-	-	18.7	17.5	30.8	29.5
% SCOD utilized †††		-	-	22.6	22.1	38.5	39.9

† Oxygen consumed was calculated based on total area under the OUR curve

†† Calculated from the straight line slope in the exponential phase

Digits in parentheses indicate entomotoxicity, SBU/10^3 spores

††† Determined by using the formula: (DS or SCOD) $_{0h}$ – (DS or SCOD) $_{48h}$ / (DS or SCOD) $_{0h}$

Table 3. Rheological parameters of fermented broths (at 48h)

Rheological Models	Model Components	Media					
		NHP	THP	NHM	THM	SIW	Soya
Bingham Law							
$\tau = \tau_o + \eta D$	η(mPa.s)	48.9	67.9	12.1	21.9	-	-
	$\tau_o(10^{-1}$ N/m$^2)$	6.93	7.28	7.83	7.77	-	-
	Confidence of fit (%)	78.8	69.4	65.1	46.2	-	-
Casson law							
$\sqrt{\tau} = \sqrt{\tau_o} + \sqrt{\eta D}$	η(mPa.s)	27.3	45.8	5.11	13	1.86	1.93
	$\tau_o(10^{-1}$ N/m$^2)$	3.37	2.65	4.96	3.67	0	0
	Confidence of fit (%)	88.7	86.6	74.4	67.1	71.8	94.2
NCA/CMA Casson							
$(1+a)\sqrt{\tau} = 2\sqrt{\tau_o} + (1+a)\sqrt{\eta D}$	η(mPa.s)	27.3	45.8	5.11	13	1.86	1.93
	$\tau_o(10^{-1}$ N/m$^2)$	1.9	1.49	2.8	2	0	0
	Confidence of fit (%)	88.7	86.6	74.4	67.1	71.8	94.2
Power law							
$\tau = K D^n$	K(mPa.s^n)	583.3	515.3	490.7	388.3	1.46	2.5
	n	0.38	0.5	0.27	0.41	1.04	0.95
	Confidence of fit (%)	84.8	88.7	84.8	82.3	91.6	95
IPC Paste							
$\eta_{10} = K R_s^n$	n_s	307.6	306.9	232.2	211.9	1.6	2.21
	η_{10}	0.62	0.5	0.73	0.59	0.04	0.05
	Confidence of fit (%)	84.8	88.7	84.8	82.3	91.6	95

Shaded cells represent poor fit of the rheological models

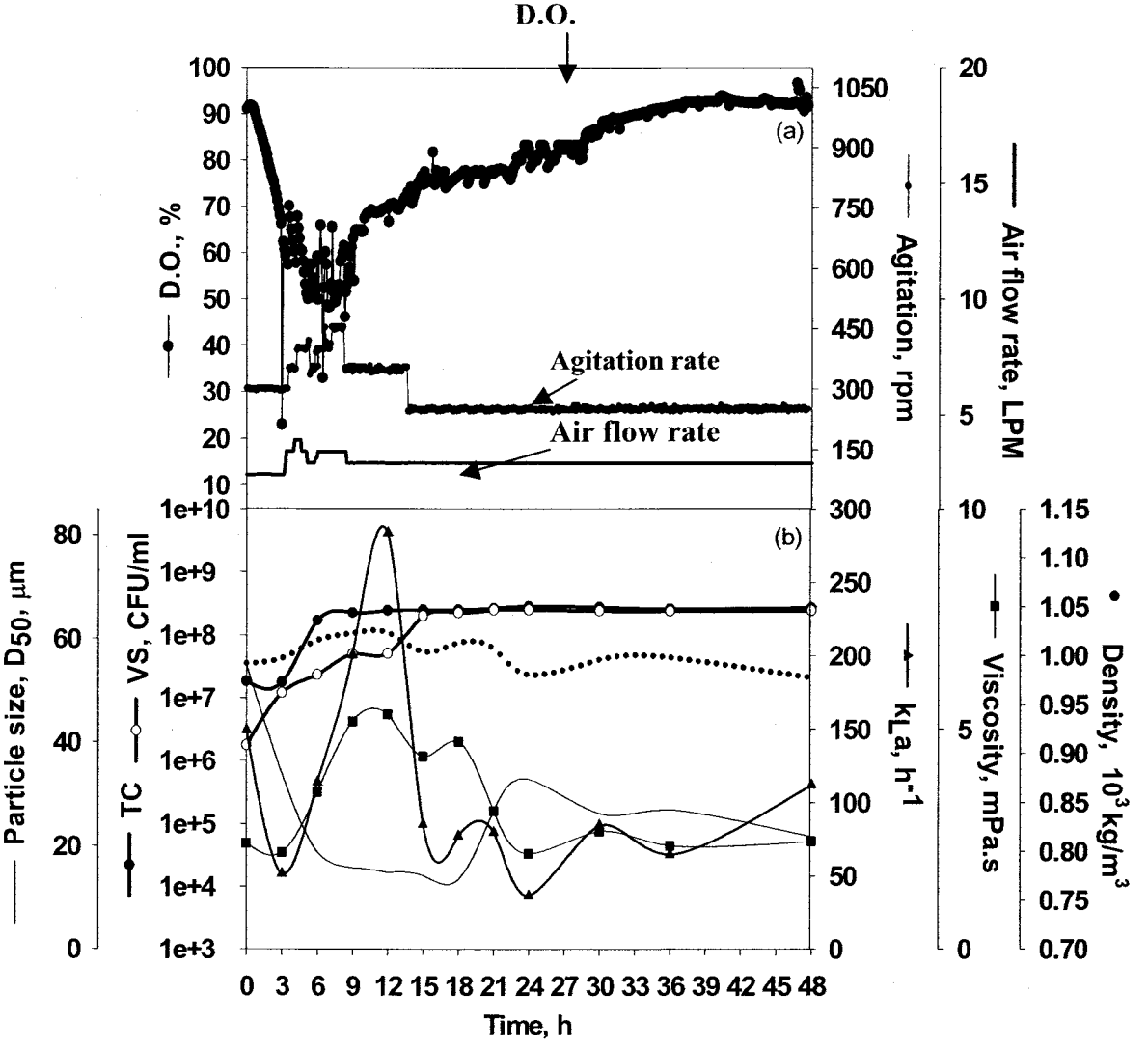


Figure 1. Bt growth (0.015 m^3 fermenter) profile in soya medium; a) process parameters, and b) performance parameters.

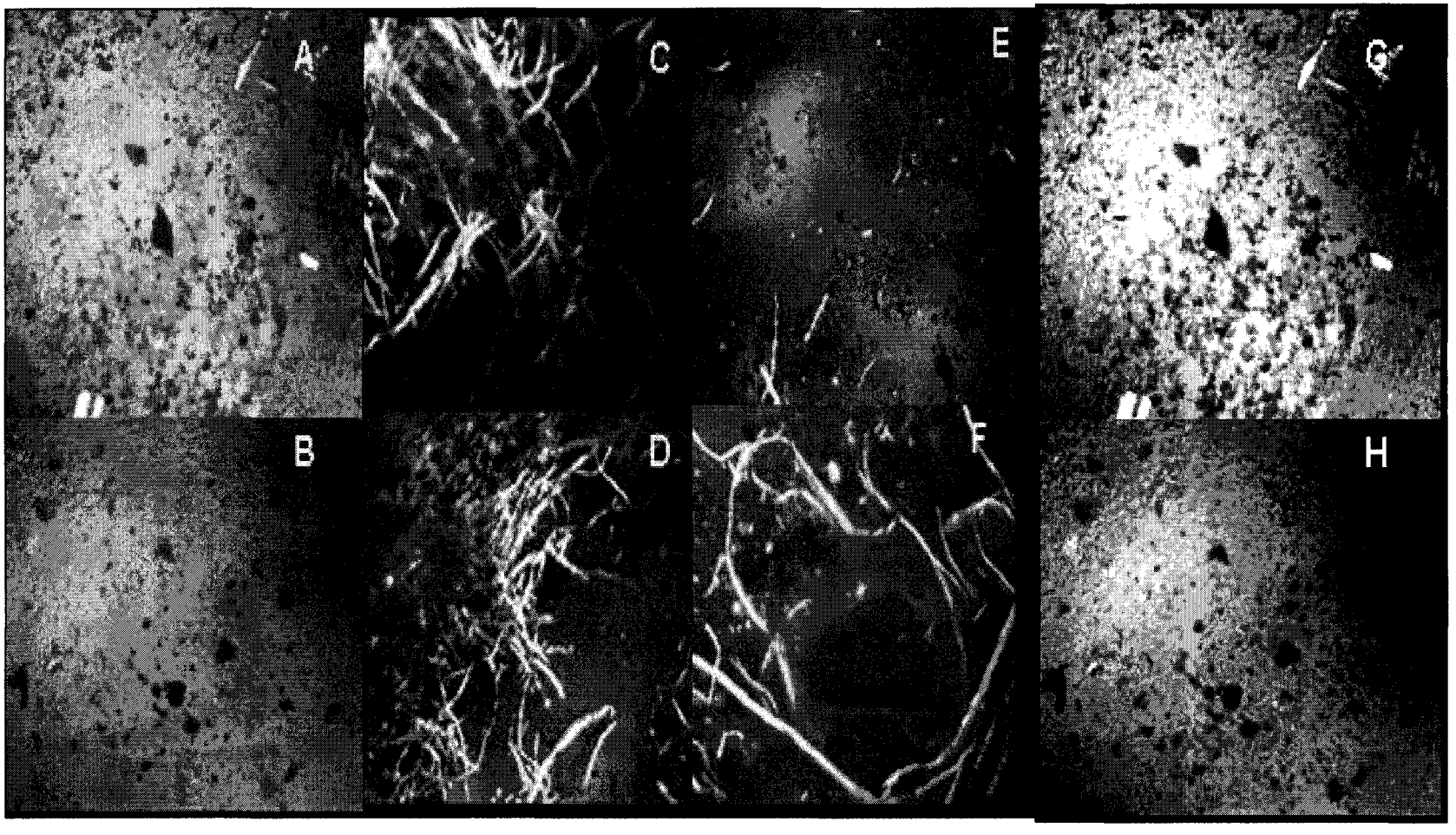


Figure 2. Morphology of different media under 40X magnification; soyameal: A after inoculation (0h) & B after fermentation (48h); primary sludge: C- after inoculation (0h) &D- after fermentation (48h); mixed sludge: E- after inoculation (0h) &F- after fermentation (48h); SIW: G- after inoculation (0h) &H – after fermentation (48h).

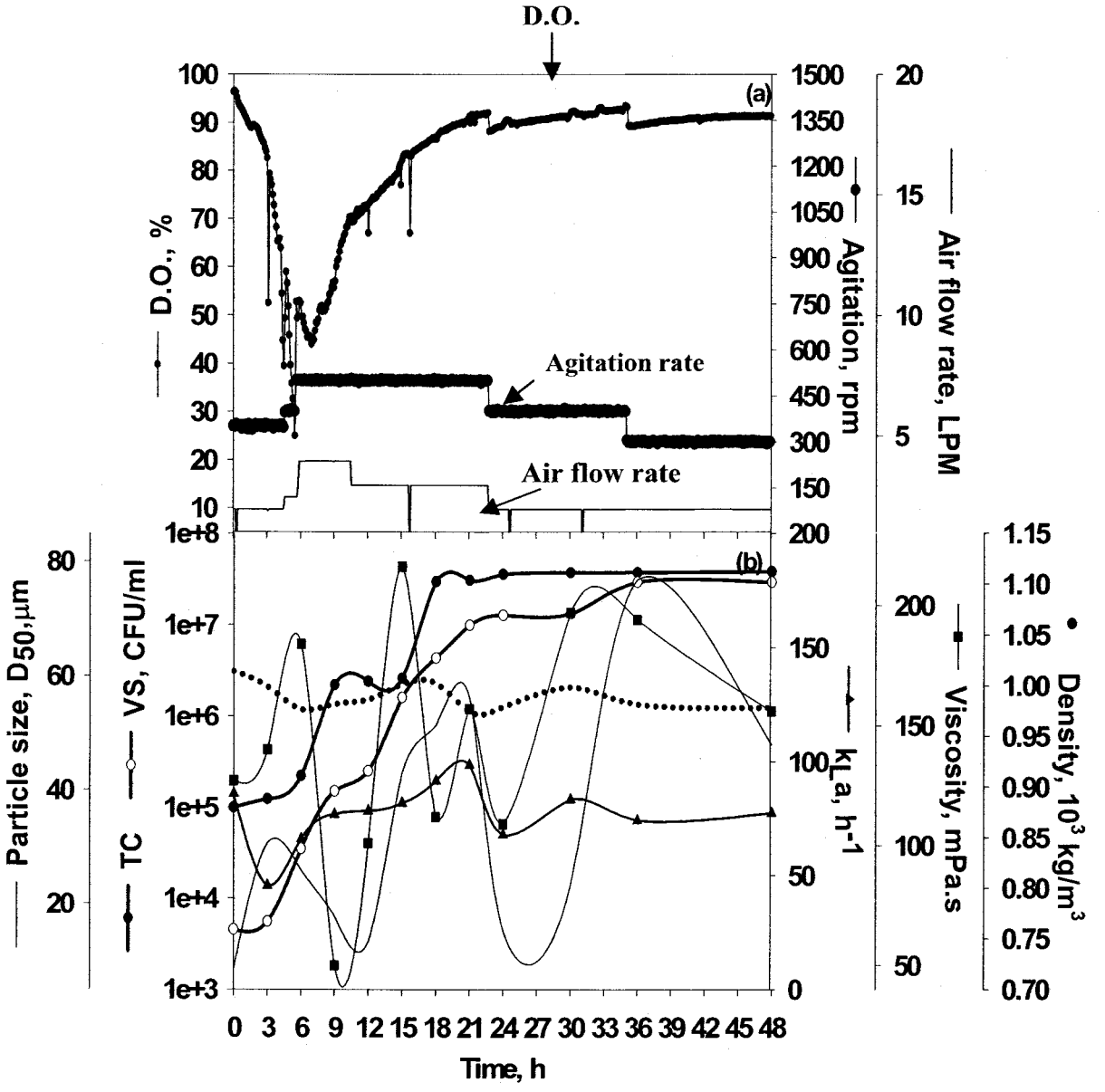


Figure 3. Bt growth (0.015 m^3 fermenter) profile in raw primary sludge; a) process parameters, and b) performance parameters.

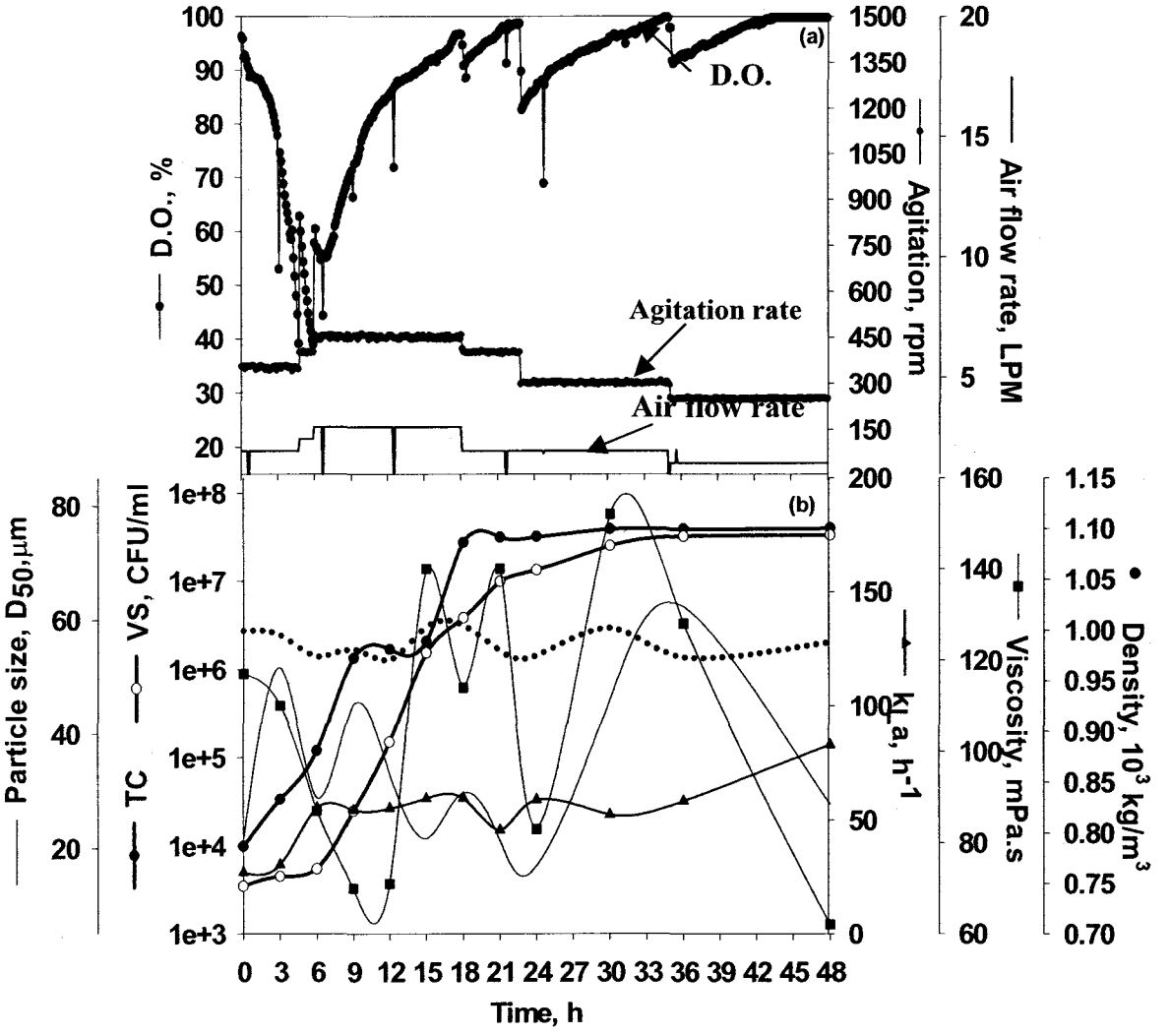


Figure 4. Bt growth (0.015 m^3fermenter) profile in hydrolyzed primary sludge; a) process parameters, and b) performance parameters.

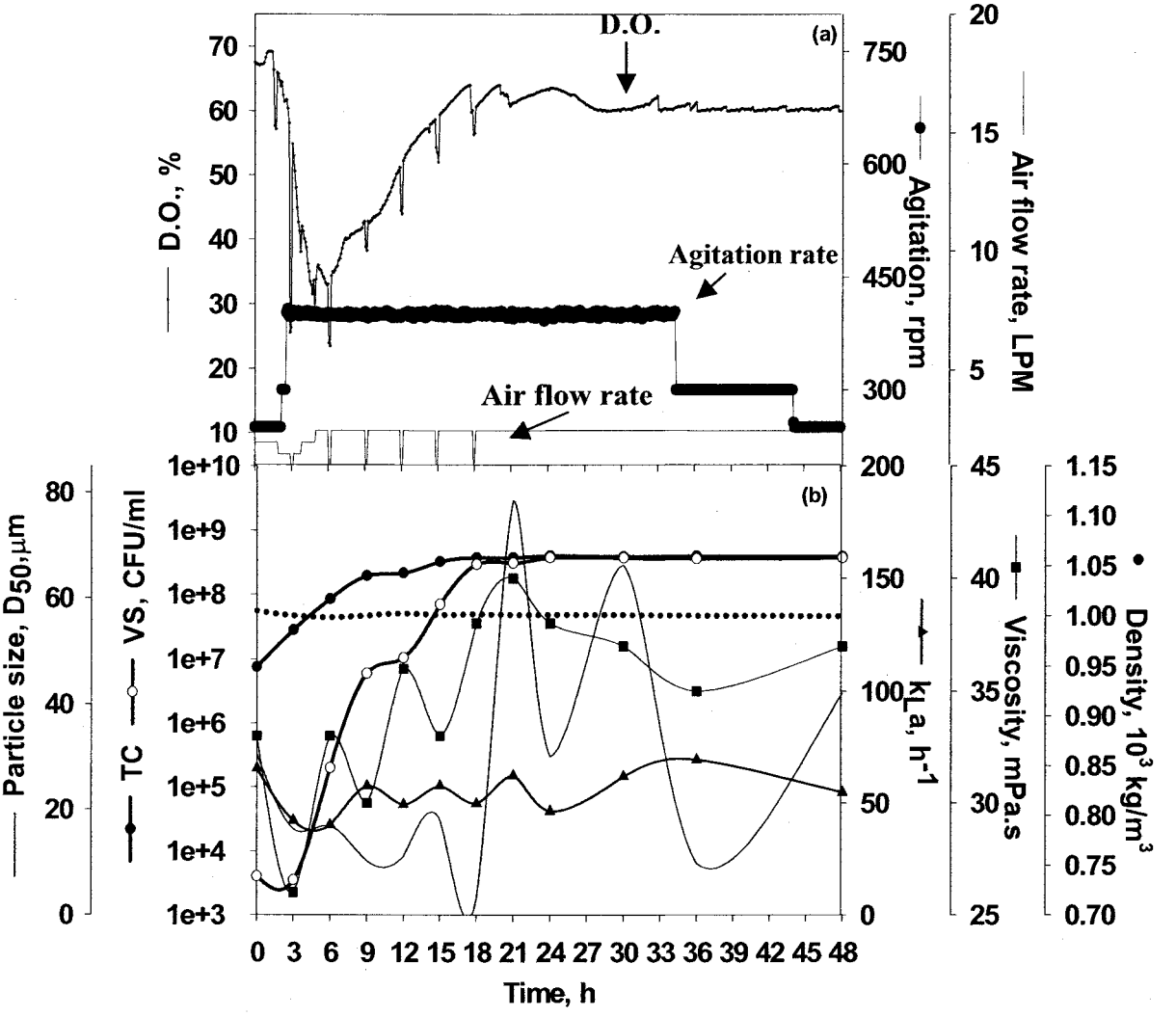


Figure 5. Bt growth (0.015 m^3 fermenter) profile in raw mixed sludge; a) process parameters, and b) performance parameters.

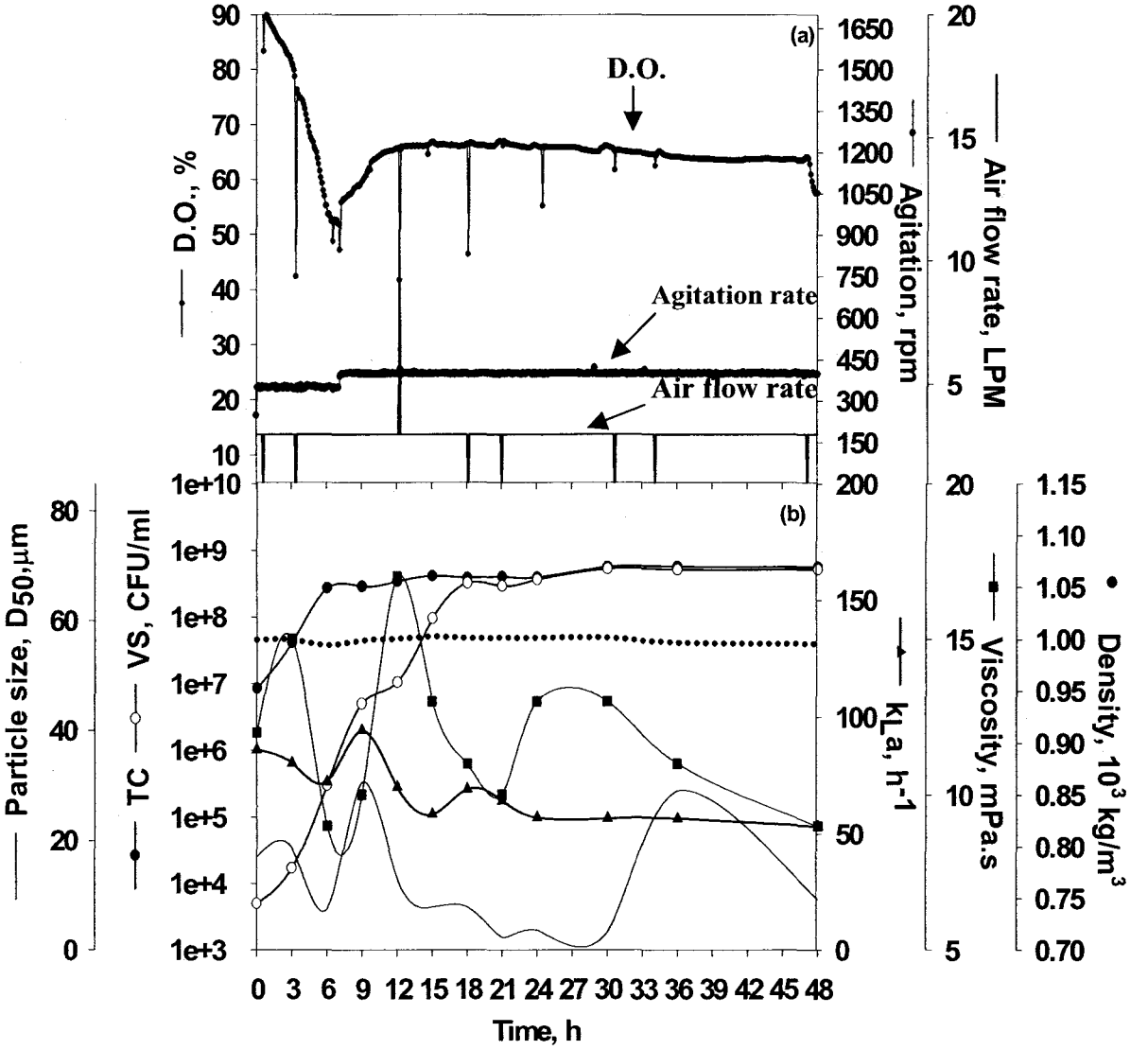


Figure 6. Bt growth (0.015 m^3 fermenter) profile in hydrolyzed mixed sludge; a) process parameters, and b) performance parameters.

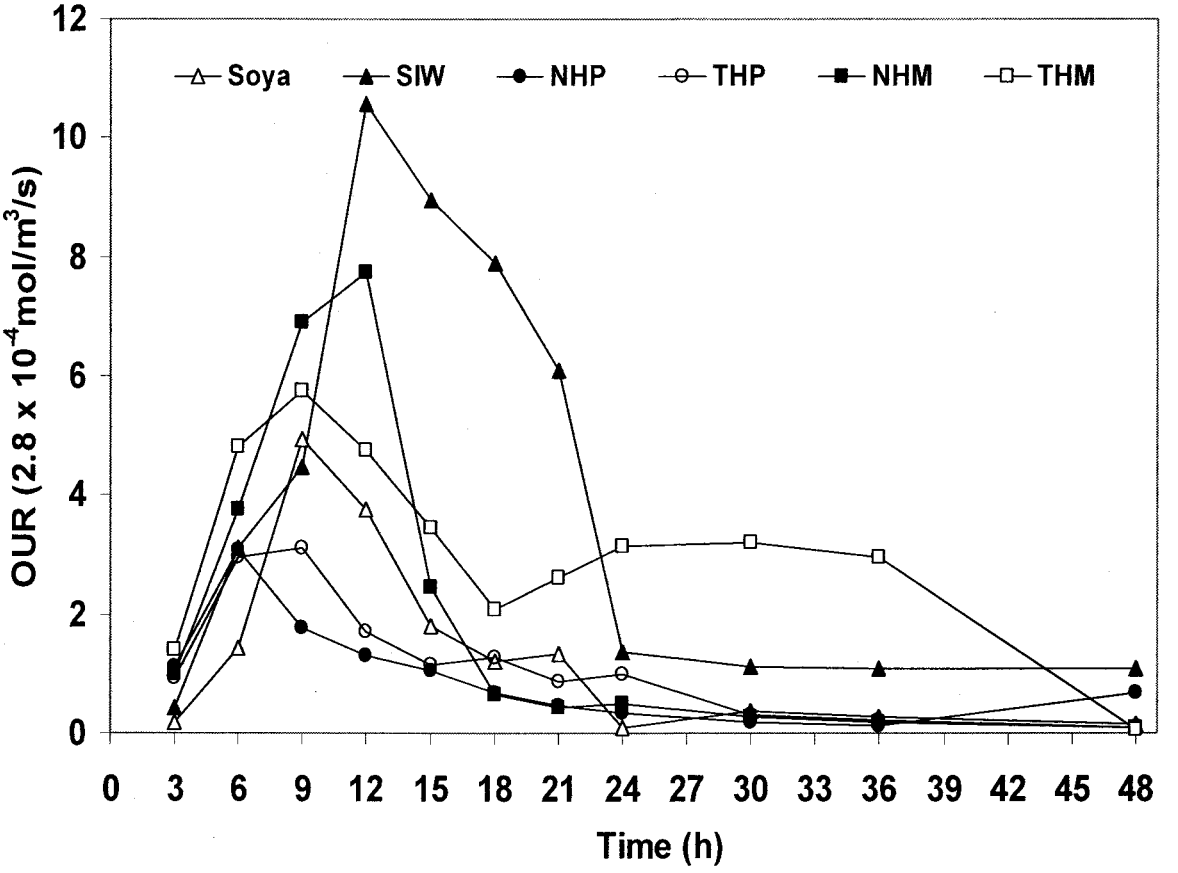


Figure 7. OUR profiles of different fermented media.

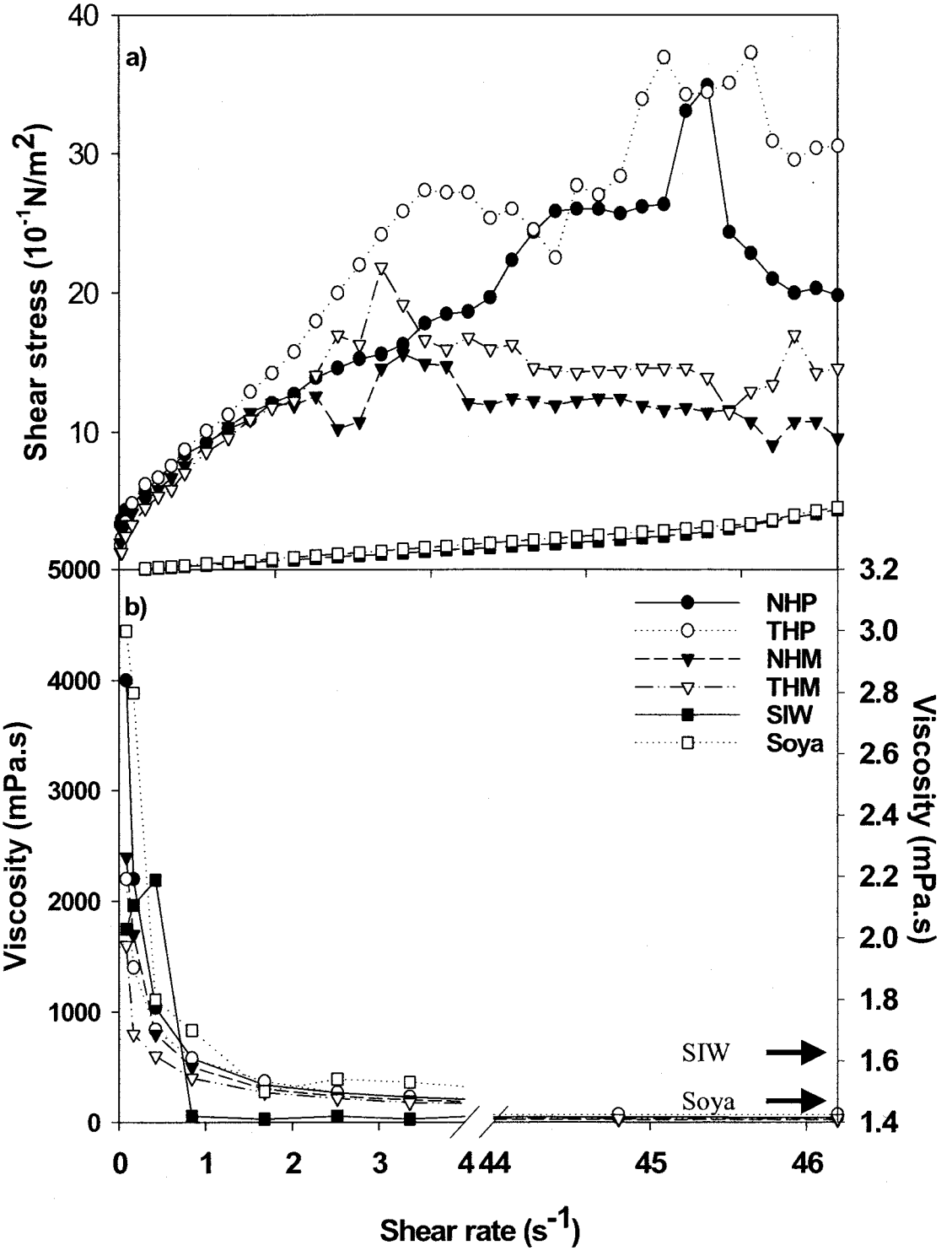


Figure 8. Rheograms and shear rate profile of different fermented broths (at 48h).

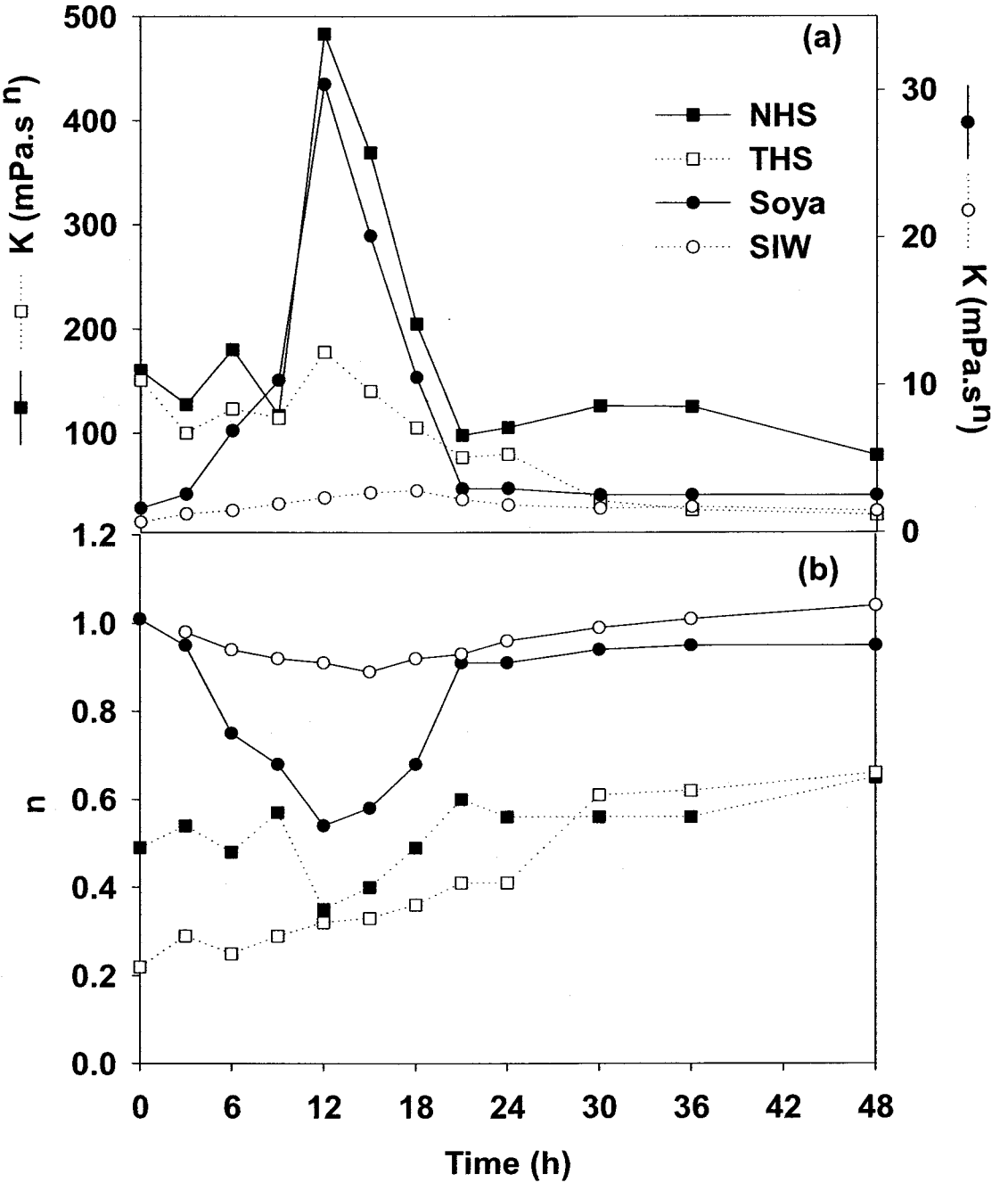


Figure 9. Consistency index (K) and flow behaviour index (n) profiles soya, secondary sludge (non-hydrolyzed, NHS); secondary sludge (hydrolyzed, THS) and starch industry wastewater fermentation.

CHAPITRE 4.

AMÉLIORATION PHYSIQUE DES BOUES D'ÉPURATION FERMENTÉES PAR Bt

Partie I

Impact of Tween 80 during *Bacillus thuringiensis* Fermentation of Wastewater Sludges

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*], J.R. Valéro[1] and R.Y. Surampalli[2]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Process Biochemistry (2005)
40: 2695-2705

Impact du Tween-80 pendant la fermentation des boues d'épuration par *Bacillus thuringiensis*

Résumé

L'effet de l'agent tensio-actif Tween 80 (0,2%, v/v) sur la production des biopesticides à base de *Bacillus thuringiensis* (Bt) en employant les boues secondaires {non-hydrolysées (NH) et hydrolysées (TH))} comme substrat de fermentation a été étudié en bioréacteurs. Les milieux contenant les boues hydrolysées ont permis d'accroître de 49 % l'entomotoxicité (Tx) de Bt alors que le nombre de cellules viables et celui des spores viables augmentent aussi par rapport à ce qui est constaté avec les boues non- hydrolysées. L'addition de Tween 80 dans les boues non hydrolysées permet d'augmenter les nombres de cellules et des spores viables de 1,67 et 4 fois respectivement. Ceci a aussi permis d'accroître le taux de croissance spécifique maximum (μ_{max}) de 0,19 à 0,24 h^{-1} et la Tx de 26,6%. Cependant, l'addition du Tween 80 aux boues hydrolysées accroît le nombres de cellules et de spores viables de 2 et 2,4 fois respectivement et le μ_{max} de 0,28 à 0,3 h^{-1} alors qu'aucun changement de Tx n'est pas noté. Le coefficient volumétrique de transfert de masse a changé de manière significative durant la fermentation alors que le taux de consommation d'oxygène (OUR) a augmenté de 5 et 3,5 fois en présence de Tween 80 dans les boues non hydrolysées et dans les boues hydrolysées, respectivement. Des variations de viscosité au cours de la fermentation ont été également observées. Ceci est dû aux effets concertés de l'augmentation du nombre de cellules et de spores viables, de la lyse de cellules, de l'agitation, de l'aération et de l'addition de substance anti-mousse. La taille des particules a diminué nettement dans tous les cas à la fin de la fermentation.

Mots-clés: *Bacillus thuringiensis*, bioréacteur, taille de particule, Tween-80, viscosité, boues d'épuration

Abstract

Effect of a surface active agent, Tween 80 (0.2 % v/v) on the changes of rheology during production of *Bacillus thuringiensis* based biopesticides using secondary wastewater sludge (non-hydrolyzed and hydrolyzed) as a raw material was studied in 15L bioreactors. Amending the non-hydrolyzed sludge with Tween 80 resulted in increase in viable cell and spore count by 1.67 and 4 times, respectively, and the maximum specific growth rate (μ_{max}) increased from 0.19 to 0.27 h^{-1}. Addition of Tween 80 to hydrolyzed sludge increased viable cell and spore count by 2 and 2.4 folds, respectively and μ_{max} increased from 0.26 to 0.3 h^{-1}. Entomotoxicity of non-hydrolyzed - Tween 80 amended sludge was 26.5 % higher than the unamended sludges, whereas hydrolyzed sludge showed no change. There were variations in viscosity during fermentation due to concerted effects of increase in viable cell and spores, cell lysis, agitation, aeration and anti-foam addition. At the end of fermentation, the viscosity decreased substantially in non-hydrolyzed sludge and reduced slightly for hydrolyzed sludge with and without Tween 80. However, viscosity was markedly reduced in the case of Tween 80 amended non-hydrolyzed sludge to a value at par with hydrolyzed sludge. The particle size decreased markedly in all cases at the end of fermentation. Overall, the volumetric mass transfer coefficient varied significantly during fermentation and oxygen uptake rate was increased by 5 and 3.5 times for Tween 80 fortified non-hydrolyzed and hydrolyzed sludge, respectively.

Keywords: *Bacillus thuringiensis*, bioreactor, fermentation, hydrolyzed, non-hydrolyzed, particle size, Tween 80, viscosity, wastewater sludges

INTRODUCTION

A microbial fermentation can be viewed as a three-phase system, involving liquid-solid, gas-solid and gas-liquid reactions. The liquid phase comprises dissolved nutrients, substrates and metabolites. The solid phase consists of individual cells, pellets, insoluble substrates, or precipitated metabolic products. The gaseous phase provides a reservoir for oxygen supply, CO_2 and other waste gases removal.

The transfer of energy, substrate and metabolite within the bioreactor are very crucial to the efficiency of the whole fermentation process. Viscosity is the most significant rheological property affecting the flow behavior of a fluid which will have a cascading impact on mixing, heat transfer, mass transfer and aeration (Parakulsuksatid, 2000; Juarez and Orejas, 2001). Especially in the case of sludge, non-Newtonian behaviour projected by viscosity and particle size can have tremendous effect on the rheology of *Bacillus thuringiensis* (Bt) fermented sludge (Brar et al., 2004 a,b). Also, wastewater sludge, in general, tends to form flocs. There is a possibility of the Bt cells getting entrenched in the sludge flocs in order to nourish from the bulk nutrition in the solid phase (Tirado-Montiel, 2001). This will result in serious oxygen transfer problems during the process of Bt fermentation of wastewater sludges and limit the production of spores and δ- endotoxin (or biopesticidal activity), an important component of entomotoxicity (Tx) of Bt as a biopesticide (Pearson and Ward, 1988). These problems could be tackled to a certain extent by the addition of surface active agents which could help reduce the surface tension at the solid-liquid interface and enhance oxygen transfer (Williams, 2002; Vasconcelosa et al., 2003). Vidyarthi et al. (2001) have already reported an increase in Tx by 24% on addition of Tween 80 in shake flask experiments. Other studies have reported the use of Tween 80 as an additive to enhance substrate assimilation during Bt fermentation in shake flask experiments (Zouari et al., 1998; Zouari and Jaoua, 1999) and also in δ-endotoxin purification and characterization studies (Maagd et al., 1999; Liu and Sengonca, 2003). There also has been extensive use of Tween 80 as wetting and suspending agent in formulations of biopesticides and also as spray mixes to improve spreading on foliage (Lescota et al., 2002; Arunsiri et al., 2003).

As deduced from several studies, Tween 80 played a dual role of increasing entomotoxicity and improving wettability and other characteristics of Bt formulations. This stimulated the possibility of combining the two actions together (i.e. enhancement of Tx value during fermentation and improving characteristics during formulation) and to verify the physical (viscosity and particle size) and Tx changes taking place during fermentation.

Therefore, this study was conducted to evaluate the effect of Tween 80 on the process parameters during Bt fermentation of wastewater sludge in the bioreactor.

MATERIALS AND METHODS

Sludge

Secondary sludge (CUQS) used in the study was obtained from CUQ (Communauté urbaine de Québec) wastewater treatment plant, Ste - Foy. The sludge was concentrated from 1.5 % to about 3.5 % (w/v) solids gradually by gravity settling and centrifugation at 7650 g for 15 minutes in a Sorvall RC 5C plus Macrocentrifuge (rotor SA-600). The characteristics of sludge used are given in Table I. Metal concentrations in sludge were found below the recommended level of guidelines of Ministère de l'Environnement de Québec, for agricultural application (Charbonneau et al., 2000). The sludge supernatant was stored in the refrigerator at 4°C and used to dilute the sludge samples as per requirements. Concentrated sludge (raw) was then homogenized using a Waring blender (3L) and mixed with the supernatant to obtain 2.5 and 5.0 % of solids concentration.

After drawing samples for all physico-chemical characterization tests, pH was adjusted to 7.0 ± 0.1 by addition of NaOH. Two types of sludges were used in the experiments – non-hydrolyzed (NH) and hydrolyzed (TH). For the sludge to be hydrolyzed, solids concentration was adjusted to 5 % v/v, taking into account 1.67 times dilution by steam, it was then transferred into a custom made mechanical steam hydrolyzer (Stainless steel (SS 316L) with pure steam injection facility; working volume: 10 liters with controlled agitation; EBR Quebec, Canada). The sludge was hydrolyzed at 140°C for 30 minutes at a pressure of 40 psig. After hydrolysis, the sludge was transferred to 15L fermenter with 10L working volume, whereas, raw sludge was transferred directly into another fermenter (15L).

Bacterial Strain

Bacillus thuringiensis var. *kurstaki* HD-1 (ATCC 33679) was used in this study. The culture conditions, maintenance, inoculum production and fermentation procedure (biopesticide production) are described elsewhere (Vidyarthi et al., 2002).

Bioreactor

Fermentation was carried out in a stirred tank bioreactor equipped with accessories and automatic control systems for dissolved oxygen, pH, antifoam, impeller speed, aeration rate

and temperature. Dimensions of the fermenter are summarized in Table II. The software (Fix 5.5, Intellution, USA) allowed automatic set-point control and integration of all stated parameters.

Initially, polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR-Canada). The oxygen probe was calibrated to zero (using N_2 degassed water) and 100 % (air saturated water). Subsequently, fermenter was charged with sludge, polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution as an anti-foam agent and 0.2% v/v Tween 80 (Poly(oxyethylene)(20)-sorbitane mono-oleate) (Difco Lab., Michigan, USA) as a surface active agent as reported in Leblanc (2003). Experiments were conducted on NH and TH sludges with or without addition of Tween 80. In-situ sterilization was carried out at 121°C for 30 minutes. When the fermenter cooled down to 30°C, the dissolved oxygen (DO) probe was recalibrated to zero by sparging N_2 gas and 100% saturation by sparging air at agitation rate of 500 rpm. The fermenter was then inoculated (2 % v/v inoculum) with acclimated pre-culture of Bt in exponential phase. In order to keep the DO above 25 % saturation, air flow rate and agitation rate were varied between 0.133-0.233 vvm and 250–700 rpm respectively. This ensured the critical DO level for Bt (Avignone-Rossa et al., 1992). The temperature was maintained at 30 ± 1°C by circulating water through the fermenter jacket. The pH was controlled at 7.0 ± 0.1 using either 4N NaOH or 4N H_2SO_4 by peristaltic pumps. Foaming during fermentation was also simultaneously controlled by PPG injection and mechanical foam disruptor (Fundafoam^{TM}).

Volumetric oxygen transfer coefficient (K_La), Oxygen Uptake Rate (OUR) and Oxygen Transfer Rate (OTR) measurements

Oxygen uptake rate was measured by conventional technique utilising "air off and on" mechanism. It is based on the dynamic method as described in Aiba et al. (1973) and k_La was measured at each sampling point.

Broth oxygen concentration was converted from % air saturation to mmol O_2/L as follows: DO electrode was calibrated in medium at 30 ± 0.1 °C and known barometric pressure and then transferred to air-saturated distilled water at known temperature and ambient pressure. This reading was used, with the known saturation concentration of oxygen in distilled water (APHA, 1995), to estimate the saturation concentration of oxygen in the fermentation medium at 30 ± 0.1°C.

Analytical details

The entomotoxicity (Tx) was determined against western spruce budworm larvae (*Choristoneura occidentalis*) using diet incorporation method (Dulmage et al., 1971). The standard deviation for Tx measurement was 9–10%. Total cell (TC) and spore (VS) concentration was determined as reported in Vidyarthi et al., 2002. The standard deviation for VC and VS measurement was 7–8%. Viscosity of sludge was determined by using a rotational viscometer (Cole-Palmer Inc.) at 30 rpm (adapted for the entire study of biopesticide production) and particle size analysis was carried out using Fritsch Laser particle sizer analyzette 22 which is based on LASER diffraction principle. During entire analysis, ultrasonic function was switched off to avoid breakage of sludge particles. The stirrer speed and recirculation pump speed were kept moderate at 250 rpm and 500 respectively to minimise the breakage/damage of sludge particles. D_{50} was chosen as the notation for average particle size as it showed the volume median diameter and this could be easily correlated with the formulation development in the downstream process and hence will serve as a better parameter of comparison vis-à-vis D32, surface area moment mean which will affect only the fermentation parameters in these studies. D_{50} was analogous to particle size expressed as diameter 50% distribution cut-off point. Standard deviation for viscosity and particle size was calculated to be 10 % based on 10 replicates.

Surface tension (ST) measurements were carried out using Sigma 70 tensiometer (KSV Instruments Ltd., Finland) by du Nouy ring method at 30°C and a cylindrical vessel of 46 mm diameter holding total volume of fermented sludge upto 30 ml was used as a sample vessel. Water was used as the reference having ST of 71.18 mN/m at 30 ± 0.1 °C.

RESULTS AND DISCUSSION

NH sludge without Tween 80

The variation of fermenter operational parameters (agitation rate, air flow rate and DO concentration) and process performance parameters (TC and VS counts, viscosity, particle size and density) for Bt grown in NH sludge without addition of Tween 80 are shown in Figures 1 a and 1b, respectively. DO levels varied from 25-75 % with the agitation rate ranging from 375 - 640 rpm and air flow rate ranging from 2.5 to 9.7 LPM (Figure 1a). There were fluctuations in all parameters in the first 9h of sludge fermentation when Bt was in active exponential phase and DO was rapidly depleted to sustain the energy metabolism. Similar trends were obtained for fermentation of sludge in bench scale fermenters by Yezza et al. (2004). After 9h, DO stabilised with minor perturbations, more so, in the stationary

phase when DO utilization was minimum due to slowing down of Bt metabolism. These results are in total agreement with the studies of Wu et al. (2002) utilizing soya meal for growth of Bt to produce thuringiensin.

TC increased from 1 x 10^6 to 6.6 x 10^8 CFU/ml until 21 h and then remained almost constant with the average concentration at 4.3 x 10^8 CFU/ml (Figure 1b). Similarly, VS count increased from 4.3 x 10^3 to 2.7 x 10^8 CFU/ml upto 21h and attained constancy at 24 h (3.3 x 10^8 CFU/ml). Tx values for all sludges at 36 and 48h are well illustrated in Table III.

The viscosity drastically decreased in the initial stages from 57 to 24 cP until 15h and further decreased slowly reaching a final value of 24 cP at 48h. A decrease until 9h was due to impeller action and hence the sludge flocs kept on breaking (active exponential phase of Bt growth seen in Figure 1b). Further, between 9-18h, the value slightly increased possibly due to various metabolites produced during exponential growth phase. Also, with the increase in time and shear rate (agitation speed), Bt fermented secondary sludge has been shown to behave as thixotropic and pseudoplastic whereby the viscosity tends to decrease (Brar et al., 2004c). Thus, controlled pH and DO conditions in the fermenter completely changed the process profile in sharp contrast to shake flask studies (Brar et al., 2004a) where viscosity increased during fermentation.

The particle size (D_{50}) decreased sharply until 9h from 24.3 to 11.6 μm and further a gradual decrease to 1.7 μm at 48h was observed. This decrease until the end of fermentation (Figure 1) could be attributed to continuous agitation (Figures 1-4). This was in sharp contrast to our previous observations in shake flask experiments where particle size increased during growth of Bt (Brar et al., 2004b).

Density almost remained constant with values ranging from 0.916 to 0.98 g/ml. Small variations in density values were a function of irregularities in sludge behaviour.

The volumetric oxygen transfer coefficient (k_La) varied from 23 to 141 h^{-1} with an increase from 0 to 3h, drastic decrease between 3 and 6h and then an increase until 15h, eventually attaining constancy until 48h. k_La decrease between 3- 6h (Figure 1b) was due to constant aeration and agitation conditions which resulted in lowering of DO of the system, as oxygen supply could not keep pace with oxygen consumption, resulting in inverse relation of k_La to OUR. Eventually, k_La started increasing as the aeration and agitation were varied to sustain DO values. In general, it is well known that k_La is dependent on many factors, including viscosity, presence of suspended solids, presence of surfactants, and ionic strength of the liquid phase (Kawase and Hashimoto, 1996).

TH Sludge without Tween 80

Figures 2a and 2b show the operational and performance parameters respectively of TH sludge fermentation. The DO varied from 25-95 %; agitation rate from 450-550 rpm and air flow remained constant at 2 LPM. As seen from the DO profiles, initial DO in TH sludge (Figure 2a) was 1.3 times higher compared to NH sludge (Figure 1a) due to lower viscosity and particle size (discussed later). The air flow rate remained constant and only agitation rate was varied to maintain DO levels. Better oxygen transfer due to low viscosity has also been reported for *Bacillus subtilis* fermentation utilising synthetic medium (Richard and Margaritis, 2003).

TC and VS increased gradually from 7.5 x 10^6 to 4.0 x 10^8 and 3.33.x 10^3 to 2.95 x 10^8 CFU/ml respectively, until 15h. Afterwards, TC and VS counts remained constant at 3.99 – 4.37 x 10^8 and 3.6 to 3.93 x 10^8 CFU/ml, respectively. TH sludge largely comprised biodegradable nutrients which resulted in higher TC and VC counts, higher μ_{max} ($0.26h^{-1}$) and Tx values as compared to NH sludge (Table III). Similar observations for μ_{max} and Tx were also made by Tirado-Montiel et al. (2001) in shake flask experiments. Higher consumption rate of nutrients led to faster appearance of stationary phase in TH sludge, and this was in accordance with previous findings in shake flask studies (Sachdeva et al., 1999).

The viscosity remained consistent from 3.6 - 3.74 cP until 6h and then kept on increasing until 12h (6.15 cP) and further decreased upto 48h to reach a value of 4.15 cP. The viscosities followed a slightly different profile than NH sludge, with a small decline in the initial 0-6h and then an upward hump at 12h and further a continuous decrease upto 48h. It was clearly established that sludge thinning during hydrolysis (viscosity reduction) was so high that despite substantial increase in cell concentration (0 to 6h), it did not affect viscosity. Although the cell concentration reached its maximum at 6h, the viscosity peak was observed at 12h probably due to late production of EPS and release of intracellular material and/or cell debris by dissociation of cells to produce spores. This was in agreement with studies carried out on *Bacillus licheniformis* fermentation (Sutherland, 1982).

D_{50} decreased gradually from 13.2 to 3.98 μm during the fermentation period. Although particle size in TH sludge followed the same profile as NH sludge, but exhibited lower magnitude, as the physical state of the fermentation medium was dissimilar. Although after hydrolysis, the particle size decreased (from 49 to 26.45 μm) at 25g/L owing to disruption of bigger flocs as reported in shake flask studies (Brar et al., 2004b). But, after sterilization due to coagulation of proteins, the particle size increased to 59 μm. These reconstituted particles

when subjected to agitation during fermentation, started breaking up resulting in decrease. Therefore, the particle size profile was linked more to the initial flocs present and possible increase due to Bt growth was not clearly marked.

Density showed not much change and ranged from 0.953 to 1.037 g/ml (Figure 2b). The density, despite, remaining constant, was higher than NH and could be due to higher concentration of dissolved solids as reported in earlier studies (Brar et al., 2004a).

k_La showed variable profile ranging from 249.2 to 11.9 h^{-1} (Figure 2b) during fermentation. Initial high value of 250 h^{-1} was observed suggesting efficient oxygen transfer due to very low initial viscosity (hydrolysis effect). Low viscosity has been found to increase effective gas-solid interfacial area and hence increase the oxygen transfer (Parakulsuksatid, 2000). The value of k_La kept on decreasing until 12h monotonically and then assumed a slower upward profile upto 15h and then almost levelled off. There could not be a proper correlation drawn with viscosity until first 6h, but later the increase in viscosity contributed towards decrease in k_La by offering oxygen transfer resistance. These results were in direct concordance with the microbial production of polysaccharides where the increase in viscosity decreased k_La by decreasing gas-liquid specific surface area (Li et al., 1995).

NH sludge with Tween 80

The variation of operational and performance variables during fermentation of NH sludge (with Tween 80) are illustrated in Figures 3a and 3b, respectively. DO ranged from 35 to 97 %; agitation rate from 300 to 600 rpm and air flow rate ranged from 0.2-2.5 LPM (Figure 3a). DO was relatively higher than NH sludge without Tween 80 as seen from Figure 1a and it required no substantial adjustments of agitation and air flow rate. This could be due to diminution of interfacial tension of liquid-liquid or liquid-solid phases (Jordan et al., 1999). The density change was minimal from 0.997 to 1.050 g/ml.

TC and VS concentrations ranged from 6.2 x 10^6 to1.1 x 10^9 and 1.2 x 10^4 to 1.1 x 10^9 CFU/ml respectively during fermentation as shown in Figure 3b with corresponding Tx values as seen in Table III. TC and VS concentrations were higher compared to NH sludge without Tween 80 which contributed to 26.5 % increase in Tx (Table III). The difference in Tx values at 36 and 48 h could be due to the fact that onset of sporulation and high spore yields is not necessarily responsible for significant production of crystal proteins which are an important control parameter of entomotoxicity (Rossa and Mignone, 1993; Paramatha, 2000). In addition, the protein crystals cannot be necessarily traced at the beginning of synthesis stage due to their relatively low concentration. Decreased ST (47 to 36 mN/m), due

to addition of Tween 80, could have also aided in proper dispersion of fermented sludge broth into the diet during bioassay ("Diet incorporation method", Dulmage et al., 1971) and hence augmented Tx.

Thus, Tween 80 played a dominant role in the improvement of process characteristics. Sludge initially contained compact flocs which probably expanded in presence of Tween 80 resulting in increase in effective surface area. The enhanced exposure of expanded flocs to heat transfer (during sterilization) resulted in breakage of flocs and hence reduced viscosity. This consequently increased oxygen transfer and enhanced availability of nutrients for Bt during fermentation.

Viscosity decreased until 3h of fermentation with an increase upto 9h (Figure 3b) and then a decrease until 18h from 12 to 6.2 cP and later levelling off to a constant value. Viscosity of NH sludge at t = 0h was almost reduced by 75% due to addition of Tween 80. The viscosity hump that appeared at 9h was due to increase in cells and probable formation of metabolites and EPS and later the values decreased owing to prolonged physical action (agitation) which started outweighing the cell growth (explained later). A similar trend has been reported for polysaccharide production in *A.pullulans* fermentation (Li et al., 1995).

D_{50} decreased upto 21 h from 257 to 11.6 μm and later remained constant. In general, similar particle size profile was observed, for all cases, due to reasons explained earlier. k_La varied from 72 to 80 h^{-1} during fermentation. It was observed that k_La (Figure 3b), started decreasing at 3h of fermentation, due to oxygen consumption patterns of Bt and then showed perturbations as a result of agitation variations to control the foam formed during fermentation as also observed by Vidyarthi et al (2000).

In addition, Tween 80 also exerts a thermodynamic influence on the system. In an attempt to reduce the overall free energy of the system, Tween 80 molecules interact with one another, forming a lipid bilayer. Such behavior allowed Tween 80 to establish a hydrophobic environment within the bulk water phase, rearranging water molecules, and providing more oxygen solubility and a reduction in overall free energy of the system. This was clearly reflected in this study where the viscosity in NH sludge with Tween 80 saddled as compared to without Tween 80. This eventually caused an increased metabolic rate of Bt which is evident from higher OUR as discussed later. The surfactant layer of Tween 80 provided a local charge at the gas bubble surface that repelled adjacent bubbles, inhibiting coalescence and enhancing mass transfer (Worden and Bredwell, 2004). Also, the increase in small bubbles increased the interfacial area per unit volume and hence k_La.

TH Sludge with Tween 80

Figures 4a and 4b show the operational and performance parameter profiles of TH sludge with Tween 80, respectively. DO was kept above 25% with varying agitation and air flow rates from 250 to 750 and 1.5 to 2.4 LPM, respectively. From 6 to 9h of fermentation, there was excessive foaming in the fermenter and this had to be controlled by adjusting the agitation and using mechanical foam breaker. With the addition of Tween 80, TH sludge (Figure 4a) showed a similar profile as NH with Tween 80 (Figure 3a) with irregular variation in DO, caused by excessive foaming during fermentation. Tween 80 exerted higher surfactant activity coupled with foaming caused by simple proteins present in TH vis-à-vis NH sludge. Also, as viscosity of TH sludge at zero hour was already reduced, addition of Tween 80 did not affect mass transfer characteristics to a large extent. These results were in close accord with the studies conducted on water-glycerol systems in a pulsed baffled reactor (Ni et al., 1997).

ST of hydrolyzed and hydrolyzed sterilized sludges after addition of Tween 80 was 40 and 31 mN/m respectively. It did not change during fermentation implying no possible production of biosurfactants by Bt and hence all change was contributed solely to addition of Tween 80. This was confirmed by TH sludge without Tween 80 where ST remained constant throughout fermentation at 40 ± 0.5 mN/m. Density of TH sludge with Tween 80 ranged from 0.997 to 1.01 g/ml.

TC and VS ranged from 6×10^6 to 9.5×10^8 and 1.3×10^3 to 9.5×10^8 CFU/ml respectively with relatively higher concentrations (2 times) in comparison to TH without Tween 80 (Figure 4b), but no significant change in Tx (Table III) was observed. Higher TC and VS could be probably due to efficient oxygen utilization by Bt cells and improved physical changes reflected in ST and viscosity. There could be a possibility that addition of Tween 80 lowered particle-particle interfacial tension and also resulted in higher oxygen diffusion and hence higher VS and Tx values. Unfortunately, Tx values did not increase proportionate to the increase in spore concentration. This fact further needs to be investigated in terms of role of other metabolites like chitinases, proteases, phospholipases and vegetative insecticidal proteins that are responsible for an integrated Tx value (Kaur, 2000). Tween 80 could have also dampened the complicated movement of interphase (solid-liquid/liquid-liquid/liquid-gas) and enhanced oxygen transfer (Parakulsuksatid, 2000). However, for TH sludge, addition of Tween 80 does not play a significant role in improving Bt productivity as the medium has been already ameliorated.

The viscosity varied from 6 to 4cP until the end of fermentation and particle size followed a similar trend ranging from 336 to 3.4 µm during the course of fermentation.

k_La varied from 28-145 h^{-1} during the course of fermentation. k_La values were relatively lower than TH sludge (without Tween 80) and were mainly due to intense foaming throughout the fermentation. Although the addition of Tween 80 altered the surface area for mass transfer in all fermentation broths, but the beneficial effects were more apparent in NH sludge. This can help in exploring the after effects of this fermentation on formulations.

Power requirements in terms of agitation and aeration rate will be lower with Tween amended sludge as high DO could be maintained at lower agitation and aeration rates. Hence, Tween 80 amendment during fermentation could lower the overall cost too, which has to be estimated for the stated fermentation process.

Viscosity and particle size Profiles

A remarkable viscosity trend was observed in all experiments (Figures 1-4), the viscosity showed an initial decrease from 0h in both NH and TH sludge (with and without Tween 80, Figures 1, 3 and 4) with the exception of TH with Tween 80 where viscosity almost remained constant for first 6h (Figure 4). Also, a hump in viscosity was observed, at 12h, for NH and TH sludge without Tween 80 (Figures 1 and 2) and at 9h for sludge with Tween 80 (Figures 3 and 4). The initial decrease could be an effect of physical action (agitation) only. As Bt cell concentration increased, it contributed towards continuous increase in viscosity. On the other hand, prolonged agitation in the fermenter tended to decrease the viscosity due to disruption of sludge flocs and new flocs that might have been generated due to new Bt cells and EPS production, as explained before. However, increase in viscosity as observed in Figures 1-4, suggested that contribution to the increased viscosity due to increased cell concentration superseded the viscosity decrease due to agitation, thus causing a net increase to attain the characteristic hump. After the hump, the viscosity started decreasing until 30h, mainly governed by physical phenomenon (agitation) and also due to pseudoplastic nature of sludges as explained earlier. Afterwards, the viscosity values further levelled off at 24, 4, 5.8 and 5 cP for NH, TH, NH (with Tween 80) and TH (with Tween 80) respectively due to stable operating conditions, specifically, agitation rate.

On the other hand, the viscosity hump appeared earlier (9h, Figures 1 and 2) where Tween 80 was amended in sludge vis-à-vis non-amended sludge (Figures 3 and 4). During the fermentation of Tween 80 fortified sludge, faster growth accompanied with intense foaming was observed which warranted rapid addition of anti-foam agent. This resulted in an early

viscosity hump in Tween 80 amended sludge. A similar relationship was also observed between anti-foam addition and viscosity and subsequent effect on mass transfer in a well stirred fermenter for the cultivation of *Pseudomonas putida* in glutamate medium (Morao et al., 1999).

Figures 5a and 5b represent the particle size distribution profile of different sludges at zero and 48h, respectively. A closer look showed the reduction in particle size as one moved from 0h to 48h which could be a manifestation of the agitation conditions in the fermenter. The particle disruption was a function of operating parameter (agitation) only and changes taking place due to fermentation were relatively insignificant. Nevertheless, there was a decrease in viscosity with particle size and similar observations could be marked in figures 2-4 with the exclusion of a single hump which mainly corresponded to anti-foam addition in these cases either due to the foam produced by abundance of soluble proteins or surfactant and the cumulative effect of both parameters (e.g. TH in Figure 4). From 0 to 48h, the curve moved more towards the lower particle size region (LHS in the Figure 5b). As lower particle size is more suitable for biopesticidal liquid suspensions, hence, the evident shift should aid in developing formulations possessing better compatibility with application equipment (Mor and Matthews, 2003).

OUR Profile

Profiles of OUR for NH and TH sludge with and without fortification of Tween 80 are presented in Figure 6. Initially, after inoculation (t = 0h), OUR was lower owing to the lower cell concentration and was in concordance with the literature studies of Bt on conventional glucose medium (Rowe et al., 2003). The OUR profile of NH sludge without Tween 80 showed a comparatively smaller increase in first 3h and reached maximum at 12h with a relatively wider time range (3h – 24h) of moderately higher values (0.4-1.8 mmol/L.h). This wider range could be correlated with slower growth for a longer period as is also clear from Figure 1. TH sludge without Tween 80 showed a similar OUR trend as in NH sludge without Tween 80.

NH sludge fortified with Tween 80 showed a continuous increase in OUR until 9h and then a continuous decline until the end of fermentation. The comparatively higher OUR values (2-5 mmol/L.h between 3-12h) than NH sludge without Tween 80 could be owing to amelioration in physics of process regime and thus better oxygen transfer during exponential phase as explained in earlier sections.

In TH sludge fortified with Tween 80, maxima of OUR was reached within initial 3h and then decreased in a stepwise manner until 15h. And later on, there was a monotonous decline in the OUR values until 48h. These specific changes in OUR could be explained on account of frequent variation in agitation and aeration rates in order to mitigate intense foaming (Figure 4a). The appealing fact was higher OUR in the case of Tween 80 fortified NH and TH sludges, which increased 5 and 3.5 times respectively.

Thus, it was evident that variations in OUR were in accordance with K_La and some irregularities could be attributed to complexity of the fermentation medium i.e. sludge and its treatment. Despite irregularities in OUR, addition of Tween 80 allowed better process performance of NH sludge and yielded higher potency of the biopesticide.

The addition of Tween 80 to NH sludge during fermentation served dual functions – improving sludge rheology and enhancing entomotoxicity at the end of fermentation. However, addition of Tween 80 created foaming problems in TH sludge and did not appreciably affect rheology and entomotoxicity too. Hence, Tween 80 should be added preferably to NH sludge to improve the physical conditions of process and entomotoxicity.

Studies of impact of Tween 80 fortified fermented sludge in comparison to non-fortified fermented sludge on formulation (wettability, dispersion, suspendibility, viscosity and particle size) are underway and field application results will be reported in near future. If Tween 80 amended fermented sludge gives same formulation characteristics as that of addition of Tween 80 during formulation (non-amended fermented sludge), it will reduce the overall cost from fermentation - through formulation- to application process stages.

Conclusions

From the aforementioned studies, following conclusions can be drawn:

1. Addition of Tween 80 altered the physico-chemical regime during sludge fermentation (oxygen transfer and nutrient assimilation).
2. Non-hydrolyzed sludge showed higher k_La with Tween 80. The hydrolyzed sludge showed no significant difference in k_La in contrast to without Tween 80 sludge.
3. Higher cell and spore concentrations were observed in non-hydrolyzed Tween 80 amended sludge.
4. Addition of Tween 80 increased entomotoxicity of non-hydrolyzed sludge by 26.5 %.

5. Fortification of Tween 80 resulted in increased maximum specific growth rate of Bt in non-hydrolyzed as well as hydrolyzed sludge.
6. Tween 80 caused reduction of interfacial tension and also markedly lowered viscosity.
7. Intense foaming was observed in hydrolyzed sludges due to dual action of surfactant property of Tween 80 and presence of simple peptides.
8. Higher oxygen uptake rates were observed for Tween 80 amended non-hydrolyzed and hydrolyzed sludges.

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STR 202047); Canada Research Chair; University of Missouri, Columbia and U.S. EPA. The views and opinions expressed in this article are those of authors and should not be construed as opinions of the U.S. Environmental Protection Agency. The authors are also thankful to Natural Sciences and Engineering Research Council of Canada and Canadian Forestry Services for providing Ph.D scholarship to Satinder K. Brar during the course of this research work.

References

Aiba S, Humphrey, AE, Millis, NF. 1973. Biochemical Engineering Academic Press, 2nd edition, New York.

APHA, AWWA, WPCF. 1995. Standard Methods for Examination of Water and Wastewaters. 18th ed. American Public Health Association, Washington, DC, USA.

Arunsiri AT, Suphantharika M, Ketunuti U. 2003. Preparation of spray-dried wettable powder formulations of *Bacillus thuringiensis* based biopesticides. J Econ Entomol 96 (2):292-299.

Avignone-Rossa C, Arcas J and Mignone C. 1992. *Bacillus thuringiensis*, sporulation and δ-endotoxin production in oxygen limited and non-limited cultures. World J of Microbiol and Biotechnol 8:301-304.

Brar SK, Verma M, Tyagi RD, Valéro JR and Surampalli RY. 2004a. Sludge based *Bacillus thuringiensis* Biopesticides. I: Rheology Studies, (unpublished).

Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. 2004b. Sludge based *Bacillus thuringiensis* Biopesticides. II: Particle size distribution (unpublished).

Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. 2004c. Comparative rheological studies of *Bacillus thuringiensis* fermented wastewater sludges for biopesticide production. (unpublished).

Charbonneau H, Hébert M, Jaouich A. 2000. Portrait de la valorisation agricole des matières résiduelles fertilisantes au Québec – partie 1 : Aspects Quantitatifs, Vecteur Environnement 33(6):30-32, 49-51.

Jordan RN, Nichols EP, Cunningham AB. 1999. The role of biosurfactant sorption on promoting the bioavailability of nutrients localized at the solid-water interface. Water Sci Technol 39 (7):91-98.

Kawase Y, Hashimoto N. 1996. Gas hold-up and oxygen transfer in three phase external-loop airlift bioreactors: non-Newtonian fermentation broths. J Chem Tech Biotechnol 65:325–334.

Juarez P and Orejas J. 2001. Oxygen transfer in a stirred reactor in laboratory scale. Latin American Applied Research 31:433-439.

Kaur S. 2000. Molecular approaches towards development of novel *Bacillus thuringiensis* biopesticides. World J of Microbiol & Biotechnol 16:781-793.

Leblanc, Maria-Eve 2003. Effets des différentes stratégies et prétraitements des biosolides municipaux sur la croissance, la sporulation, l'entomotoxicité de *Bacillus thuringiensis* var. *kurstaki*. Master's thesis, Institut Nationale de la Recherche Scientifique-Eau, Terre et Environnement, Université du Québec, Canada.

Lescota RD, Mookerjee PK, Misselbrook J, Peterson RF Jr. 2002. Pesticidal formulation. U. S. Patent No. 20020114821.

Li GQ, Qiu HW, Zheng ZM, Cai ZL, Yang SZ. 1995. Effect of fluid properties on mass transfer in a bioreactor. J Chem Tech Biotechnol 62:385-391.

Liu B, Sengonca C. 2003. Conjugation of δ-endotoxin from *Bacillus thuringiensis* with abamectin of *Streptomyces avermitilis* as a new type of biocide, GCSC-BtA, for control of agricultural insect pests. Anzeiger für Schädlingskunde 76(2):44-49 (in English).

Maagd RAD, Bakker P, Staykov N, Dukiandjiev S, Stiekema W, Bosch D. 1999. Identification of *Bacillus thuringiensis* delta-endotoxin Cry1C Domain III amino acid residues involved in insect specificity. Appl and Environmental Microbiol 5(10):4369-4374.

Mor SG, Matthews GA. 2003. Recent developments in sprayers for application of biopesticides- an overview. Biosystems Engineering 84(2):119–125.

Morão A, Maia CI, Fonseca MMR, Vasconcelos JMT, Alves SS. 1999. Effect of antifoam addition on gas-liquid mass transfer in stirred fermenters. Bioprocess and Biosystems Engineering 20(2):165–172.

Ni X, Gao S, Santangeli L. 1997. On the effect of surfactant on mass transfer to water-glycerol solutions in a pulsed baffled reactor. J Chem Tech Biotechnol 69:247-253.

Parakulsuksatid P. 2000. Utilization of microbubble dispersion to increase oxygen transfer in pilot scale baker's yeast fermentation unit. Master's Thesis, Faculty of Virginia, USA.

Paramatha, R.B. 2000. Hyper-production of insecticidal crystal protein (δ-endotoxin) by *Bacillus thuringiensis* var. *israelensis* is not related to sporulation-specific biochemical functions.Curr. Microbiol., 41, 187–191.

Pearson D, Ward OP. 1988. Effect of culture conditions on growth and sporulation of *Bacillus thuringiensis subsp. israelensis* and development of media for production of the protein crystal endotoxin. Biotechnol Lett 10(7):451–456.

Rossa, A.C. , Mignone, C. 1993. δ-endotoxin activity and spore production in batch and fed-batch cultures of *Bacillus thuringiensis*. Biotechnol. Lett., 15, 295–300.

Richard A, Margaritis A. 2003. Rheology, oxygen transfer, and molecular weight characteristics of poly(glutamic acid) fermentation by *Bacillus subtilis*. Biotechnol Bioeng 82:299-305.

Rowe GE, Margaritis A, Wei N. 2003. Specific Oxygen Uptake Rate variations during batch fermentation of *Bacillus thuringiensis* subspecies *kurstaki* HD. Biotechnol Prog 19:1439-1443.

Sachdeva V, Tyagi RD, Valéro JR. 1999. Factors affecting the production of *Bacillus thuringiensis* biopesticides. Devel Microbiol 3:363-365.

Sutherland IW. 1982. Biosynthesis of microbial exopolysaccharide. Adv Microb Physiol 23:79– 150.

Tirado-Montiel ML, Tyagi RD, Valéro JR. 2001. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticide. Water Res 35(16):3807–3816.

Vasconcelosa JMT, Rodriguesa JML, Orvalhoa SCP, Alvesa SS, Mendes RL, Reis A. 2003. Effect of contaminants on mass transfer coefficients in bubble column and airlift contactors. Chemical Engineering Science 58:1431–1440.

Vidyarthi AS, Desrosiers M, Tyagi RD, Valéro JR. 2000. Foam control in biopesticide production from sewage sludge. J Ind Microbiol Biotechnol 25:86-92.

Vidyarthi AS, Tyagi RD, Valéro JR, Surampalli RY. 2002. Studies on the production of *B.thuringiensis* based biopesticides using wastewater sludge as a raw material. Water Res 36(19):4850-4860.

Worden RM, Bredwell MD. Gas-Liquid mass transfer in production of large-scale bioproducts: application to synthesis gas processing. http://www.egr.msu.edu/biotech/gas.htm. [cited 11 May,2004].

Williams JA. 2002. Keys to bioreactor selection. Chem. Eng. Progress 98(3):34–41.

Wu WT, Hsu YL, Ko YF, Yao LL. 2002. Effect of shear stress on cultivation of *Bacillus thuringiensis* for thuringiensin production. Appl Microbiol. Biotechnol 58:75–177.

Yezza A, Tyagi RD, Valéro, JR, Surampalli RY. 2004. Scale-up of biopesticide production process using wastewater sludge as a raw material. J Industrial Microbiol and Biotechnol 31: 545-552.

Zouari N, Dhouib A, Ellouz R, Jaoua S. 1998. Nutritional requirements of a strain of *Bacillus thuringiensis* subsp *kurstaki* and use of gruel hydrolysate, for the formulation of a new medium for deltaendotoxin production. Appl Biochem. Biotechnol 69:41–52.

Zouari N, Jaoua S. 1999. The effect of complex carbon and nitrogen, salt, Tween 80 and acetate on delta-endotoxin production by a *Bacillus thuringiensis* subsp *kurstaki*. J of Industrial Microbiol and Biotechnol 23:497–502.

Table I. Characteristics of secondary CUQ sludge

Parameter	Concentration	Parameter	Concentration
TS (g/l)	20.4	Ca (mg/kg)	14762
TVS (g/l)	15	Cd (mg/kg)	0.39
SS (g/l)	17.5	Cr (mg/kg)	24
VSS (g/l)	13.5	Cu (mg/kg)	189
C_t (mg/kg)	298402	Ni (mg/kg)	20.3
N_t (mg/kg)	41780	Fe (mg/kg)	12001
P_t (mg/kg)	7975	K (mg/kg)	9084
$N\text{-}NH_3$ (mg/kg)	979	Pb (mg/kg)	27.6
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$ (mg/kg)	16	S (mg/kg)	4779
$P\text{-}PO_4^{3-}$ (mg/kg)	5489	Zn (mg/kg)	301
Al (mg/kg)	5749	Na (mg/kg)	1289

Table II. Dimensions of bench scale bioreactor

Parameters	Value(s)
Total volume	15
Working volume	10
Six blade Rushton turbines	3
Impeller diameter, D_i (m)	0.1
Tank diameter, D_t (m)	0.20
D_t / D_i	2
Liquid height, H_L (m)	0.3
H_L / D_t	1.5

Table III. Comparative rheology and potency of fermented sludge with different treatment conditions

Time (h)	Parameter(s)	NH	TH	NH (with Tween 80)	TH (with Tween 80)
36	Viscosity (cP)	13	3.61	5.4	3.98
	D_{50} (µm)	5.27	5.09	9.4	3.4
	Tx (IU/µL)	12254	18100	14489	18562
	TC (CFU/ml)	5.4×10^8	2.9×10^8	1.14×10^9	6.5×10^8
	VS (CFU/ml)	3.35×10^8	2.8×10^8	1.08×10^9	6.3×10^8
48	Viscosity (cP)	24	4.09	5.79	5
	D_{50} (µm)	1.7	3.98	10.8	4.2
	Tx (IU/µL)	12710	18960	16090	19021
	TC (CFU/ml)	5×10^8	3.97×10^8	1.15×10^9	9.5×10^8
	VS (CFU/ml)	3×10^8	3.93×10^8	1.13×10^9	9.39×10^8
	Maximum Specific growth rate* (μ_{max},h^{-1})	0.19	0.26	0.27	0.30

*** Calculated from the straight line slope in the exponential phase**

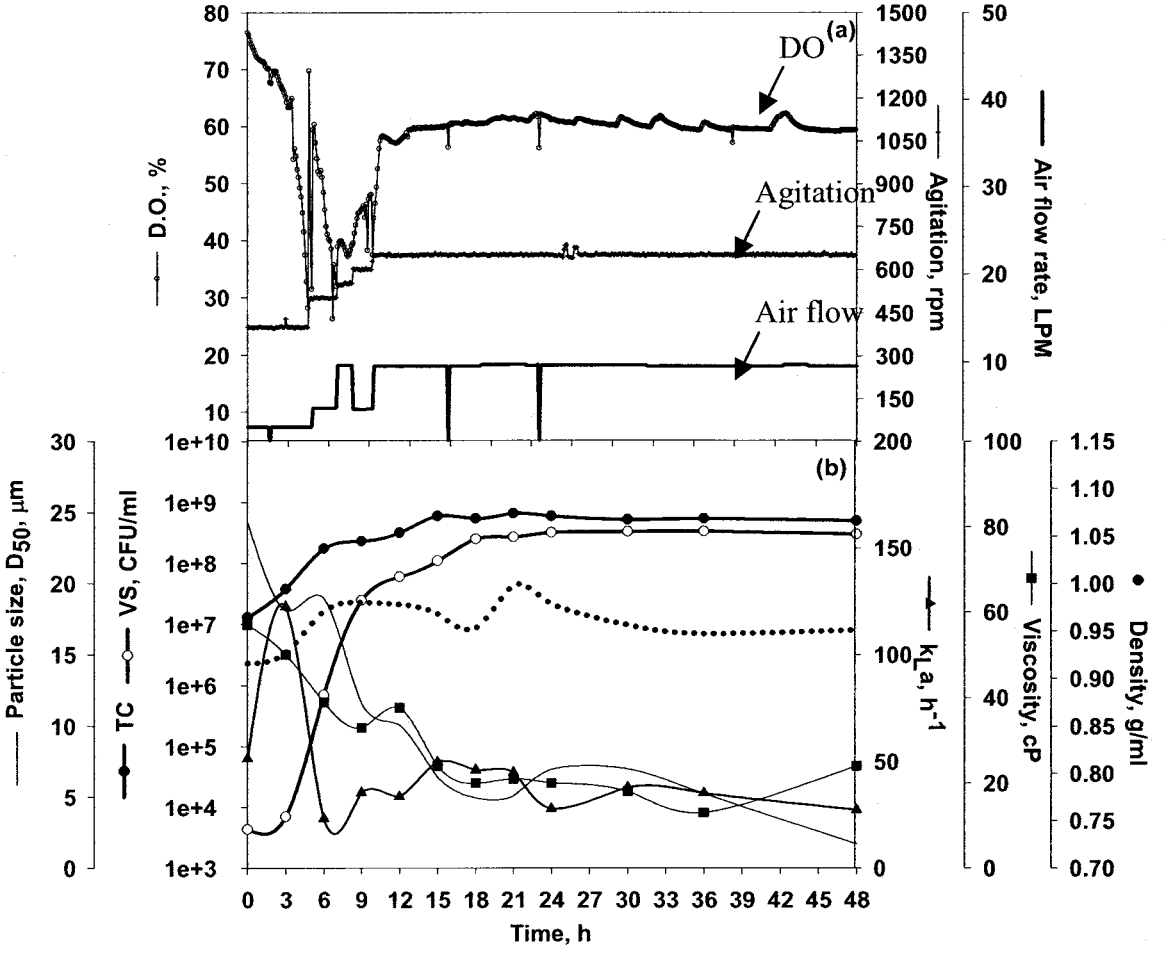


Figure 1. Non-hydrolyzed sludges without Tween 80: a) Operational parameters of fermenter; b) Rheology and performance parameters

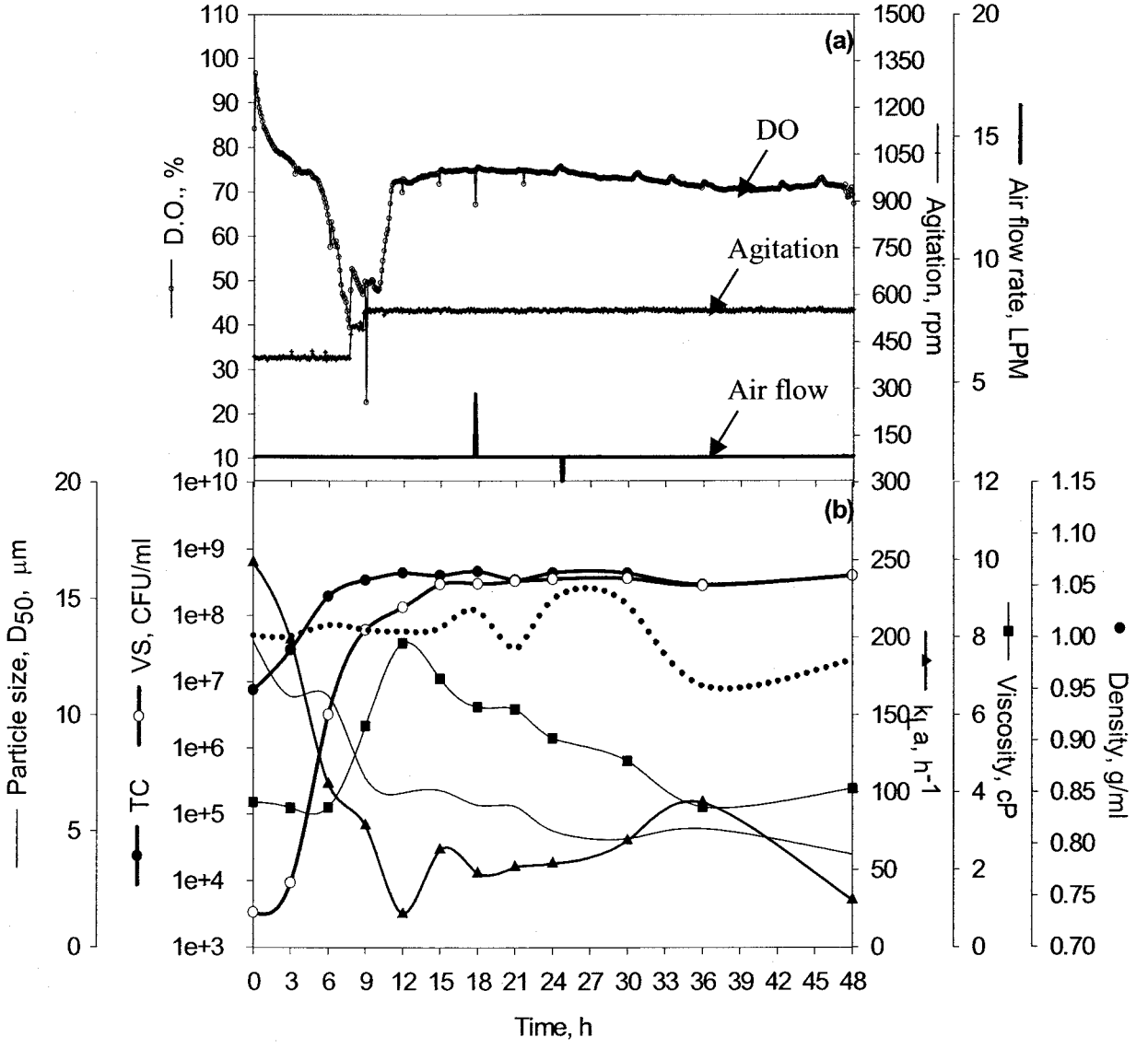


Figure 2. Hydrolyzed sludges without Tween 80: a) Operational parameters of fermenter; b) Rheology and performance parameters

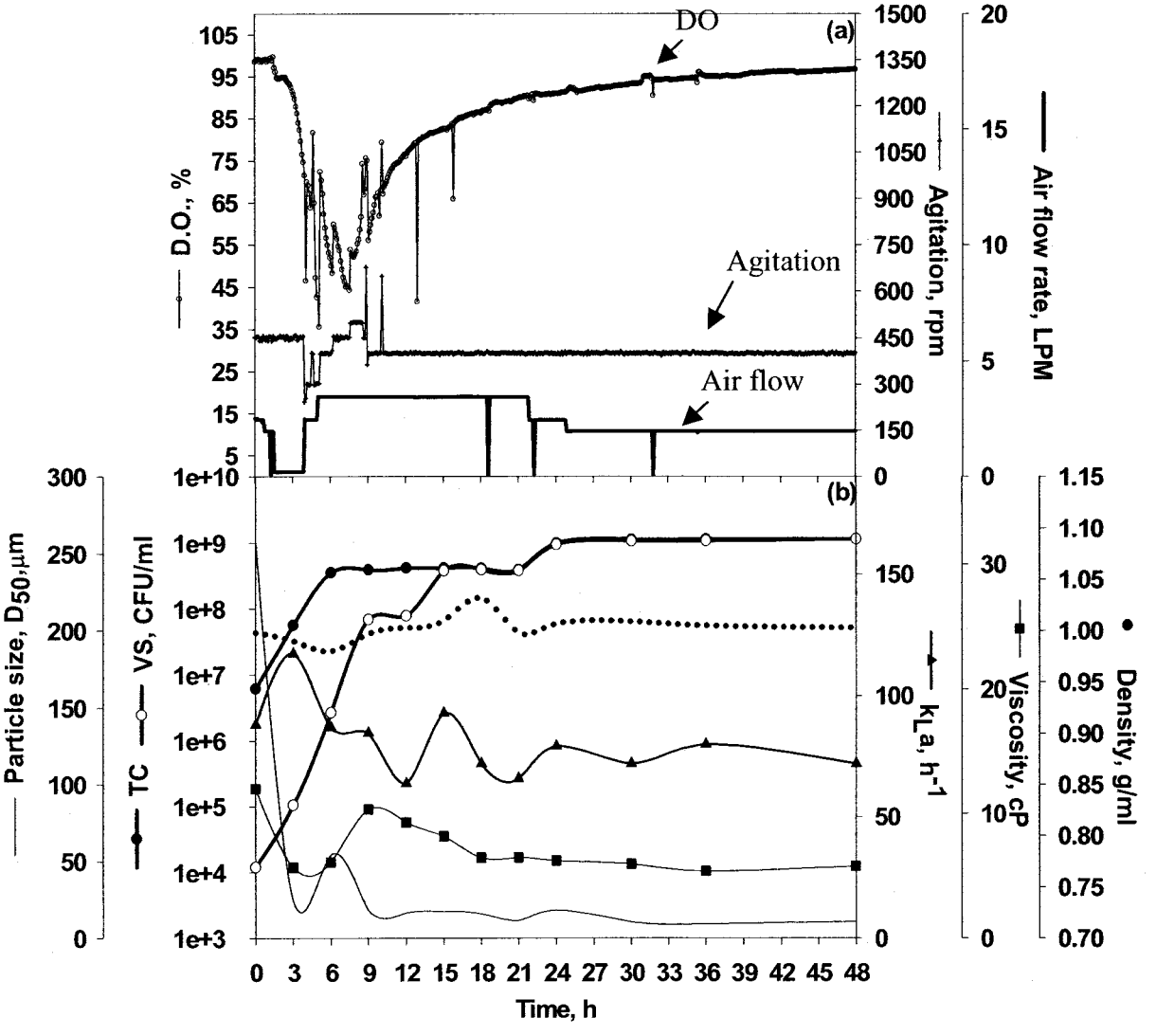


Figure 3. Non-hydrolyzed sludges with Tween 80: a) Operational parameters of fermenter; b) Rheology and performance parameters

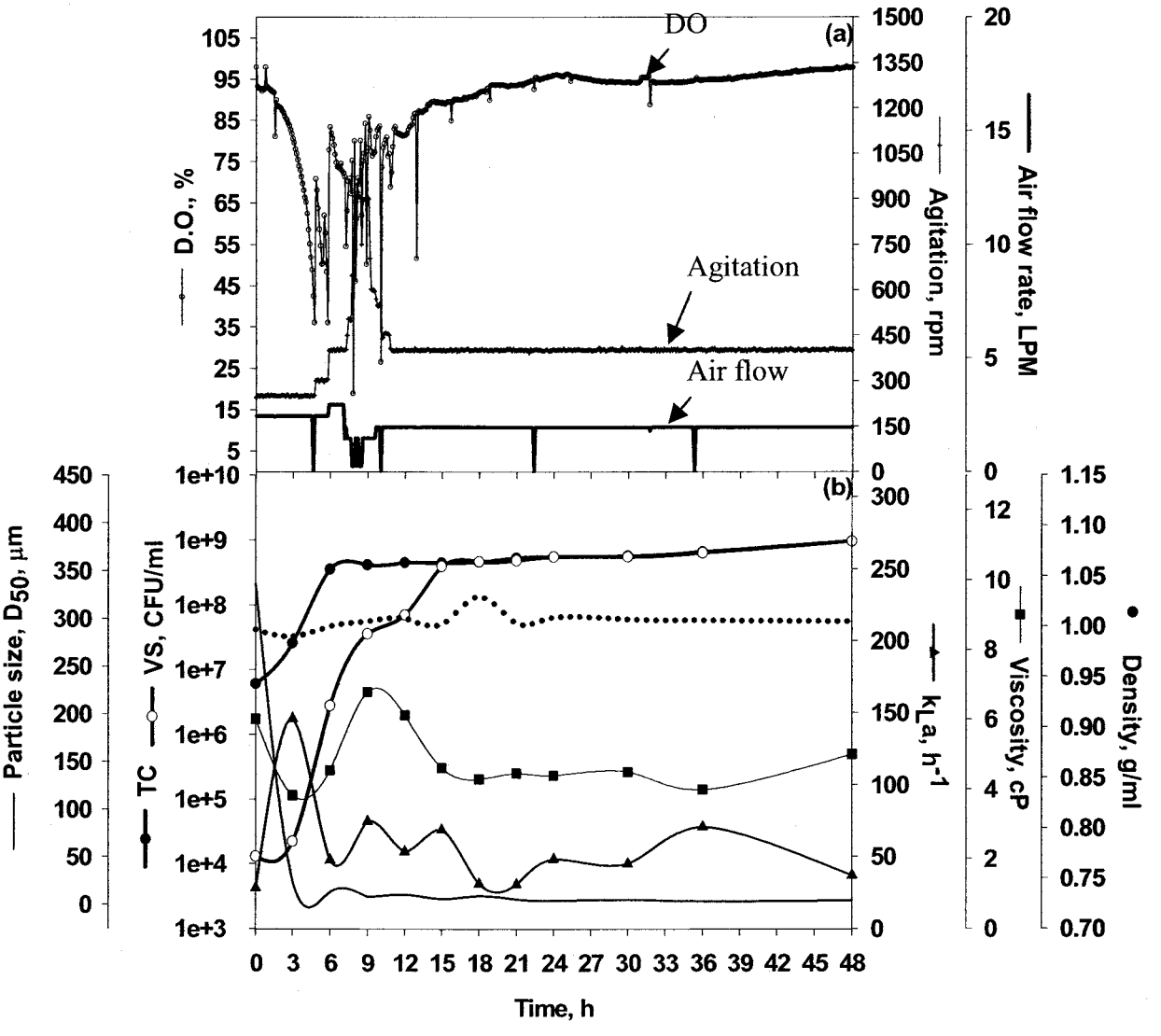


Figure 4. Hydrolyzed sludges with Tween 80: a) Operational parameters of fermenter; b) Rheology and performance parameters

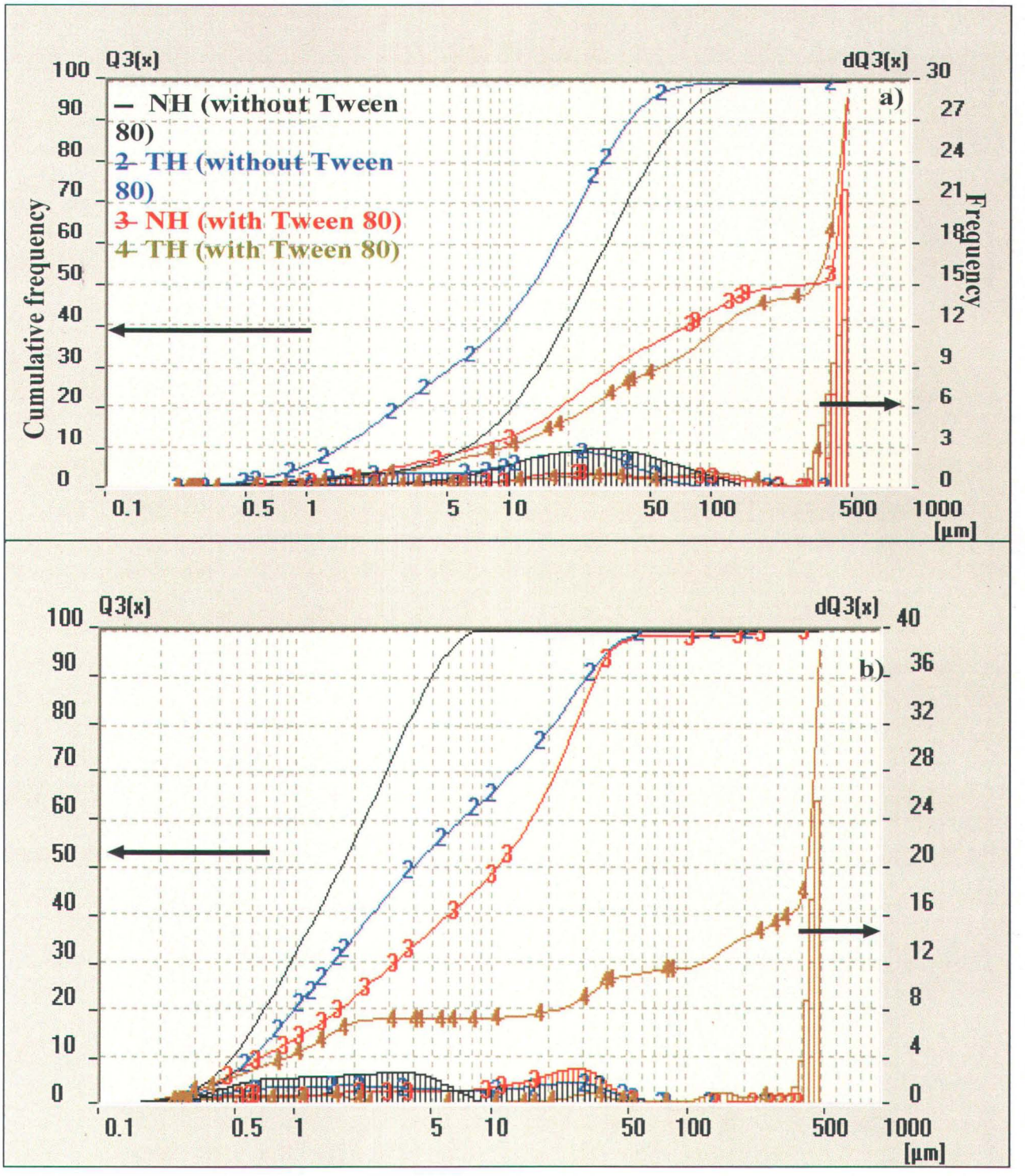


Figure 5. Particle size distribution of different sludges: a) At zero hours after inoculation; b) at 48 hours. (Lines represent cumulative frequency and curved bars - frequency)

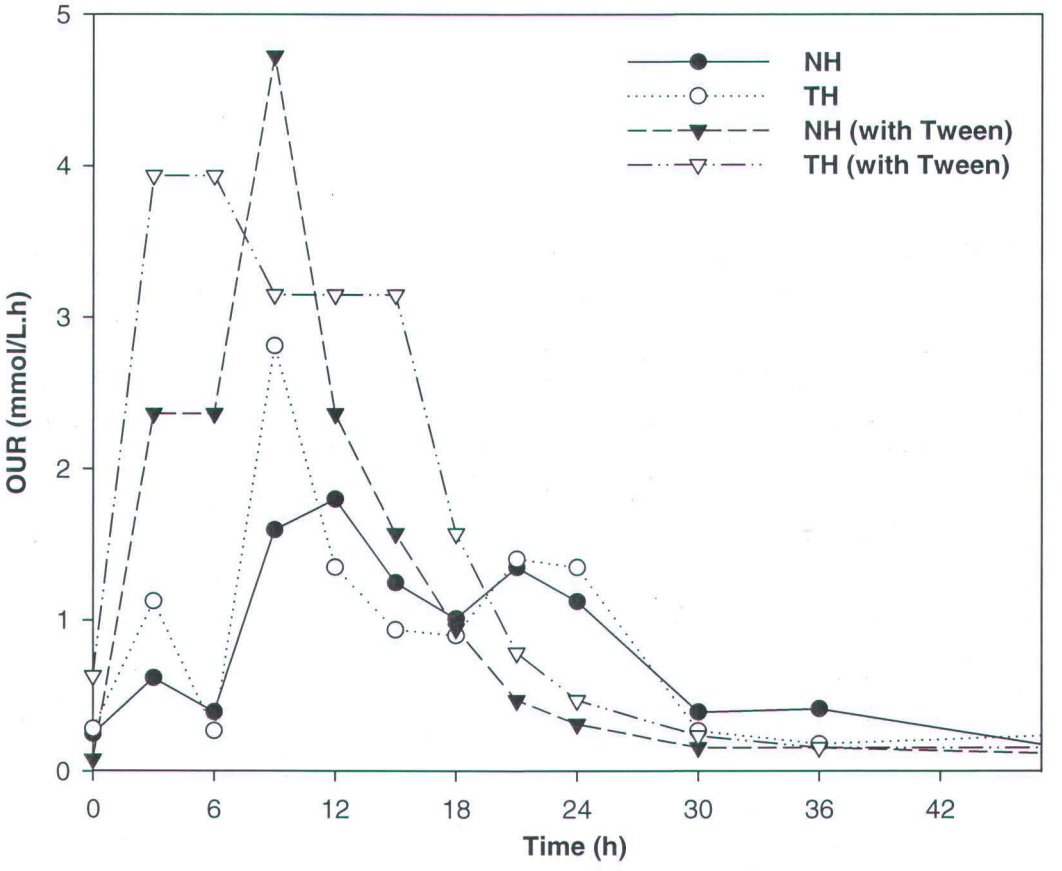


Figure 6. OUR profiles during fermentation of sludge at different treatment conditions

Partie II

Ramification of Solids Concentration on Entomotoxicity and Enzyme Activity of *Bacillus thuringiensis* Fermented Wastewater Sludge

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*], J.R. Valéro[1] and R.Y. Surampalli[2]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Bioresource Technology
(Under Review)

Ramification de concentration en solides sur l'activité enzymatique et l'entomotoxicité des boues d'épuration fermentées par *Bacillus thuringiensis*

Résumé

Cette étude a examiné la possibilité de produire les enzymes protéase et chitinase de manière concomitante avec la production de biopesticides à partir de solutions de boues d'épuration fermentées par *Bacillus thuringiensis* à des concentrations en solides de 30 g/l. Les propriétés rhéologiques des boues d'épuration ont été modifiées avec l'addition de Tween-80 (0,2 % de v/v). L'addition de Tween 80 aux boues non-hydrolysées a augmenté le nombre de cellules et de spores viables de 1,6 et de 1,3 fois, respectivement. Le taux de croissance spécifique maximum h^{-1} (μ_{max}) passait de 0,17 à 0,22, alors que l'entomotoxicité (Tx) augmentait de 29,7 %. De même, le coefficient volumétrique de transfert de masse (k_La) a montré des variations significatives pendant la fermentation et le taux de consommation d'oxygène a doublé. Alors que l'activité protéolytique et l'entomotoxicité ont augmenté, l'activité chitinolytique a diminué en présence du Tween-80. Les courbes d'entomotoxicité spécifique en relation avec la concentration en spores viables obéissent à la loi de puissance, et une relation linéaire a été mise en évidence entre le Tx et l'activité des protéases. La viscosité a varié pendant la fermentation et le volume des particules a augmenté dans les boues contenant du Tween-80, alors que la taille des particules (D_{50}) a diminué de manière régulière jusqu'à la fin de la fermentation.

Mots-clés: *Bacillus thuringiensis*; bioréacteur; 30 g/L matières en suspension; taille de particule; Tween-80; viscosité; boues d'épuration brutes.

Summary

This study investigated the possibility of production of *Bacillus thuringiensis* based biopesticides from raw wastewater sludge at higher solids concentration (30 g/L) concomitant with enzymes (protease and chitinase) production. The rheology of wastewater sludge was modified with addition of Tween-80 (0.2 % v/v). The fortification of raw wastewater sludge resulted in 1.6 and 1.3 folds increase in cell and spore count, respectively. The maximum specific growth rate (μ_{max}) augmented from 0.17 to 0.22 h^{-1} and entomotoxicity (Tx) increased by 29.7 %. Meanwhile, volumetric mass transfer coefficient (k_La) showed major variations during fermentation and oxygen uptake rate increased by 2 folds. The proteolytic activity increased while chitinase decreased for Tween amended wastewater sludge, but the entomotoxicity increased. The specific entomotoxicity followed Power law when plotted against spore concentration and the relation between Tx and protease activity was linear. The viscosity varied and volume percent of particles increased in Tween-80 amended wastewater sludge and particle size (D_{50}) shifted to lower particle size region at the end of fermentation.

Keywords: *Bacillus thuringiensis*; Fermenter; 30 g/L suspended solids; Particle size; Tween-80; Viscosity; Raw wastewater sludge

Introduction

The production of *Bacillus thuringiensis* (Bt) based biopesticides has observed several leaps over the last five decades in terms of economical raw material; enhanced biopesticidal potential and high performing formulations. In this context, wastewater sludge as a raw material for Bt production has seen immense progress as it offers various advantages: zero cost raw material, perennial availability; higher entomotoxicity (Tx) with low spore concentration; low variability of sludge in terms of nutrients; higher entomotoxicity of crystal protein produced in sludge vis-à-vis conventional semi-synthetic soyameal medium; stable formulations and significant contribution to sustainable sludge management (Tirado-Montiel *et al.* 2001; Lacchab *et al.* 2001; Vidyarthi *et al.* 2002; Yezza *et al.* 2004, 2005a,b; Brar *et al.* 2004; Barnabe *et al.* 2005).

The nutrients for Bt growth which are ingrained in sludge solids (adsorbed and/or embedded) are usually present in the form of flocs. It has been already established that 20-25 g/L is the optimal suspended solids (SS) concentration for Bt fermentation. At higher SS (>25 g/L), there is a problem of oxygen transfer due to viscosity increase which limits the formation of spores and crystal protein, important contributors to Tx (Tirado-Montiel *et al.* 2001; Vidyarthi *et al.* 2002; Brar *et al.* 2005a). At lower SS (<20 g/L SS), lower Tx was observed due to nutrient limitation (Lacchab *et al.* 2001). The barrier of high SS was minimized through sludge hydrolysis and a comparatively higher Tx was achieved (Barnabe 2005; Brar *et al.* 2005b). Meanwhile, fortification of raw sludge (25 g/L SS) with Tween-80 also increased the Tx by 29.9% in shake flask as well as in fermenter (Vidyarthi *et al.* 2001; Brar *et al.* 2005b). The increased Tx was due to improved volumetric oxygen transfer rate (k_La) and higher oxygen uptake rate (OUR) compared to non-Tween amended sludge. It is also important to add that fortification of hydrolyzed sludge at 25 g/L SS (TH-25) did not enhance the Tx value. This suggested that the medium was already simplified and hence Tween-80 did not aid in physical amelioration.

Furthermore, after fermentation, the Bt fermented broth is concentrated by centrifugation followed by re-suspension of the centrifugate (or pellet) obtained in the supernatant to attain desired Tx value. The concentrated product is then transformed to formulation where different additives are added to achieve necessary physical/chemical properties (high

suspendibility, low viscosity, phagostimulation, UV protection, stickiness to plant or tree leaves) of the final product. Tween as one of the additives is added to increase wettability and suspendibility of the product (to enhance ease of field application). Tween has been already proved to enhance oxygen transfer and substrate assimilation during Bt growth and endotoxin synthesis (Brar *et al.* 2004). Further, addition of Tween to fermentation medium could serve two purposes: 1) increase suspendibility of the final formulated product and, 2) facilitate dissolution of centrifuged pellet in supernatant which otherwise may be difficult to solubilize.

In further pursuit of the above mentioned research, this study focuses on use of Tween-80 to improve the non-hydrolyzed sludge medium properties at 30 g/L SS. Hydrolyzed sludge was not explored as there was no marked improvement in Tx and also intense foaming problem was encountered with Tween-80 amendment at 25 g/L SS. This study was based on the following hypothesis: if there was an increase in Tx for Tween amended sludge, the hydrolysis step could be eliminated and hence total cost of Bt production may be lowered.

Bt synthesizes different types of proteases during the post-exponential and stationary phases, required to hydrolyse complex proteins in order to satisfy its nutritional needs (Liu & Tzeng 2000). Proteases are also required during sporulation for spore formation and some Bt proteases may also hydrolyze the crystal proteins lowering Tx (Oppert 1999). Additionally, chitinases have been known to affect the Tx of Bt through synergism. During penetration into the insect midgut, chitin of peritrophic membrane needs to be hydrolyzed to facilitate action of δ-endotoxin aided by chitinases (Liu *et al.* 2002). Wastewater sludge is complex and may comprise cell wall debris of fungi and residual crustacean shells which may act as an inherent source of chitin and stimulate chitinase production. This necessitates investigation of chitinase production during Bt fermentation of wastewater sludge which has not been reported so far as well as proteases which form an important component of Bt metabolism as well as mode of action.

Thus, principal objectives of these investigations are to study the effect of Tween-80 amendment on: a) physical-biological parameters of Bt fermentation of non-hydrolyzed sludge at 30 g/L SS (higher value); b) chitinase, protease and Tx profiles of Bt fermentation.

Materials and methods

Sludge sampling

The raw wastewater sludge was acquired from Eastern secondary facility of Quebec city wastewater treatment plant (Quebec, Canada) and characterization results are presented in Table 1. The physical-chemical characteristics were determined according to Standard Methods (APHA 1998). The sludge was concentrated from 1.7 % to about 3.0 % (w/v) SS by gravity settling followed by centrifugation at 7650 g for 15 minutes in a Sorvall RC 5C plus Macrocentrifuge (rotor SA-600, DuPont, Canada). Concentrated sludge (raw) was then homogenized using a Waring blender (3L) to attain 3.0 % SS concentration.
The sludge samples at initial pH of 6.1 ± 0.1 were adjusted to 7.0 ± 0.1 by addition of 4N NaOH. The study comprised two sludges at SS of 30 g/L- non-hydrolyzed without Tween-80 (NH-30, control) and non-hydrolyzed with Tween-80 (NHT- 30).

Microorganism

Bacillus thuringiensis var. *kurstaki* HD-1 (ATCC 33679) was used in this study. The culture conditions, maintenance, inoculum production and fermentation procedure (biopesticide production) are described elsewhere (Vidyarthi *et al.* 2002).

Fermenter

The fermentation of sludges with or without addition of Tween 80 was carried out in stirred tank bioreactor equipped with accessories and automatic control systems for dissolved oxygen, pH, antifoam, impeller speed, aeration rate and temperature. The programmable logic control (PLC) with software (iFix 3.5, Intellution, USA) was used to control fermenter parameters and data acquisition.

Initially, polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR-Canada). The oxygen probe was calibrated to zero (using N_2 degassed water) and 100 % (air saturated water). Subsequently, fermenter was fed with sludge, polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution as an anti-foam agent and 0.2% v/v Tween 80 (Poly(oxyethylene)(20)-sorbitane mono-oleate) (Difco Lab., Michigan,

USA) as a surface active agent. *In situ* steam sterilization was carried out at 121°C for 30 min. When the fermenter cooled to 30°C, the dissolved oxygen (DO) probe was recalibrated as stated earlier at agitation rate of 500 rpm. The fermenter was then inoculated (2 % v/v inoculum) with sludge acclimated pre-culture of Bt in exponential phase (10-12h). In order to keep the DO above 25 % saturation, air flow rate and agitation rate were varied between 0.13-0.23 vvm and 250–700 rpm, respectively. The temperature was maintained at 30 ± 1°C by circulating water through the fermenter jacket. The pH was maintained at 7.0 ± 0.1 by peristaltic pump operated automatic 4N NaOH or 4N H_2SO_4 addition. The foaming was controlled by PPG injection and mechanical foam disruptor (Fundafoam^{TM}).

Oxygen uptake rate (OUR) was measured by conventional technique utilizing "air off and on" method based on the dynamic gassing out technique (Aiba *et al.* 1973). Volumetric oxygen transfer coefficient, k_La was measured at each sampling point as per earlier studies (Brar *et al.* 2005a).

Physical parameters measurement

Viscosity of sludge was determined by using a rotational viscometer (Cole-Palmer Inc., Canada) at a shear rate of 36.71 s^{-1} (adapted and optimized for the entire study of biopesticide production). Particle size analysis was carried out using Fritsch Laser particle sizer analyzette 22 (Fritsch GmbH, Germany) as per the details described in Brar *et al.* (2005b). Surface tension (ST) measurements were carried out using Sigma 70 tensiometer (KSV Instruments Ltd., Finland) by du Nouy ring method at 30°C as per details in Brar *et al.* (2005b).

Protease and chitinase assay

Samples collected from the fermenter were centrifuged at 7650 g for 20 min at 4°C. Proteolytic activity was determined according to modified Kunitz (1947) method on one part of supernatant (in triplicate).

Likewise, other part of supernatant (in triplicate) was utilized for determination of chitinase activity by measuring the reducing end group, N-acetyl glucosamine (NAG), degraded from colloidal chitin as described by Ueda and Arai (1992). The assay mixture consisted of 0.5 ml

of enzyme solution (sample), 1ml 0.05 % diacetyl glycol chitin in 50 mM acetate buffer (pH = 4.0 ± 0.1). After incubation at 50 ± 1°C for 30 min in a recirculating water bath, the reaction was terminated by heating in boiling water for 5 min. Subsequently, 2.0 ml of 1.5 mM potassium ferricyanide reagent (0.5 g potassium ferricyanide in 1L of 0.5M sodium carbonate) was added. Later, the samples were transferred again into boiling water for 15 minutes and set aside to attain room temperature. As fermented sludge supernatant was turbid, the samples were filtered using a 0.45 μm filter and optical density of clear solution was measured immediately by a spectrophotometer (Varian Cary 100 Bio) at 420 nm. The chitinase activity was calculated using a standard curve obtained from known concentrations of NAG (0–0.15 mg).

One unit of chitinase activity is defined as the amount of enzyme solution capable of releasing reducing ends corresponding to 1 μg NAG from chitin at pH 4.0 and 50°C in one minute. Negative control tubes contained all components except substrate and blanks contained all components except the enzyme. Standard deviation for all reported results was upto 8%.

Cell, spore count and bioassay

Total cell (TC- vegetative cells and spores) count was determined as in earlier studies (Vidyarthi *et al.* 2002). Viable spore (VS) concentration was evaluated by heat shock treatment at 80°C for 10 min in a silicone oil bath and later the samples were cooled (5 min) before spreading onto tryptic soya agar plates. The standard deviation for TC and VS measurement was 7–8%.

The entomotoxicity (Tx) was determined against spruce budworm larvae (*Choristoneura fumiferana*) using diet incorporation method (Vidyarthi *et al.* 2002). Tx was expressed in relative spruce budworm units (SBU) and was compared with commercial formulation Foray 76B (Abbott Labs, Chicago, USA) at a potency of 20.1×10^9 IU/L (International Units per Litre) measured against cabbage looper (*Trichoplusia ni*). SBU in this study was equivalent to 75-80 % of international units (measured against cabbage looper). The standard deviation for Tx measurement was 9–10%.

Results and discussion

NH-30 sludge without Tween 80 (NH-30)

The fermenter operational (agitation rate, air flow rate and DO concentration) and process (TC and VS counts, k_La, viscosity and particle size) profiles for Bt grown in NH-30 sludge are presented in Figs. 1 a and 1b, respectively. First 9h of sludge fermentation depicted variations in all parameters when Bt was in active exponential phase and DO depleted rapidly to sustain the energy metabolism. However, the DO stabilized in the stationary phase (after 18h) with minor perturbations. Interestingly, the Bt fermentation followed diauxic growth with first phase from 0 to 12 h and second phase from 12 to 48h (Figure 1b). This was in accord with earlier study where diauxic growth was observed when Bt was grown on mixed substrates (different combinations of complex proteins and simple sugars and vice versa) due to probable transient accumulation of acids such as gluconate, 2-keto gluconate, α-ketoglutarate or pyruvate in the culture medium that required a short lag phase before they were completely oxidized (Ribbons 1969). Thus, the presence of different degrees of biodegradable material (simple and complex) in sludge could have contributed to diauxic Bt growth in wastewater sludge (Tirado-Montiel *et al.* 2001).

TC increased from 2.85×10^5 to 2.6×10^8 CFU/ml until 18 h, giving a final value of 2.9×10^8 CFU/ml at 48h (Figure1b). Similarly, VS count increased from 5.9×10^2 to 1.32×10^8 CFU/ml upto 21h with an average of 1.55×10^8 CFU/ml at 48 h with corresponding Tx values (10789 and 11632 SBU/μL at 36 and 48 h, respectively) as given in Table 2. The TC and spore counts for NH-30 (2.9×10^8 and 1.55×10^8, respectively) were lower at the end of fermentation (48h) in comparison to NH-25 sludge (TC and VS of 5×10^8 and 3×10^8 CFU/ml, respectively) with lower Tx (11632 SBU/μL) when compared to Brar *et al.* (2005a) (12710 SBU/μL). The higher SS resulted in comparatively higher viscosity and hence low oxygen transfer. Higher osmotic pressure at increased SS would have also negatively affected the nutrient transfer across the Bt cell membranes causing lower Tx. Further, at higher SS, substrate inhibition phenomenon may also play a role as excess of substrate may interfere with enzyme activities affecting Bt metabolism.

k_La increased between 3- 9h (Figure 1b) despite the aeration and agitation rates being constant. This could be due to gradual decrease in viscosity and particle size because of

constant agitation. Further, the k_La variations from 9 to 24h were inversely related to viscosity and particle size changes, which occurred due to cell lysis during sporulation and also breakage of sludge flocs caused by agitation. However, after 24h, k_La increased upto 36h and then decreased towards the end of fermentation (48h). This was possibly due to the attainment of stationary phase so that TC and VS counts stabilized and agitation rate was constant at increased aeration rate. The irregularities in k_La may also be due to frequent anti-foam addition and extracellular polymeric substances (EPS) from sludge or produced by Bt. The viscosity decreased substantially in the first 9h and thereafter the changes were less important. The initial decrease may be due to breaking of wastewater sludge floc binding of extracellular polymeric substances and further during Bt growth, due to poor physical conditions (high viscosity and particle size) of NH-30. Moreover, there was less intense foam when compared to hydrolyzed sludge which reduced the requirement of anti-foam (one of the major causes for viscosity increase with a hump, discussed later). There was no characteristic hump of viscosity as observed for non-hydrolyzed sludge at 25 g/L SS (NH-25) and hydrolyzed sludge (TH-25) so that viscosity decreased continuously due to agitation conditions and inherent rheological nature of sludge which was pseudoplastic (Brar *et al.* 2005a).

NH-30 Sludge with Tween 80 (NHT-30)

Different process and fermentation parameters illustrated in Figure 2a showed that the DO varied from 95-35 %. The initial DO in NHT-30 sludge (Figure 2a) was 1.2 times higher in comparison to NH-30 sludge at similar aeration rate (2.5 LPM) and lower agitation rate (450 and 250 rpm for NH-30 and NHT-30, respectively; Figs. 1a and 2a). The increase in DO for NHT-30 despite higher agitation rate for NH-30 could be brought about by the action of Tween-80. Tween is well known to decrease the surface tension at solid-liquid interface (Jordan *et al.* 1999).

TC and VS increased gradually from 3×10^6 to 4.5×10^8 and 6×10^2 to 1.6×10^8 CFU/ml, upto 21h and 24h, respectively as seen in Figure 2b. Later, TC and VS counts remained steady at 4.7×10^8 and 1.9×10^8 CFU/ml (48h), respectively. Presence of Tween resulted in better assimilation of nutrients due to enhanced oxygen transfer, lower viscosity and hence higher TC and VS counts, higher μ_{max} (0.22 h^{-1}) and Tx values (29.7 % increase) as compared

to NH-30 sludge (Table 2). Bt growth was normal growth phase (improved physical nature of otherwise complex medium resulted in ease of nutrient assimilation by Bt cells) and diauxic growth pattern was absent when compared to NH-30.

The Tx (16552 SBU/μL, Table 2) was slightly higher than Tween amended NH-25 (16090 SBU/μL, Table 2), but lower than hydrolyzed sludge (TH-25 and THT-25, with Tx of 18960 and 19021 SBU/μL, respectively) reported by Brar *et al.* (2005a). However, the specific Tx was higher for NHT-30 in relation to NHT-25 (Table 2). It may be due to the fact that for NHT-25, there was 3 times increase in VS which resulted in higher Tx, but for NHT-30, the VS concentration almost remained same and yet the Tx increased. This throws light on the possibility of formation of other virulence factors in high amounts so that Tx was higher. Bt synthesizes various virulence factors, namely, vegetative insecticidal proteins (VIPs), phospholipases, cytolytic proteins, chitinases and antibiotics that enhance Tx by synergism (Chang *et al.* 2001). Interestingly, the moderately higher Tx value of NHT-30 will lead to utilization of raw sludge at higher solids and may have positive effects on formulations (discussed later). Meanwhile, the moderately low Tx value of NHT-30 than TH-25 could be due to higher nutrient availability in TH sludge (physico-chemical and rheological modifications) when compared to Tween amendment (physical and rheological modification).

In the beginning, at 3h, k_La was higher (42.7 h^{-1}, Figure 2b) when compared to NH-30 sludge (32.3 h^{-1}, Figure1b) and was attributed to efficient oxygen transfer due to very low initial viscosity and particle size caused by Tween-80. The k_La decreased from 3 to 6h as viscosity increased slightly (0.5 units) and then increased 6 to 9h with slight constancy until 12 h as the agitation rate was adjusted to keep up the DO level. Meanwhile, when the viscosity increased from 12-15h (hump in Figure 2b), the k_La decreased afterwards, and the viscosity followed a decreasing profile. Further, k_La also started increasing partially affected by the decreasing particle size (rheology of the medium). Low viscosity has been found to increase oxygen transfer by reducing surface tension at solid –liquid interface (Brar *et al.* 2005a). Decrease in ST (48 to 37 mN/m) of NHT-30 by Tween-80 also improved oxygen transfer and enhanced availability of nutrients during Bt fermentation. Zouari *et al.* (2002) have also reported the increase in production yield of delta-endotoxins by Bt strains in gruel and fish meal media by surfactant addition.

The viscosity decreased slightly (28 to 25 cP) until 12h and it was followed by a characteristic hump at 15h (Figure2b). The initial decrease in viscosity could be an effect of physical action (agitation). As Bt cell concentration increased, it contributed towards continuous increase in viscosity. On the other hand, prolonged agitation in the fermenter tended to decrease the viscosity due to disruption of sludge flocs and new flocs that might have been generated. Increased viscosity due to increased cell concentration (9-15h) superseded the viscosity decrease due to agitation, causing a net increase to attain the characteristic hump. In the post-hump region (after 15h), the viscosity started decreasing, mainly governed by physical phenomenon (agitation) and also due to pseudoplastic nature of sludges (Brar *et al.* 2005a). Further, after 18h, TC reached a maximum, while sporulation continued. At 24h, both the viscosity decrease and VS stabilized. Hence, viscosity decrease between 15-24 h could be attributed to cell lysis or EPS degradation. The hump appeared later than NHT-25 sludge where it was observed at 9h (Brar *et al.* 2005a) which could be due to lower μ_{max} (0.22) in comparison to NH-25 (μ_{max}=0.24) and hence slower growth.

OUR profile

The profiles of OUR for NH-30 and NHT-30 are presented in Figure 3. Initially, at 3h, OUR was lower for NH-30 owing to lower DO consumption which was in agreement with the literature studies of Bt on conventional glucose medium (Rowe *et al.* 2003). The OUR profile of NH-30 sludge showed a comparatively smaller increase in first 6h and reached maximum at 12h (peak values -1.5 mmol/L.h). Meanwhile, the OUR profile was different from NH-25 where the values were between 0.4-1.8 mmol/L.h (Brar *et al.* 2005a). The moderately lower OUR peak value for NH-30 than NH-25 could be due to the increase in solids concentration and hence limitation in oxygen transfer.

NHT-30 sludge showed a continuous increase in OUR from 3h to 12h and then a continuous decline until the end of fermentation (15 to 48h). The OUR values (2.5-3.6 mmol/L.h between 9-12h were higher than NH-30 sludge that persisted for a longer period of time which could be owing to amelioration in physical properties of process regime and thus better oxygen transfer during exponential phase as explained in earlier sections. However, the OUR was lower than NHT-25 sludge (2-5 mmol/L.h between 3-12h) and TH-25 sludge (2-4 mmol/L.h) which may be attributable to simplicity of the broth (NHT-25 and TH-25) in

terms of rheology and nutrients when compared to NHT-30 where higher SS predominated as a limiting factor in enhancing OUR. Nevertheless, higher OUR in Tween amendments may be due to the surfactant layer of Tween-80 that provided a local charge at the gas bubble surface and repelled adjacent bubbles, inhibiting coalescence and enhancing oxygen transfer. Additionally, the increase in small bubbles increased the interfacial area per unit volume and hence k_La.

Normally, the variations in OUR were in concordance with k_La and some irregularities could be attributed to complexity of the fermentation medium. Thus, addition of Tween-80 increased TC and VS count of NHT-30 sludge and yielded biopesticide with higher potency. Power requirements in terms of agitation rate will also be lower as higher DO was observed at lower agitation rates. Hence, Tween-80 amendment during fermentation could lower the overall cost of the fermentation process by reducing energy consumption.

Particle size profile

The particle size decreased all through the fermentation in both NH-30 and NHT-30 (Figs. 1b and 2b) due to the physical agitation conditions which resulted in break-up of larger sludge flocs. Figs. 4a and b represent the particle size distribution profile of NH -30 and NHT-30 sludge, respectively before and after fermentation where the shift in volume percent of particles from larger (0h) to smaller particle size (48h) was observed. The cumulative frequency curve was less steep with smaller slope and more volume percent of particles i.e. 23% for NHT-30 in comparison to NH-30 (12%, Figure 4a). This suggested that Tween played an important role in increasing the volume percent of particles towards lower particle size region. It was possible that there was steric hindrance resulting from the adsorption of Tween-80 (polymer with long chains), which was soluble in the dispersing medium. Moreover, when sludge particles (flocs) come closer, the solvated chains would interact to prevent irreversible agglomeration. Furthermore, this type of repulsion will play an important role in aqueous formulations by stabilizing them.

Protease and chitinase activity

Figure 5a presents the protease and chitinase activity profiles of Bt fermented NH-30 and NHT-30 sludge. It was observed that protease activity (PA) increased until 30h (1.2 IU/ml)

and then gradually decreased with and without Tween amendment. During early part of exponential phase (0-6h), proteases are secreted to metabolize organic compounds (proteins) which may be related to extracellular proteases. The protease production in the medium towards later stage (6-30h) was to hydrolyse complex proteins in the raw material, which was possibly required for active synthesis of proteins for formation of spore coats and cortex (Sharipova *et al.* 2002). Later stage enzymes may be intracellular proteases which play a pivotal role in Bt metabolism and sporulation and may be adsorbed to spore, crystal protein, SS and extracellular polymers. In fact, *Bacillus* proteases, especially, the intracellular serine protease A (IspA) have been proved to be involved in the formation of spores with the turnover of intracellular proteins with lesser known role in processing of crystal proteins (Chen *et al.* 2003). A decrease in PA after 30h of fermentation (Figure 5a) could be a manifestation of nutrient limitation and denaturation of enzyme (auto-digestion of protease and proteolytic attack by other proteases such as intracellular protease released after cell lysis) (Yezza *et al.* 2005b). However, it remains to be verified whether the cessation of PA towards the end was a function of nutrient limitation and/or protease denaturation or perhaps both. As shown in Figure 5b, PA followed an exponential relationship with total cell count (0-30 h) in concordance with earlier studies at 10-25 g/L SS (Yezza *et al.* 2005 b) as well as by other media, namely, soya and starch industry wastewater (data derived from Brar *et al.* 2005 a,b).

Likewise, the chitinase activity showed same production pattern as proteolytic activity with a peak of 76 U/ml and 57 U/ml for NH-30 and NHT-30 sludge, respectively, at 18h of growth and then it started decreasing (Figure 5a). Meanwhile, unlike protease profile, no exponential relation was observed between chitinase activity and TC. High chitinase activity in the fermented broth is desirable as it will enhance the Tx through synergistic action by aiding in penetration of chitin bound insect midgut epithelial layers (Liu *et al.* 2002). Normally, chitinase production in most of the chitinase producing bacteria is inducible by chitin, chito-oligosaccharides, or even N-acetyl glucosamine (Arora *et al.* 2003). In the present case, the chitinase production could be induced by the presence of fungal cell wall debris and or residual crustacean shells as mentioned earlier. Otherwise, chitinase production could be constitutive. It has been already reported that glucose medium (without chitin) caused single point mutation in the promoter gene of chitinase and shifted the synthesis from inducible to constitutive mode (Arora *et al.* 2003).

Meanwhile, Tween-80 increased the PA and decreased chitinase activity (Figure 5a). In the presence of Tween-80, despite amelioration of medium rheology, the secretion of protease was higher. On the contrary, Zouari *et al.* (2002) observed that Tween amendment of gruel and fishmeal medium during Bt fermentation decreased PA and enhanced the concentration of δ-endotoxins. The situation can be analyzed in two ways. Firstly, PA increased from 0-30h (Figure 5a) which might have aided in sporulation in formation of spore cortex and exosporium (discussed later) and hence Tween-80 would have further assisted in release of proteins from substrates. Secondly, PA decreased (30-48h, Figure 5a) so that PA would not have caused degeneration of crystal proteins (increasing stability or making crystal proteins resistant) and hence Tx increased which was about 80-90 % (Figure 6, discussed later). Thus, Tween amendment in sludge might have increased Tx value due to: a) comparatively lower degeneration of crystals (as discussed above) and; b) higher concentration of δ-endotoxins or other virulence protein factors such as VIPs, chitinases and phospholipases. The decrease in chitinase on Tween amendment could be due to the fact that it is not required in normal metabolic course of Bt metabolism and further the mRNA of this enzyme may be unstable. Feng *et al.* (2003) observed that mannase activity was reduced in presence of different substrate conditions due to lowered mRNA stability. Meanwhile, regardless of the decrease in chitinase activity and constant spore concentration after 15h for both NH-30 and NHT-30 (Figure 5), the Tx increased. This may be attributed to the secretion of other virulence factors (discussed earlier). Tx increase may also be attributed to matured spores, more toxic spores, complete crystal proteins, overexpression of *cry* gene (number of genes may be higher per plasmid which may increase Tx of crystal proteins), enhanced crystal protein structure and overall mode of action (synergy between different virulence factors) in insect midgut.

Entomotoxicity (Tx) profiles

Fermentation Tx Trends

The entomotoxicity (Tx) profiles of NH-30 and NHT-30 during fermentation are presented in Figure 6. The Tx increased from 5842 to 11632 SBU/μL and from 8802 to 16521 SBU/μL for NH-30 and NHT-30 with fermentation period, respectively following a logarithmic law. It was interesting to note that VS count increased exponentially with concomitant increase in Tx value until 24 h and also during the stationary state (24-48h), despite the stabilization of

VS concentration (1.42 x 10^8 and 1.64 x 10^8 CFU/ml for NH-30 and NHT-30, respectively) as seen in Figs. 1b, 2b and Figure6. To recall, generally, the sporulating bacteria undergo seven distinct physiological stages accompanied by biochemical changes to form spores: pre-septation (I), septation (II), engulfment (III), cortex formation (IV), spore coat formation (V), spore maturation (VI) and free spore formation through lysis of the mother cell (VII) (Bulla *et al.* 1980). Crystal protein is primarily formed during the spore engulfment (stage III) and cortex formation (stage IV) stages of the sporulation process and attains full size at time when the exosporium appears as reported by Liu and Tzeng (2000). During growth ($t \leq 24h$), spores were continuously formed (some were released into the medium - free spores and some did not release-bound spores) inside the cell but the process of spore maturation probably continued after 24h releasing crystal proteins and other synergistic compounds in the medium. Thus, the initial Tx increase with VS increase (0-24h) may be contributed to: a) entomotoxicity of partially matured spores and some matured spores which may contain more virulence factors (discussed earlier) and; b) secondary metabolites like VIPs. Later Tx increase, despite VS stabilization (24-48h) was probably a sum of various events: maturation of spores, release of crystal proteins (affecting shape and size, completion or incompletion of structure) into the fermented medium and other secondary metabolites (e.g. antibiotics, enzymes) causing a synergistic effect. If less virulence factors are built-in the proteinic layers of the spore during stage V, the spore will be less entomotoxic. Similarly, if stage VI is curtailed, the subsequent sporulation events contributing to its properties of resistance (spore survival) and increase in insecticidal activity will not occur. These observations were also supported by Bulla (1980) and Aronson (1995) who emphasized that stages IV, V and VI of the sporulation are very significant for maturity of the spore and affect shape and size of the crystal proteins and consequently insecticidal activity (preventing septicemia in insect).

Another important factor to consider is that at the end of fermentation, there was no appreciable difference between VS of NHT-30 and NH-30 (Table 2) yet, the Tx was substantially higher (29.7%) for NHT-30. This may be due to the fact: a) crystal proteins synthesized during NHT-30 fermentation were probably more toxic in comparison to NH-30; b) there was higher production of VIPs and other virulence factors (enzymes/antibiotics) and; c) possible selection of cells containing more plasmids, more *cry* genes per plasmid and/or Bt I and Bt II promoters as reported by Oppert (1999). In this concern, Icgen *et al.* (2002) argued that starch and dextrin, in comparison to other synthetic media, yielded higher levels

of spores (1.4 to 3.7 x 10^{-1} sporulation frequency, ratio of heat resistant spores to viable cells), but relatively low toxin. Moreover, it has been reported in literature that if the gene coding for molecular chaperone responsible for crystal formation is altered which may occur sometimes during sporulation phase due to absence of packaging (virulence) factors in some media, the crystal proteins may be less toxic as the relative amount of Cry protoxin may be lower (Chang *et al.* 2001). Chang *et al.* (2001) also reported that the relative amount of Cry1Da1 protoxin in inclusions was twofold lower when cells were sporulated in Luria-Bertani medium than when cells were sporulated in a glucose-yeast extract medium. Thus, the differences in the initial transcription rates, translation efficiencies, δ-endotoxin composition of an inclusion, and quantitative plasmid composition of cells and overexpression of *cry* genes might have contributed to higher Tx of NHT-30.

Correlation of Tx with VS

The specific Tx (spTx, Tx per 1000 spores) profiles of NH-30 and NHT-30 with VS are presented in Figure 7. The spTx followed power law for NH-30 and NHT-30 of the form: $spTx = a\ (VS)^{b}$ with correlation coefficients (R^2) of 0.99, "a" and "b" are proportionality constants. Greater the value of "b", the spTx will be higher with increasing VS and more pronounced are effects of VS formation on Tx. On the other hand, "a" is a direct measure of spTx at a given VS value. The constants "a" and "b" depended on characteristics of fermentation medium and pre-treatment method (physical and/or chemical). Interestingly, these results were in concordance with other data (Figure 7) on wastewater and wastewater sludge at lower SS concentration (< 30 g/L); pilot plant and soyameal medium (Yezza *et al.* 2004, 2005 a,b; Brar *et al.* 2005a,b).

The spTx decreased rapidly in first 24h when spore concentration increased exponentially (time is denoted on the Figure 7b by dashed line). However, in the later half of fermentation period, spTx decreased less rapidly. In the first 24h, higher percentage of cells produced spores, but they were not released into the medium which tended to decrease the spTx rapidly. Meanwhile, other virulence components, namely, VIPs, enzymes and soluble proteins were released into the medium which contributed to the initial higher spTx. The soluble proteins produced during exponential growth are subsequently used for the synthesis of toxin crystal and other virulence factors, namely, VIPs, chitinases, phospholipases and

antibiotics. Meanwhile, in the later half (24-48h), spores matured, crystal proteins were released and even secondary metabolites were generated contributing cumulatively to net Tx effect causing less rapid decrease in spTx. Thus, it becomes important to measure Tx in supernatant during fermentation to ascertain the quantity of soluble proteins which are toxic and contribute to Tx. Further, the spTx was higher for NHT-30 suggesting that either the crystal protein was more toxic or other virulence components (enzymes and antibiotics) were synthesized in significant concentrations in the medium. If less virulence factors were built in the proteinic layers of the spore during stage V of sporulation, the spores may be less entomotoxic (as discussed earlier).

When the Tx data were plotted versus VS, they did not yield exponential law as observed by Yezza *et al.* (2005b).Thus, it seems that the exponential law was valid only until 25 g/L SS and beyond this concentration, the law was invalid. As described earlier, this could be due to the fact that Tx may not solely be a manifestation of sporulation and crystal protein and is generally a sum of all virulence parameters which may not be produced to a large extent at 30 g/L SS. At increased SS concentration, there was also fermentation stress (low oxygen transfer, high osmotic pressure and high viscosity) that might have caused a difference in spore entomotoxicity and synthesis of other virulence factors.

Further, the maturity of the spore (as discussed earlier) is very important factor of Tx. The slow growth in sludge medium (μ_{max} – 0.3 h^{-1}) when compared to soya (μ_{max} – 0.52 h^{-1}) might have created favourable conditions for spores to reach their full maturity before cellular lysis (Vidyarthi *et al.* 2002) causing higher Tx. Further, Barnabe (2005) showed that ratio of Tx/purified spores (at the end of fermentation, 48h) was 0.03-0.048 in sludge when compared to soyameal (0.013). This suggested that spores were more entomotoxic which along with crystal proteins and other virulence factors gave higher Tx in sludge. In fact, spore-crystal protein mixtures have been found to have great synergistic effect rather than separate (Asano *et al.* 2000; Chang *et al.* 2001). Barnabe (2005) also observed that the ratio Tx/mg soluble proteins after separating spores and crystal proteins from the fermented broth (48h) was 11.5-28 in sludge in contrast to soya (1.5). The soluble proteins comprised VIPs and various enzymes, namely, chitinases, phospholipases present in the soluble phase (supernatant) of the fermented broth which have strong virulence effect and synergize Tx. It has been also reported that the individual *cry* genes are transcribed at different rates in several media,

resulting in unequal amounts of the protoxins in inclusions and also perhaps absence of other cytolytic factors which, if normally bound to the protoxin help in their stability and toxicity (Aronson 1995). Hence, irrespective of the type of medium, fermenter size, pre-treatment or suspended solids concentration, the specific Tx followed power law.

Correlation of Tx with protease activity (PA)

The Tx profiles of NH-30 and NHT-30 with PA are presented in Figure 8. The Tx values were linearly correlated with PA until 30h, irrespective of Tween addition (protease reached maximum concentration at 30h, Figure5a) for NH-30 and NHT-30. When these correlations were extended to other already reported media based on wastewater sludge, there was a strong concordance (Yezza *et al.* 2005 a,b). The crystal protein formation needed higher protein quantity that was probably provided by hydrolysis of complex proteins (mediated by proteases) in the residual complex substrate (sludge). It was observed that at peak PA (30 h), about 80-90% of Tx was already formed in the broth at 30h as also reported earlier (Yezza *et al.* 2004, 2005 a,b). The protease decreased after 30h (Figure 5) and relative Tx increase was about 10-20%. Thus, PA could serve as signature of indirect prediction of Tx of Bt and termination of fermentation. The slight increase of 10-20% in Tx in later stages (30-48h) may be due to prolongation of stages III and IV of sporulation (discussed earlier) so that more complete spores and crystal proteins were released with enhanced functionalities which increased Tx.

Recapitulation and Possible Repercussions

The study suggested that Tween addition increased entomotoxicity (of otherwise complex wastewater sludge medium) at higher SS (30 g/L) as it improved rheology (viscosity, particle size, surface tension); enhanced μ_{max} and OUR. Although sludge pre-treatment (hydrolysis, TH-25) resulted in increase in Tx (18960 SBU/μL) by 49 % when compared to NH-25 (12710 SBU/μL, Brar *et al.*, 2005b) in comparison to 29.7% increase in Tx for NHT-30 (16552 SBU/μL) in comparison to NH-30 (11632 SBU/μL) sludge, yet the effect could be comparable (Table 2). In this light, careful cost economics needs to be done to evaluate the balance between pre-treatment costs; use of higher sludge solids concentration; relative gain of Tx and stable formulation development. It is also possible that Tween fortification of

sludge before fermentation enhances the re-suspension of centrifugate (pellet or thick paste) during downstream processing which otherwise is difficult to re-suspend as the solids usually get compacted creating difficulty in re-suspension (Brar *et al.* 2006).

Moreover, when Tween-80 amended sludge fermented broth was used directly for formulation development, the dispersibility values (70-90%) were similar to that of the fermented product (65-93%) where Tween-80 was amended (after Bt fermentation) as an adjuvant during formulation development (Brar *et al.* 2004). Thus, addition of Tween to sludge before fermentation, as stated earlier will serve various functions: a) enhance oxygen transfer rate and hence entomotoxicity during fermentation and; b) as an aid to re-suspend thick paste and pellet after centrifugation; c) as a dispersant (wetting agent) in the formulated product; d) solids can act as carriers during spray drying for development of powders; and e) minimize risks of contamination due to adjuvant addition later in formulations. The development of Bt dry powders usually requires silica and other bulky materials as carriers to protect Bt components from heat during drying as well as enhancing stability of dry formulations (Burges 1998).

Furthermore, NHT-30 fermented broth during centrifugation may give better Tx recovery as presence of higher solids will aid in adsorption of different Tx components on solids and hence efficient recovery. The NHT-30 formulation may also enhance UV resistance in the field due to presence of higher solids with moderately larger particle size that may protect spores and crystal protein from degradation and higher concentration of chromophores (humic and fulvic acids; UV absorbers) when compared to NHT-25 and TH-25 sludge. Globally, bioprocessing of raw sludge at higher solids concentration by using Tween-80 as medium amendment agent would aid in handling of higher solids, decrease in waste and global carbon sequestration (mitigating greenhouse emissions). This will further assist in the noble venture of sustainable waste management.

Conclusions

From the aforementioned studies, following conclusions can be drawn:

1. Higher entomotoxicity (29.7 %) and cell/spore concentration was observed in Tween amended non-hydrolyzed sludge vis-à-vis non-hydrolyzed sludge at higher suspended solids concentration.

2. Addition of Tween-80 to non-hydrolyzed sludge at even higher solids increased k_La, cell and spore concentration, Tx, maximum specific growth rate and oxygen uptake rate when compared to unamended sludge.
3. Protease and chitinase activity declined in the presence of Tween-80 and the entomotoxicity increased.
4. The entomotoxicity, irrespective of the type of medium, fermenter size, pre-treatment or suspended solids concentration followed power law when specific entomotoxicity was plotted against spore concentration and the relation between entomotoxicity and protease activity was linear.
5. Increase in Tx during growth and non-growth period was important for overall biopesticidal activity of Bt.
6. The entomotoxicity was related linearly with the protease activity and at peak value of protease (30h), about 80-90% of entomotoxicity was expressed.

Abbreviations: Bt, *Bacillus thuringiensis*; DO, dissolved oxygen; EPS, extracellular polymeric substances; k_La, volumetric mass transfer coefficient; μ_{max}, maximum specific growth rate; NAG, N-acetyl glucosamine; NH-25, non-hydrolyzed sludge at 25 g/L SS; NH-30; non-hydrolyzed sludge at 30 g/L SS; NHT-25; Tween amended non-hydrolyzed sludge at 25 g/L SS; OUR, oxygen uptake rate; PA, protease activity; spTx, specific entomotoxicity (Tx/1000 spores); SS, suspended solids; ST, Surface tension; TC, Total cells; Tx, entomotoxicity; TH-25, Hydrolyzed sludge at 25 g/L SS; TH-30, Hydrolyzed sludge at 30 g/L SS; THT-25, Tween amended hydrolyzed sludge at 25 g/L SS; THT-30, Tween amended hydrolyzed sludge at 30 g/L SS; VS, viable spores.

ACKNOWLEDGEMENTS

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STR 202047); Canada Research Chair; University of Missouri, Columbia and U.S. EPA. The views and opinions expressed in this article are those of authors and should not be construed as opinions of the U.S. Environmental Protection Agency. The authors also thank Dr. Simon Barnabé for reading the manuscript and providing valuable suggestions in the preparation of manuscript. The authors are also thankful to Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Services and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing Ph.D scholarship to Satinder K. Brar during the course of this research work.

REFERENCES

Aiba, S., Humphrey, A.E. & Millis, N.F. 1973. Biochemical Engineering Academic Press, 2nd edition, New York.

APHA, AWWA, WPCF. 1998. Standard Methods for Examination of Water and Wastewaters. 20th edn. (Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. eds.), American Public Health Association, Washington, DC, USA.

Aronson, A.I. 1995. The protoxin composition of *Bacillus thuringiensis* insecticidal inclusions affects solubility and toxicity. *Applied Environmental Microbiology* **61,** 4057–4060.

Arora, N., Ahmad, T. Rajagopal, R. & Bhatnagar, R.K. 2003. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis* HD-1. *Biochemical Biophysical Research Communications* **307**, 620–625.

Asano, S., Ogiwara, K., Indrasith, L.S., Takahashi, M., Suzuki, N. & Hori, H. 2000. Synergism of spore on insecticidal activity of δ-endotoxin of *Bacillus thuringiens*is against the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae) is not observed at late stage in bioassay. *Applied Entomology Zoology* **35** (4), 583-590.

Barnabé, S. 2005. Hydrolyse et oxydation partielle des boues d'épuration comme substrat pour produire *Bacillus thuringiensis* HD-1. Ph.D thesis, Institut National de la Recherche Scientifique, Eau, Terre et Environnement, Université du Québec, Québec, Canada.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. & Banerji, S. 2004. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations.*Water Science and Technology* **50**(9), 229-236.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. & Surampalli, R.Y. 2005a. Sludge based *Bacillus thuringiensis* biopesticides: Viscosity Impacts. *Water Research* **39**, 3001-3011.

Brar, S.K., Verma, M., Barnabé, S., Tyagi, R.D., Valéro, J.R. & Surampalli, R.Y. 2005b. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges. *Process Biochemistry* **40**, 2695–2705.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. & Surampalli, R.Y. 2006. Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge. *Water Research* **40**, 1310-1320.

Bulla, L.A., Bechtel, D.B., Kramer, Y.I., Shethna, Y.I., Aronson, A.I. & Fitz-James, P.C. 1980. Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*. *CRC Critical Reviews in Microbiology* **8**, 174-204.

Chang, L., Grant, R. & Aronson, A. 2001. Regulation of the packaging of *Bacillus thuringiensis* δ-endotoxins into inclusions. *Applied Environmental Microbiology* **67**(11), 5032-5036.

Chen, Fu-Chu, Shen, Li-Fen, Tsai, Ming-Chu & Chak, Kin-Fu. 2003. The IspA protease's involvement in the regulation of the sporulation process of *Bacillus thuringiensis* is revealed by proteomic analysis. *Biochemical Biophysical Research Communications* **312**, 708–715.

Feng, Y., Heb, Z., Onga, S.L., Hua, J., Zhang, Z. & Nga, W.J. 2003. Optimization of agitation, aeration, and temperature conditions for maximum β-mannanase production. *Enzyme Microbiology Technology* **32**, 282–289.

Içgen, Y., Içgen, B. & Özcengiz, G. 2002. Regulation of crystal protein biosynthesis by *Bacillus thuringiensis*: II. Effects of carbon and nitrogen sources. *Research Microbiology* **153**, 605–609.

Jordan, R.N., Nichols, E.P. & Cunningham, A.B. 1999. The role of biosurfactant sorption on promoting the bioavailability of nutrients localized at the solid-water interface. *Water Science and Technology* **39**(7), 91-98.

Kunitz, M. 1947. Crystalline soybean trypsin inhibitor. *Journal of General Physiology* **30,** 291-310.

Lachhab, K., Tyagi, R.D. & Valéro, J.R. 2001. Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids concentration. *Process Biochemistry* **37**, 197-208.

Li, G.Q., Qiu, H.W., Zheng, Z.M., Cai, Z.L. & Yang, S.Z. 1995. Effect of fluid properties on mass transfer in a bioreactor. *Journal of Chemical Technology and Biotechnology* **62**, 385-391.

Liu, C.M. & Tzeng, Y.M. 2000. Characterization study of the sporulation kinetics of *Bacillus thuringiensis*. *Biotechnology and Bioengineering* **68**(1), 11-17.

Liu, M., Cai, Q.X., Liu, H.Z., Zhang, B.H., Yan, J.P. & Yuan, Z.M. 2002. Chitinolytic activities in *Bacillus thuringiensis* and their synergistic effects on larvicidal activity. *Journal of Applied Microbiology* **93**, 374–379.

Oppert, B. 1999. Protease interactions with *Bacillus thuringiensis* insecticidal toxins. *Archives of Insect Biochemistry and Physiology* **42**, 1-12.

Ribbons, D.W. 1969. Automatic assessment of respiration during growth in stirred fermentors. *Applied Microbiology* 438-443.

Rowe, G.E., Margaritis, A. & Wei, N. 2003. Specific oxygen uptake rate variations during batch fermentation of *Bacillus thuringiensis* subspecies *kurstaki* HD. *Biotechnological Progress* **19**, 1439-1443.

Sharipova, M.R., Balaban, N.P., Gabdrakhmanova, L.A., Shilova, M.A., Kadyrova, Y.M., Rudenskaya, G.N. & Leshchinskaya, I.B. 2002. Hydrolytic Enzymes and Sporulation in *Bacillus intermedius*. *Microbiology* **71**, 420–424.

Tirado-Montiel, M.L., Tyagi, R.D. & Valéro, J.R. 2001. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticide. *Water Research* **35**(16), 3807–3816.

Ueda, M. & Arai, M. 1992. Purification and some properties of chitinase from *Aeromonas* sp. No. 10S-24. *Bioscience Biotechnology and Biochemistry* **56**, 460–464.

Vidyarthi, A.S., Tyagi, R.D. & Valéro, J.R. 2001. Effect of surface active agents on the production of biopesticides using wastewater sludge as a raw material. *Water Science and Technology* **44**(10), 253–259.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R. & Surampalli, R.Y. 2002. Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as a raw material. *Water Research* **36**(19), 4850-4860.

Yezza, A., Tyagi, R.D., J.R. & Surampalli, R.Y. 2004. Scale-up of biopesticide production process using wastewater sludge as a raw material. *Journal of Industrial Microbiology and Biotechnology* **31**, 545-552.

Yezza, A., Tyagi, R.D., J.R. & Surampalli, R.Y. 2005a. Production of *Bacillus thuringiensis* based biopesticides in batch and fed cultures using wastewater sludge as a raw material. *Journal of Chemical Technology and Biotechnology* **80**, 502-510.

Yezza, A., Tyagi, R.D., J.R. & Surampalli, R.Y. 2005b. Correlation between entomotoxicity potency and protease activity produced by *Bacillus thuringiensis var. kurstaki* grown in wastewater sludge. *Process Biochemistry* **41**, 794-780.

Zouari, N., Achour, O. & Jaoua, S. 2002. Production of delta-endotoxin *by Bacillus thuringiensis* subsp *kurstaki* and overcoming of catabolite repression by using highly concentrated gruel and fish meal media in 2 and 20dm^3 fermenters. *Journal of Chemical Technology and Biotechnology* **77**, 877–882.

Table 1. Characteristics of raw secondary CUQ sludge

Parameter	Concentration	Parameter	Concentration
Total solids (TS) (g/L)	22.5±1.1	Ca	15238±551
Total volatile solids (TVS) (g/L)	15±1.1	Cd	0.27±0.1
Suspended solids (SS) (g/L)	17.0±1.3	Cr	31±3.4
Volatile suspended solids (VSS) (g/L)	12.7±1.5	Cu	203±87.7
Total Carbon	301202±2056	Ni	18.4±5.3
Total nitrogen	43280±289	Fe	11789±768
Total phosphorus	7658±302	K	8976±1324
$N\text{-}NH_3$	892±121	Pb	24.8±3.8
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$	18±2.3	S	5021±459
$P\text{-}PO_4^{3-}$	4987±238	Zn	264±151
Al	5023±428	Na	1332±697.4

All concentrations are in mg/kg, unless stated otherwise.
± represents standard error.

Table 2. Comparative physical parameters and potency of fermented sludge

Time (h)	Parameter(s)	NH-30	NHT-30
36	Viscosity (cP)	17.4	18.6
	D_{50} (µm)	5.2	2.63
	Tx (SBU/µL)	10789 (74.4)	15598 (82.1)
	TC (CFU/ml)	2.8×10^8	4.9×10^8
	VS (CFU/ml)	1.45×10^8	1.9×10^8
48	Viscosity (cP)	15.43	13.7
	D_{50} (µm)	5.8	2.38
	Tx (SBU/µL)	11632 (75)	16552 (82.6)
	TC (CFU/ml)	2.9×10^8	4.7×10^8
	VS (CFU/ml)	1.55×10^8	2×10^8
	Maximum Specific growth rate† (μ_{max},h^{-1})	0.17	0.22

Parameters ††	**NH-25**††	**TH-25**††	**NHT-25**††	**THT-25**††	**TH-30**††
TC (CFU/ml)	5×10^8	3.97×10^8	1.15×10^9	9.5×10^8	3.97×10^8
VS (CFU/ml)	3×10^8	3.93×10^8	1.13×10^9	9.39×10^8	3.93×10^8
Tx (SBU/µL)	12710(42.3)	18960(48.2)	16090(14.23)	19021(20.25)	19000 (48.3)
μ_{max} (h^{-1})	0.19 h^{-1}	0.28	0.24	0.30	0.43

† Calculated from the straight line slope in the exponential phase

Digits in parentheses represent the entomotoxicity, $SBU/10^3$ spores

The standard deviation has been already incorporated in the final values.

††Shaded cells present the data derived from earlier studies (Barnabe, 2005; Brar *et al.*, 2005b).

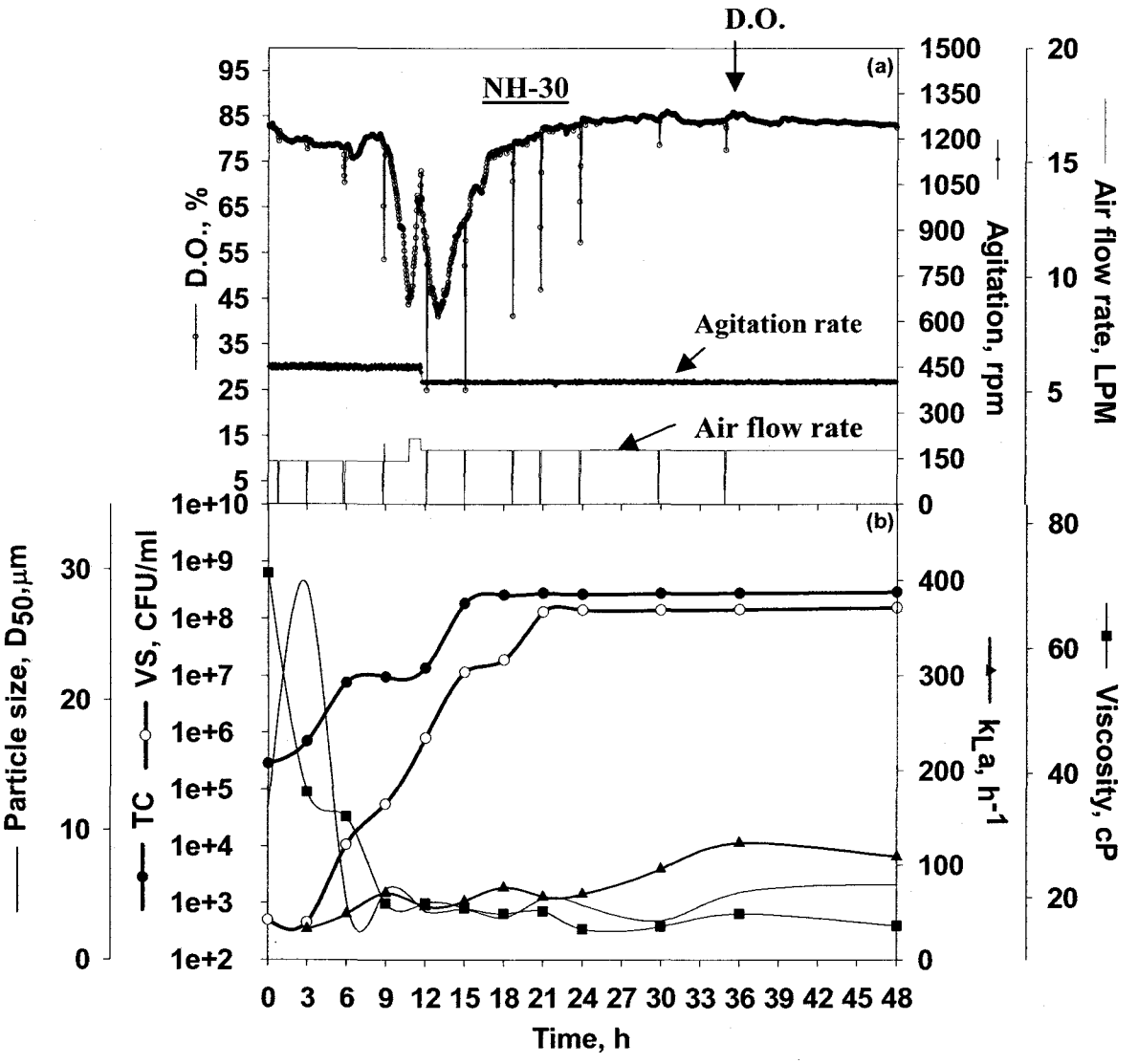


Figure1. Bt fermentation profiles (15L fermenter) of non-hydrolyzed sludge (without Tween-80):a) Operational parameters and; b) Growth parameters.

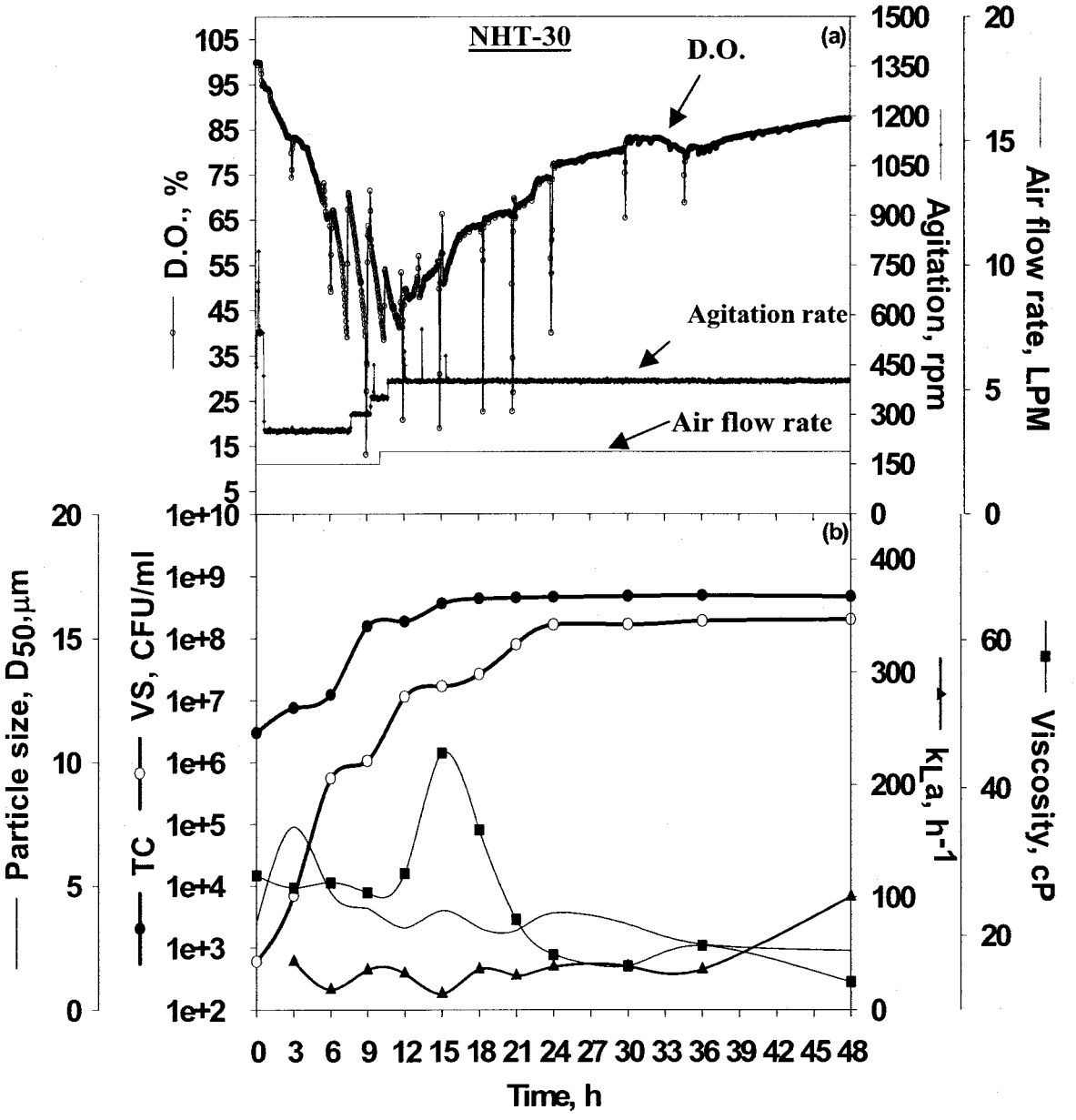


Figure 2. Bt fermentation profiles (15 L fermenter) of non-hydrolyzed sludge (with Tween-80): a) Operational parameters and; b) Growth parameters.

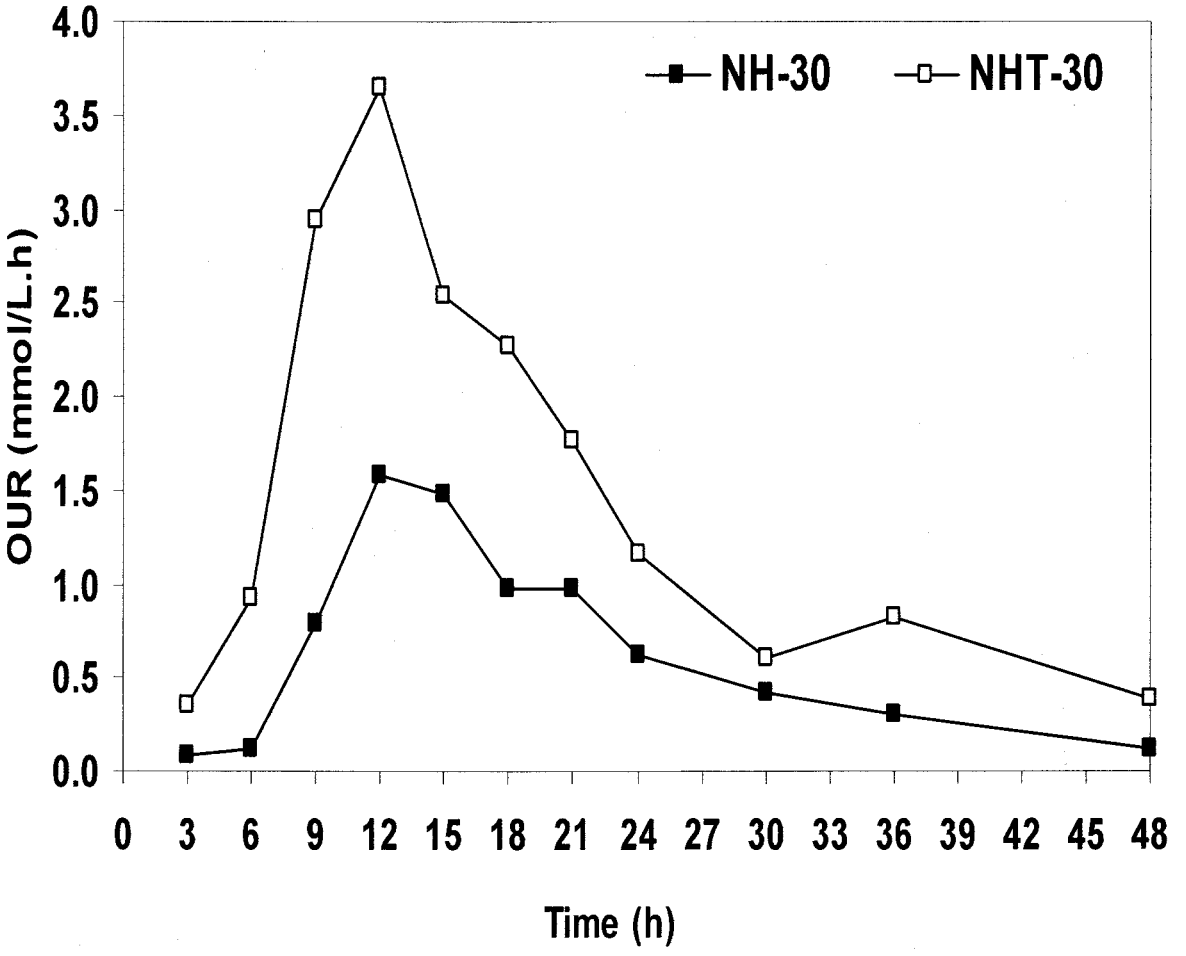


Figure 3. OUR profile of non-hydrolyzed sludge (with and without Tween-80) at SS=30 g/L.

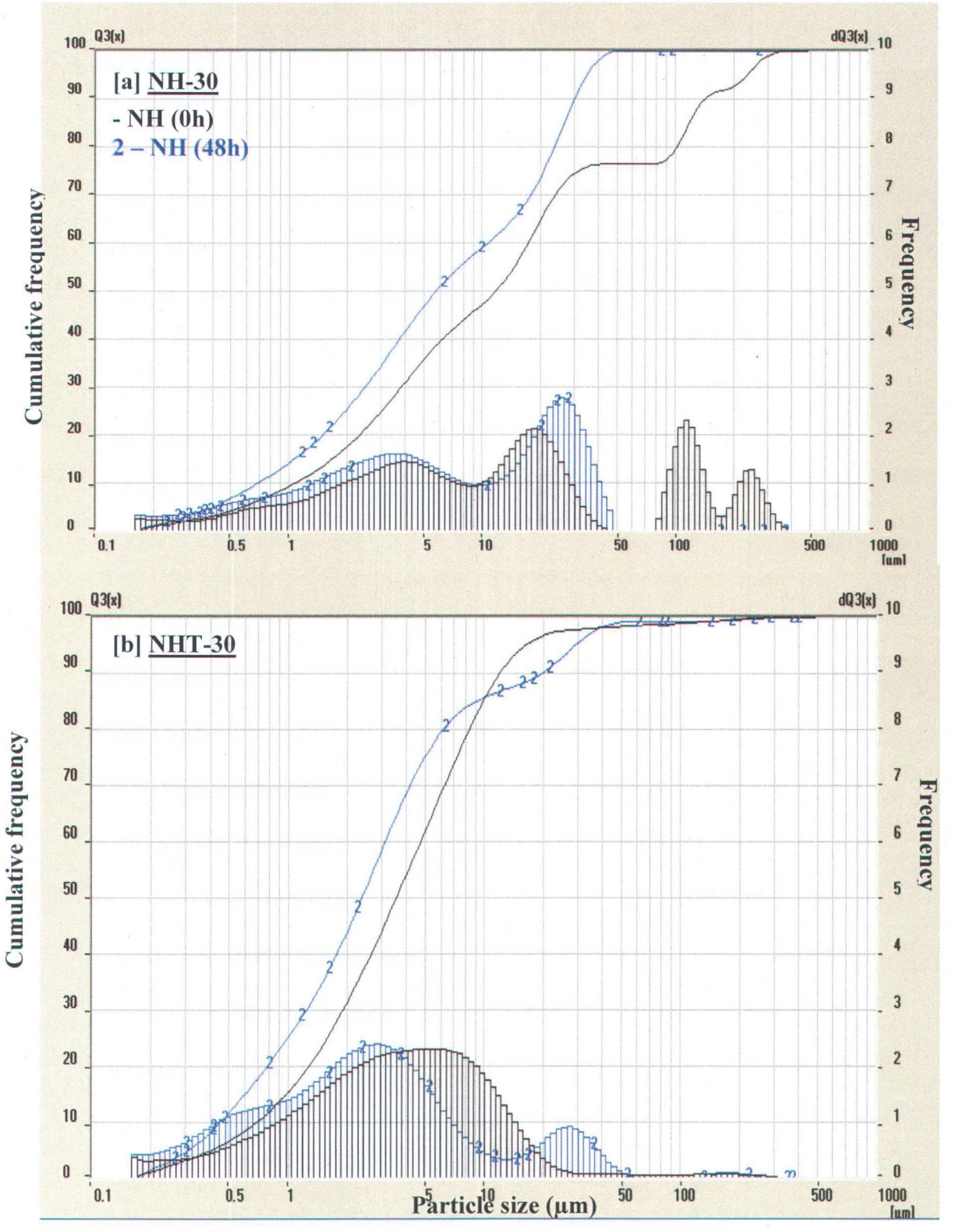
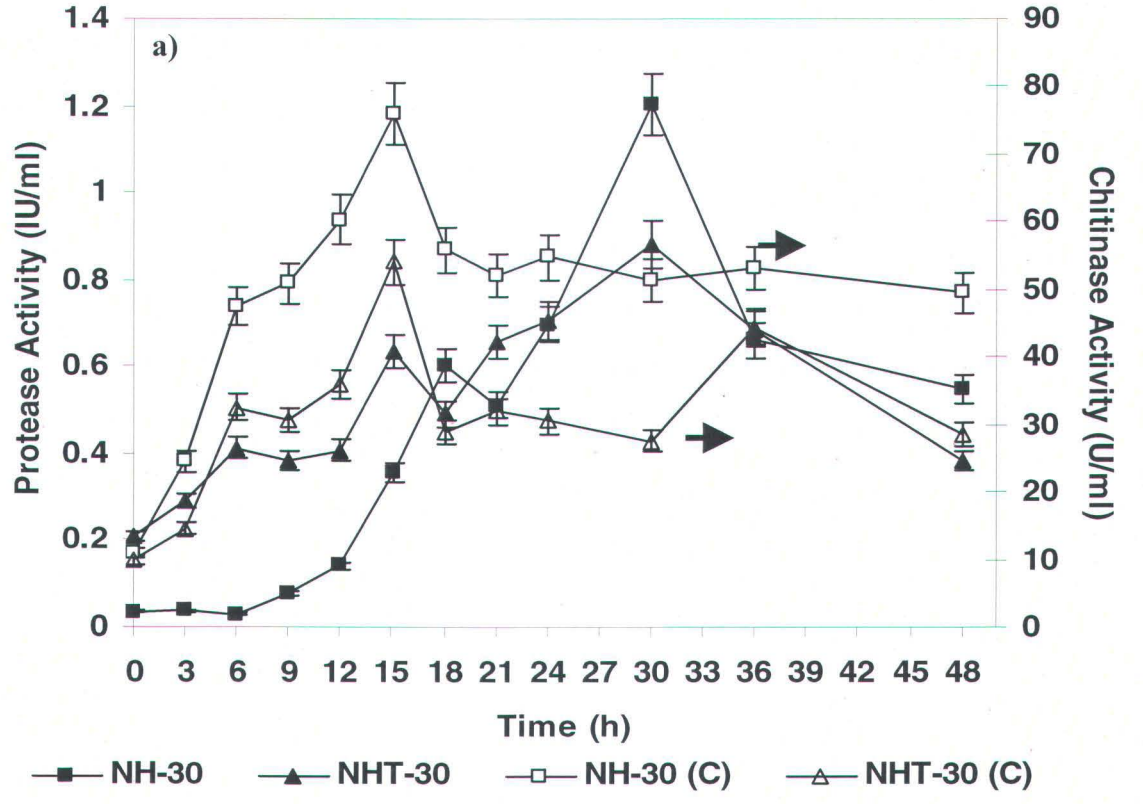


Figure 4. Comparison of particle size distribution before and after fermentation for non-hydolyzed sludge; [a] without Tween-80 addition and [b] with Tween-80 addition.



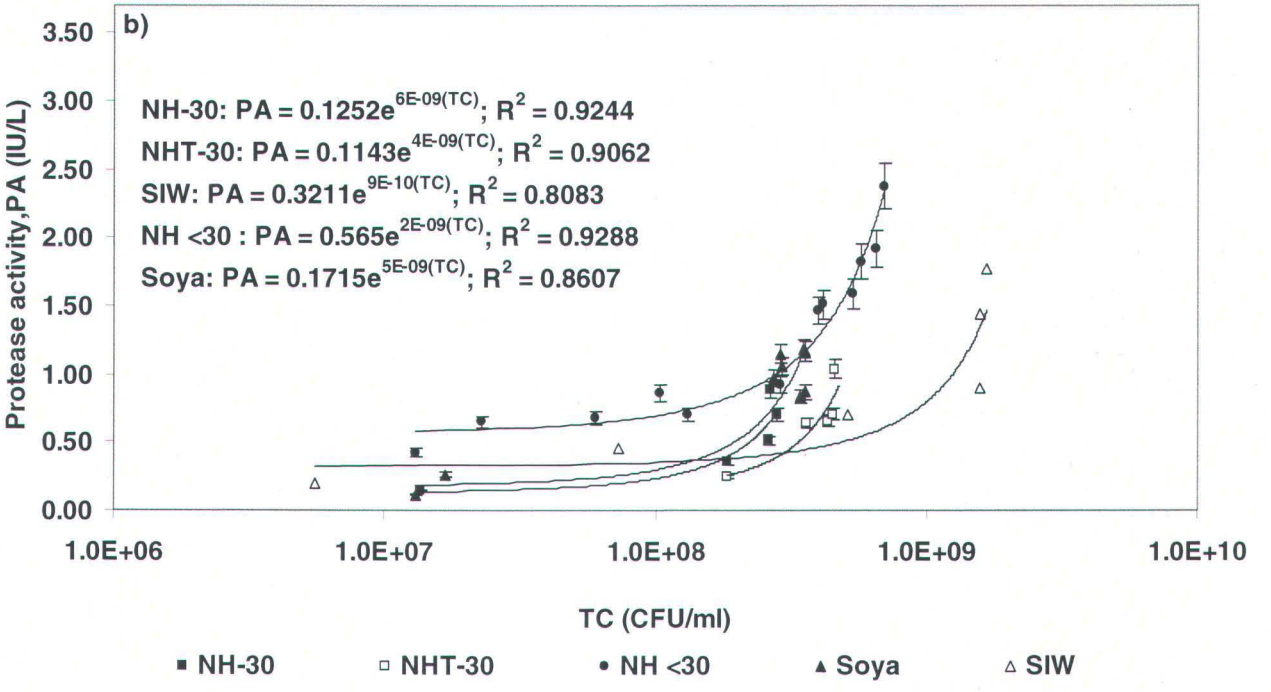


Figure 5. a) Chitinase and protease activity profiles of non-hydrolyzed Bt fermented broths (with and without Tween-80) and; b) correlation of protease activity with TC. "C" in parentheses represents chitinase activity.

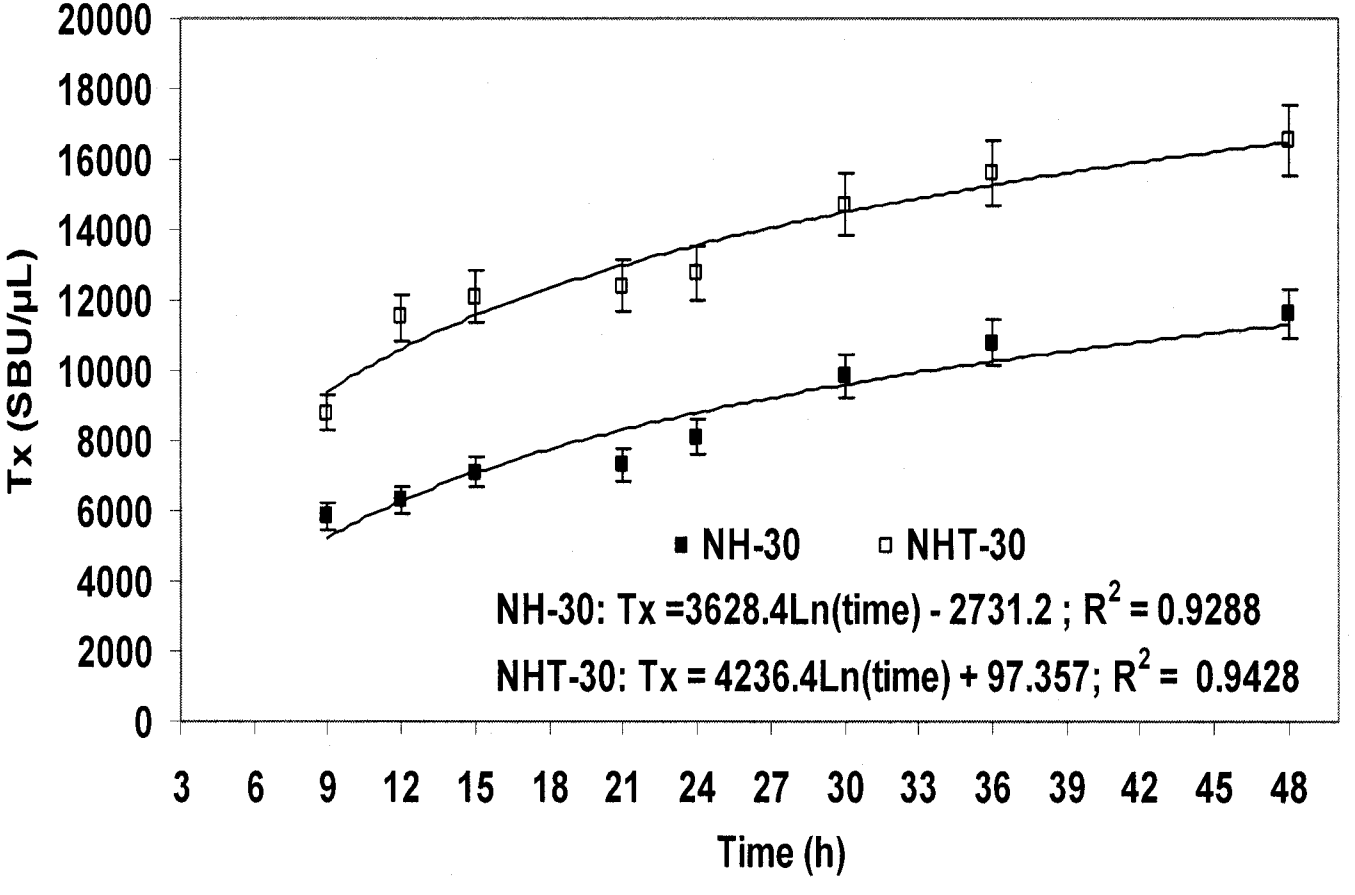


Figure 6. Entomotoxicity profiles of NH-30 and NHT-30 with fermentation time. The number after the hyphen denotes the suspended solids concentration.

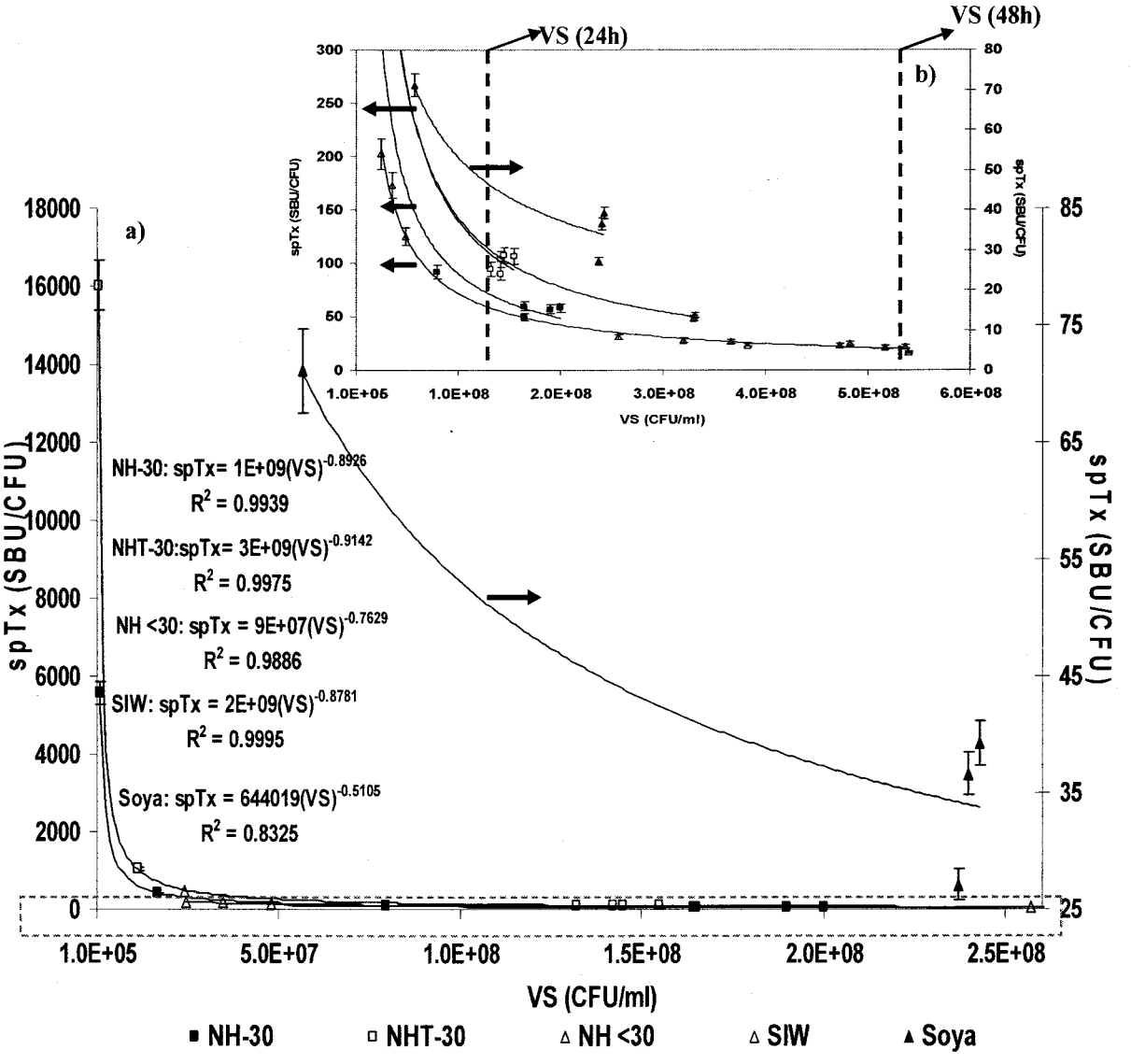


Figure 7. a) Correlation profiles of spTx (Tx/1000 spores) with VS (inset "b" shows higher resolution of the dotted area). The data represented as NH<30 have been derived from Yezza *et al.* (2004, 2005 a,b) and comprises NH -15, NH-20, NH-25 and NH-25-150L data. The starch industry wastewater (SIW) was derived from Brar *et al.* (2005a,b). The number after the hyphen denotes the suspended solids concentration.

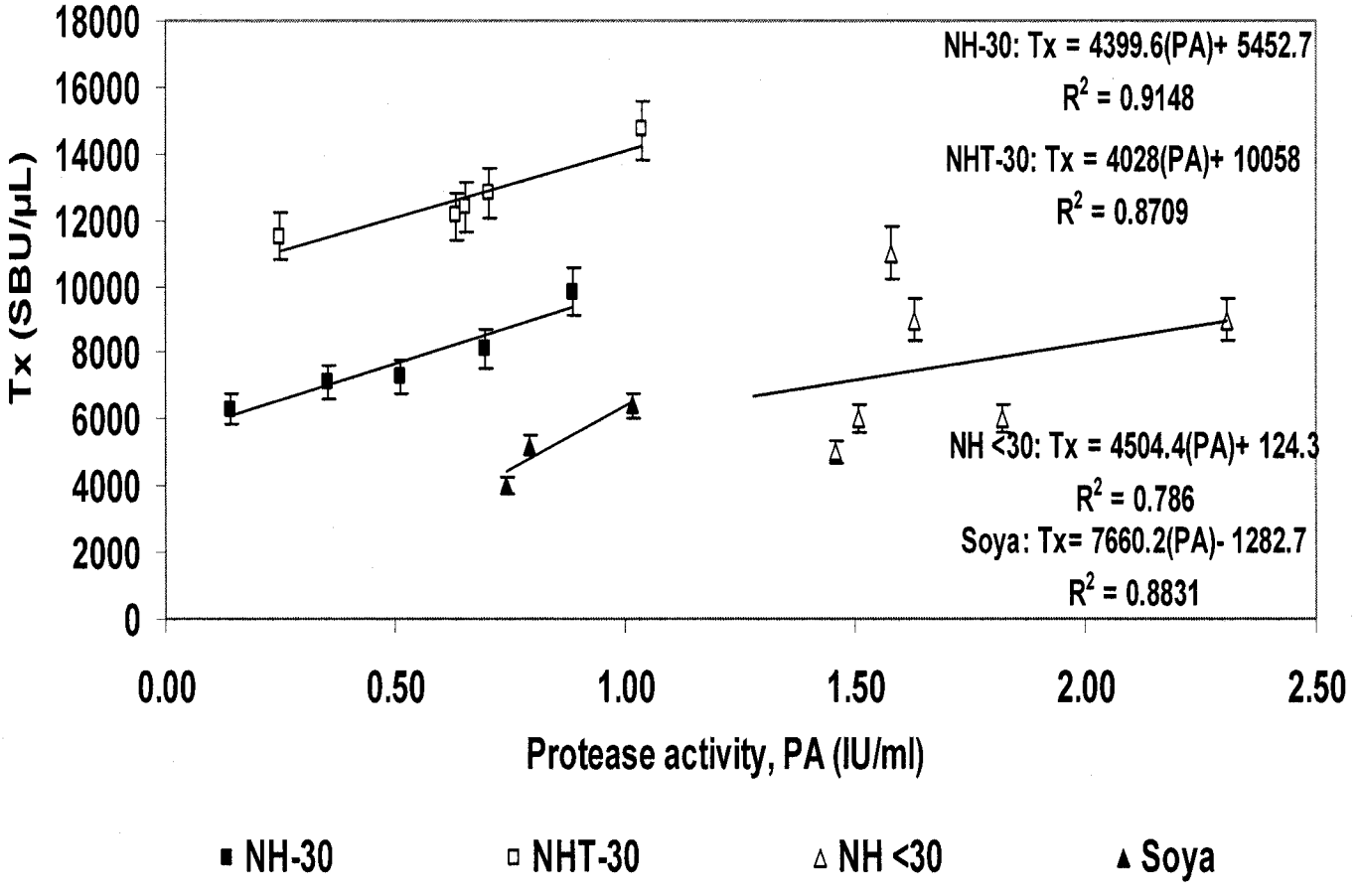


Figure 8. Correlation profiles of Tx with protease activity. The number after the hyphen denotes the suspended solids concentration.

CHAPITRE 5.

PROCÉDÉS EN AVAL DES BOUILLONS FERMENTÉS PAR Bt

Partie I

Efficient Centrifugal Recovery of *Bacillus thuringiensis* Biopesticides from Fermented Wastewater and Wastewater Sludge

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*], J.R. Valéro[1] and R.Y. Surampalli[2]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Water Research (2006)

40:3010-3020

Fermentation des eaux usées et des boues d'épuration par *Bacillus thuringiensis* : Récupération de l'entomotoxicité par centrifugation des bouillons fermentés

Résumé

Des études ont été entreprises sur les possibilités d'augmenter l'entomotoxicité par centrifugation des bouillons fermentés des eaux usées de l'industrie d'amidon (SIW) ou des boues d'épuration (brutes -NH et hydrolysées –TH, respectivement) par le *Bacillus thuringiensis* (Bt) en comparaison avec le milieu semi-synthétique à base de soya. Des facteurs influençant l'entomotoxicité (Tx) tels que la concentration en solides, le pH, la température et la force centrifuge ont été étudiés. La concentration par centrifugation à environ 100 g/l de solides n'a pas augmenté la Tx. L'efficacité de la centrifugation (récupération de Tx) était plus élevée à un pH 4 et à une température 20 °C pour les bouillons fermentés des SIW (98 %), pour les boues d'épuration (98 et 97,8 % pour non-hydrolysées et hydrolysées, respectivement) et pour le bouillon issu de la fermentation du milieu à base de soya (83 %). Pour une récupération maximale de l'entomotoxicité (SIW – 95% ; NH – 90% ; le TH – 98% et le soya – 78 %), la force centrifuge et le temps requis étaient respectivement de 48000 g et de 30 minutes. Les pertes dans l'efficacité de récupération étaient inférieures pour SIW et les boues d'épuration par rapport au milieu à base de soya avec la force centrifuge normalement utilisée commercialement (9000 g). La détermination des vitesses de sédimentation pour différents bouillons fermentés ont permis d'établir le facteur Σ dans le cas d'une centrifugation commerciale continue à une capacité donnée et par conséquent de simuler les besoins en demande électrique. Il a été ainsi établi que la demande en alimentation électrique pour une récupération efficace de l'entomotoxicité était plus élevée pour le milieu conventionnel à base de soya par rapport aux autres bouillons ayant fait l'objet de cette étude.

Mots-clés: *Bacillus thuringiensis*, centrifugation, entomotoxicité, eaux usées/boues d'épuration, facteur de Sigma, surnageant

Abstract

Studies were conducted on harvesting of *Bacillus thuringiensis* (Bt) based biopesticides from fermented broths of starch industry wastewater (SIW), wastewater sludge (raw and hydrolyzed – NH and TH, respectively) and semi–synthetic soyameal to enhance entomotoxicity by centrifugation. Pertinent factors influencing entomotoxicity (Tx) - solids concentration, pH, temperature and centrifugal force were investigated. The centrifugate solids concentration beyond 100 g /l did not enhance Tx, instead caused pellet formation. Centrifugation efficiency (Tx recovery) was higher at pH 4 and temperature 20°C for starch wastewater (98 %), wastewater sludge (98 and 97.8 % for non-hydrolyzed and hydrolyzed, respectively) and soya broth (83 %). For maximum entomotoxicity recovery (SIW - 95%; NH – 90%; TH – 98% and soya – 78%), the centrifugal force and time required was 48000 g and 30 min, respectively. Losses in recovery efficiency were lower for SIW and wastewater sludge in comparison to soya on adopting commercially recommended centrifugal force of 9000 g. The settling velocity computations for different fermented broths enabled calculation of Σ factor for continuous commercial centrifuge of a given capacity and hence simulation of power requirements. It was established that power requirements for a given entomotoxicity recovery efficiency were highest for conventional medium (soya) in comparison to other waste based fermented broths.

Keywords: *Bacillus thuringiensis*, Centrifugation, Entomotoxicity, Wastewater/wastewater sludge, Sigma factor, Supernatant.

1. Introduction

Bacillus thuringiensis (Bt) are spore forming, soil inhabiting, Gram-positive bacteria widely used in agriculture, forests and public health sector as biopesticides. This microorganism is characterized by the production of crystal-shaped parasporal inclusion bodies having known insecticidal activities (Agaisse and Lerecluse, 1996). There have been extensive studies carried out on improvement of the biological efficacy of Bt based biopesticides by utilizing alternative substrates and cost-effective additives for formulations to enhance entomotoxicity (Tirado-Montiel et al., 2001; Zouari et al., 2002).

Of all the pursuits to achieve good yield of biopesticide production, wastewater sludge as a potential substrate has come out to be an economically feasible option (Sachdeva et al., 2000). Wastewater sludge is considered a global problem and its sustainable management via value-addition is an alternative efficacious route to other disposal options, namely, landfilling and incineration. Thus, Bt based biopesticide production from sludge or wastewater would be a resourceful clean-up option as well as augmentation path for commercialization of economical biopesticides. Biopesticide production from starch industry wastewater (SIW) and wastewater sludge (WWS) has been extensively researched from process point of view-pre-treatment (improvement of substrate complexity), medium amelioration agents (enhancing nutrient assimilation), optimizing process parameters, scale-up and formulation development studies (Lachhab et al., 2001; Tyagi et al., 2001; Vidyarthi et al., 2002; Brar et al., 2004; Yezza et al., 2004, Barnabé et al., 2005).

The penultimate step in production of biopesticides is recovery of broth components (crystal protein, spores, vegetative insecticidal proteins, enzyme systems like proteases, chitinases, phospholipases) in a judiciously productive manner to develop formulations henceforth (Bernhard and Utz, 1993; Rowe and Margaritis, 2004). In earlier studies on downstream processing of biopesticides, lactose-acetone technique was used for spore and crystal toxin recovery, this method was highly laborious and low yielding (Dulmage, 1970). This paved way to the centrifugation techniques with an extra step of spray drying to obtain dry powder for solid formulations (Zamola et al., 1981; Rojas et al., 1996; Teera-Arunsiri et al., 2003). Lately, several advances have taken place in the field of downstream processing techniques for biopesticide recovery. However, use of advanced methods like ultrafiltration,

microfiltration and vacuum filtration to separate insoluble solids (active ingredients) from the soluble liquid (inert) fraction of the harvest liquor still remains a cost-intensive option (Gulati et al., 2000) and hence centrifugation could be used as a simple, cost-effective and versatile technique.

Moreover, centrifuges have been used commercially for recovery of Tx components from Bt fermented synthetic medium (no reported data), but there are no existing studies on centrifugation of Bt fermented wastewater and wastewater sludge. Furthermore, no data exists on exact Tx losses during centrifugation of Bt fermented synthetic media. At this juncture, study on optimization of centrifugation technique is necessary to concentrate Bt fermented SIW and WWS.

Thus, the present study investigates the factors affecting centrifugation performance (solids concentration, pH, temperature, centrifugal force) to recover Tx from fermented SIW, WWS and semi-synthetic commercial medium to utilize the broth further either for spray drying (powder) or liquid formulations directly.

2. Materials and Methods

2.1 Bacterial strain

Bacillus thuringiensis var. *kurstaki* HD-1 (ATCC 33679) was used in this study. The culture conditions, maintenance, inoculum production, and fermentation (biopesticide production) are described elsewhere (Vidyarthi et al., 2002).

2.2 Bt production media

During the course of this study, three different media were used for Bt growth: conventional synthetic medium based on soybean meal (control) that comprised (g/l): soybean meal, 15.0; glucose, 5.0; starch, 5.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.02; $CaCO_3$, 1.0; secondary sludge (WWS) obtained from Communauté Urbaine du Québec wastewater treatment plant at Ste-Foy, Québec and starch industry

wastewater (SIW) from ADM-Ogilvie (Candiac, Québec, Canada). The WWS and SIW were immediately utilized (within 1-2 weeks) for fermentation as long term storage even at 4°C would lead to deterioration (slow endogenous respiration of microbes).

2.3 Characterization of wastewater (WW)/wastewater sludge

Total solids (TS), total volatile solids (TVS), suspended solids (SS) and volatile suspended solids (VSS); ammonia nitrogen ($N\text{-}NH_4^+$); pH; total Kjeldahl nitrogen (TKN); total phosphorus; metals concentration (Al, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb and Zn) were determined according to Standard Methods (APHA et al., 1998). Physico-chemical characteristics of the WW (wastewater) and WWS are presented in Table 1.

2.4 Solids amendment and pre-treatment procedure

The sludge was concentrated from approximately 1.6 % to 5 % (w/v) suspended solids by gravity settling followed by centrifugation at 7650 g for 15 minutes at 20 ± 1 °C in a Sorvall RC 5C plus Macrocentrifuge. The sludge supernatant was stored in the refrigerator at 4 ± 1°C and used to dilute the sludge samples as per requirements. The concentrated sludge was homogenized using a Waring blender for different solids concentration.

For steam hydrolysis, 5 % w/v solids concentration sludge (considering 1.67 times dilution by steam; empirically obtained factor) was transferred into a custom made 15 L mechanical steam hydrolyzer (stainless steel (SS 316L) with superheated steam injection facility; working volume: 10 L with controlled agitation; EBR Quebec, Canada). The sludge was hydrolyzed at 140 ± 1°C for 30 minutes at a pressure of 40 psig (Barnabé et al., 2005). Raw and hydrolyzed sludge were referred to as NH and TH, respectively.

2.5 Fermenter set-up

A stirred tank 15 L fermenter (Biogenie Inc., Quebec) equipped with accessories, computer coupled programmable logic controller (iFix 3.5, Intellution) for dissolved oxygen, pH, anti-foam, impeller speed, aeration rate, and temperature was utilized for fermentation as per the procedure detailed in Brar et al. (2005). The volumetric mass transfer coefficient (k_La) was measured by dynamic gassing out method (Aiba et al. 1973).

2.6 Harvesting Techniques

2.6.1 Post-fermentation step

Temperature of fermented broths (NH, TH, SIW and soya) was gradually lowered from 30 ± 1 to 25 ± 1°C. Individually sterilized 4M H_2SO_4 and 4M NaH_2PO_4 (in a 1:1 ratio) were utilized to lower the pH from 7 ± 0.1 to 4.5 ± 0.1 via automated peristaltic pumps integrated into the fermentation set-up. Moreover, addition of NaH_2PO_4 served as a buffering agent (usually added to formulated products). Later, the broth was collected aseptically in sterile HDPE containers (12 l capacity, VWR Canlab, Canada), sealed with Parafilm™ and stored in a freezing chamber (DuPont, USA) at -20°C until use for formulation.

2.6.2 Optimization of solids concentration, pH and temperature

The fermented broth (frozen broth was brought to room temperature) was centrifuged at 9000 g at 20 ± 1°C and pH 4.0± 0.1 to attain solids concentrations of 25, 40, 60, 70, 100, 120, 140 160 g /l and corresponding viable spore (VS) and entomotoxicity (Tx) was measured for each of the fermented concentrated broths (NH, TH, SIW and soya).

A portion of fermented broth was adjusted to different pHs (4, 5, 7 and 8.5) and concentrated to 70 g/l to study effects of pHs on centrifugation at 20 ± 1 °C and 9000 g. Particle size of the broth at different pH was measured by LASER diffraction technique (Brar et al., 2004). For temperature studies, another portion of the fermented broth was centrifuged to 70 g/l at different centrifuge temperatures (4, 10, 20 and 30°C), pH 4.0±0.1 and 9000 g. The efficiency was analyzed by determining VS (VS_c) and Tx (Tx_c) in the centrifugate.

2.6.3 Centrifugation technique - Tx losses

To ascertain the losses of Tx in the supernatant during the centrifugation process, fermented broth samples (30 ml each) were collected in centrifuge tubes (50 ml volume, Nalgene, USA) with screw caps. The samples were centrifuged at different centrifugal forces: 3000, 6000, 9000, 12000, 24000, 30000, 36000, 48000 and 50000 g for 30 min at pH 4.0 for all broths at temperature 20± 1°C. Respective supernatants were drawn to determine VS and Tx. At the determined optimal (48000 g) and commercial centrifugal force (9000g, Lisansky et al.,

1993), centrifugation was carried out at different time intervals (5, 10, 20, 30, 45, 60, 90 and 120 min) and Tx and VS losses were determined. Tx losses were estimated with respect to 48 h fermented broth with corresponding Tx of 9.54, 12.7, 16.5 and 18.9 x 10^9 SBU/l (spruce budworm units/litre, also explained in section 2.8) for soya, NH, SIW and TH, respectively. A factor, entomotoxicity recovery efficiency (ERE) was defined as:

$$\text{ERE (\%)} = \left(\frac{Tx_{(feed)} - Tx_{(supernatant)}}{Tx_{(feed)}} \right) \times 100 \qquad (1)$$

2.7 Viable spore (VS) count

The procedure specified in Vidyarthi et al (2002) was utilized for VS count with a small modification of subjecting samples to heat treatment ("heat shock") in a silicone bath (Thermo-Lift, Buchler Instruments, USA) at 80 ±1°C for 10 min and then cooling in an ice bath for 5 min before plating on tryptic soya agar. Similar heat treatment has been carried out by Fedhila et al (2002) for counting Bt spores. The standard deviation for spore count was 8.0 %.

2.8 Bioassay

The entomotoxicity (Tx) was evaluated by bioassays using eastern spruce budworm larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) in second instar, provided by Natural Resources Canada (Sault Ste-Marie, Ontario). The larvae were raised on an artificial diet for 7 days to obtain the third and fourth instar (L3-L4) larvae. The bioassays were conducted using the diet incorporation method (Beegle, 1990). In this technique, 1.5 ml of appropriately diluted Bt samples of fermented wastewater sludge/wastewater/soya were incorporated into 30 ml of molten agar based diet (at 60 ± 1°C). The composition of the artificial diet is described elsewhere (Tirado-Montiel et al., 2001). Afterwards, the mixture was distributed in aliquots of 1 ml in twenty 15 x 45 mm glass vials (VWR Canlab, Canada) with perforated plastic cap.

Sixty vials containing above mentioned 1 ml artificial diet (C1) were used as a control, meanwhile another control contained sterilized WWS and SIW (C2). Once the diet in the

vials solidified and cooled down, one L3-L4 larva was introduced into each vial and left for feeding adlibitum for 7 days at 25 ± 1°C. Mortality was monitored after 7 days. If mortality in control vials was higher than 10%, the analysis was repeated.

Tx of sample preparations was obtained by comparing the final mortality (percentages) of spruce budworm larvae with that of a standard commercial product (Foray 76B, Abbott Laboratories, Chicago, IL) and expressed as relative spruce budworm units (SBU/µl). Foray 76 B contained spores and crystals of Bt var. *kurstaki* at a potency of 20.1 x 10^9 IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni*). On comparison of Tx of Bt fermented sludge samples, it was found that SBU reported in this study was 20-25 % higher than IU. The standard deviation for Tx measurement was 8–10 %.

3. Results and Discussion

3.1 Solids content

Profiles of entomotoxicity in centrifugate (Tx_c) and VS in centrifugate (VS_c) versus solids concentration during harvesting of diverse types of fermented broths are given in Figs.1 (a and b). The Tx_c increased linearly with TS with a maximum value of 32 BSBU/l (Billion Spruce budworm units/l) for WWS and 25.4 and 18.4 BSBU/l for SIW and soya, respectively at 160 g/l (16 % solids). The broth turned into a thick paste at 70 g /l for NH and TH and at 100 g/l for soya and SIW, further concentration of solids was impractical in all broths for formulation purpose. Henceforth, all broths were slurried at 70 g/l TS and it was chosen as a reference TS. The Tx_c of 17, 17.3, 24.7 and 25.5 x 10^9 SBU/l was observed for broths of soya, NH, SIW and TH respectively at 70 g/l (Fig. 1a).

In addition, Tx recovery at 100 g/l solids (limiting TS for soya and SIW; Tx_c increased nominally with further increase in TS) followed the order TH>SIW>NH>soya. The Tx was much higher for NH, TH and SIW than reported values for different commercial fermented media at the same solids concentration (Lisansky et al., 1993). As said before, the experiment was terminated at 100 g/l (in fact at 70 g/l for comparison) due to difficulty in handling the concentrated broth (pellet formation) which involved resuspension into saline solution with 0.2 % v/v Tween 80 to determine Tx. Hence, Tx values could be an overestimation as Tween would expand the surface of crystal protein resulting in higher

surface-volume contact and enhancing Tx relative to non-Tween amended samples (Burges, 1998). Additionally, control run on sludge supernatant showed zero Tx and ruled out any entomotoxic effect of sludge as established by earlier studies (Tirado-Montiel et al., 2001; Vidyarthi et al., 2002).

The increase in Tx_c with TS was not as marked as VS_c. This could be due to losses of complementary components, namely, vegetative insecticidal proteins (VIPs) and various enzymes like chitinases, proteases, phospholipases and some unknown antibiotics which play a major role as synergizers in enhancing Tx (Porcar and Juarez-Pérez, 2003).

In order to further minimize the losses in Tx at higher TS (for example- 70 g/l; Fig.1a) in the future, a sequential centrifugation-ultrafiltration scheme could be employed. The supernatant obtained will be subjected to ultrafiltration with the size cut-off of the smallest size metabolites (e.g. $\leq$ 30 kDa, namely, chitinase, VIP, proteases) of interest in the broth.

3.2 pH and temperature

The Tx_c and VS_c at various harvesting pHs and temperatures for different fermented broths at TS of 70 g/l are presented in Figs. 2 (a and b), respectively. Temperature 20°C and pH 4 was found to be optimal for harvesting broth solids of NH, TH, SIW and soya. Acidic pH conditions were found to be better, as the sedimentation velocity would have enhanced due to neutralization of negative charges on the protein surface, which promoted the interaction between charges and particle size increments (Atkinson and Daoud, 1976). Studies were conducted only until pH 8.5 as above this pH, alkaline protease enzyme present in the broth could become active causing early degradation of crystal protein and loss of Tx (Donovan et al., 1997). Moreover, proteases characterized from wastewater sludges have been found to be highly active from pH 8-10 (Tyagi et al., 2001). Henceforth, pH 4.0±0.1 was adopted for subsequent experiments.

Furthermore, better Tx_c at lower pH (around 4) could be due to the fact that this pH was near to isoelectric point of bacteria (pH 2-4) (Rojas et al., 1996); at this pH, broth particle surfaces would carry only slightly negative charge. With increasing pH values, the floc system will be increasingly negatively charged and similar charges in the floc structure would cause

repulsion. This will lower the possibility of adsorption of spores and δ-endotoxin on the flocs in the broth and cause suspension resulting in poor harvesting. Additionally, lower pH would ensure less contamination problems (Burges, 1998) and lowering of viscosity due to compactness of the floc structure (Sanin, 2002). The lower viscosity would enhance sedimentation rate (according to Stoke's law, viscosity and sedimentation velocity are inversely related). Moreover, an acidic pH will curb cleavage of inter-chain disulfides and autolysis (factors detrimental for stability of Bt crystal) and hence eliminate the possibility of solubilization of crystal protein. In solution, crystal proteins have been reported to undergo conformational transitions in response to changes in pH which may affect their overall potency (Rajamohan et al., 1998).

Despite the advantages offered by lowered pH, namely, lowered surface charges, lowered viscosity, better sedimentation, and less contamination, pH studies were not carried out below pH 4.0. This was due to the reason that lowered pH (< 4.0) may cause inactivation of crystal protein by affecting functional groups and influencing overall handling during formulation development (necessitate use of acid resistant process equipments). Further, much lower pH (< 4.0) later in formulations may also cause corrosion of loading tanks in aeroplanes.

The decrease in Tx_c and VS_c in a polynomial fashion (quadratic Equations, Fig. 2b) could be due to the fact that decrease in temperature would increase viscosity and decrease sedimentation. Although, increase in temperature would decrease viscosity, in turn this should increase the sedimentation rate, yet the Tx_c and VS_c decreased at 30°C for all fermented broths. This may be due to inactivation of enzymes and other virulent factors at relatively higher temperature, which otherwise contribute to Tx.

As seen in Fig. 2 (a and b), lower Tx_c and VS_c for soya could be due to lower particle size of 2 μm which caused discrete and lower settling of broth solids. Meanwhile, the particle size of NH, TH and SIW was 17.8, 8.6, and 4.1 μm, respectively. The bigger particle size (floc structure) in wastewater sludges could serve as a positive parameter promoting efficient centrifugation. The soya broth behavior is in agreement with the results obtained by Rojas et al. (1996) while using a disc-stack centrifuge separation technique for Bt fermented molasses and corn steep liquor.

Eventually, considering the direct relationship between sedimentation velocity and particle size (Stoke's law -sedimentation velocity is directly proportional to square of particle size), centrifugal force required to recover all broth components (discussed later) would decrease with increase in particle size (due to expansion of flocs at higher pH). Accordingly, sedimentation rate order would be NH>TH>SIW>soya, requiring higher centrifugal force for SIW and soya to achieve higher Tx recovery. The marginal increase in particle size at pH 8.5 for all broths (NH, TH, SIW and soya - 18.8, 10.2, 6.2 and 4.4 μm, respectively) enhanced sedimentation velocity and therefore marginal increase in Tx recovery as per Stoke's law should have occurred. Further, crystal protein and spores might be encased in the flocs/adsorbed further strengthening relevance of particle size (normally observed in WW/WWS fermented broths where samples need to be vortex mixed with glass beads in the sample). Nevertheless, there was no increase in Tx_c and/or VS_c with pH (Fig. 2a), which could be due to decrease in particle density. This is based on the assumption that particle size increased with no increase in particle density due to formation of voids in the floc structure and not deposition/aggregation of floc mass.

3.3. Centrifugal force

The ERE profiles for different fermented broths along with sedimentation velocity (v_g) at pH 4.0±0.1 and temperature of 20±1°C for different relative centrifugal force (RCFs) are illustrated in Fig. 3. The v_g is a measure of the rate at which the WW/WWS solids will move in response to centrifugal force generated in the centrifuge and is a function of aggregates (size and shape of flocs, in this case) and interactions with aqueous phase and was calculated using Equation 3 (discussed later). It was observed that percent ERE increased with increase in centrifugal force at constant time (30 min) of centrifugation and vice versa (Fig. 3). Increase in centrifugal force also resulted in lowering of dissolved solids (percent lowering of dissolved solids was 50, 37, 20 and 14% for TH, SIW, NH and soya, respectively when going from 4000 to 50 000 g) in the supernatant and increase in ERE as seen from Fig. 3. Furthermore, the v_g (particle characteristic) was inversely related to centrifugal speed which determines RCF (Fig. 3, also presented in Equation 3 later) so that particles having high sedimentation velocity were settled at lower RCF. The v_g calculations based on Equation 3 (discussed later) were obtained by integrating the settling rate dr/dt of fermented medium particulates between the limits R_0 and R_1 (maximum and minimum radius of the centrifuge

rotor, respectively) (Harrison et al., 2002). Thus, the effect of particle size was implicitly expressed in the v_g term. It was also noteworthy that although ERE increased with increase in centrifugal force, still VS recovery did not increase proportionately (data unreported). This was probably owing to differences in size (smaller size of crystal protein will result in weaker drag force, hence better settling) despite the contrast in densities (usually, spores of Bt have density of 1.35 kg/l and crystal density – 1.30 kg/l as reported by Burges, 1998).

The studies at 9000 g (commercially recommended RCF, Lisansky et al., 1993), in a time period of 2 h, showed ERE of 70.5, 81.5, 80.6 and 43 % for NH, TH, SIW and soya, respectively as seen in Fig. 4a. The ERE was higher for SIW, NH and TH broths in comparison to soya, probably due to floc adsorption phenomenon (described earlier). Moreover, the v_g (particle characteristic) was inversely related to centrifugation time (Equation 3, discussed later) so that the higher sedimentation velocity particles settled in shorter time when compared to lower v_g particles. Studies at lower RCF would ensure lower power consumption and mitigate overall cost of biopesticide (Zamola et al., 1981). However, at 9000 g, still there were non-negligible Tx losses in supernatant, which necessitates investigation of ultrafiltration technique for supernatant and/spray drying/differential centrifugation of the broth to obtain improved Tx yields.

The ERE almost stabilized at 48000 g (Fig. 4b). The time required to achieve 70% ERE for NH and TH broth was 5 minutes, 20 min for SIW and 30 min for soya (Fig. 4b). However, this high RCF at commercial scale could be detrimental to the operation by increasing: wear and tear, shearing effect on flocculated particles, and degree of difficulty in scrolling settling solids, due to increasing slippage forces. Above all, presently this RCF is practically impossible at commercial scale. Hence, in addition to ultrafiltration, differential centrifugation could be explored as a potential technique, facilitating scale-up.

The time profile at 48000 g (Fig. 4b) showed lower recovery time for NH and TH due to probability of adhesion of spores and parasporal inclusions onto fermented sludge flocs. On the contrary, more likelihood of Tx losses in the supernatant of SIW and soya was due to smaller particle size (4.1 and 2 μm for SIW and soya, respectively) and tendency of the broth to become rheopectic (viscosity increased by 20 and 15 mPa.s units for SIW and soya, respectively at a small shear rate of 7.34 s^{-1}) and hence thickening with time. Nevertheless,

this method yielded excellent results without aid of any flocculants and filter aids commonly employed in conventional processes (Rojas et al., 1996; Burges, 1998). Centrifugation in this case, was more advantageous due to nature of the medium, SIW, NH and TH, which possess flocs probably serving as adsorbents as well as protectants for crystal protein in comparison to commercial soya medium.

Interestingly, the ERE profiles at different RCFs, 9000 g and 48000 g followed logarithmic law (y = a ln(x) + b, Figs. 3, 4a and 4b). Hence, for any increase in ERE, increase in time and RCF will be logarithmic. Therefore, desired time and RCF for a given fermented broth needs to be determined precisely to make the centrifugation process practically feasible. The constants "a" and "b" will depend on physical characteristics of fermented broths like viscosity and particle size. Additionally, v_g was inversely related to time as seen in Figs. 4a and 4b.

Further, a brief analysis of losses of recovery efficiency, $\left(\frac{Tx_{(9000\,g)} - Tx_{(48000\,g)}}{Tx_{(feed)}}\right)\times 100$ in both commercial (9000 g) as well as laboratory (48000 g) RCF, after 2 h of centrifugation, showed relative loss in efficiency in the order: soya>NH>TH>SIW with relative values (%) being 48.7, 21.3, 15 and 9.8, respectively. Thus, the order of suitability for centrifugation of different broths was SIW>TH>NH>soya.

3.4 Translation of batch centrifuge data to continuous commercial scale centrifuge

Laboratory scale centrifugation study can serve as a database to design large-scale continuous centrifuge or to know the performance of an existing centrifuge vis-a-vis type of the fermented broth to be centrifuged. In general, continuous commercial centrifuges are designed on a mechanical basis and cannot be modified easily, whereas, in the absence of laboratory centrifugation studies, centrifuge design is impossible.

The performance of a commercial continuous centrifuge is governed by the following Equation:

$$Q = v_g \, \Sigma \qquad (2)$$

The settling velocity (v_g) of the particle contained in the broth is a function of the particle and fluid characteristics only and is independent of a specific centrifuge, and is defined as;

$$v_g = \frac{g \ln(R_o / R_1)}{t\omega^2} \quad (3)$$

The angular velocity "ω" is given by 2πn.

The Sigma factor (Σ) corresponds to the capacity of the centrifuge (design characteristic) to handle a particular broth and is independent of particle and fluid characteristics and has been reported in literature as the most quantitative approach for a feasible centrifuge scale-up operation (Maybury et al., 2000; Harrison et al., 2002). The "Σ" ultimately determines the best design criteria for a large-scale centrifuge (for a disc bowl centrifuge "Σ" is given by $2\pi N\omega^2 (R^3_0 - R^3_1) \cot(\theta)/3g$ and has dimensions of L^2). Moreover, determination of "Σ" would assist in scale-up of the centrifuge as it would set-up the specifications on hand. Conclusively, a centrifuge could be selected of required "Σ" value based on "v_g" of the broth and the process requirements of "Q".

The batch centrifuge data from this study were used to determine the most suitable settling velocity (v_g, optimal with respect to Tx recovery, Equation 2). The recovery efficiency varied with the type of raw material used for Bt fermentation and centrifugation time (Fig. 4a). At commercially recommended RCF of 9000 g and in a given time, the Tx removal efficiency followed the order: TH> SIW> NH >soyameal (Fig. 4a). For the laboratory centrifuge used in this study, with R_o = 10.7 cm and R_1 = 3.26 cm and at 8671 rpm (9000 g), the v_g (based on relative % ERE) computed for different fermented media (Equation 3) at commercial RCF are presented in Table 2. The v_g varied for different fermented broths according to the relative centrifugation time (Table 2). The recovery was better for TH as it had the highest settling velocity (Table 2). The lower v_g for NH in relation to TH fermented sludge as observed in this study will impose higher "g" for a similar Tx recovery for NH.

Feed flow rate "Q" to the centrifuge is based on three parameters – plant Tx capacity; Tx value in final fermented broth and required recovery efficiency and the respective values are denoted in Table 2. The computation of "Q" was based on medium capacity Bt production plant (3×10^7 BIU/annum, Rowe and Margaritis, 2004). Consequently, "v_g" and "Q" were utilized to compute "Σ" by using Equation 2. Different centrifugation time for each raw material was selected based on average centrifugation efficiency (ERE, in this case) to be 60-

70% (Lisansky et al., 1993). This fact is also supported by the curves that tend to flatten towards the end (60-70% ERE, Fig. 4a). Moreover, enhanced value of ERE above 70% may impose additional power requirements (increased Σ values, discussed later).

Furthermore, these data are crucial for selecting a suitable "type" and "capacity" of a commercial centrifuge based on "Σ" factor as discussed earlier (Equation 2). The data on higher Tx recovery of TH with respect to NH (Table 2 and Fig. 4a) was also in concordance with the viscosity of 25.13 and 17.8 mPa.s obtained for Bt fermented NH and TH broths, respectively (Brar et al., 2005).

The centrifugation requirements based on "Σ" factor (calculated from Equation 2) for commercial RCF (9000 g, Table 2) were in the order: soya > NH > SIW > TH. Henceforth, a commercial centrifuge of 127683 m^2 ("Σ") based on this study will provide upto 70% ERE for non-conventional Bt fermented broths viz. NH, TH and SIW. In contrast, soyameal broth required a much higher "Σ" factor (Table 2).

3.4.1 Power requirements for centrifuge operation

In order to calculate the power requirements imposed by different broths, it was necessary to establish a relationship between ERE and Σ. These relations were based on the laboratory scale data of 9000 g used in this study. As already discussed, ERE varied logarithmically with time of centrifugation (Fig. 4a), which gave a general Equation:

$$ERE = a \ln(t) + b \quad (4)$$

where; "a" and "b" = proportionality constants which depended on fermented broth characteristics and were evaluated for each broth (Fig. 4a). Equation 4 can be rearranged as;

$$t = e^{(ERE-b/a)} \quad (5)$$

Further, considering Equation 3,

$$v_g = \frac{c}{t} \quad (6)$$

where; $c = \frac{g ln(R_o / R_1)}{(2\pi n)^2}$. Moreover,

$$Q = \frac{Plant\ Tx\ Capacity}{\left(\frac{ERE}{100}\right) \times Tx_{feed}} = \frac{K}{ERE} \tag{7}$$

Where; $K = \frac{Plant\ Tx\ Capacity \times 100}{Tx_{feed}}$

Now, combining Equations 2, 5, 6 and 7,

$$\Sigma = \frac{c}{3600} * \frac{e^{\frac{(ERE-b)}{a}}}{ERE} \tag{8}$$

Equation 8 was used to simulate Σ values corresponding to ERE required for different fermented broths (Fig. 5a). Further, the power requirements were calculated for respective Σ values based on motor current (Amp) and bowl speed (corresponding to different Σ) curves provided by GEA-Westfalia Inc. (personal communication) and is presented in Fig. 5b. As the sigma factor would vary for different fermented broths, it will determine the power consumption to achieve required ERE. Consequently, to accomplish the required ERE, Σ and power requirements will follow the order: soya>NH>SIW>TH.

The results from this study would aid in developing a strategy for downstream processing of Bt based biopesticides from various fermented (synthetic media, wastewater and wastewater sludge) broths. Centrifuged solids shall be further employed in development of liquid formulations and as slurry for spray drying to obtain dry powders and thus the different parameters affecting floc characteristics and Bt adsorption will gain importance after formulation development (Brar et al., 2004). It was noteworthy that centrifugation alone without addition of any filter aids (normally required in sludge dewatering and also used in synthetic media concentration) could be successfully employed for higher potency recovery along with broth solids, especially, in the case of wastewater and wastewater sludge in comparison to commercial soya medium. Moreover, despite the increase in sedimentation during harvesting, the filter aids will dilute the broths in terms of entomotoxicity. Further, during formulation development, the filter aids would make handling difficult due to enhanced viscosity and later would decrease effective droplet size during spraying (field application). To overcome these problems, ultrafiltration will be considered to recover entomotoxicity losses in supernatant and the broth flux rate will be established by the entomotoxicity components in the broth. In this context, wastewater and wastewater sludge

biopesticides would have an edge over conventional biopesticides due to simple recovery steps and higher potency product boosting their entry into world pesticide market.

Further, information regarding optimal RCFs and centrifugation times for different broths based on this study forms the basis for translation of results from batch to continuous centrifuge by carrying out studies (performance test) on various process parameters (flow rate, and flow geometry) required in commercial centrifuges. Additionally, the supernatants could be concentrated by ultrafiltration to ameliorate entomotoxicity recovery efficacy, which will help in an overall economics evaluation of downstream processing.

4. Conclusions

The results suggested that the entomotoxicity recovery was higher for wastewater and wastewater sludges relative to semi-synthetic soyameal medium without addition of any additives, contributing to overall economy of separation process. The centrifugate solids concentration beyond 100 g /l did not enhance Tx, instead caused pellet formation and thus 70 g/l was adopted as reference. The laboratory scale maximum recovery was obtained at 48000 g. The wastewater sludge, starch industry wastewater and soya broths yielded higher entomotoxicity recovery at pH 4 and temperature 20°C at 70 g/l total solids. The settling velocity calculations based on entomotoxicity recovery efficiency and feed flow rate to the centrifuge aided in sigma factor computations. The centrifugation requirements based on "Σ" factor for commercial centrifugal force of 9000 g followed the order: soya > NH > SIW > TH. It was also established that for a given entomotoxicity recovery efficiency, the power requirements for a commercial centrifuge for a given capacity plant followed the order: soya>NH>SIW>TH.

Nomenclature	
Bt	*Bacillus thuringiensis*
DO	Dissolved oxygen
ERE	Entomotoxicity recovery efficiency
kDa	Kilo Dalton
NH	Non-hydrolyzed/raw wastewater sludge
RCF	Relative centrifugal force
SBU/l	Spruce budworm units/litre
SIW	Starch industry wastewater
SS	Suspended solids
TH	Hydrolyzed wastewater sludge
TKN	Total Kjeldahl nitrogen
TS	Total solids
TVS	Total volatile solids
Tx	Entomotoxicity
Tx_c	Entomotoxicity in centrifugate
VIP	Vegetative insecticidal proteins
VS	Viable spores
VS_c	Viable spores in centrifugate
VSS	Volatile suspended solids
WW	Wastewater
WWS	Wastewater sludge
Symbols	
g	Acceleration due to gravity (9.81 m/s^2)
ω	Angular velocity (radians/seconds)
θ	Angle at which discs are tilted in a disc bowl centrifuge
t	Centrifugation time (seconds)
Σ	Centrifuge parameter, dimension of $L^2(m^2)$
Q	Flow rate (L/d)
L	Length (m)
R_o	Maximum radius of rotor on the bottle top (cm)
R_l	Minimum radius at the liquid interface (cm)
N	Number of discs in a disc bowl centrifuge
n	Revolutions per minute (rpm)
v_g	Settling velocity (m/s)

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. We are also thankful to Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Service and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing scholarship to Satinder K. Brar. We express our thanks to Mr. Pierre Tardif of GEA-Westfalia Inc. for providing us valuable technical information on commercial centrifuge.

References

Agaisse, H., Lerecluse, D., 1996. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? J. Bacteriol. 177, 6027–32.

Aiba, S., Humphrey, A.E., Millis, N.F., 1973. Biochemical Engineering, second ed. Academic Press, New York.

APHA, AWWA, WPCF 1998. In: Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. (Eds.) Standard Methods for Examination of Water and Wastewaters. 20th edn., American Public Health Association, Washington, DC, USA.

Atkinson, B., Daoud, I.S., 1976. Microbial flocs and flocculation in fermentation process engineering. Adv. Biochem. Eng. 4, 41-124.

Barnabé, S., Verma, M., Tyagi, R.D.,Valéro, J.R., 2005. Culture media for increasing biopesticide producing microorganism's entomotoxicity, methods of producing same, biopesticide producing microorganisms so produced and method of using same. US patent International application PCT/CA2005/000235.

Beegle, C.C., 1990. Bioassay methods for quantification of *Bacillus thuringiensis* δ-endotoxin, Analytical Chemistry of *Bacillus thuringiensis.* In: Hickle LA, Fitch WL (eds), Analytical Chemistry of *Bacillus thuringiensis.* American Chemical Society, USA, pp14–21.

Bernhard, K., Utz, R., 1993. Production of *Bacillus thuringiensis* for industrial and commercial uses. In: Entwistle, P.F., Cory, J.S., Baily, M.J., Higgs, S. (eds), *Bacillus thuringiensis*: an environmental biopesticide: theory and practice. Wiley, Great Britain, pp 255–267.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Banerji, S., 2004. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations. Water Sci Technol. 50(9), 229–236.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. 2005. Sludge based *Bacillus thuringiensis* biopesticides: viscosity impacts. Water Res. 39, 3001-3011.

Burges, H.D. (ed.) 1998. *Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments*, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Donovan, W.P., Tan, Y., Slaney, A.C., 1997. Cloning of the *nprA* gene for neutral protease A of *Bacillus thuringiensis* and effect of in vivo deletion of *nprA* on insecticidal crystal protein. Appl. Environ. Microbiol. 63, 2311–2317.

Dulmage, H.T., 1970. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* var. *alesti.* J. Invertebr. Pathol. 15, 232–239.

Fedhila, S., Msadek, T., Nel, P., Lereclus, D., 2002. Distinct *clpP* genes control specific adaptive responses in *Bacillus thuringiensis.* J. Bacteriol. 184(20), 5554–5562.

Harrison, R.G., Todd, P., Rudge, S.R., Petrides, D., 2002. Bioseparations Science and Engineering. Oxford University Press. 432 pp.

Gulati, R., Saxena, R.K., Gupta, R., 2000. Fermentation and downstream processing of lipase from *Aspergillus terreus.* Process Biochem. 36, 149–155.

Lachhab, K., Tyagi, R.D., Valéro, J.R., 2001. Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids concentration. Process Biochem. 37(2), 197–208.

Lisansky, S.G., Quinlan, R.J., Tassoni, G., 1993. The *Bacillus thuringiensis* Production Handbook, CPL Press, Newbury, pp124.

Maybury, J.P., Hoare, M., Dunnill P. 2000. The use of laboratory centrifugation studies to predict performance of industrial machines: studies of shear-insensitive and shear-sensitive materials. Biotechnol.Bioeng. 67(3), 265-273.

Porcar, M., Juarez-Pérez, V., 2003. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol. Rev. 26, 419-432.

Rajamohan, F., Lee, M.K., Dean, D.H., 1998. *Bacillus thuringiensis* insecticidal proteins: molecular mode of action. Progr Nucleic Acid Res. Mol. Biol. 60, 1–27.

Rojas, J.V., Gutierrez, E., De la Torre, M., 1996. Primary separation of the entomopathogenic products of *Bacillus thuringiensis.* Biotechnol Prog.12, 564–566.

Rowe, G.E., Margaritis, A., 2004. Bioprocess design and economic analysis for the commercial production of environmentally friendly bioinsecticides from *Bacillus thuringiensis* HD-1 *kurstaki.* Biotechnol. Bioeng. 86 (4), 377–388.

Sachdeva, V., Tyagi, R.D., Valéro, J.R., 2000. Production of biopesticides as a novel method of wastewater sludge utilization/disposal. Water Sci Technol. 42, 211–216.

Sanin, F.D., 2002. Effect of solution physical chemistry on the rheological properties of activated sludge. Water SA 28(2), 207–211.

Teera-Arunsiri, A., Suphantharika, M., Ketunuti, U., 2003. Preparation of spray-dried wettable powder formulations of *Bacillus thuringiensis*-based biopesticides. J. Econ. Entomol. 96(2), 292–299.

Tenney, M.W., Stumm, W., 1965. Chemical flocculation of microorganisms in biological waste treatment. JWPCF 37(10), 1370–1388.

Tirado-Montiel, M.L., Tyagi, R.D., Valéro, J.R., 2001. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. Water Res. 35 (16), 3807–3816.

Tyagi, R.D., Foko, V.S., Barnabé, S., Vidyarthi, A.S., Valéro, J.R., 2001. Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using sewage sludge as raw material. Water Sci Technol. 46 (10), 247–254.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., 2002. Studies on the production of *B-thuringiensis* based biopesticides using wastewater sludge as a raw material. Water Res. 36 (19), 4850–4860.

Yezza, A., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y., Smith, J., 2004. Scale-up of biopesticide production processes using wastewater sludge as a raw material. J. Ind. Microbiol. Biotechnol. 31, 545-552.

Zamola, B., Valles, P., Meli, G., Miccoli, P., Kajfez, F., 1981. Use of the centrifugal separation technique in manufacturing a bioinsecticide based on *Bacillus thuringiensis.* Biotechnol. Bioeng. XXIII, 1079–1086.

Zouari, N., Achour, O., Jaoua, S., 2002. Production of delta-endotoxin *by Bacillus thuringiensis* subsp. *kurstaki* and overcoming of catabolite repression by using highly concentrated gruel and fish meal media in 2 and 20 dm^3 fermentors. J. Chem. Technol. Biotechnol. 77, 877–882.

Table 1. Characteristics of wastewater/wastewater sludge

Parameter (s)	Secondary sludge		Starch industry wastewater	
TS (g/l)	23	±1.8	17.3	±1.2
TVS (g/l)	18	±1.2	14.2	±1.1
SS (g/l)	16	±1.2	2.4	±1.2
VSS (g/l)	14	±2	2.4	±1.4
pH	5.5	±0.1	3.0	±0.1
Concentration (mg/kg TS)				
C	293607	±5658	656234	±6986
N_t	44403	±495.6	44068	±1625
P_t	8867	±156.8	33858	±2126
$N\text{-}NH_3$	986	±236.4	111.2	±63.4
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$	16.7	±1.3	5	±1.26
$P\text{-}PO_4^{3-}$	5437	±357	15321	±2456
Al	5463	±423	57001	±3422
Ca	13298	±568.4	12018	±326
Cd	3.49	±1.1	0.62	±0.21
Cr	28	±1.3	1.5	±0.06
Cu	385	±136	346.5	±169.5
Fe	12301	±592	8061.9	±758.6
K	1086	±369	22744	±3332
Pb	31	±5.3	30	±5.8
S	4485	±632	2298.3	±63.6
Zn	301	±182	248.1	±76.2
Na	1249	±315	2185.7	±233
Ni	11.42	±4.6	n.d.	-

n.d. – not detectable

Table 2. Scale-up parameters of commercial centrifuge (9000 g) for different substrates

Parameters	NH	TH	SIW	Soyameal
Tx (BIU/L) †	12.7	18.96	16.5	9.54
Commercial Tx (BIU/d) ††	9.1×10^4	9.1×10^4	9.1×10^4	9.1×10^4
Centrifugation time (min)	60	45	60	120
% ERE	66	76.8	72.7	42.3
v_g (m/s)	3.93E-09	5.24E-09	3.93E-09	1.96E-09
Q (m^3/s) †††	5E-04	3.51E-04	2.6E-04	1.04E-03
Σ (m^2)	127683	55248	89378	530961

† Corresponds to Tx of different fermented broths at 48 h of fermentation (feed to centrifuge).

†† Typical Tx of a medium scale commercial plant assumed to operate 24 h/day, 330 days per year at a production scale of 3×10^7 BIU per annum for batch as well as fed-batch Bt fermentation (Rowe and Margaritis, 2004).

††† For a realistic approach, centrifugation time must be considered in order of hours and not days, hence transformation of "Q" to m^3/s, where 6h was assumed to be the operational time for centrifuge

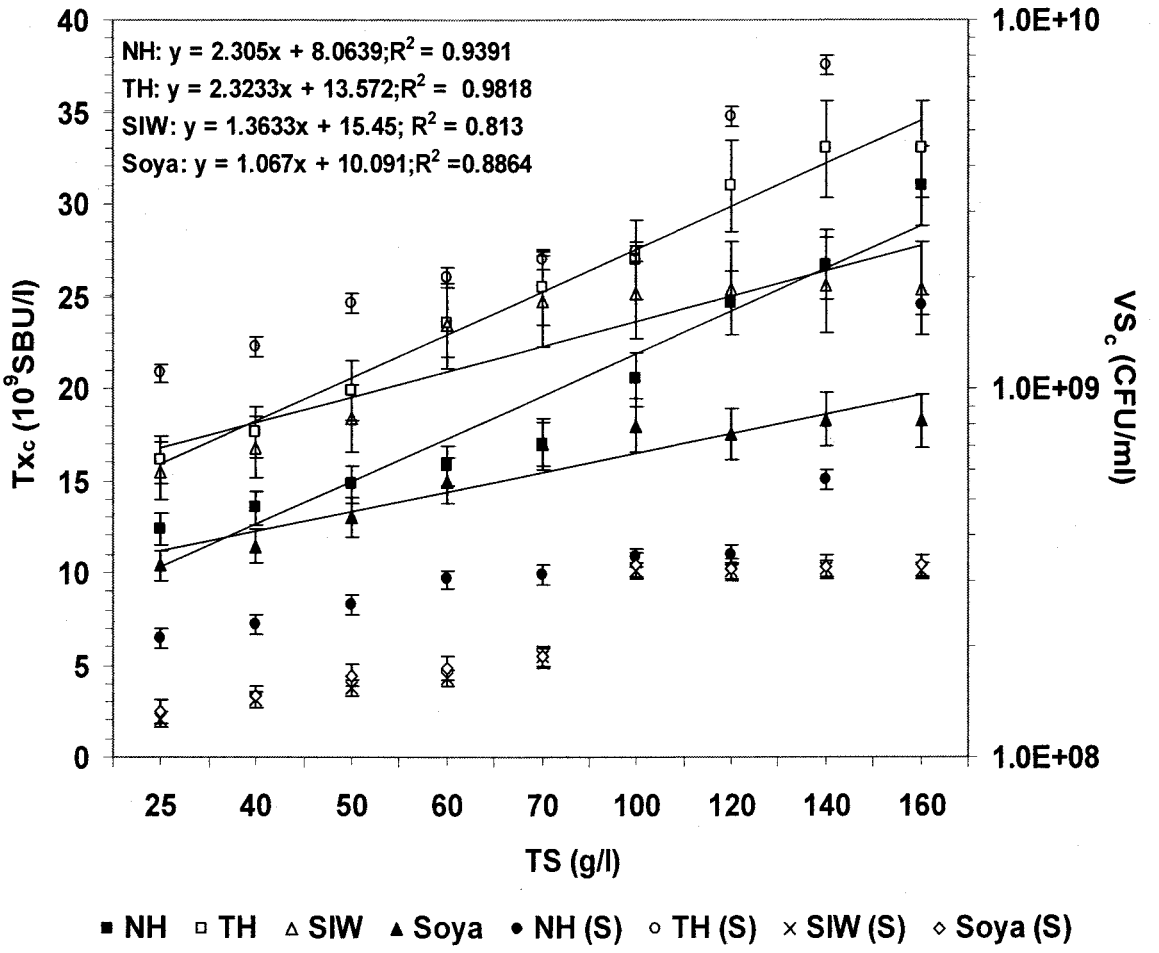


Fig. 1. VS and Tx in centrifugate at different solids concentration ("S" represents VS); continuous lines represent Tx_c profiles and scatter points represent VS_c profile (regression equations represent relations between Tx_c and TS).

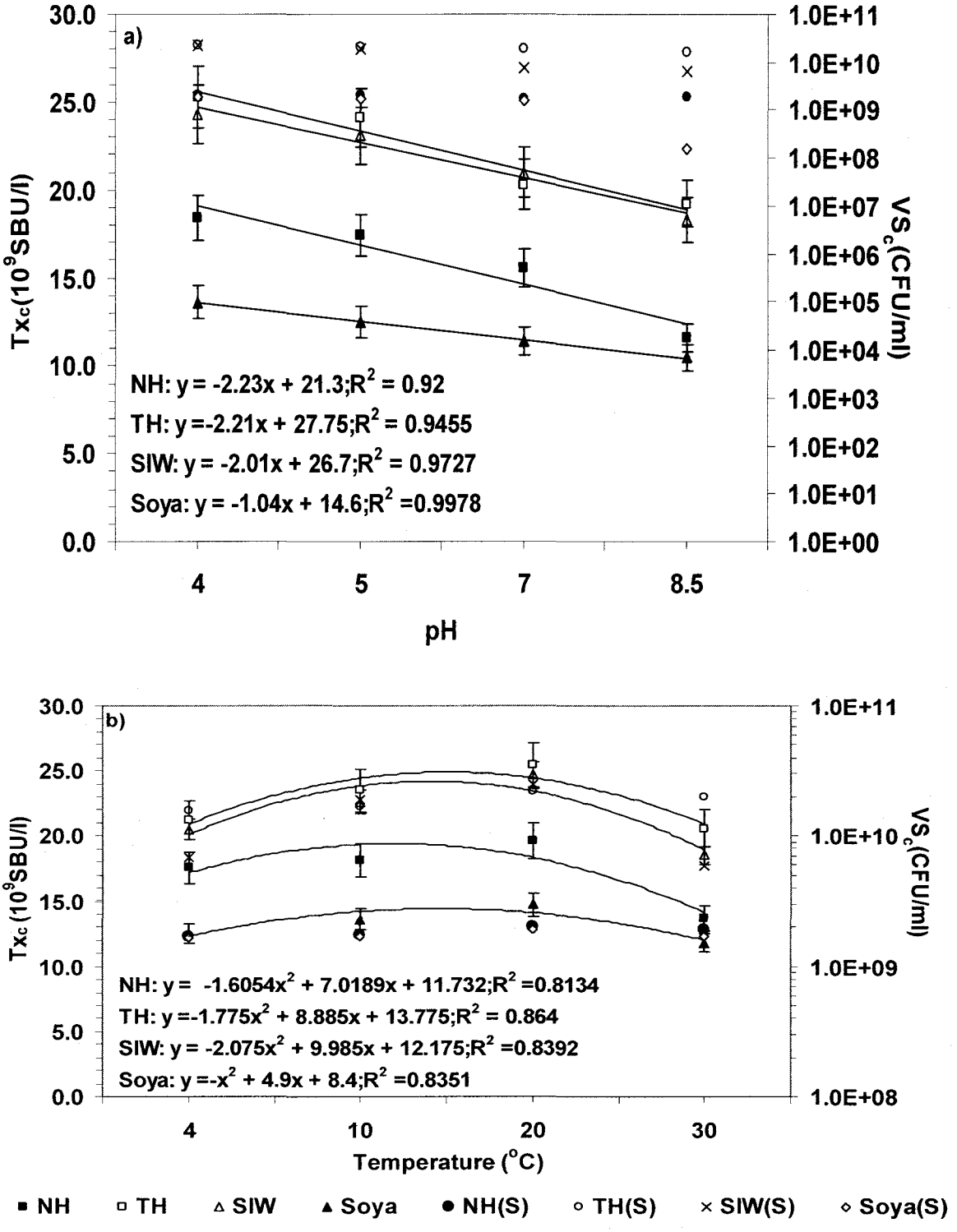


Fig. 2. Tx and VS in centrifugate at different: (a) pH; (b) temperatures ("S" represents VS). The regression data belong to Tx_c (value on which biopesticidal potential is based).

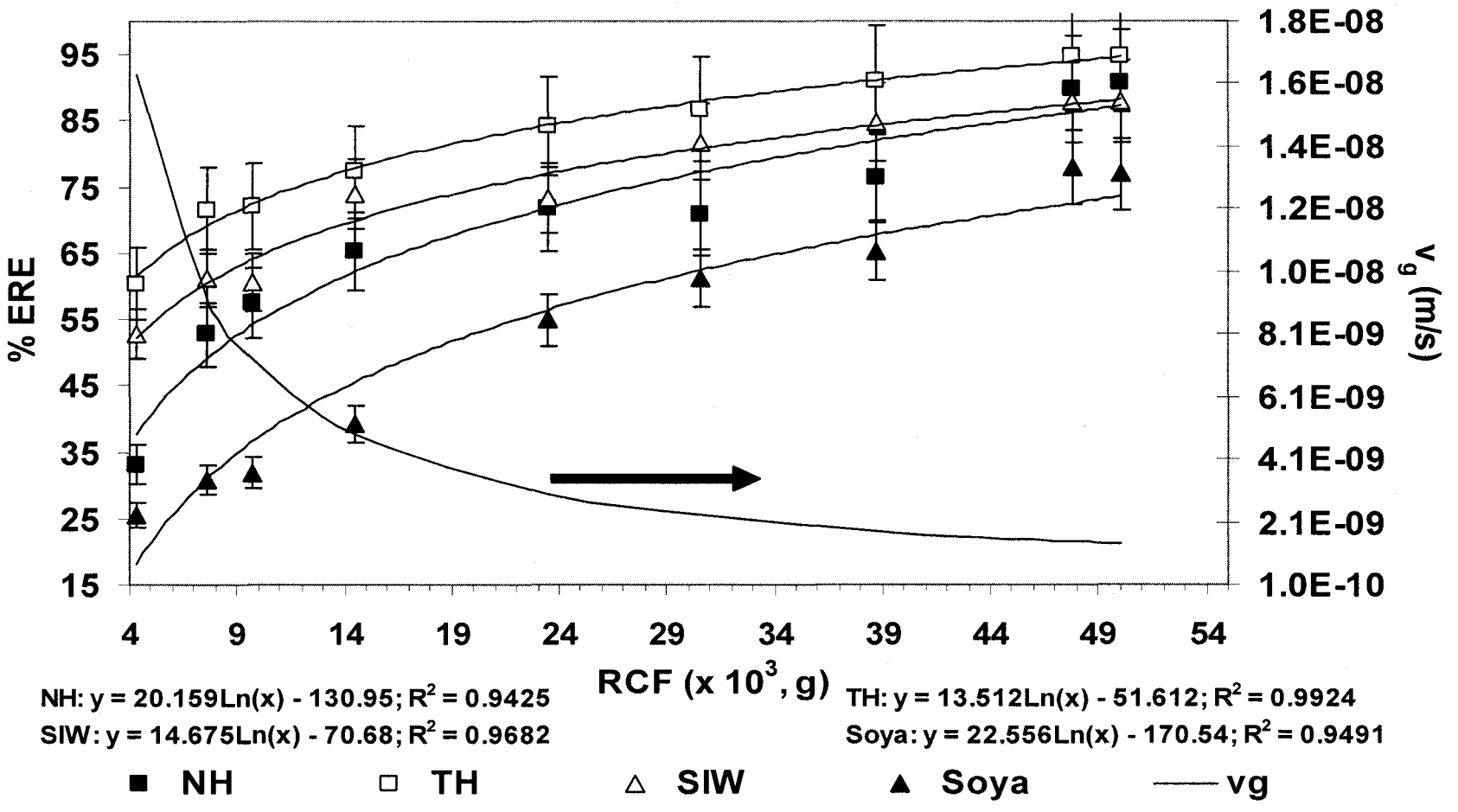


Fig. 3. ERE profiles for different fermented broths at different RCFs (regression equations represent relations between % ERE and RCF)

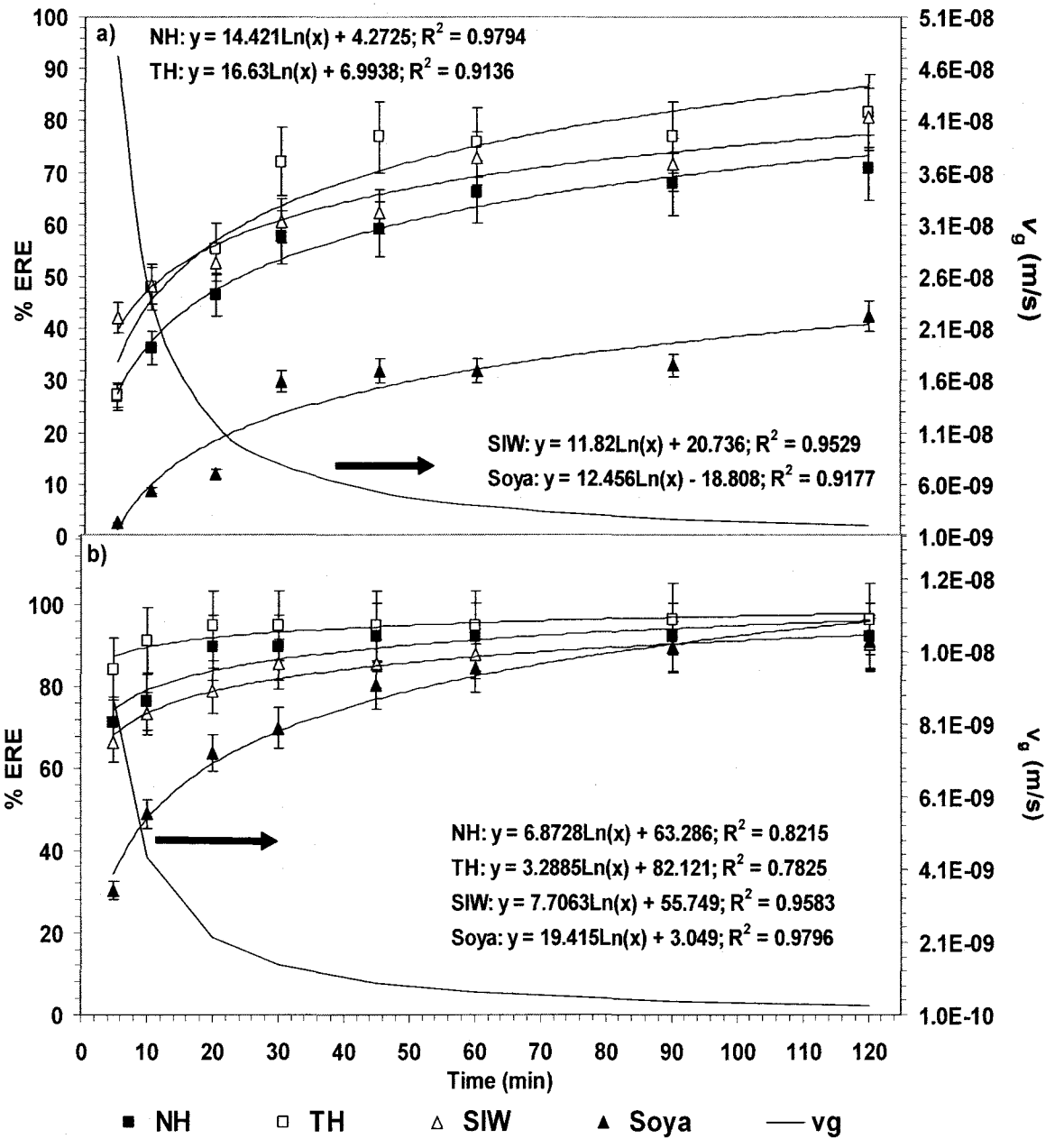


Fig. 4. ERE profiles for different fermented broths; a) at 9000 g and b) at 48000 g (regression equations represent relations between % ERE and RCF).

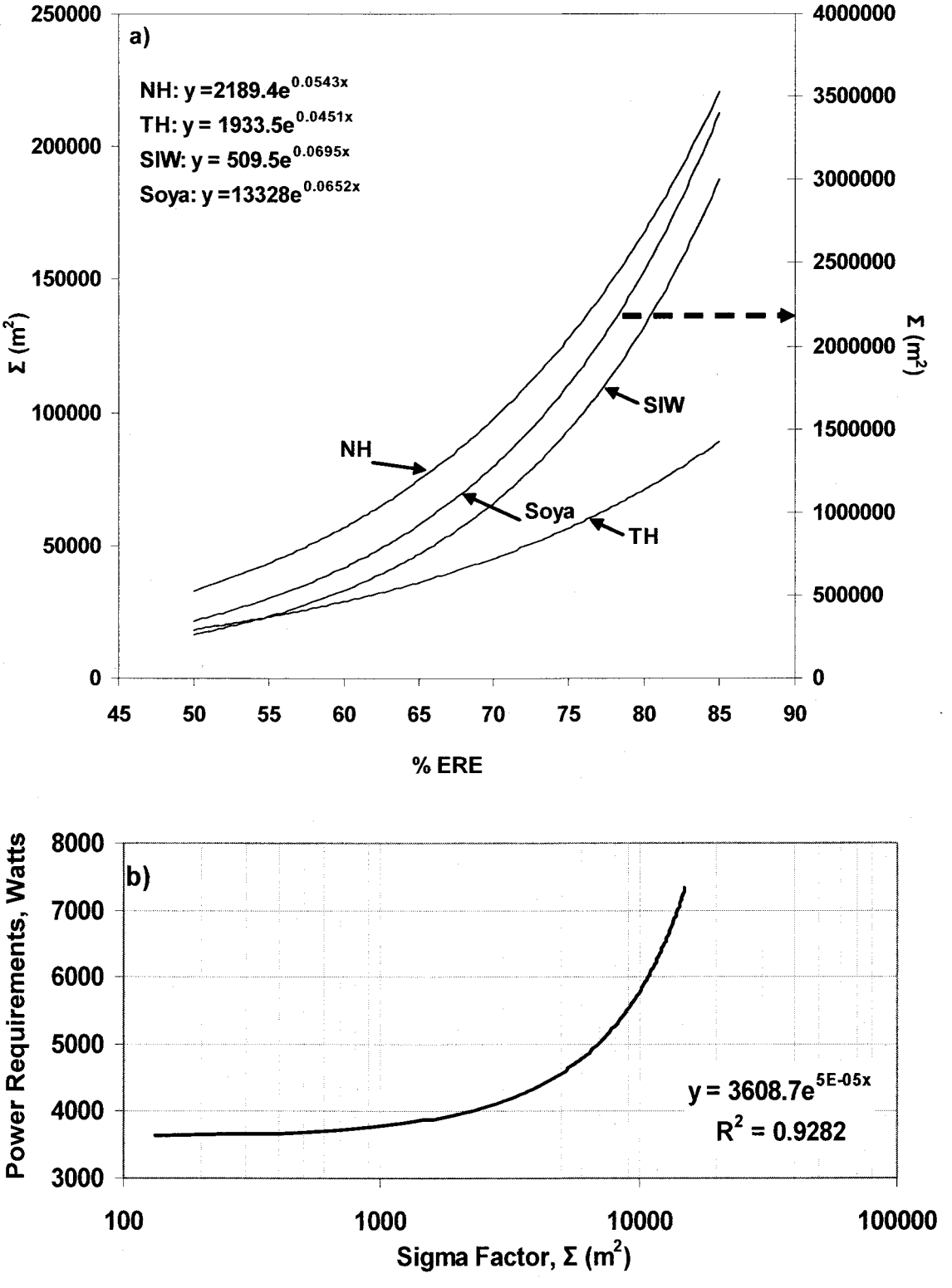


Fig. 5. Sigma profiles for different fermented broths a) Sigma versus ERE and b) Power required versus sigma.

Partie II

Presence and Characterization of Chitinases present in *Bacillus thuringiensis* Fermented Wastewater and Wastewater Sludge

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*], J.R. Valéro[1] and R.Y. Surampalli[2]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[2]USEPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Environmental Technology

(Under review)

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. et Surampalli, R.Y. (2008). *Bacillus thuringiensis* fermentation of wastewater and wastewater sludge - presence and characterization of chitinases. *Environ. Technol.* 29(2): 161-170. http://dx.doi.org/10.1080/09593330802028550

CHAPITRE 6.

DÉVELOPPEMENT DES FORMULATIONS AQUEUSES

Partie I

Recent Advances in Downstream Processing and Formulations of *Bacillus thuringiensis* based Biopesticides - Review

Satinder K. Brar[1], Mausam Verma[1], R.D. Tyagi[1*] and J.R. Valéro[1]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

Process Biochemistry (2006)

41:323-342

Développements des procédés en aval et des formulations de biopesticides à base de *Bacillus thuringiensis* – revue de littérature

Résumé

Des insecticides microbiens à base de *Bacillus thuringiensis* (Bt) sont utilisés intensivement depuis quatre décennies en raison de leur efficacité entomotoxique et de leur innocuité envers la santé humaine et l'environnement. Toutefois, dans plusieurs régions du monde, l'emploi de Bt est souvent limité par les coûts élevés de production et du développement de formulation commerciale. Divers intrants alternatifs pour la fermentation ont remplacé avec succès les milieux synthétiques coûteux, mais les coûts des procédés de formulation ne sont pas encore véritablement pris en considération. L'efficacité de la récupération de Bt lors de la récolte après la fermentation a un impact direct sur la commercialisation du produit et sur sa transformation ultérieure lors du développement de la formulation. La formulation est un lien crucial entre la production du Bt et son application et influence l'aspect économique, la durée de conservation, la facilité d'application et l'efficacité sur le terrain. Divers facteurs environnementaux comme les rayons ultraviolets, la pluie, le pH, la température et la physiologie du feuillage peuvent influencer l'efficacité des formulations de Bt. Des préparations de Bt ont été développées pour contrer certains effets néfastes des facteurs environnementaux. Les suspensions conventionnelles peuvent être remplacées par des suspensions avancées comme des microencapsulations ou des microgranules de Bt pour préserver son entomotoxicité. Cet article passe en revue les plus récents développements des procédés en aval et des formulations des biopesticides à base de Bt tout en tenant compte de l'effet des différents paramètres environnementaux. De plus, à l'aide de résultats saillants, la mise en marché éventuelle de préparations alternatives de Bt en utilisant des boues d'épuration et des eaux usées comme substrat est également discutée.

Mots-clés: *Bacillus thuringiensis*; biopesticides; effets sur l'environnement; formulations; microencapsulations; eaux usées; boues d'épuration.

Abstract

Bacillus thuringiensis (Bt) has been extensively used for four decades in biopesticidal formulations due to its safe environmental and human health records. The widespread use of Bt is often challenged by production as well as formulation costs. Various alternative local media have successfully replaced costly synthetic media, but the actual constraint is embedded in harvesting and formulation costs. Harvesting efficacy governs the marketability of a product by affecting potency and aiding in further processing during formulation development. Formulation is a crucial link between production and application and dictates economy, longer shelf life, ease of application and enhanced field efficacy. There are various environmental factors like ultraviolet radiation, rain, pH, temperature and foliage physiology which impede efficacy of Bt formulations. There have been developments of various formulations depending on application target and feasibility – solid and liquid to overcome the adverse environmental effects. Conventional formulations have been substituted by advanced versions like microencapsulations and microgranules to enhance residual entomotoxicity. This article reviews recent advances in downstream processing and formulations of Bt based biopesticides incorporating effect of different environmental parameters. In addition, probable inclusion of alternative Bt formulations from fermented wastewater and wastewater sludge in the future also has been included with inputs on their advantages with some salient results.

Keywords: *Bacillus thuringiensis*; Biopesticides; Environmental effects; Formulations; Microencapsulations; Wastewater; Wastewater sludge

1. Introduction

Agriculture and forests form an important resource to sustain global economical, environmental and social system. Their protection against pests is a priority and due to the adverse impact of chemical insecticides, use of biopesticides is increasing [1]. A number of biopesticides (bacteria, fungi, virus, pheromones, plant extracts) have been already in use to control various types of insects responsible for the destruction of forests and agricultural crops. *Bacillus thuringiensis* (Bt) based biopesticides are especially of utmost importance and occupy almost 97 % of the world biopesticide market [2,3]. A biological pesticide is effective only if it has a potential major impact on the target pest, market size, variability of field performance, cost effectiveness, end-user feedback and a number of technological challenges namely, fermentation, formulation and delivery systems [4,5]. Development cost, time and ease of registration and potential growing market in contrast to chemical pesticides make biopesticides interesting proponents to investigate.

Bt is a Gram positive, spore forming bacteria that has insecticidal properties (also called "entomotoxicity") affecting a selective range of insect orders, namely, Lepidoptera, Diptera and Coleoptera [6,7]. Despite, extensive research in the field of Bt biopesticides, many formulations do not deliver effectively in field owing to variable environmental stress (for example, forestry and agriculture). Another reason could be adoption of integrated approach which can play an important role in biopesticide development, in other words, tailoring fermentation and harvesting processes to produce higher potency efficacious formulations. Biopesticide research has been comprehensively detailed in Burges [6], but there are recent advances, which have taken place henceforth. Meanwhile, wastewater (WW) and wastewater sludge (WWS) based Bt formulations also need to be elaborated and discussed due to their inherent positive features (discussed later).

Genetic engineering may play a complementary role in the development of more efficacious formulations by facilitating greater toxin production, broadening the host range, and enhancing germination, sporulation and expanding Bt spectrum, yet the role of formulations with conventional strains cannot be overlooked [8,9]. Genetically engineered microorganisms are beyond the scope of this review article. Several core areas that need to be addressed before the biopesticides can penetrate the international market are activity spectrum,

persistence and recycling, improvement of formulations by using conventional and simple adjuvants/additives which are not cost intensive [10].

Ever since the entry of first commercial product of Bt named "Sporeine" in France in 1938, there has been continuous rise in development of advanced products. In this light, present review discusses various advances in Bt harvesting technologies as well as formulations. Furthermore, environmental factors like UV, rain, pH and temperature plaguing field stability of Bt formulations are explored along with an alternative WW/WWS based Bt formulation.

2. Harvesting techniques

The final fermented Bt broth comprises of spores, cell debris, inclusion bodies, enzymes and other residual solids, which needs to be recovered efficiently to be utilized in subsequent formulation step [11,12]. Generally, depending on the desired entomotoxicity of final product and scale of production, the processing required varies significantly. Key factors governing the choice of harvesting strategy include process throughput, physical characteristics of product and impurities and desired end-product concentration [13].

Most commercial Bt products contain insecticidal crystal proteins (ICP), viable spores, enzyme systems (proteases; chitinases; phospholipases), vegetative insecticidal proteins and many unknown virulent factors along with inerts/adjuvants. Earlier, lactose-acetone technique was used as a method to recover Bt spores with measurable losses [14]. However, use of advanced methods like ultracentrifugation, microfiltration and vacuum filtration to separate insoluble solids (active ingredients) from soluble liquid (inert) fraction of the harvest liquor, has resulted in efficient recovery of the active ingredient (a.i.) of various biotechnological products [15,16]. Co-expression of heterologous chitinase genes in Bt has also been demonstrated to increase insecticidal activity of the bacterium [17,18] and hence judicious recovery of a.i. from entire broth is necessary to enhance potency of the product.

Harvesting microorganisms from submerged fermentation is often difficult due to low concentration of products, their thermolabile nature and in some cases, poor stability. Stabilizing adjuvants may have to be incorporated in post-harvest operation to prevent spore mortality and/or germination. Rapid drying or addition of specific biocidal chemicals may be

required to prevent growth of microbial contamination in the broth or centrifuge slurry [19]. This method could find utility only for small volumes of broth liquid. Even, foam flotation process has been applied to obtain crystal-enriched suspensions of Bt in which gelatin caused spores to be selectively entrained in the foam and thereby separated from suspensions [20]. However, this method involved post-process laborious steps incorporating various chemicals.

Some authors have utilized method of spray drying for large broth volumes [21]. This drying can be preceded by thickening of the fermentation liquid by centrifugation and filtration using filter aids like celite, superfloc etc. to reduce handling volume [22,23]. In the spray drier, water is removed from the broth slurry as it passes through the heated inlet (150-200°C). The resulting powder coats the walls and collects in the spray dryer [24]. Although not a physical loss, yet measurable bioactivity diminished by spray drying process due to the continuous exposure of the bioactive components to the high temperatures [25].

An efficient recovery of active spore-crystal complex of Bt was reported by Rojas et al. (1996) by using either a disk-stack centrifuge or a rotary vacuum filter with spore recovery efficiency higher than 99% [26]. Nevertheless, the concentration of dry solids produced by filtration (31.5 %) was superior to centrifugation (7.5 %). Similar study on Bt var. Berliner was carried out by using a continuous centrifuge with recovery rate of 85-90 % and decrease in separation efficiency with increase in flow rates [27].

At the final stage after fermentation, lactose (5%) was sometimes added as a cryoprotectant to prevent clumping during storage and lactose-acetone co-precipitation could be used as a sequential step to centrifugation to achieve higher δ-endotoxin recovery efficiency [28]. The final products (powder/suspension) are suitably formulated as aqueous (flowable) or oil concentrate, spraying powder, or granulates. Nevertheless, literature on harvesting methods is very scarce as most of the Bt commercial production is carried out by industries and hence separation process is proprietary and secure.

Although harvesting is considered as an important step that may augment or suppress biopesticidal activity, still current techniques based on centrifugation – conventional, differential and density gradient and spray drying suffer inefficient δ-endotoxin recovery and inherent losses.

2.1 Advances in downstream processing

Pre-existing techniques in food industry and biotechnology could be adapted to biopesticide production with modifications. Micellar-enhanced ultrafiltration (MEUF) was a recently proposed technique to separate dissolved organic compounds like thuringiensin from aqueous streams [29]. In this process, surfactant was added to an aqueous stream containing organic solute to form micelles to separate target compound for subsequent purification. The separation depended on ultrafiltration (UF) membrane, type and concentration of surfactant, set of experimental conditions (such as pH, ionic strength, temperature, and trans-membrane pressure, etc.) [30].

In-situ product removal (ISPR), the retrieval of a biochemical product from the vicinity of a cell during active fermentation has been suggested as possible method of protein removal in various commercial processes [31]. However, there has been no reported application of the ISPR process in biopesticide production. Cross-flow microfiltration (CFM) has been utilized lately for extraction of all kind of proteins and harvest of recombinant yeast [32,33]. Lee et al. (2004) used CFM for extraction of proteins resistant to attack by proteases present in the cell wall [34]. But, this method is highly specific and also recommends recovery of purified product and may not find use in the biopesticide recovery.

Among all advances, centrifugation appears to be a viable alternative and with further advances in design and speed, it could serve as better equipment for downstream processing of biopesticides. The viability has been validated by studies on centrifugal processing of cell debris and inclusion bodies from recombinant *Escherichia coli* [35].

Thus, main objective of a good harvesting technique is to minimize number of unit operations involved in the process, reducing overall process and validation costs while also simplifying ease and economy of process automation. This is a key issue for development of an integrated biopesticide production facility governing total cost of the product.

3. What and why formulations?

The problems of stability of biopesticides both during storage and after application have stalled biopesticide development to a great extent [36]. At this juncture, formulation development can play a key role addressing four major objectives which can serve as benchmarks for success; 1) Stabilize microbial agents during distribution and storage; 2) Aid Handling and Application of the product; 3) Protect agent from adverse environmental factors; and 4) Enhance activity of microbial agents in field. Principally, a formulation comprises a.i. (fungi, bacteria, virus, nematodes etc.) and additives (various to maintain status quo of a.i.) to fulfill aforestated objectives and give SHAPE to biopesticides.

Commercial biopesticides must be economical to produce, have persistent storage stability, high residual activity, be easy to handle, mix, and apply, and provide consistently effective control of the target pest [37-40]. The global objective is to enhance widescale use of commercial biopesticides which may not be as effective as their chemical counterparts, but with time they may become as effective. Meanwhile, producers may have to evaluate their level of pest control to achieve economical crop yields [36].

Additionally, formulation addresses the problems: speed of kill; loss of field activity via persistence of detrimental environmental conditions comprising sunlight, adverse moisture (dry or wet), rains, wind, plant characteristics such as leaf chemistry, and microbial growth of competing organisms; poor palatability and modification of application techniques by use of adjuvants and /inerts through stable chemistry [41]. Hence, formulation, a mandatory pre-requisite for all biopesticides, bridges fermentation and field application.

3.1 Challenges in formulations

Biopesticides pose a different set of challenges due to their inherent nature - particulate suspensions; thermally more susceptible; prone to contamination and pest specific and hence need to be designed accordingly. Meanwhile, most of the challenges have been taken care of in the present Bt biopesticide scenario, which has changed the "stand-alone" concept of chemical pesticides. Thus, extensive exploration of Bt biopesticides and continuous advances

since early 20th century have contributed significantly to their widespread acceptance and use in comparison to other biopesticides.

3.2 Adjuvants/additives

They are chemically and biologically active compounds that can alter the formulation physics and kill the targeted species without harming other insects (i.e. enhance its selectivity; [42]) and reduce the effective biopesticide dose required [43]. Registration agencies like U.S. Environmental Protection Agency (US EPA) regulate the inclusion of certain ingredients in adjuvant formulations and hence testing of adjuvant and/inerts is restrained within the list 4A and 4B comprising minimal risk and no risk inert ingredients respectively [44]. For example, in the past, xylene was used as a preservative in Bt formulations, but its adverse environmental impacts later led to the withdrawal of this concept [45,46]. This onus ropes in inclusion of mainly biodegradable adjuvants/additives enhancing eco-friendliness of biopesticides.

Key factors in selection of appropriate adjuvants are summarized in Table 1 and details of different adjuvant/additives with their functions and examples are illustrated in Table 2.

Selection of adjuvant/additive is governed by the type of formulation desired and follows the same criteria for all media based Bt formulations. The characteristics and composition of biopesticidal formulations vary with type of habitat (foliage; soil; water; warehouse; size), pathogen (type; characteristics; regeneration mechanism and factors), rheology of technical material (viscosity; particle size; density), insect species (feeding habits; feeding niche; life cycle), mode of action (oral/ contact); host-pathogen-environment interactions (behavioral changes; resistance; stability), mode of application (aerial; land) and application rate (L/ha and kg/ha). It must be emphasized that the final product is determined by the specific medium used in fermentation, fermentation conditions and post-fermentation processing.

Broadly, formulations can be classified into dry solid (dusts, granules, powders and briquettes) and liquid (termed "suspensions"; oil or water based and emulsions) formulations [47]. Different formulations of Bt registered in Canada/North America with their potency

details are listed in Table 3. This list is not exhaustive as there are numerous unregistered Bt formulations worldwide, especially, in developing countries.

3.3 Dry solid products

3.3.1 Dusts

Dusts are formulated by the sorption of an a.i. onto a finely ground, solid inert such as talc, clay, or chalk, with particle size ranging from 50-100 μm. Although, finer particles adhere better, at the same time, they pose serious inhalation hazard for the user and drift hazard for the sprayer. Couch and Ignoffo [48] have listed different types of inert fillers used with insect pathogens with different formulations normally containing <10% of microorganism (a.i.) by weight.

Particle size (0.5 to 50 μm), bulk density (0.5 – 0.6 g/cm^3) and flowability are important control parameters [49]. During application, smaller particles collect on target surfaces and large ones fall off and lack stickiness. Thus, stickers (adherents) and desiccants (prevent caking) are commonly employed. Bt dusts have been employed in stored bulk grains to control surface-dwelling lepidopteran pests [50]. Early work also reported higher control of loopers, semi-loopers and cabbage pests with Bt dusts when compared to conventional sprays [51]. The higher control could be due to uniform coverage on underleaf surface and protection from sunlight, however, windy conditions would support liquid sprays.

Bt dusts have been widely used in the control of corn borer larvae. However, with the development of granules (discussed later), use of dusts was restricted owing to adverse health impacts (respiratory) on the end user [52]. Still, their usage continues unregulated in developing nations [53]. Recently, two formulations of *Bacillus thuringiensis* Berliner (wettable powder and dust) to control the larvae of *Lobesia botrana* Denis and Schiffermueler (Lepidoptera: Tortricidae) were tested in Greece. Dusts provided maximum kill in contrast to wettable powder [54].

3.3.2 Granules

The granules comprise discrete masses of 5-10 mm^3 formulated by using carriers like clay minerals, starch polymers, dry fertilizers and ground plant residues [55]. Concentration of organisms in granules is 5-20 %. There are three types of granules: 1) exterior granules – microbes attached to outer surface of a carrier by a sticker; 2) exterior granules without stickers and 3) incorporated granules – all constituents mixed into a paste to from matrix and later sieved (after processing for granulation) to desired size. Normally, they are employed in agricultural crops, e.g. cabbage, corn etc [56].

Granular ingredients are retained pasty so that the material can be pressed through a granulation die. They are made by mixing together all ingredients into a liquid carrier and then extruded. There have been various types of granules – wheat meal granules [57]; corn meal baits; granules formed with gelatinized cornstarch or flour [25,58-63]; casein [64]; gluten [65]; cottonseed flour and sugars [66]; gelatin or acacia gum [67]; sodium alginate and paraffin [68]; diatomaceous earth [69] and semolina [70] commonly employed as formulations. In a recent advancement in granular formulations, solutions containing powder formulations of *Bacillus thuringiensis* var. *israelensis* (Bti) or *Bacillus sphaericus* were transformed into ice pellets (named IcyPearls). This technique encompassed various advantages over Bti sand granules: 1) Bti ice pellets melted on the water surface and released the microbial crystals; 2) there was no loss of Bti by friction in the spraying equipment; and 3) the ice formulation resulted in increased swath widths, significantly reducing application costs [71]. However, practical diverse application of such formulations in tropical countries with peak temperatures remains a big quest.

Technology of granules has paved way to agglomerates or encapsulated versions which can give better protection against UV radiation, rain and wind.

3.3.3 Briquettes

They are large blocks with sizes ranging from 100-250 μm and possess same carriers as in granules presenting no drift problems. Bti based briquette formulations are largely utilized in public health sector for control of mosquitoes.

Various formulations of Bti have given higher rates of kill and sustained persistence, in several cases, ranging up to two months in single application [68,72,73]. Briquettes have been normally made using organic polymers like polyvinyl alcohol to give floatability causing sustained release of toxins over several months [74-77]. Mostly floating type formulations with carrier materials such as wheat flour are common with Bti [75] and also buoyant forms which have self-encapsulating abilities [78] and gypsum has been used to enhance sustained release of toxins [79].

3.3.4 Wettable powders (WPs)

They consist of 50-80% technical powder, 15-45 % filler, 1-10% dispersant and 3-5 % surfactant by weight to achieve a desired potency formulation (measured in International Units). Fillers are hydrophilic and usually contain silica which resists cake formation and friability during grinding. However, silica content must be kept low to avoid abrasion of formulation equipment [80]. Dispersant must be added to retain suspension in dispersion; surfactants to overcome surface tension at liquid-solid interface (excess may lead to foam generation and partitioning of spores therein). Normally, particles settle rapidly, hence spray tanks fitted with agitators are usually used.

Among the dried formulations of biopesticides, much attention has been paid to WPs because of their longer shelf life, good miscibility with water and ease of application as sprays with conventional equipment [80]. Despite the advantages, literature is not abundant for Bt WP products, only WP formulation for nuclear polyhedrosis virus of *Anticarsia gemmatalis* was shown to be evaluated by Medugno et al. [81]. Recently, Teera-Arunsiri et al. [82] have reported development of Bt ssp. *aizawai* based WP with 55% suspendibility, 24s for wetting time, and 5.69 x 10^4 CFU/ml of LC_{50} value against *Spodoptera exigua* larvae. The authors used fish meal as a growth substrate and locally available tapioca starch as a sticker and vegetable oil as surfactant. Many Bt WP products that find use in gardens and agriculture could also be located on company website of Valent Biosciences and Novartis Corporation [83,84].

Although there are lots of advantages rendered by dry formulations, yet they encompass various problems as well, as reported in Table 4.

3.4 Liquid suspensions

3.4.1 Suspension concentrates (flowables)

They are suspensions of particulates in liquids, with 10-40% microorganism, 1-3% suspender ingredient, 1-5% dispersant, 3-8% surfactant, and 35-65% carrier liquid (oil or water). The products are prevented from settling due to reversible agglomeration by dispersants; surfactants act as wetting agents and spreaders, and generally, non-ionic ones (derivatives of poloxyethylene and polyoxypropylene) are preferred along with water soluble sunscreens [6]. Based on type of carrier liquid, surfactants are characterized by hydrophile-lipophile balance (HLB) whereby lower HLB is suitable for water-in-oil and vice versa for oil-in-water based suspensions [85,86].

Formation of biopesticide concentrates in oil increased sedimentation and hence wet milling of product was recommended to retain suspendibility. Biodegradable oils, for instance, vegetable oils are preferred and a detailed listing of ecologically benign oils is given by Underwood [87]. The clear difference between water and oil based formulations could be easily understood from Figure 1.

In forestry, generally, ultra low volume (ULV) sprays (required dispersion rate of 20 BIU/ha in a final volume of 2.5L/ha) are preferred to deliver higher Bt concentrations [88]. Earlier, the suspension concentrates were ready-to-use formulations requiring just the addition of sticker (0.06 %) which minimized spraying cost by eliminating mixing time and maximizing payload [88]. Some authors had also suggested addition of sorbitol as a dispersing agent, which enhanced density of Bt formulations resulting in concentrated suspensions (reducing loading costs) and acting as an anti-freeze as well as anti-evaporant further structuring formulations [89]. The success rates of Bt formulations led to more than 70% use of Bt biopesticides in North America in 1990s for forest Lepidoptera pests control compared to less than 5% in 1981 [90]. However, nowadays, the trend is to achieve maximum kill with minimum dosage (maximum efficacy per droplet) and in this context, a study carried out in Quebec, Canada established 30 BIU/ha (BIU - billion international unit) as the optimal dosage at 1.5 L/ha spray volume by using a high potency (20 BIU/L) product [91]. This strategy will help curb transportation costs enhancing commercial viability of the Bt

formulations. Liquid formulations of *B. sphaericus* have also been successfully employed in rice fields, ponds and other water sources against mosquitoes from the genus *Culex* [92,93].

3.4.2 Emulsions

They comprise of liquid droplets dispersed in another immiscible liquid (dispersed phase droplet size ranges from 0.1 - 10 μm), e.g. oil-in-water (normal emulsions) and water-in-oil (invert emulsions). The emulsions do not encounter sedimentation problems, but creaming and layer separation are common [94]. In biopesticide jargon, they are referred to as suspo-emulsions. As oil is external phase in invert emulsions, losses due to evaporation and spray drift are minimal [95,96]. However, lower shelf stability and phytotoxicity may affect the overall performance of the emulsions.

In 1980s, ULV sprays in forests comprised oil emulsions, but lately it has been replaced with aqueous emulsions [6]. Presently, oil emulsions find restricted use in household or agricultural sprays [44,97]. Probable reasons for their discontinued use in forests could be: increase in drift velocity which may fall across large distances affecting habitations; creaming problem during storage and lower biodegradability in contrast to water formulations.

3.4.3 Encapsulations

They are recent advances in bioinsecticidal formulations and provide protection from extreme environmental conditions (UV radiation, rain etc.) and enhanced residual stability due to slow release of formulations (sustained delivery). They are usually liquid suspensions with possibility of powders and granules too. Microbial propagules (e.g., Bt) are encapsulated in a coating (capsule) made of gelatin, starch, cellulose and other polymers and even microbial cells (also referred to as "ghost encapsulations") [98-101].

Initial encapsulations were an extension of chemical pesticides and involved addition of clays, matrices such as polyvinyl propylene and polyvinyl alcohol [102,103]. This led to the exploitation of biopolymers to make the products eco-friendly [104-106].

Conventionally, autoencapsulated (biological origin) formulations against European corn borer (*Ostrinia nubilalis*), were made by mixing starch powder and sugar [104,107]. Fine, encapsulated products can be sprayed in any volume as the pathogen is held tightly to additives causing less wastage [108,109]. Bok et al. [110] suggested use of carbohydrate rich biopolymeric gels for construction of these matrices and showed 50 % mortality of diamondback moth even after 15 days. Elsewhere, Cheung and Hammock [111] emulsified crystal and solubilized Bti toxin with Freund incomplete adjuvant. The encapsulation of lipophilic material altered buoyancy of the toxin and reversed sensitivities of the mosquito larvae towards Bti toxin. Attachment to latex beads was also considered to enhance entomotoxicity of Bti formulations [112].

The starch encapsulated granules have been successfully scaled up, but there were losses owing to decrease in residual activity due to rain and UV radiation [113]. Eventually, a modified method developed using solvents showed sustained release of Bt over 12 d [114]. When the formulations were tested in field, it was observed that there was no change in activity during dry season, but during rains, rest of the formulations lost their activity with respect to starch formulations [115]. To take into account pH effects normally encountered in field, addition of gluten to the starch matrix aided in retaining the larval mortality of O. nubilalis > 90% over a pH 5-11 and also resisted 5 cm rain [116]. This provided a broader pH range for the formulations to act and protected δ-endotoxin by competing with proteases at alkaline pH and secondary plant compounds such as tannins [117]. Ramos et al. [118] have discussed use of different biopolymers for granular formulations of Bt. There have been several studies on incorporation of lignin into microcapsules to further extend residual activity and it was observed that activity remained 75 % after 7 days versus 53 % for commercial Dipel formulation [119, 120]. Moreover, the maize flour microcapsule formulation offered increased protection from UV radiation and improved rainfastness of Bt during simulated rainfall. Likewise, Côté et al. [121] reported an extended period of mortality of *Choristoneura rosaceana* with bioencapsulated formulation (rice flour based) in comparison to conventional DiPel WP formulation. Alginate coated Bti formulations were also used to control mosquitoes [122]. In some formulations, polyethylene glycol or ethylene glycol was used as an encapsulating binder with anti-freeze properties for better dispersion [123-125].

Another recent advance in encapsulations is production of hydrocapsules that are of shellcore type (water based), consisting of a polymer membrane surrounding a liquid center. These shells are produced by using UV radiation initiated free-radical copolymerization of functionalized prepolymers (silicones, urethanes, epoxys, polyesters, etc.) and/or vinyl monomers such as acrylates for better dispersion and UV radiation protection [126,127].

Encapsulation in the form of microcapsules has been extensively exploited to give smaller size, highly efficient fungal biopesticide formulations [122]. This technology could be extended to Bt suspensions, which would enhance aerial dispersion onto foliage and feeding, by larvae.

Liquid formulations have several advantages over dry formulations (Table 4), and especially encapsulated products lower the cost substantially due to close juxtaposition of various mandatory additives like UV radiation screens and stickers in micro-quantities. In addition to these conventional categories, there are several advances in formulations, which include sustained release and use of synergists, or phagostimulants to enhance the otherwise normal entomotoxicity.

3.5 Advances in formulations (for improved delivery)

The effectiveness of Bti, on aquatic organisms, is generally dependent on bioavailability of the material which can be problematic in aqueous or sub-surface environments. For example, entomopathogen may not remain in the region of interest, where the aquatic organisms are located, for a length of time sufficient to provide complete treatment. Akin to solid formulations, controlled release formulations comprising different carriers like polymers, charcoal, petroleum coke, coke from coal, woody ring portions of corn cob etc. can be used with a coating of fatty acids and alcohols for regulating sustained release rate and profile into target environment [128,129]. The protein-polysaccharide complex (PPC) composition containing water-soluble cellulose derivatives, seaweed polysaccharides such as alginate and carrageenin, seed mucilaginous polysaccharides. Complex plant exudate polysaccharides such as gum Arabic, tragacanth, guar gum, pectin, ghatti, and microbially synthesized polysaccharides such as xanthan gum have also been one of the choices for controlled release formulations [130].

In field application, issues like coverage and application volume affect deposit structure of Bt formulations. Deposit efficacy has been a favorite and most controversial topic of researchers [89]. As droplet size is related to its radius by third order exponent, it significantly affects deposits and in turn governs framework of new formulations. While application methodologies producing large number of small droplets (spinning disk, rotary cage, etc) often improve coverage, a concomitant increase in efficacy is not necessarily observed [131,132].

3.6 Booster formulations (enhanced entomotoxicity)

These formulations fall into a different class as there is enhancement of entomotoxicity by either stimulant or synergistic action. It all began with preliminary gustatory stimulation studies of baculovirus based biopesticides for bollworm control, which yielded encouraging three folds increase in entomotoxicity [133]. Initial phagostimulant studies were mainly sugar based derived from pure sugars viz. lactose, sucrose and others [59,134]. With the progress in understanding of the actual and specific action of these adjuvants, newer options of amino acids, starch, ascorbic acid and non-conventional ones like cucurbitacins and garbanzo beans were explored [60,135,136]. As interest escalated in these phagostimulants, some commercial ones like Coax, Entice, Gusto Konsume, Mo-Bait were introduced which increased feeding responses of pests delivering higher entomotoxicity products in the field [137,138]. Semiochemical-based toxic baits are another category of these types of formulations where a volatile attractant or floral extract is added to formulations to fool the pests. Lance and Sutter [139] formulated a bait containing carbaryl, cucurbitacin, and several non-pheromone volatile attractants in dry, bran/ starch based carrier. Unfortunately, the formulation sank to the ground after 4-9 days yielding negative results. In yet another study, bait composed of a commercial phagostimulant (Coax, Konsume, or Nu-Lure) and one of several insect growth regulators (IGR's) was tested on fall armyworm, *Spodoptera frugiperda* and this yielded encouraging results especially with control rate of 90-95% and reducing adult numbers than alternating swaths [140,141]. Lately, addition of 675 µg/l monosodium glutamate to commercial formulation of Bt ssp. *kurstaki*, (DiPel®2X DF), lowered LC_{50} from 450 to 150 µg/l ($P < 0.05$, Lethal Ratio Significance Test), indicating its potential to enhance entomotoxicity and economy of Bt based formulations [142].

Moreover, lytic enzyme like chitinase could also increase entomotoxicity by perforating the peritrophic membrane barrier in the larval midgut and thus increasing accessibility of Bt δ-endotoxin molecule to its receptor on epithelial cell membranes [143]. In fact, synergism between chitinase and Bt is as old as 1970s as also confirmed by growth studies of different subspecies of Bt on chitin [144]. Larvae of spruce budworm, *Choristoneura fumiferana*, died more quickly when exposed to chitinase-Bt mixtures rather than Bt or chitinase alone [145,146]. Similar observations were made against the mortality of gypsy moth (*Lymantria dispar*) larvae and the toxic effect was correlated with enzyme activity [147,148]. Different crude chitinase preparations and endochitinases have constantly enhanced entomotoxicity of Bt [149-151].

There are other kinds of compounds that augment the entomotoxicity of Bt, antibiotics like zwittermicin, which are similar in structure to the synergist molecule [152,153]. Particles such as latex beads that adsorb solubilized polypeptides have even been used to raise the entomotoxicity of Bti crystal proteins [154]. Additionally, some optical brighteners like Tinopal LPW, known to be domain of viral biopesticides, when combined with Bt enhanced entomotoxicity and caused mortality of Colorado potato beetle larvae in relatively lesser time [155].

Hence, addition of different phagostimulants, antibiotics, optical brighteners and more recently chitinase producing Bt hold the key to future Bt formulations.

4. Environmental effects

As seen earlier, Bt formulations suffer short field persistence owing to adverse environmental factors, which needs to be understood completely before developing new formulations.

4.1 Sunlight/UV radiation

Natural sunlight, especially UV radiation portion of the spectrum: UV-B (280-310 nm) and UV-A (320-400 nm) is mainly responsible for inactivation of insect pathogens. Several authors have proposed the involvement of chromophores (exogenous, possibly endogenous too) derived from fermentation media, which after cell lysis, adsorbed onto Bt crystal

proteins [156-158]. These chromophores, absorbing at 300-380 nm, so far uncharacterized, passed excited, electronic-state energy to oxygen molecules, in turn converted to the highly reactive singlet, or free radical state. In this state, the singlet oxygen attacked indole side chains of tryptophan residues on the protein toxin, resulting in loss of insecticidal activity [157,158]. Cohen et al. [158] suggested the use of cationic moieties such as acriflavin and rhodamine B to transfer energy from excited tryptophan and act as UV radiation blocker.

Effect of UV radiation also varies under different climate conditions, for e.g., half-life of Bt in USA on cotton plants was 30-48h and in Egypt on castor plants was 19-40h [159,160]. Earlier studies by Morris [161] used water soluble dyes, namely, DS 49 (benzophenone) and erio acid red (sodium salt of formyl-m-benzenedisulphonic acid) which increased the residual activity of Bt on white spruce trees by 2.9-fold. Dunkle and Shasha [58,162] described a starch encapsulated Bt technique containing UV radiation screens against sunlight. Ragaei [160] utilized 2% solution of Congo Red which increased residual activity on castor plants 3.3-fold. Bartelt [59] demonstrated that if Coax (CCT, Lichtfield Park, AZ, USA), (a proprietary product used for corn borer larvae) was incorporated in water dispersible granules; only 25 % of the entomotoxicity was lost in greenhouse tests. Subsequent work carried out by McGuire [104], documented a similar response by corn borer larvae under field conditions. Bt granules (size, 150-210 μm) with wheat meal used both as a carrier and a feeding stimulant, against *Earias insulana* in cotton, gave excellent results against UV radiation, dew etc. [57]. The benefits of flour over starch include lower cost and the protein may also act as a feeding stimulant, and/or a sunlight screen [60]. Various organic compounds like benzaldehyde, cinnamaldehyde, salicylaldehyde, methylene blue and yeast extract when employed as sunscreens for *B. sphaericus*, showed elimination of larvicidal activity after 12 h UV irradiation [163].

Also, when efficacy of melanin for the protection of mosquito larvicidal activity of Bt against UV radiation was studied, the bioassays confirmed an important role of melanin as a photoprotective agent [164]. The biggest problem with melanin usage would be dissolution of the pigment on exposure to rain. Recently, there has been a study reported on Btk mutant producing melanin which on irradiation at 254 nm and 366 nm, showed higher entomotoxicity than parent strain [165,166]. Thus, utilization of such mutants with increased UV radiation resistance can aid in developing stable Bt formulations for field application.

Based on the reported findings as outlined in Table 5, screening agents that absorb in 300 - 400 nm range should enhance the persistence of foliar applied Bt products [161]. Several studies have been carried out with the incorporation of various UV radiation screens like Congo Red, folic acid, molasses, lignin, alginate, cellulose, shellac yeast, p-amino benzoic acid with mixed results on UV radiation protection [47,65,116,119,162,167-172]. Certain fluorescent brighteners, especially compounds of stilbene type, specifically, derivatives of 4,4'-diamino-2,2'-stilbene disulfonic acid and their salts enhanced biological activity up to 1000-fold and protected Bt from UV radiation exposure [173].

Further, UV radiation agents performed well with water based Bt suspensions as discussed earlier. Recently, a state-of-the-art technology has been developed involving double sheath - a water-soluble protective coating and a hydrophobic oil carrier (suspending agent), for application to a target substrate. When such a formulation was applied to a target substrate, oil component facilitated dispersal of formulation over the substrate, resulting in adherence and subsequent UV radiation protection [174].

Nevertheless, encapsulation of biopesticidal materials in a matrix has been considered as the most effective form of formulation and as mentioned earlier, various coating agents have been explored to achieve the same. Lately, there has been focus on utilization of protection as well as sustained release through microencapsulated formulations [175-177]. Thus, UV radiation will have serious repercussions on the product stability, which is considered in development of advanced Bt formulations.

4.2 Rainfall and Dew

Rainfall is another important natural component that would affect the persistence of biopesticides on foliage leading to wash off before action. Behle et al. [116] reported that 3 cm of rain reduced efficacy by 20 percent in Bt biopesticides. However, Ferro et al. [117] demonstrated that little to no loss in activity of Bt ssp. *tenebrionis* was observed after 2.5 cm of simulated rain if rain fell after the insecticidal application had dried (approximately 15 min) [178]. Heavy dew also decreased Bt efficacy on foliage. Rainfall may be even more

important in product degradation than light when foliage is shaded from direct sunlight (forestry, whorl of corn plants) [179].

Several different approaches to formulation appear to have improved the rainfastness of Bt products. Experimental cornstarch based formulations showed increased activity following rainfall, possibly because of the ability of cornstarch to stick onto crop foliage [115] and similar results were shown by maize flour microcapsules [120]. Similarly, *Pseudomonas fluorescens* cells genetically engineered to produce Bt endotoxins showed improved rainfastness, possibly due to the presence of polysaccharides, proteins, and glycoproteins present in the *Pseudomonas* cell wall that help it adhere to crop foliage [6]. Conventional Bt products, in contrast, are composed of lysed spores, cell debris and crystals, and thus cannot take advantage of any cell wall adhesiveness possessed by *Bacillus* cells.

This drawback has fuelled the development of new commercial products within the past several years. For foliar applications, Mycogen Corporation's development of the CellCap7 encapsulation system has improved the stickiness of spray applications, thus enhancing rainfastness [99-101]. From a practical approach, it has been common practice among producers to add sugar, molasses, oils or commercially available sticking agents to their Bt spray tanks. The improved performance may be due to the sticking ability imparted by these additives and there is a tendency to use multi-purpose agents for better economy. Bacterial ghosts, which represent empty cell envelopes of Gram-negative bacteria, have been applied successfully as vaccine candidates or as potential drug carriers [180]. Such envelopes from the plant-adhering bacterium *Pectobacterium cypripedi* possess high sticking abilities and have been used as effective carriers for chemical pesticides with 60% retention of toxicity after a heavy simulated rain (84 mm) [181]. Therefore, in future similar approach could be adopted for Bt based biopesticides.

In toto, rains can significantly affect the activity of biopesticides by wash-off before action and the need to add stickers has to be studied comprehensively. Perhaps, encapsulated formulations could provide a better solution.

4.3 pH

Literature indicates that Bt activity is stable above pH 3 and below 11 [80,182]. As the length of exposure increased, the sensitivity of Bt to extreme pHs also increased. Similarly, as temperature increased, Bt sensitivity to extreme pHs also increased. It is likely that the

optimal pH range for Bt is considerably narrower than pH 3 to 11 under typical field conditions where high temperatures frequently prevail [167].

Normally, commercial Bt products are buffered in the region of pH 4-5, to inhibit growth of most microbial contaminants and possible breakdown of crystal proteins by alkaline proteases that can lead to lowering of activity [131]. In practice, few end-users are plagued with highly acidic water, but water pHs of 10 are unusual in some regions. For most commercial Bt products, end-users are advised to add buffering agents to their spray tanks when water pH is higher than 9 due to utilization of available water resources (already at alkaline pH). Recently, wheat gluten based formulations have been developed that could sustain the entire pH range 3-11 [64,65].

Effects of pH are mostly encountered while loading the spray mixtures into containers (aluminum or iron) which could get corroded and afflict effective field application of the biopesticides [6] and this necessitated use of buffering agents. The pH has been also found to ruin the formulation performance during storage (action of proteases) as well as application (tank corrosion) and post-application (foliage action discussed later) stage.

4.4 Temperature

Temperature plays an important role in shelf life as well as post-application persistence of Bt formulations. It has been observed that temperatures lower than 10°C and higher than 30°C may have deleterious effects on the activity of bacterial pathogens over an extended period of time [157]. This may be caused by degradation of the a.i. by heat, or more likely, by reduced feeding of insects because of high or low temperature extremes [183]. In crop protection and vector control, temperature extremes are commonly encountered. For example, applications of Bti made against mosquitoes, in Canada, are used in early spring, when water temperatures are near 10-15°C when larval feeding and digestive activity is low to allow significant activity of Bt spores and crystals [184]. The feeding rates of mosquito larvae are similarly reduced by temperatures of 5°C [185].

Higher temperatures have also resulted in decreased activity of Bt products, especially in the tropics, where temperatures frequently exceed 30°C [161]. In addition to possible inactivation of the toxin, high temperatures resulted in decreased feeding rates for many insects and enhanced deleterious effects of pH [182]. A method to counteract the effect of adverse temperatures is to select Bt strains active over a wide range of temperatures and use

of encapsulated formulations. Hence, **both extremes of temperature can decrease the residual activity of Bt formulations.**

4.5 Foliage

Biological activity of Bt formulations on foliage is short-lived, with half-life ranging from one to two days on unshaded foliage [156] and 20 to 30 days on shaded foliage [186]. In addition to environmental factors (such as sunlight and rain), two other factors – leaf expansion and the presence of secondary plant compounds have a significant impact on persistence of Bt on foliage. Specifically, volatiles like aldehydes, ketones, carboxylic acids and their derivatives present on these leaves have an antibiotic effect on Bt spores and sometimes, cause of its inactivation or delay in normal growth [187-189].

During evaluation of bacterial persistence, leaf growth and expansion are normally overlooked parameters. For fast growing plants such as leafy vegetables, tomatoes, potatoes or cotton, it takes only a few days for bacterial formulations to become significantly diluted by the development of new, untreated leaf area [190]. In fact, it has been reported that Bt products appear to persist longer in forests (residual activity of 20 - 30 days) than in agricultural settings (hours to few days for row crops) [86,191]. The reasons are unclear but it could be caused by shading from direct sunlight, as well as lack of leaf expansion in forest fauna. This "dilution" effect may be one of the most important reasons that improved bacterial persistence observed in laboratory tests conducted on artificial diet or leaf discs does not translate to outdoor field experiments. Hence, in addition to influencing the outcome of field tests, the effect of leaf growth and expansion should be taken into account when product rates, application timing and application frequency protocol is developed, with higher rates and increased application frequencies recommended for fast growing agricultural crops.

Certain chemicals present on the phylloplane and soil can also affect the efficacy of product, for instance cotton exudates Mg^{2+}, Ca^{2+} and Mn^{2+} as carbonates and bicarbonates raise the pH as high as 10 or 11, and this could affect performance of the formulations [192]. As Bt is endotoxin is a digestive tract poison, leaf extract would have the least effect [6]. To overcome pH problems, casein, gluten and tannic acid based formulations were developed [64,65,193]. **Thus, the most effective way to counteract foliage pH is by applying well planned spray timing strategy and incorporating buffers either in the formulation or in the tank mix.**

Extensive review by Joung and Côté [194] has established that Bt would not have any significant impact on the non-target microflora in soil, water and foliage under field conditions.

5. Wastewater/wastewater sludge based Bt formulations

Wastewater/wastewater sludge (WW/WWS) has been successfully used as a raw material for Bt biopesticide production with lower process costs [195-205]. However, the formulation costs are yet to be accounted for in the overall Bt production process. The WW/WWS based Bt biopesticides encompass advantages and drawbacks over commercial biopesticides. Two principal problems associated with the use of wastewater sludge are the presence of toxic heavy metals and pathogens. As sludges are completely sterilized prior to Bt fermentation, all pathogens are eliminated. Further, sludges used for Bt production meet the regulatory criteria prescribed by the Quebec Environment Ministry [206] and about 50% of total sludge (1million tons of dry sludge solids per year) produced in Canada meets the regulatory criteria. This mitigates the problem of metals. The quantity of Bt based sludge to be sprayed in field (about 50g for a potency of 76B) would be very low compared to the amount of sludge permitted for agriculture land application (15–30 tons per hectare depending on N, P, K content) and hence the risk of metal contamination is almost nil [205].

The harvesting methods for WW/WWS based Bt biopesticides may not require filter aids as sludge contains flocs (average particle size of non-hydrolyzed, hydrolyzed sludge and was 17.8, 8.6, 4.1 and 2 μm, respectively), which will behave as adsorbents for spores and crystal protein during centrifugation and as protectants during adverse spray drying conditions [207,208]. However, advanced harvesting techniques (for example, UF) might need further research as membrane fouling and cake formation could occur due to complex rheology (under investigation in our laboratory). Furthermore, higher entomotoxicity was obtained in Bt fermented sludge as compared to semi-synthetic medium [200-205]. This may require moderately concentrated broths for formulation amendment reducing solids concentration and hence increasing suspendibility.

Furthermore, during Bt formulation development, Bt fermented sludge produced chitinases, which would enhance entomotoxicity (data unpublished) and there would be no need to add external source of chitinases as done in several synthetic media derived formulations (discussed earlier). The WW/WWS based Bt formulations possess higher rainfastness property (preliminary studies have yielded encouraging results, data unreported)

as there is presence of extra-cellular polymeric substances (EPS) known to play an important role in biofilm formation [209]. The EPS could act as stickers resulting in better adherence. Moreover, due to the natural encapsulation of spores and crystals in sludge flocs, the Bt formulations were resistant to variable temperatures [210].

As is evident from the Figure 2, WW/WWS formulations even without addition of UV radiation screens, performed better than commercial formulation. The half-lives of formulations followed the order: raw sludge>hydrolyzed sludge >starch wastewater>soya and were reported to be (in days) 11.14>9.51>9.02>2.8. $T_{0.5}$ (half-life) of non- hydrolyzed sludge was higher probably due to the flocs (average particle size, 17.8 μm) that provided protective sheath around crystal protein and spores reducing their inactivation and hence better residual entomotoxicity. Hydrolyzed sludge was better than starch wastewater and soya probably due to two reasons: higher floc size (8.6 μm) which formed a protective sheath (discussed earlier) and lower protease activity (1.1 IU/ml) as compared to starch wastewater (1.8 IU/ml) and soya (1.3 IU/ml). The high protease activity in latter formulations would have caused inactivation of crystal protein, hence decreased entomotoxicity and lower half-life.

Thus, UV resistance results gave **better performance of the sludge based Bt formulations in comparison to half-life of conventional Bt formulations** in field conditions ranging from 16 h to 2 d [169]. Additionally, domestic sludge has been reported to have components with chromopheric compounds or auxochromes (majority of fulvic, humic and hymathomelanic acids) with absorbance at 350 nm [211]. These components would have acted as natural UV radiation screens in sludge based formulations. This warrants further studies to validate the effect of UV radiation screens in formulations (conclusively, addition of UV radiation screens must further extend the residual entomotoxicity). Hence, sludge based Bt biopesticides can be an important addition to Bt product profile as they would be more cost effective than the synthetic medium based biopesticides.

6. Recapitulation and research priorities

The biopesticide production is an integrated and interlinked process. Hence, rational biopesticide development/delivery would comprise formulation type; synergy with application equipment; spatial targeting and timing of application, in addition to use of smart alternatives like cost-effective media based formulations (sludge based formulations, discussed earlier). Despite extensive literature on formulations, no study has been reported on

either the formulations of Bt fermented products based on waste/agricultural by-products and/or a complete process from fermentation until formulation.

Rheology of fermented broth will be decisive in downstream processing steps to give a consistent product, which will exert considerable influence on application and ultimately, the efficacy. Despite considerable research, it is not yet fully comprehensible if there is a dependence of the biological properties of a microbial biopesticide formulation upon its physical properties, yet they are interdependent [6]. Thus, development of formulations must take into account the biotic (spore concentration and entomotoxicity) and abiotic factors (UV radiation, temperature, pH, rain, foliage and others) for better delivery. Furthermore, different parameters and additives for specific type of formulation (e.g. to avoid cake formation in dry products, hygroscopic material must be avoided). Also, possibility of contamination and degradation of crystal protein by proteases during storage has to be minimized (pH changes) and hence strongly acidic solutions are preferred. Distinct features suggest a practical place for both solid and liquid Bt formulations: in fact, both sell nearly equally.

Within the limits of biological requirements of Bt, as well as the economic reality of production cost, the fermentation process has to be tailored to meet the needs of subsequent formulations.

7. Conclusions

Bt based biopesticidal formulations will find wider application in future by adopting simple harvesting methods and robust and economical choice of various additives for different formulations. In general, there are two types of formulations – solid and liquid, including advances like encapsulations and both have a distinct application and hence equal markets. Various environmental factors, namely, UV radiation, temperature, wind, pH and rain influence the field efficiency of Bt formulations. From late 90s to recently, Bt formulation trends have progressed in the direction of "maximum efficacy per drop" resulting in high potency concentrates requiring lower spray volumes. Wastewater and/wastewater sludge based formulations may hold the key to various problems encountered by commercial medium based formulations aiding in two ways – sustainable reuse of wastes and enhanced penetration of Bt biopesticides into global pesticide market. This will also greatly expand the repertoire of commercial Bt product types.

Acknowledgements

The authors are sincerely thankful to Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. We are also thankful to the Natural Sciences and Engineering Research Council of Canada and Canadian Forestry Service for providing Ph.D scholarship to Satinder K. Brar during the course of this research work.

References

[1] Marrone PG. Microbial pesticides and natural products as alternatives. Outlook Agric 1999; 28(3): 149-154.

[2] Cannon RJC. Prospects and progress for *Bacillus thuringiensis* based pesticides. Pestic Sci 1993; 37: 331-335.

[3] Chapple AC, Bateman RP. Application systems for microbial pesticides: necessity not novelty. In "*Microbial Insecticides: Novelty or Necessity?*" *British Crop Protection Council Proceedings/Monograph Series* No. 68 1997; pp181-190.

[4] Jacobsen BJ, Backman PA. Biological and cultural plant disease controls: Alternatives and supplements to chemicals in IPM systems. Plant Dis 1993; 77: 311-315.

[5] Copping L. The Biopesticides Manual. 333 pp. British Crop Protection Council, UK, 1998.

[6] Burges HD. (ed.) *Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments*, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1998.

[7] Chapple AC, Downer RA, Bateman RP. Theory and practice of microbial insecticide application. In: Lacey, L.A., Kaya, H.A. (eds.). Field Manual of Techniques in Invertebrate Pathology. Kluwer, Dordrecht 2000; pp 5-37.

[8] Parekh S, Vinci VA, Strobel RJ. Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 2000; 54: 287–301.

[9] Cords H, Fisher RA. Stable concentrated bacterial insecticide suspensions. US Patent 3,271,243, 1966.

[10] Priest FG. Biological control of mosquitoes and other biting flies by *Bacillus thuringiensis* and *Bacillus sphaericus*. J Appl Bacteriol 1992; 72:357-369.

[11] Bernhard, K, Utz, R. Production of *Bacillus thuringiensis* for industrial and commercial uses. In: Entwistle, P.F., Cory, J.S., Baily, M.J., Higgs, S., editors, *Bacillus thuringiensis*: an environmental biopesticide: theory and practice. Wiley Pubs. Great Britain. 1993; pp 255-267.

[12] Rowe GE, Margaritis A. Bioprocess design and economic analysis for the commercial production of environmentally friendly bioinsecticides from *Bacillus thuringiensis* HD-1 *kurstaki.* Biotechnol Bioeng 2004; 86 (4): 377-388.

[13] Keller, K Friedmann T, Boxman A. The bioseparation needs for tomorrow. TRENDS in Biotechnol 2001; 19 (11): 438-441.

[14] Dulmage HT. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis var. alesti.* J Invertebr Pathol 1970; 15: 232-239.

[15] Christy C, Vermant S. The state-of-the-art of filtration in recovery processes for biopharmaceutical production. Desalination 2002; 147: I-4.

[16] Darnon E, Morin E, Belleville MP, Rios GM. Ultrafiltration within downstream processing: some process design considerations. Chem Eng Processing 2003; 42: 299-309.

[17] Lertcanawanichakul M, Wiwat C. Improved shuttle vectors for expression of chitinase gene in *Bacillus thuringiensis*. Lett Appl Microbiol 2001; 2: 123–128.

[18] Arora N, Ahmad T, Rajagopal R, Bhatnagar RK. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis* HD-1. Biochem Biophy Res Commun 2003; 307: 620–625.

[19] Soper RS, Ward MG. Beltsville Symposia in Agricultural Research. Biol Cont in Crop Production 1981; 5: 161-180.

[20] Sharpe ES, Herman AI, Toolan SC. Foam flotation process for separating *Bacillus thuringiensis* sporultion products. United States Patent 1981; 4,247,644.

[21] Zhou X, Chen S, Yu Z. Effects of spray drying parameters on the processing of a fermentation liquor. Biosystems Eng 2004; 88 (2): 193–199.

[22] Bonnefoi A. Method for producing biological pest destroying agents. US Patent 1963; 3,071,519.

[23] Cords H, Fisher RA. Stable concentrated bacterial insecticide suspensions. US Patent 1966; 3,271,243.

[24] Taborsky V. Small-scale processing of microbial pesticides. FAO Agricultural Services Bulletin No. 96, Food and Agriculture Organization of the United Nations, Rome, 1992.

[25] Tamez-Guerra P, McGuire MR, Medrano-Roldan H, Galan-Wong LJ. Sprayable granule formulations of *Bacillus thuringiensis*. J Econ Entomol 1996; 89: 1424-1430.

[26] Rojas JV, Gutierrez E, De la Torre M. Primary separation of the entomopathogenic products of *Bacillus thuringiensis*. Biotechnol Prog 1996; 12: 564-566.

[27] Zamola B, Valles P, Meli G, Miccoli P, Kajfez F. Use of the centrifugal separation technique in manufacturing a bioinsecticide based on *Bacillus thuringiensis*. Biotechnol Bioeng 1981; XXIII: 1079-1086.

[28] Dulmage HT, Rhodes RA. Production of pathogens in artifical media. Pp.507-540. In Microbial control of insects and mites. H.D Burges and N.W. Hussy, eds. Academic Press. New York. 1971.

[29] Tzeng YM, Tsun HY, Chang YN. Recovery of thuringiensin with cetylpyridinium chloride using micellar-enhanced ultrafiltration process. Biotechnol Prog 1999; 15: 580-586.

[30] Markels JH, Lynn S, Radke CJ. Cross-flow ultrafiltration of micellar surfactant solutions. AIChE J 1995; 41(9): 2058-2066.

[31] Agrawal A, Burns MA. Application of membrane-based preferential transport to whole broth processing. Biotechnol Bioeng 1996; 55(4): 581-591.

[32] Persson A, Jonsson AS, Zacchi G. Use of microfiltration as first step in recovery of protein from a fermentation broth. Appl Biochem Biotechnol 2004;112 (3): 151-162.

[33] Russotti G, Osawa AE, Sitrin RD, Buckland BC, Adams WR, Lee SS. Pilot scale harvest of recombinant yeast employing microfiltration: a case study. J Biotechnol 1995; 42: 235-246.

[34] Lee CT, Morreale G, Middelberg APJ. Combined in-fermenter extraction and cross-flow microfiltration for improved inclusion body processing. Biotechnol Bioeng 2004; 85(1): 103-113.

[35] Wong HH, O'Neill BK, Middelberg AP. Centrifugal processing of cell debris and inclusion bodies from recombinant *Escherichia coli*. Bioseparation 1996; 6 (6): 361-372.

[36] Tsuji K. Recent trends in pesticide formulations. In C.L. Foy, D.W. Pritchard, and G.B. Beestman Eds., Formulation Science Volume I., Association of formulation Chemists, 1997; pp. 53-83.

[37] Cross JV, Poloneko DR. An industry perspective on registration and commercialization of biocontrol agents in Canada. Can J Plant Pathol 1996; 18: 446-454.

[38] Frey PM. Biocontrol agents in the age of molecular biology. TRENDS in Biotechnol 2001; 19(11): 432-433.

[39] Matthews GA. Pesticide Application Methods, 2nd edn., Longman Scientific and Technical, Harlow, 1992.

[40] Hall FR, Chapple AC, Taylor RAJ, Downer RA. Biorational Pest Control Agents Formulation and Delivery, ACS Symposium Series 595, 1995; 68-78.

[41] Hall FR, Menn JJ. Biopesticides : Use and Delivery, Humana Press, New Jersey, USA, 1999.

[42] Rodham DK, Wang Y, Cantwell JB, Winn PD, Foundling J. Formulating microbial biocontrol agents. Pestic Sci 1999; 55: 340-342.

[43] Behle RW, Tamez-Guerra P, Shasha BS, McGuire MR. Formulating bioinsecticides to improve residual activity, Formulations Forum "99, March 3-5, 1999; Orlando, FL, USA.

[44] U.S. EPA. Inert ingredients in pesticide products: Policy statement; Revision and modification of lists, 52 FR 13305 and 54 FR 48314, 1989, November 22.

[45] Fortin, C, Lapointe, D, Charpentier, G. Susceptibility of brook trout (*Salvelinus fontinalis*) fry to a liquid formulation of *Bacillus thuringiensis* serovar. *israelensis* (Teknar®) used for blackfly control. Can J Fish Aq Sci 1986 ; 43: 1667-1670.

[46] Organic Materials Review Institute (OMRI). Comments on the National Organic Program Rule Proposed by the US Department of Agriculture TMD-94-00-2. 1998, Eugene, OR: OMRI.

[47] Rhodes DJ. Formulation of Biological Control Agents, in Exploitation of Microorganisms (ed. D.G. Jones), Chapman and Hall, London, 1993; pp 411-439.

[48] Couch TL, Ignoffo CM. *Formulation of insect pathogens, in microbial control of pests and plant diseases*, (ed. Burges, H.D.), Academic Press, London 1981; pp 621-634.

[49] Hewitt AJ. The effect of tank mix and adjuvants on spray drift. *In:* McMullan, P.M. (ed.) Adjuvants for Agrochemicals: Challenges and Opportunities. Proceedings of the Fifth International Symposium on Adjuvants for Agrochemicals, Chemical Producers Distributors Association, Memphis, TN, 1998; pp. 451-462.

[50] McGaughey WH. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Sci 1985; 229: 193-195.

[51] Sundara-Babu PC, Krishnan A. Preliminary studies on the effect of Thuricide® for control of the semi-looper, *Achaea janata,* on the castor bean plant, *Ricinus communis*. J Invertebr Pathol 1970; 15: 129-130.

[52] Lynch RE, Robinson JF, Berry EC. European corn borer: Yield losses and damage resulting from a simulated natural infestation. J Econ Entomol 1980; 73: 141–144.

[53] Harris J, Dent D. Priorities in Biopesticide Research and Development in Developing Countries, CABI Publishing, Wallingford, UK, 2000.

[54] Ifoulis AA, Savopoulou-Soultani M. Biological control of Lobesia botrana (Lepidoptera: Tortricidae) larvae by using different formulations of Bacillus thuringiensis in 11 vine cultivars under field conditions. J Econ Entomol. 2004 Apr; 97(2):340-343.

[55] Green JM. Adjuvant outlook for pesticides. Pesticide Outlook. October, 2000; 196-199.

[56] Mayer W, Wassmer C, Doerr S. Solid formulations. United States Patent 2000; 6,030,924.

[57] Navon A, Keren S, Levski S, Grinstein A, Riven Y. Granular feeding baits based on *Bacillus thuringiensis* products for the control of lepidopterous pests. Phytoparasitica 1997; 25 (Suppl.):101S-110S.

[58] Dunkle RL, Shasha BS. Starch encapsulated *Bacillus thuringiensis:* a potential new method for increasing environmental stability of entomopathogens. Environ Entomol 1988; 17: 120-126.

[59] Bartelt RJ, McGuire MR, Black DA. Feeding stimulant for the corn borer (Lepidoptera: Pyralidae): additives to a starch based formulation for *Bacillus thuringiensis*. Environ Entomol 1990; 19(1):182-189.

[60] Gillespie RL, McGuire MR, Shasha BS. Palatability of flour granular formulations to European corn borer larvae (Lepidoptera: Pyralidae). J Econ Entomol 1994; 87: 452-457.

[61] Tamez-Guerra P, Castro-Franco R, Medrano-Roldan H, McGuire MR, Galan-Wong LJ, Luna-Olvera HA. Laboratory and field comparisons of strains of *Bacillus thuringiensis* for activity against noctuid larvae using granular formulations (Lepidoptera). J Econ Entomol 1998; 91: 86–93.

[62] Yang C-C, Pan I-H, Chen M-H, Kao S-S, Tsai Y-S. Method for the preparation of a biocidal formulation containing starch and *Bacillus thuringiensis*. United States Patent 1994; 5,352,597.

[63] Shasha BS, McGuire MR. Adherent starch granules. United States Patent 1999; 5,997,945.

[64] Behle RW, McGuire MR, Shasha BS. Extending the residual activity of *Bacillus thuringiensis* with casein based formulations. J Econ Entomol 1996; 89:1399-1405.

[65] Behle RW, McGuire MR, Gillespie RL, Shasha BS. Effects of alkaline gluten on the insecticidal activity of *Bacillus thuringiensis*. J Econ Entomol 1997a; 90:354-360.

[66] Ridgway RL, Illum RR, Farrar RR Jr, Calvin DD, Fleischer SJ, Inscoe MN. Granular matrix formulation of *Bacillus thuringiensis* for control of the European corn borer (Lepidoptera: Pyralidae). J Econ Entomol 1996; 89 (5):1088-1094.

[67] Maldonado Blanco MG, Galan Wong LJ, Rodriguez Padilla C, Quiroz Martinez H. Evaluation of polymer-based granular formulations of *Bacillus thuringiensis israelensis* against larval Aedes aegypti in the laboratory. J Am Mosq Control Assoc. 2002 Dec; 18(4):352-358.

[68] Sulaiman S, Jeffery J, Sohadi AR, Yunus H, Busparani V, Majid R. Evaluation of Bactimos wettable powder, granules and briquets against mosquito larvae in Malaysia. Acta Trop, 1990; 47:189-95.

[69] Quimby Jr. PC, Caesar AJ, Birdsall JL, Connick, Jr. WJ, Boyette CD, Zidack NK, Grey WE. Granulated formulation and method for stabilizing biocontrol agents. United States Patent 2002; 6,455,036.

[70] Andersch W, Hain R, Kilian M. Granulates containing micro-organisms. United States Patent 1998; 5,804,208.

[71] Becker N. Ice granules containing endotoxins of microbial agents for the control of mosquito larvae-a new application technique. J Am Mosq Control Assoc. 2003 Mar; 19(1):63-66.

[72] Skovmand O, Sanogo E. Experimental formulations of *Bacillus sphaericus* and *B. thuringiensis* israelensis against *Culex quinquefasciatus* and *Anopheles gambiae* (Diptera: Culicidae) in Burkina Faso. J Med Entomol 1999; 36: 62-67.

[73] Gunasekaran K, Prabakaran G, Balaraman K. Efficacy of a floating sustained release formulation of *Bacillus thuringiensis* subsp. *israelensis* in controlling *Culex quinquefasciatus* larvae in polluted water habitats. Acta Tropica 2002; 83: 241-247.

[74] Levy R, Nichols MA, Miller TW Jr. Culigel superabsorbent polymer controlled-release system: application to mosquito larvicidal bacilli. *Proceedings and Abstracts, Vth International Colloquium on Invertebrate Pathology and Microbial Control,* Adelaide, Australia, 1990; pp 107.

[75] Mittal PK. Biolarvicides in vector control: challenges and prospects. J Vector Borne Dis 2003; 40(1-2): 20-32.

[76] Bradbury RS, Quinlan RJ, Most BH. Pesticidal composition for water treatment. United States Patent 1989; 4,865,842.

[77] Aly C, Mulla MS, Schnetter W, Xu BZ. Floating bait formulations increase effectiveness of *Bacillus thuringiensis* var. *israelensis* against Anopheles larvae. J Am Mosq Control Assoc. 1987 Dec;3(4):583-588.

[78] Goldberg LJ. Carrier and dispersal mechanism for a microorganic larvicide. United States Patent 1980; 4,187,290.

[79] Yang KW, Junginger GJ, Mockridge RG, Pearce, III RC. Compositions of gypsum pesticide briquets. United States Patent 1998; 5,747,054.

[80] Jones KA, Burges HD. Formulation of bacterial, viruses and protozoa to control insects. In: Burges, H.D. (Ed.), Formulation of Microbial Biopesticides: Beneficial Micro-organisms, Nematodes and Seed Treatments. Kluwer Academic Publishers, Dordrecht, Netherlands, 1998; pp. 7–127.

[81] Medugno CC, Ferraz JMG, Maia AHN, Freitas CCL. Evaluation of a wettable powder formulation for the nuclear polyhedrosisvirusof *Anticarsia gemmatalis* (Lep.: Noctuidae). Pest Sci 1997; 51: 153-156.

[82] Teera-Arunsiri A, Suphantharika M, Ketunuti U. Preparation of spray-dried wettable powder formulations of *Bacillus thuringiensis*-based biopesticides, J Econ Entomol 2003; 96 (2): 292-299.

[83] Valent Biosciences website, http://www.valent.com/agricultural_products/, cited, 2 January, 2004.

[84] Novartis Corp. website, http://www.novartis.com/, cited, 2 January, 2004.

[85] Hazen JL. Adjuvants - terminology, classification, and chemistry. Weed Technol 2000; 14: 773-784.

[86] Witt WW. Adjuvants. University of Kentucky College of Agriculture, Agripedia. http://www.ca.uky.edu/agripedia/pls404/adjuvant.htm. 2001(cited 3 November, 2003).

[87] Underwood AK. Adjuvant trends for the new millennium. Weed Technol 2000; 14: 765-772.

[88] Valéro JR. Three Years of Experimental Aerial Application of *Bacillus thuringiensis* at 2.01/ha against *Choristoneura fumiferana*, Information Report LAU-X-89B, Forestry Canada, Quebec, Canada, 1989.

[89] Smirnoff WA, Valéro, JR. Characteristics of a highly concentrated *Bacillus thuringiensis* formulation against spruce budworm, *Choristoneura fumiferana* (Lepidoptera:Tortricidae). Can Ent 1983; 115: 443-444.

[90] Cibulsky RJ, Devisetty BN, Melchior GL, Melin BE. Formulation and application technologies for microbial pesticides: review of progress and future trends. J Test Eval 1993; 21: 500-503.

[91] Bauce E, Carisey N, Dupont A, van Frankenhuyzen K. *Bacillus thuringiensis* subsp. *kurstaki* aerial spray prescriptions for balsam fir stand protection against spruce budworm (Lepidoptera: Torstricidae). J Econ Entomol 2004; 97(5):1624-1634.

[92] Lacey LA. Production and formulation of *Bacillus sphaericus*. Mosquito News 1984; 153-159.

[93] Cyanamid. *Acrobe® Biolarvacide: Technical Information, Field Trial Summary,* American Cyanamid Company, Wayne, New Jersey, 1992.

[94] Narayanan KS, Ianniello RM, Pritchard DW. UV protective aqueous emulsion and emulsifiable solids for cosmetic and agrichemical formulations. United States Patent 1997; 5,597,574.

[95] Aven M, Hasui H, Motoyoshi M. Non-aqueous concentrated spreading oil composition. United States Patent 2002; 6,387,848.

[96] Aven M. Non-aqueous suspension concentrate. United States Patent 2000; 6,165,940.

[97] Langley J, Symes KC. Polymeric compositions and methods of producing them. US Patent 5,744,152, 1998.

[98] Bok SH, Lee HW, Son KH, Kim SU, Lee JW, Kim DY, Kwon YK. Process for preparing coated microbial pesticides and pesticides produced therefrom. US Patent 5,273,749, 1993.

[99] Barnes AC, Cummings SG. Cellular encapsulation of biological pesticides. United States Patent 1987a; 4,695,462.

[100] Barnes AC, Cummings SG. Cellular encapsulation of pesticides produced by expression of heterologous genes. United States Patent 1987b; 4,695,455.

[101] Barnes AC, Edwards DL. Cellular encapsulation of biologicals for animal and human use. United States Patent 1989; 4,861,595.

[102] Ahmed SM, Nagamma MV, Majumdar SK. Studies on granular formulation of *Bacillus thuringiensis* Berliner. Pestic Sci 1973; 4:19-23.

[103] Baker CA, Brooks AA, Greenlay AZ, Hennis JMS. Encapsulation method. European Patent 1987; 0320483.

[104] McGuire MR, Shasha BS, Lewis LC. Field evaluations of granular starch formulations of *Bacillus thuringiensis* against *Ostrinia nubilalis* (Lepidoptera: Pyralidae), J Econ Entomol 1990; 83: 2207-2210.

[105] Baker CA, Brooks AA, Greenley RZ, Henis JM. Encapsulation of biological material in non-ionic polymer beads. United States Patent 1992; 5,089,407.

[106] Clark FM, Offit PA, Speaker TJ. Aqueous solven encapsulation method, apparatus and microcapsules. United States Patent 2003; 6,531,156.

[107] Shasha BS, Dunkle RL. Starch encapsulation of entomopathogens. US Patent 4 859 377, 1989.

[108] Eden J, Trksak R, William R. Starch based particulate encapsulation process. US Patent 4 755 397, 1988.

[109] Shasha BS, McGuire MR. Adherent, autoencapsulating spray formulations of biocontrol agents. US Patent 5 06 197, 1991.

[110] Bok SH, Kim SU, Kwon YK. Bioencapsulated biopesticides and process for the manufacture thereof. Canadian Patent 1994; CIPO 2,118,267.

[111] Cheung PYK, Hammock BD. Micro-lipid-droplet encapsulation of *Bacillus thuringiensis* subsp. *israelensis* δ-endotoxin for control of mosquito larvae. Appl Environ Microbiol 1985; 50 (4): 984-988.

[112] Schnell DJ, Pfannenstiel MA, Nickerson KW. Bioassay of solubilized *Bacillus thuringiensis* var. *israelensis* crystals by attachment to latex beads. Sci 1984; 223: 1191-1193.

[113] Shasha BS, McGuire MR. Slow release formulations of pesticides. In D.G. Chasin and L.E. Bode (eds.), Pesticide formulations and application systems. American Society for Testing and Materials, Philadelphia, 1992.

[114] McGuire MR, Shasha BS. Adherent starch granules for encapsulation of insect control agents, J Econ Entomol 1992; 85(4): 1425-1433.

[115] McGuire MR, Shasha BS, Lewis LC, Nelson TC. Residual activity of granular starch encapsulated *Bacillus thuringiensis.* J Econ Entomol 1994; 84: 631-637.

[116] Behle RW, McGuire MR, Shasha BS. Effects of sunlight and simulated rain on the residual activity of *Bacillus thuringiensis* formulations. J Econ Entomol 1997b; 90:1506-1516.

[117] Navon A. Control of Lepidopteran pests with *Bacillus thuringiensis.* In: P.F. Entwistle, J.S. Cory, M.J. Bailey, and S. Higgs [ed.], *Bacillus thuringiensis*, an environmental biopesticide: theory and practice, Wiley, New York, 1993; pp. 125-146.

[118] Ramos LM, McGuire MR, Galan Wong LJ. Utilization of several biopolymers for granular formulations of *Bacillus thuringiensis.* J Econ Entomol 1998; 91: 1109-1113.

[119] Shasha BS, McGuire MR, Behle RW. Lignin-based pest control formulations. US Patent 5 75 0467, 1998.

[120] Tamez-Guerra P, McGuire MR, Behle RW, Shasha BS, Galan-Wong LJ. Assessment of microencapsulated formulations for improved residual activity of *Bacillus thuringiensis.* J Econ Entomol 2000; 93: 219–225.

[121] Côté JC, Vincent C, Son KH, Bok SH. Persistence of insecticidal activity of novel bioencapsulated formulations of *Bacillus thuringiensis* var. *kurstaki* against *Choristoneura rosaceana* [Lepidoptera: Tortricidae]. Phytoprot 2001; 82: 73-82.

[122] Winder RS, Wheeler JJ, Conder N, Otvos IS, Nevill R, Duan L. Microencapsulation: a strategy for formulation of inoculum. Biocont Sci Technol 2003; 13: 155-169.

[123] Lew CW, Branly K, Gaytan J. Encapsulation with water soluble polymer. United States Patent 1997a; 5,643,351.

[124] Lew CW, Branly K, Gaytan J. Encapsulation with water soluble polymer. United States Patent 1997b; 5,599,583.

[125] Owaga M, Inoue M, Ohtsubo T, Kawada H. Solid insect growth regulating compositions. United States Patent 1998; 5,707,639.

[126] Lechelt-Kunze C, Simon J, Zitzmann W, Kalbe J, Muller H-P, Koch R. Biological material embedded in hydrogels, a process for the embedding thereof, and its use as artificial seed. United States Patent 2000; 6,164,012.

[127] Toreki W, Manukian A, Strohschein R. Hydrocapsules and method of preparation thereof. United States Patent 2004; 6,780,507.

[128] Kotz ME, Van der Zwan JJ. Controlled release agglomerated carrier. United States Patent 1986; 4,563,34.

[129] Levy R. Controlled delivery compositions and processes for treating organisms in a column of water or on land.United States Patent 1999; 6,001,382.

[130] McArdle B. Herbicidal and insecticidal protein-polysaccharide delivery compositions and methods for controlling plant and insect populations. United States Patent 1998; 5,747,416.

[131] Dubois NR, Reardon RC, Mierzejewksi K. Field efficacy and deposit analysis of *Bacillus thuringiensis,* Foray 48B, against Gypsy moth. J Econ Entomol 1993; 86 (1): 27-33.

[132] Ebert TA, Taylor RAJ, Downer RA, Hall FR. Deposit structure and efficacy of pesticide application. 1: Interactions between deposit toxicant concentration and deposit number. Pestic Sci 1999; 55: 783-792.

[133] Ignoffo CM, Hostetter DL, Smith DB. Gustatory stimulant, sunlight protectant, evaporation retardant: three characteristics of a microbial insecticidal adjuvant. J Econ Entomol 1975; 69(2): 207-210.

[134] Allsopp PG. Sugars, amino acids, and ascorbic acid as phagostimulants of larvae of *antitrogus parvulus* and *lepidiota negatoria* (coleoptera: scarabaeidae). J Econ Entomol 1992; 85: 106-111.

[135] Bartlet E, Parsons D, Williams IH, Clark S J. The influence of glucosinolates and sugars on feeding by the cabbage stem flea beetle, *Psylliodes chrysocephala.* Entomologia Experimentalis et Applicata 1994; 73: 77-83.

[136] Lopez JD Jr, Shaver TN, Lingren PD. Evaluation of feeding stimulants for adult *Helicoverpa zea*, pp. 920-924. In: Proceedings, Beltwide Cotton Production and Research Conferences, National Cotton Council, Memphis, TN, 1994.

[137] Farrar RR Jr, Ridgway RL. Enhancement of activity of *Bacillus thuringiensis* Berliner against four Lepidopterous insect pests by nutrient based phagostimulants. J Entomol Sci 1995; 30: 29-42.

[138] Lopez JD Jr, Lingren PD. Feeding response of adult *Helicoverpa zea* (Lepidoptera: Noctuidae) to commercial phagostimulants. J Econ Entomol 1994; 87: 1653-1658.

[139] Lance DR, Sutter GR. Field tests of a semiochemical-based toxic bait for suppression of corn rootworm beetles (Coleoptera: Chrysomelidae). J Econ Entomol 1992; 85: 967-973.

[140] Chandler LD. Use of feeding stimulants to enhance insect growth regulator-induced mortality of fall armyworm (Lepdioptera: Noctuidae) larvae. Florida Entomol 1993; 76: 316-326.

[141] Chandler LD. Comparison of insecticide-bait aerial application methods for management of corn rootworm (Coleoptera: Chrysomelidae). Southwestern Entomol 1998; 23:147-159.

[142] Pszczolkowski MA, Brunner JF, Doerr MD, Brown JJ. Enhancement of *Bacillus thuringiensis* with monosodium glutamate against larvae of obliquebanded leafroller (Lep.:Tortricidae). J Appl Entomol 2004; 128(7): 474.

[143] Kramer KJ, Muthukrishnan S. Insect chitinases: molecular biology and potential uses as biopesticides. Insect Biochem Molec Biol 1997; 27(11): 887-900.

[144] Smirnoff WA, Valéro, JR. Determination of the chitinolytic activity of nine subspecies of *Bacillus thuringiensis*. J Invertebr Pathol 1977; 30: 265-266.

[145] Smirnoff WA. Three years of aerial field experiments with *Bacillus thuringiensis* plus chitinase formulation against the spruce budworm. J. Invertebr Pathol 1974; 24(3): 344-348.

[146] Morris ON. A 2-year study of the efficacy of *Bacillus thuringiensis*-chitinase combinations in spruce budworm (*Choristoneura fumiferana*) control. Can Entomol 1976; 108: 3225-3233.

[147] Daoust RA. Mutualistic interactions in the microbiological control of *Lymantria dispar* (L). Ph.D Dissertation, University of Massachusetts, Amherst, 1978.

[148] Gunner HB, Met MZ, Berger S. Chitinase producing Bt strains. In: Microbial Control of Spruce budworms and gypsy moths. Eds. D.G. Grimble and F.B. Lewis, pp. 102-108. U.S. Forestry Service, GTR-NE-100, 1985.

[149] Martins EMF, Guzzo SD, Palazzo DA, Harakava R. Metabolic alterations detected in Natal orange trees infected by *Xylella fastidiosa*. Summa Phytopathologica, 1999; 25: 288-293.

[150] Wiwat C, Thaithanun S, Pantuwatana S, Bhumiratana A. Toxicity of chitinase-producing *Bacillus thuringiensis* ssp. *kurstaki* HD-1 (G) toward *Plutella xylostella*. J Invertebr Pathol 2000; 76(4): 270-277.

[151] Liu M, Cai QX, Liu HZ, Zhang BH, Yan JP, Yuan ZM Chitinolytic activities in *Bacillus thuringiensis* and their synergism effects on larvicidal activity. J Appl Microbiol 2002; 93: 374–379.

[152] Manker DC, Lidster WD, Starnes RL, MacIntosh SC. Potentiator of *Bacillus* pesticidal activity. Patent Coop. Treaty WO94/09630, 1994.

[153] Broderick NA, Goodman RM, Raffa KF, Handelsman J. Synergy Between Zwittermicin A and *Bacillus thuringiensis* subsp. *kurstaki* Against Gypsy Moth (Lepidoptera: Lymantriidae). Environ Entomol 2000; 29(1): 101-107.

[154] Visser B, van Workum M, Dullemans A, Waalwijk C. The mosquitocidal activity of *Bacillus thuringiensis* var. *israelensis* is associated with Mr 230 000 and 130 000 crystal proteins. FEMS Microbiol Lett 1986; 30: 211–214.

[155] Martin PAW. A Stilbene Optical brightener can enhance bacterial pathogenicity to gypsy moth (Lepidoptera: Lymantriidae) and Colorado potato beetle (Coleoptera: Chrysomelidae). Biocont Sci Technol 2004; 14(4): 375-383.

[156] Pusztai M, Fast P, Gringorten L, Kaplan H, Lessard T, Carey PR. The mechanism of sunlight mediated inactivation of *Bacillus thuringiensis* crystals. Biochem J 1991; 273: 43-47.

[157] Ignoffo CM. Environmental factors affecting persistence of entomopathogens. Florida Entomol 1992; 75: 516-525.

[158] Cohen E, Rozen H, Joseph T, Braun S, Margulies L. Photoprotection of *Bacillus thuringiensis* var. *kurstaki* from ultraviolet irradiation. J Invertebr Pathol 1991; 57: 343-351.

[159] Beegle CC, Yamamoto T. Invitation paper (C.P. Alexander Fund): History of *Bacillus thuringiensis* Berliner research and development. Can Entomol 1992; 124: 587-616.

[160] Ragaei M. Studies on the effect of *Bacillus thuringiensis* on the greasy cutworm *Agrotis ypsilon* (Rott.) PhD Thesis, University of Cairo, 1990.

[161] Morris O. Protection of *B. thuringiensis* from inactivation by sunlight. Can Entomol 1983; 115: 1215–1227.

[162] Dunkle RL, Shasha BS. Response of starch encapsulated *Bacillus thuringiensis* containing UV screens to sunlight. Environ Entomol 1989; 18: 1035-1041.

[163] Çökmüs C, Sayar AH, Saçilik SC, Osmanagaoglu Ö, Berber Í. Effects of UV-light on *Bacillus sphaericus* and its protection by chemicals. J Basic Microbiol 2002; 40(4): 215–221

[164] Liu YT, Sui MJ, Ji DD, Wu IH, Chou CC, Chen CC. Protection from ultraviolet irradiation by melanin of mosquitocidal activity of *Bacillus thuringiensis* var. *israelensis*. J Invertebr Pathol 1993; 62: 131-136.

[165] Patel KR, Wyman JA, Patel KA, Burden BJ. A mutant *Bacillus thuringiensis* producing a dark-brown pigment with increased UV resistance and insecticidal activity. J Invertebr Pathol 1996; 67: 120–124.

[166] Saxena D, Ben-Dov E, Manasherob R, Barak Z, Boussiba S, Zaritsky A. A UV tolerant mutant of *Bacillus thuringiensis* subsp. *kurstaki* producing melanin. Curr Microbiol 2002; 44: 25–30

[167] Salama HS, Morris ON. The use of Bt in developing countries, pp. 237-254. *In* P.F. Entwistle, J.S. Cory, M.J. Bailey, and S.Higgs (eds.), *Bacillus thuringiensis*, An Environmental Biopesticide: Theory and Practice. John Wiley and Sons, Chichester, England, 1993.

[168] McGuire MR, Shasha BS, Eastman CE, Oloumi-Sadeghi H. Starch and flour based sprayable formulations- effect on rainfastness and solar stability of *Bacillus thuringiensis*. J Econ Entomol 1996; 89: 863-869.

[169] Ragaei M. Laboratory evaluation of shellac as ultraviolet screen for the *Bacillus thuringiensis* var. *entomocidus* against *Spodoptera littoralis* larvae. *Anz. Schädlingskunde,Pflanzenschutz,Umweltschutz* 1998; 71: 132-134 (article available in English).

[170] Hobbs DG, Campbell BJ, Lidster WD. Ultra violet radiation lignin protected pesticidal compositions. US Patent 5,994,266, 1999.

[171] Wirtz KR, Lebo SE, Sanford ME. Method for providing a stable protective coating for UV sensitive pesticides. United States Patent 5,939,089, 1999.

[172] De Vlieger JJ, Smits PH. Composition for controlling pests, process for the preparation thereof and use thereof in crop protection. United States Patent 1998; 5,807,566.

[173] Shapiro M, Dougherty E, Hamm JJ. Compositions and methods for biocontrol using fluorescent brighteners. United States Patent 1992; 5,124,149.

[174] Leland JE, Mullins DE, Vaughn LJ. Water soluble UV-protective coatings for biological pesticides and process for making same.US Patent 20040038825, 2004.

[175] Yu JY, Lee, WC. Microencapsulation of pyrrolnitrin from *Pseudomonas cepacia* using gluten and casein. J Ferment Bioeng 1997; 84 (5): 444-448.

[176] Ramstack JM, Herbert PF, Strobel J, Atkins TJ. Preparation of biodegradable microparticles containing a biologically active agent. US Patent 5,650,173, 1997.

[177] Fowler JD, Feinstein BE. Insecticidal matrix and process for preparation thereof. U.S. Patent 5,885,603, 1999.

[178] Ferro DN, Slocombe AC, Mercier CT. Colorado potato beetle (Coleoptera: Chrysomelidae): residual mortality and artificial weathering of formulated *Bacillus thuringiensis* subsp. *tenebrionis*. J Econ Entomol 1997; 90: 574-582.

[179] van Frankenhuyzen K, Nystrom C. Residual toxicity of a high potency formulation of *Bacillus thuringiensis* to spruce budworm. J Econ Entomol 1989; 82: 868-872.

[180] Paukner S, Kohl G, Jalava K, Lubitz W. Sealed bacterial ghosts- -Novel targeting vehicles for advanced drug delivery of water-soluble substances. J Drug Targeting 2003; 11: 151-161.

[181] Hatfaludi TS, Liska M, Zellinger D, Ousman J Pa, Szostak M, Ambrus ARD, Jalava K, and Lubitz W. Bacterial ghost technology for pesticide delivery. J Agric Food Chem 2004; 52: 5627-5634.

[182] Nishiitsutsuji-Uwo J, Ohsawa A, Nishimura MS. Factors affecting the insecticidal activity of delta endotoxin of *Bacillus thuringiensis*. J Invertebr Pathol 1976; 29:162-169.

[183] Han EN, Bauce E. Dormancy in the life cycle of the spruce budworm: physiological mechanisms and ecological implications. Recent Res Devel Entomol 2000; 3: 43-54.

[184] Molloy DP. Progress in the Biological Control of Black Flies with *Bacillus thuringiensis israelensis*, with emphasis on temperate climates, pp. 161-186. *In* H. deBarjac and D.J. Sutherland (eds.), Bacterial Control of Mosquitoes and Black Flies. Rutgers University Press, New Brunswick, NJ, 1990.

[185] Becker N, Margalit J.. Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies, pp.147-170. In: P.F. Entwhistle, J.S. Cory, M.J. Bailey, and S. Higgs, editors. *Bacillus thuringiensis*, An Environmental Biopesticide: Theory and Practice. John Wiley and Sons, Chichester, England, 1993.

[186] Beckwith RC, Stelzer MJ. Persistence of *Bacillus thuringiensis* in two formulations applied by helicopter against western spruce budworm (Lepidoptera: Tortricidae) in north central Oregon. J Econ Entomol, 1987; 80: 204-207.

[187] Smirnoff WA. Effects of volatile substances released by foliage of *Abies balsamea.* J Invertebr Pathol 1972; 19(1): 32-35.

[188] Maksymiuk B. Occurrence and nature of antibacterial substances in plants affecting *Bacillus thuringiensis* and other entomogenous bacteria. J Invertebr Pathol 1970; 15(3): 356-371.

[189] Ferry N, Edwards MG, Gatehouse JA, Gatehouse, AMR. Plant–insect interactions: molecular approaches to insect resistance. Current Opinion in Biotechnol 2004; 15: 155–161.

[190] Ali A, Young SY. Effects of rate and spray volume of *Bacillus thuringiensis* var. *kurstaki* on activity against *Heliothis virescens* and persistence in cotton terminals. J Econ Entomol 1993; 86: 735-738.

[191] Fuxa JR. Fate of released entomopathogens with reference to risk assessment of genetically engineered microorganisms. Entomol Soc Amer Bull Winter 1989.

[192] Gringorten JL, Mile RE, Fast PG, Soho SS, van Frankenhuyzen K. Suppression of *Bacillus thuringiensis* d-endotoxin activity by low alkaline pH. J Invertebr Pathol 1992; 60: 47-52.

[193] Gibson DM, Gallo LG, Krasnoff SB, Ketchum RE. Increased efficacy of *Bacillus thuringiensis* subsp. *kurstaki* in combination with tannic acid. J Econ Entomol. 1995 Apr;88(2):270-277.

[194] Joung, KC, Côté, JC. A review of the environmental impacts of the microbial insecticide *Bacillus thuringiensis*. Agriculture and Agri-Food Canada, Technical Bulletin No. 29 2000, 16 pages. Copies available at : Horticultural R&D Centre web site at http://res2.agr.ca/stjean/crdh.htm.

[195] Sachdeva V, Tyagi RD, Valéro JR. Production of biopesticides as a novel method of wastewater sludge utilization/disposal. Water Sci Technol 2000; 42: 211-216.

[196] Tirado-Montiel ML, Tyagi RD, Valéro JR. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. Water Res 2001; 35 (16): 3807–16.

[197] Tirado-Montiel ML, Tyagi RD, Valéro JR, Surampalli RY. Production biopesticides using wastewater sludge as a raw material – Effect of process parameters. Water Sci Technol 2003; 48 (8): 239-246.

[198] Lachhab K, Tyagi RD, Valéro JR. Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids concentration. Process Biochem 2001; 37 (2): 197-208.

[199] Tyagi RD, Foko VS, Barnabé S, Vidyarthi AS, Valéro JR. Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using sewage sludge as raw material. Water Sci Technol 2001; 46 (10): 247-254.

[200] Vidyarthi AS, Desrosiers M, Tyagi RD, Valéro JR. Foam control in biopesticide production from sewage sludge. J Ind Microbiol Biotechnol 2000; 25: 86–92.

[201] Vidyarthi AS, Tyagi RD, Valéro JR. Effect of surface active agents on the production of biopesticides using wastewater sludge as a raw material, Water Sci Technol 2001;44 (10): 253–259.

[202] Vidyarthi AS, Tyagi RD, Valéro JR, Surampalli RY. Studies on the production of *B-thuringiensis* based biopesticides using wastewater sludge as a raw material. Water Res 2002; 36 (19): 4850-4860.

[203] Yezza A, Tyagi RD, Valéro JR, Surampalli RY. Bioconversion of industrial wastewater and wastewater sludge into *Bacillus thuringiensis* based biopesticides in pilot plant fermentor. Biores Technol 2006b; 26, 1850-1857.

[204] Yezza A, Tyagi RD, Valéro JR, Surampalli RY, Smith J. Scale-up of biopesticide production processes using wastewater sludge as a raw material. J Ind Microbiol Biotechnol 2004b; DOI 10.1007/s10295-004-0176-z.

[205] Yezza A, Tyagi RD, Valéro JR, Surampalli RY Production of *Bacillus thuringiensis* based biopesticides in batch and fed-batch cultures using wastewater sludge as a raw material. J Chem Technol Biotechnol 2004c; 80: 502-510.

[206] MENV. Guide sur la valorisation des matières résiduelles fertilisantes: Critères de références et normes réglementaires. Direction du Milieu Rural, Environnement Québec, Canada, 138 pp ; 2004.

[207] Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. Sludge based *Bacillus thuringiensis* biopesticides: viscosity impacts. Water Res 2005; 39: 3001-3011.

[208] Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge. Water Res 2006, 40, 1310-1320.

[209] Sutherland IW. Exopolysaccharides in biofilms, flocs and related structures. Water Sci Technol 2001; 43(6): 77–86.

[210] Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations. Water Sci Technol 2004; 50(9): 229-236.

[211] Manka J, Rebhun M, Mandelbaum A, Bortinger A. Characterization of organics in secondary effluents. Environ Sci Technol 1974; 8 (12): 1017-1020.

[212] Tu M, Randall JM. Weed Control Methods Handbook, The Nature Conservancy, Ohio, USA, 2003.

[213] Lisansky SG, Quinlan RJ, Tassoni G. The *Bacillus thuringiensis* Production Handbook, CPL Press, Newbury, 1993; pp.124.

[214] Prabakaran G, Padmanabhan V, Balaraman K. Development of a self floating slow release formulation of *Bacillus thuringiensis* var. *israelensis* and its larvicidal activity. Indian J Exp Biol 2001 Jan; 39(1):82-84.

[215] Angus TA. Use of methyl cellulose in laboratory tests of bacterial pathogens of insects. Can Ent 1954; 86: 206.

[216] Angus TA. Potential usefulness of vinyl latices as stickers. Can Ent 1959; XCI: 254-255.

[217] Jankevica J, Zarins I. Biological control of *Malacosama neustria* L. population with Latvian isolate of nuclear polyhedrosis virus, In: Microbial insecticides: novelty or necessity? Symposium Proceedings No. 68 (ed. H.F. Evans), British Crop Protection Council, Farnham, 1997; pp 285-288.

[218] Tamez-Guerra P, Garcia-Gutierrez C, Medrano-Roldan H, Galan-Wong LJ, Sandoval-Coronado CF. Spray dried microencapsulated *Bacillus thuringiensis* formulations for the control of *Epilachna varivestis*. Mulsant Southwest Entomol 1999; 24: 37-48.

[219] Carr ME, Doane WM, Wing RE, Bagley EB. Starch encapsulation of biological active agents by a continuous process. U.S. Patent 1993; 5,183,690.

[220] Morris ON, Converse V, Kanagaratnam P. Chemical additive effects on the efficacy of *Bacillus thuringiensis* Berliner subsp. *kurstaki* against *Mamestra configurata* (Lepidoptera: Noctuidae). J Econ Entomol 1994; 88: 815-824.

[221] McMullan PM. Utility adjuvants. Weed Technol 2000; 14: 792-797.

[222] Montermini A, Nanni C, Boselli M. Integrated defence of poplar: two years trials against *Hyphantrea cunea* (Drury) with a new microbial formulation of *Bacillus thuringiensis* var. *kurstaki* distributed by helicopter. *ANNP – BCPC Second International Symposium on pesticide application techniques*. Strasbourg 22nd, 23rd and 24th September 1993; pp 433-436.

[223] Ejiofor AO, Okafor N. Formulation of a flowable liquid concentrate of *Bacillus thuringiensis* serotype H-14 spores and crystals as mosquito larvicide. J Appl Bacteriol 1991; 71: 202-208.

[224] Yang C-C, Pan I-H, Chen M-H, Kao S-S, Tsai Y-S. Anti-ultraviolet biocidal composition. United States Patent 1995; 5,427,787.

[225] Smith KL, Herbig SH. Labile insecticide compositions. United States Patent 1998; 5,750,126.

[226] Lebo Jr. SE, Detroit WJ. Method for microencapsulation of agriculturally active substances. United States Patent 1996; 5,552,149.

[227] Bohm HA, Friend DR. Microencapsulated insecticidal pathogens. United States Patent 1990; 4,948,586.

[228] McGuire MR, Streett DA, Shasha BS. Evaluation of starch encapsulation for formulation of grasshopper (Orthoptera: Acrididae) entompox viruses. J Econ Entomol 1991; 84:1652-1656.

[229] Shapiro M, Vaughn JL. Enhancement in activity of homologous and heterologous baculoviruses infectious to cotton bollworm (Lepidoptera: Noctuidae) by an optical brightener. J Econ Entomol 1995; 88 (2): 265-269.

[230] Munson D, Lew CW, Gaggero JM, Branly K. Aqueous solven encapsulation method, apparatus and microcapsules. United States Patent 1996; 5,571,522.

[231] Alexander SR, Aref AA, Smith MS. Palatable solid pesticidal compositions of ethylene and vinyl acetate copolymer. United States Patent 1992; 5,135,744.

[232] Tamez-Guerra P, McGuire MR, Behle RW, Shasha BS, Pingel RL. Storage stability of *Anagrapha falcifera* nucleopolyhedrovirus in spray-dried formulations, J Invertebr Pathol 2002; 79 (1): 7-16.

[233] Berto P, Dickburt C, Lepoivre P. Biopesticide compositions. European Patent 1 238 587 A2, 2002.

[234] Gaudet, MD, Puritch GS. Fatty acid salt enhancement of bacterial insecticide. United States Patent 1989; 4,826,678.

[235] Marshall LGI. Biological control agent biocarriers and method of formation. US Patent 5,888,500, 1999.

[236] Yardin MR, Kennedy IR, Thies JE. Development of high quality carrier materials for field delivery of key microorganisms used as biofertilisers and biopesticides. Rad Phy Chem 2000; 57: 565-568.

[237] Vidyasekaran P, Muthamilan M. Development of formulations of *Pseudomonas fluoroscens* for control of chickpea wilt. Plant Dis 1995; 79 (8): 782-786.

[238] Drop data analysis. http://www.dropdata.net/download/PestiSci_L3_6.PDF, cited 4 March, 2004.

[239] Levy R. Flowable insecticidal delivery compositions and methods for controlling insect populations in an aquatic environment. United States Patent 1991a; 4,985,251.

[240] Levy R. Terrestrial delivery compositions and methods for controlling insect and habitat-associated pest populations in terrestrial environments. United States Patent 1991b; 4,983,390.

[241] Levy R. Insecticidal delivery compositions and methods for controlling a population of insects in an aquatic environment. United States Patent 1998; 5,824,328.

[242] Henderson; Jack A. Insecticide carriers and insecticides. United States Patent 1994; 5,326,560.

[243] Puritch GS, McHarg D, Bradbury R, Mason W. Supersorbent material as pesticide potentiator. United States Patent 1991; 5,037,654.

[244] Losel P, Penners G, Weissmuller J. Insecticidal attract-and-kill formulations. United States Patent 1998; 5,707,638.

[245] McKibben GH, Dickens JC, Smith JW. Plastic bait composition for attracting and killing crop pests. United States Patent 1994; 5,290,556.

[246] Sundaram A, Leung JW, Devisetty BN. Rainfastness of *Bacillus thuringiensis* deposits on conifer foliage. *Pesticide Formulations and Application Systems: 13th Volume, ASTM STP 1183*, Eds. Berger, Bala, Devisetty and Hall, American Society for Testing and Materials, Philadelphia. 1993; pp 227-241.

[247] Raun ES, Sutter GR, Revelo MA. Ecological factors affecting the pathogenicity of *Bacillus thuringiensis* var. *thuringiensis* to the European corn borer and fall armyworm. J Invertebr Pathol 1966; 8: 365-375.

[248] Cantwell GE, Franklin BA. Inactivation by irradiation of spores of *Bacillus thuringiensis* var. *thuringiensis*. J Invertebr Pathol 1966; 8: 256-258.

[249] Griego VM, Spence KD. Inactivation of Bacillus thuringiensis spores by ultraviolet and visible light. Appl Environ Microbiol 1978; 35(5): 906-910.

[250] Burges HD, Hillyer S, Chanter DO. Effect of ultraviolet and gamma rays on the activity of delta endotoxin protein crystals of *Bacillus thuringiensis*. J Invertebr Pathol 1975; 25: 5-9.

[251] Morris ON Ed. Report of the 1989 CANUSA Cooperative *Bacillus thuringiensis* Spray Trials. FMDX-40, Forest Pest Mgmt. Inst., Sault Ste.Marie, Ontario, Canada, 1980.

[252] Ignoffo CM, Garcia C. UV photo–inactivation of cells and spores of *Bacillus thuringiensis* and effects of peroxidase on inactivation. Environ Entomol 1978; 7: 270-272.

Table 1 Screening factors to choose an adjuvant/additive

Formulation Type
Solid, liquid, encapsulated forms
Target(s)
Pest species (type)
Developmental stage
Dense or sparse growth? (Low/ high volumes of spray?)
Barriers to penetration (Waxy, hairy or thick leaves, sediments?)
Method of application (aerial/terrestrial; foliar spray, boom and nozzle spray, hydraulic) and timing
Volume application rate and spray droplet size spectrum
Environment
Site conditions (Aquatic or terrestrial? In sensitive areas?)
Current conditions (Air temperature? Windy? UV radiation? Rain?)
Water chemistry (Hard or soft water? Low or high pH?)
Other(s)
Product interactions or compatibility issues
Order of incorporation into the tank mix
Avionics use and buffer zone width

Modified from Tu and Randall [212]

Table 2 Different types of adjuvants/additives used in microbial formulations

Adjuvants/ additives	Function(s)	Example(s)	Reference (s)
Dispersant	Dispersion of formulation into dispersant medium	Amylose; Aluminium silicate; Sodium starch glycolate;	[213,214]
Surfactants and wetters	Enhance the emulsifying, dispersing, spreading, sticking or wetting properties of the biopesticide (includes spray modifiers)	Ethoxylates (Tween/Triton series); polyethylene glycol	[48,213]
Stickers and spreaders	Adhesion of pesticides onto the foliage protecting from rain wash-off and spreading evenly for maximum coverage	Gelatin; gums; molasses; skimmed milk; proprietary like Nufilm and chevron; vegetable gels; vegetable oils; waxes; water-soluble polymers	[8,37,64,65, 116,137,215-218]
Drift control agents/anti-evaporants/humectant	Reduce spray drift, which most often results when fine (< 50μm diameter) spray droplets are carried away from the target area by breezes, including those caused by the vehicle carrying the spray equipment and control of foam while mixing	Polyacrylamides, polysaccharides, and certain types of gums, sorbitol, sucrose, molasses, polyglycol, molasses, glycerol	[64,89,117, 219,220]
Thickening agents	Modify the viscosity of spray solutions and are used to reduce drift, particularly for aerial applications	Water swellable polymers producing a "particulate solution," hydroxyethyl celluloses, and/or polysaccharide gums	[48,215,220]
pH Buffers	Adjust or buffer pH; improve the dispersion or solubilization in the formulation, control its ionic state and increase adjuvant compatibility	Sodium phosphate; Potassium phosphate	[214,221,222]
Defoaming and antifoam agents	Reduce surface tension, physically burst the air bubbles, and/or otherwise weaken the foam structure	Dimethopolysiloxane-based; silica; alcohol and oils	[58,223]

Adjuvant/ additive	Function(s)	Example(s)	Reference (s)
UV radiation screens	Protect from the deleterious effect(s) of sunlight by forming a protective layer on the formulations	Congo Red; folic acid; lignin; molasses; p-Aminobenzoic acid; alkyl phenols	[25,64,65,224-228]
Phagostimulants	Stimulate feeding of formulations by pests	Corn meal; sucrose; wheat germ; corn germ; soya flour; casein, edible oil, glutamate, molasses	[104,115,228-231]
Synergists	Multiple modes of action; generally complements various formulation components	Sorbitol; sorbic acid; sodium phosphate; stilbene; Tinopal; silicate; protease inhibitors, oleic acid, linoleic acid	[98,232-234]
Anti-microbial agents	Suppresses the growth of other microorganisms, retaining formulation purity	Sorbic acid; propionic acid; crystal violet	[82,235]
Carriers	Aid in delivery of formulation to target	Alginate; carrageenan; peat, acrylate and acrylamide supersorbents, diatomaceous earth	[236-243]
Binders	For binding the particulates in granules together	Gums; molasses; PVP, resins	[66,82]
Suspending agents	Keep the formulation in suspension	Sorbitol; soya polysaccharides; starch glycolates; sucrose	[131,238]
Attractants	Act as baits to attract target pests	Pheromones, cucurbitacin and various alkaloids; plastisol (PVC and cotton seed oil)	[244,245]
Multi-purpose	Perform various functions at the same time	Molasses; starch, lignin	[107,113,119,246]

Table 3 Products (formulations) registered for commercial use in Canada/North America

Industry/ Company	Product	Active Ingredient(s)	Entomotoxicity	Registration Year	Expiry Year	Target Pest(s)
Valent Biosciences Corporation	Dipel WP (Wettable Powder)		16000 BIU/mg	1972	2006	Spruce budworm; gypsy moth; bagworm; spring & fall cankerworms and cabbage looper
	Thuricide 48LV (Liquid suspension)	*Bacillus thuringiensis berliner ssp kurstaki*	12.7 BIU/L	1984	2007	Bagworm; elm spanworm; fall spanworm; gypsy Moth; spring & fall cankerworm; spruce budworm; jack pine budworm
	Vectobac-200G Larvicide (Granules)		200 ITU/mg	1984	2007	
	Teknar Granules Larvicide		260 AAU / mg	1986	2004	Mosquitoes
	Vectobac 200G (Granules)	*Bacillus thuringiensis israelensis*	200 ITU/mg	1986	2007	
	Vectobac 600L (Aqueous suspension)		600 ITU/mg	1986	2004	Fungus gnats
	Teknar HP-D Larvicide (Aqueous suspension)		3000 AAU/mg	1986	2004	Mosquitoes & Black flies
	Novodor Flowable Concentrate	*Bacillus thuringiensis ssp. tenebrionis*	3.6 %	1995	2004	Colorado potato and elm leaf beetle

Table 3 Continued

Industry/ Company	Product	Active Ingredient(s)	Entomotoxicity	Registration Year	Expiry Year	Target Pest(s)
Valent Biosciences Corporation	Foray 48B		12.7 BIU/L	1997	2004	Spruce budworm (Eastern & Western); gypsy moth; jackpine budworm; eastern hemlock looper; whitemarked tussock moth and forest tent caterpillar whitemarked tussock moth forest tent caterpillar and satin moth
	Foray 48BA Low Volume		12.7 BIU/L	1997	2007	
	Foray 76B (Aqueous concentrate)		20.0 BIU/L	1997	2007	
	Foray 96B		25.4 BIU/L	2003	2004	
	Dipel 2X DF (Dry Flowable (Wettable Granules)	*Bacillus thuringiensis berliner ssp kurstaki*	32,000 IU/mg	2000	2007	spruce budworm; gypsy moth; bagworm; spring and fall cankerworm;fall webworms; elm spanworm; tent caterpillar; cabbage looper; leafroller and diamondback moth
Certis USA LLC	Thuricide-HPC High Potency Aqueous Concentrate		4.2 BIU/L	1972	2007	Spruce budworm; gypsy moth; bagworm; spring & fall cankerworms and cabbage looper
AFA Environment Inc.	Aquabac II XT (Liquid suspension)	*Bacillus thuringiensis israelensis (Bacillus thuringiensis serotype H-14)*	1.28 BITU/Kg	2003	2007	Mosquitoes
	Aquabac 200G - 10/14 (Granules)		0.20 BITU/Kg	2001	2006	
	Aquabac 200G (10/14) (5/8)		200 ITU/mg	2001	2006	

Table 3 Continued

Industry/ Company	Product	Active Ingredient(s)	Entomotoxicity	Registration Year	Expiry Year	Target Pest(s)
AFA Environment Inc.	Aquabac XT	*Bacillus thuringiensis israelensis*	1200 ITU/mg	2001	2006	Mosquitoes and blackflies
Abbott Laboratories Ltd.	Dipel 176 (Emulsifiable suspension)		16.9 BIU/L	1988	2007	Forest tent caterpillars; gypsy moth spruce budworms; hemlock looper
Woodstream Canada Corporation	Safer's BTK (Liquid concentrate)	*Bacillus thuringiensis berliner ssp kurstaki*	12.7 BIU/L	1996	2006	Gypsy moth; tent caterpillar and cabbage looper
AEF Global Inc.	Bioprotec Aqueous Biological (Aqueous suspension)		12.7 BIU/L	2000	2007	Gypsy moth; eastern spruce budworm ;western spruce budworm; jack pine budworm; forest tent caterpillar; eastern hemlock looper; bagworm; elm spanworm; fall spanworm; spring & fall cankerworm; satin moth and white marked tussock moth
	Bioprotec CAF Aqueous		12.7 BIU/L	2001	2004	
	Bioprotec HP		17 5000 IU/mg	2002	2004	
	Bioprotec ECO		12.7 BIU/L	2003	2007	

(Source: Modified from http://eddenet.pmra-arla.gc.ca; cited 07 June, 2004, Pest Management Regulatory Authority, Canada).
ITU – International Toxic Units; IU – International Units; BITU – Billion International Toxic Units ; AAU - Aedes Aegypti Units (1 ITU = 2.5 AAU)
IU – It refers to standardized potency (by bioassay) of different marketed Bt products against Bt var. *thuringiensis* E-61 standard from Institut Pasteur, Paris, France , assigned a potency of 1000 International Units (IU) per mg. Bioassay is carried out against mediterranean flour moth (*Ephestia kuhniella*) in Europe and in US, a primary reference standard of Bt HD-1-S-1971 strain with an assigned potency of 18000 IU/mg is being used, bioassayed against cabbage looper (*Trichoplusia ni*).

Table 4 General advantages and disadvantages of different formulations

	Dry solid formulations	**Liquid suspensions**
Advantages	**Are** ready-to-use types like dusts, granules, briquettes and applicable with simple equipments. **Granules** show less drift and applicable to hidden foliage too. **WPs** are easy to transport, store and apply when required; low risks of operator safety. **Cost** effective - transportation costs are low. **Do not** need high quantity of preservatives.	**Emulsion** concentrates and suspension concentrates hold high a.i., bulk storage not necessary. **ULV** concentrates can be used without mixing. **Encapsulated** suspensions increase residual toxicity and decrease user hazard. **Development** process is without harsh conditions of drying – higher recovery. **Used** in agriculture, gardens and forests. **Less** expensive to apply.
Disadvantages	**Dusts** are subject to drifts and pose user hazards. **Some** WPs could clog sprayers. **Formulation** involves harsh spray drying steps – loss of a.i. **Use** restricted to gardens, agriculture and water streams. **More** expensive to apply	**Subject** to deterioration on long storage. **a.i.** may settle out of emulsions and suspensions, at times. **Sometimes**, require complex spray equipments, e.g., in forestry.

Table 5 Key findings on effects of UV radiation on entomopathogenic activity of Bt

Wavelength	Test substance	Effect	Reference (s)
250 nm	spores	lethal	[247-249]
253.7 nm	spores; crystals	lethal to spores and no effect on crystals	[250]
330 nm; 400 nm	spores; crystals	lethal	[65,119]
300- 380 nm	crystal	inactivation of protein	[58,156]
sunlight (300-750 nm)	spores	rapid inactivation	[161,251]
sunlight (300-750 nm)	spores; crystals	spores more sensitive than crystals, but both inactivated in 3 days	[168,252]
visible light (400-750 nm)	spores; crystals	no effect	[156,161]

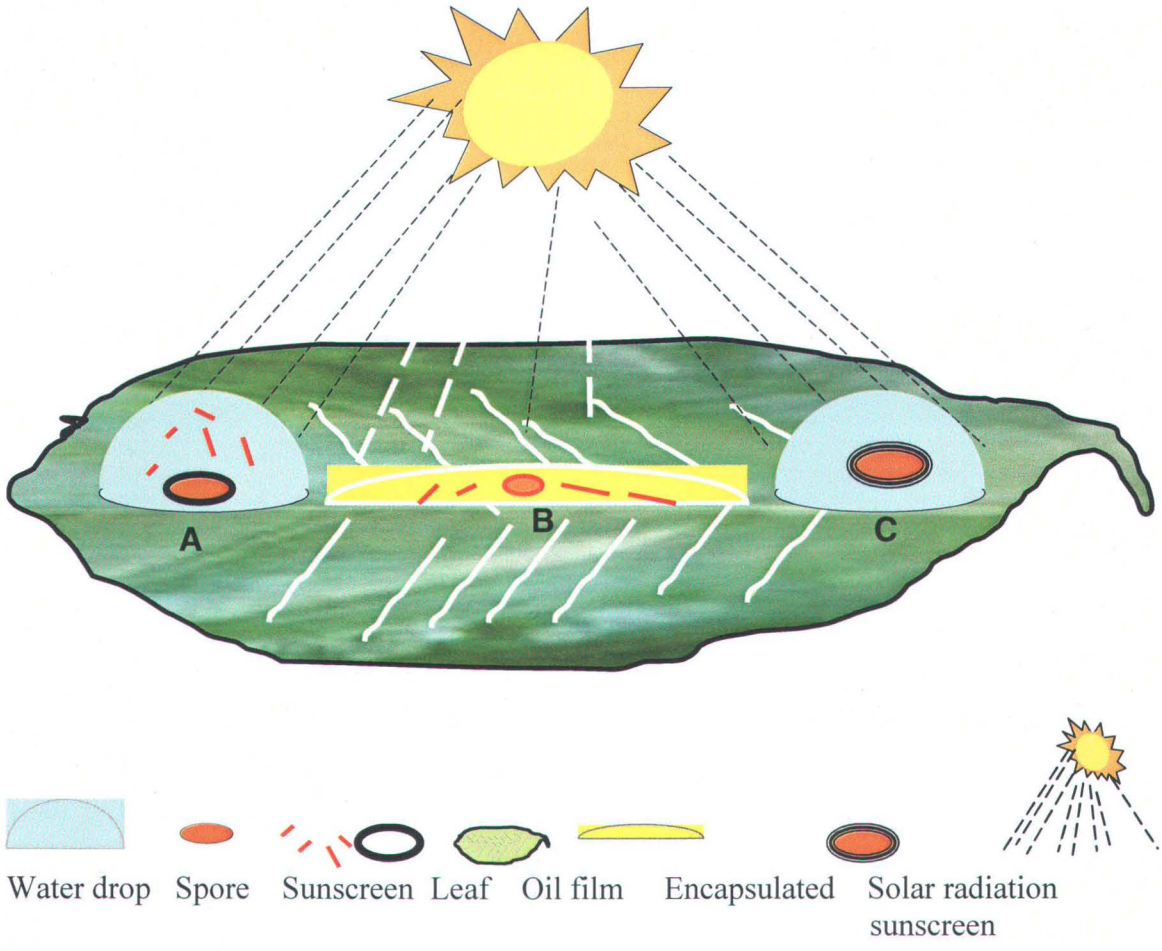


Fig. 1. Water and oil-based formulations on interaction with leaf (hydrophobic) surfaces.

A. Water soluble sunscreen; water droplet forms a large contact angle on the hydrophobic surface. As the droplet dries the water-soluble sunscreen concentrates around the spore [6, 119]. Water based formulations will cause minimum drifts owing to density effects during field spray.

B. Oil soluble sunscreen; oil droplet forms a thin film and small contact angle on the hydrophobic surface. As the oil droplet spreads over the surface, the oil-soluble sunscreen spreads with the oil exposing the spore [6].

C. Water medium and sunscreen is encapsulated to give a long term UV radiation protection (suggested approach).

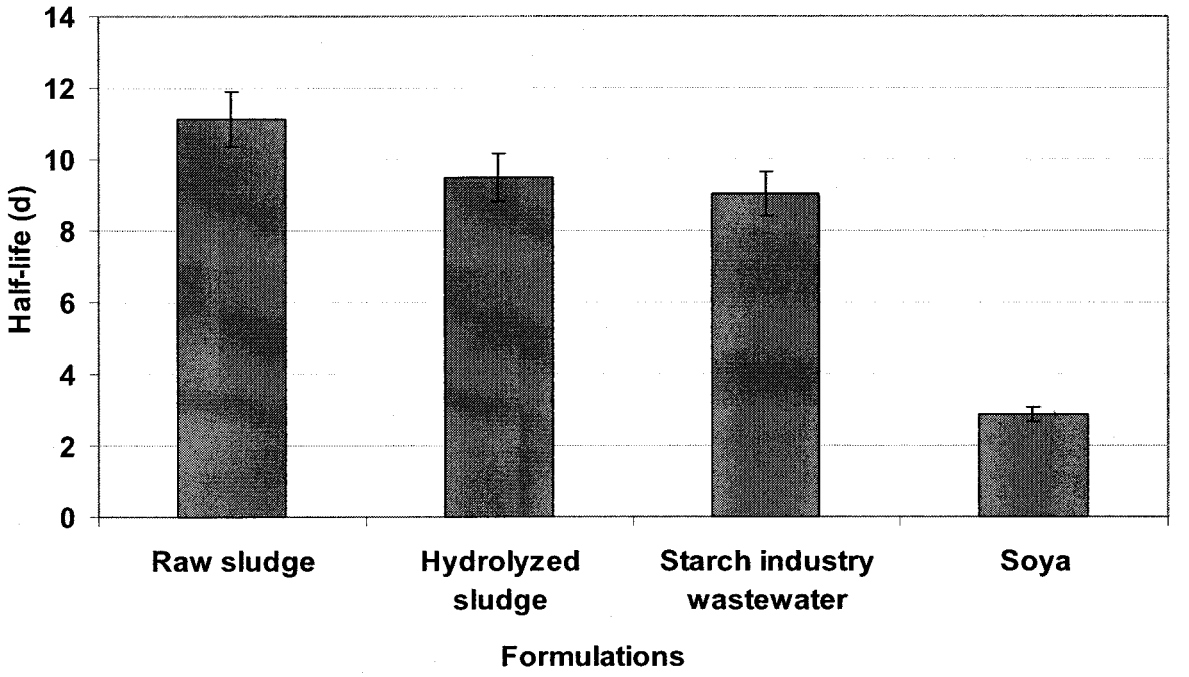


Fig. 2. Half lives for different Bt formulations (without UV radiation screen) on UV radiation exposure

Partie II

Bacillus thuringiensis Proteases: Production, Sporulation and Synergism – Review

Satinder K. Brar[1], M. Verma[1], R.D. Tyagi[1], R.Y. Surampalli[2], S. Barnabé [1] and J.R. Valéro[1]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9
[2]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Process Biochemistry (2007)

doi:10.1016/j.procbio.2007.01.015

Protéases produire par *Bacillus thuringiensis* : production, sporulation et synergisme - Revue de littérature

Résumé

Certaines sous-espèces de *Bacillus thuringiensis* (Bt) produisent, pendant leur croissance des métalloprotéases et des protéases alcalines sérines (protéases endogènes). Ces enzymes ont un effet sur la sporulation du Bt et sur son potentiel entomotoxique contre différents ordres d'insectes. La production des protéases de Bt a été étudiée en employant des substrats conventionnels et des substrats alternatifs et en considérant les répercussions vis-à-vis les formulations de Bt et leur action sur la mortalité des larves des insectes cibles. Un rapport entre l'activité protéolytique et le nombre total de cellules viables durant la fermentation de Bt a été établi tout en explorant la possibilité d'employer l'activité des protéases produites par le bacille comme un indicateur potentiel du pouvoir insecticide. En général, les protéases influencent le pouvoir insecticide de deux manières, soit en hydrolysant les pro-toxines inactives en des fragments dont certains sont des toxines actives (suite à l'action endogène des protéases de Bt ou à l'action exogène des protéases intestinales produites par les larves d'insectes ciblés), soit par la dégradation des pro-toxines en fragments n'ayant parfois pas d'activité insecticide (normalement sous l'action endogène des protéases de Bt). En fait, l'activité protéolytique des protéases endogènes (intra et extracellulaire) est ambigüe et soulève plusieurs questions sur leur rôle dans l'action entomotoxique du bacille. Cette revue de littérature examine les diverses écoles de pensées (traditionnelles et récentes) qui tentent de résoudre l'énigme des interactions des protéases de Bt avec les cristaux protéiques insecticides produits par Bt à différents niveaux (sporulation et action insecticide).

Mots-clés: *Bacillus thuringiensis;* cristal protéique insecticide; protéases; sporulation; eaux usées; boues d'épuration

Abstract

Bacillus thuringiensis (Bt) subspecies produces metalloproteases and serine alkaline proteases (endogenous) which affect sporulation and entomotoxicity against different insect orders. The production of Bt proteases is investigated in conventional medium and alternative substrates with future repercussions on Bt formulations and larval mortality. Relationship between protease activity and total cell count during Bt fermentation has been discussed while protease activity as a potential indicator of entomotoxicity has also been explored. In general, the proteases influence entomotoxicity in two divergent ways- processing of inactive protoxins to active toxin fractions (by endogenous Bt as well as exogenous larval midgut proteases) and; degradation of protoxins to fragments which sometimes lack insecticidal activity (usually by Bt proteases). In fact, the function of endogenous (intra and extracellular) proteases is ambiguous and has been raising serious questions on their role in larval mortality. The review explores various schools of thoughts (traditional as well as advanced) to solve the enigma of protease interactions with crystal toxins at different levels (sporulation and insecticidal action).

Keywords: *Bacillus thuringiensis;* insecticidal crystal protein; proteases; sporulation; wastewater; wastewater sludge

1. Introduction

Bacillus thuringiensis (Bt) is a spore forming bacterium that produces a potent insecticidal crystalline protein (ICP) making it a successful biopesticide. The ICPs are also referred to as Cry proteins and contain δ-endotoxins, which cause mortality of insects belonging to different orders, namely, diptera, coleoptera and lepidoptera [1,2].

Bt is also an excellent source of protease enzymes. The proteases are considered key players in a wide range of biological processes, such as, cell cycle regulation, cell growth and differentiation and sporulation. The secreted proteases could be intracellular and/extracellular. Intracellular proteases are vital to sustain various cellular and metabolic processes, such as, sporulation and cell differentiation, protein turnover, enzyme maturation and hormones and also in protoxin activation of Bt based biopesticides [3,4]. Extracellular proteases carry out protein hydrolysis in fermented media and enable the cell to absorb and/or adsorb and utilize hydrolytic products. Alkaline serine proteases are the most dominant group of proteases produced by bacteria, fungi, yeast, viruses, protozoa, and actinomycetes and Bt, in general [5]. Most commercial serine proteases, mainly, neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*.

Proteases have incited the interest of many researchers for a long time and they have been extensively studied for production from different sources, cellular role, downstream processing and characterization [6-8]. However, Bt proteases have been studied in literature only from the point of view of their role in insecticidal activity.

Despite the literature on mass production of proteases and their role in general, the proteases still remains a Pandora's box. There is a need to establish their precise role in spore formation, synergy with insect entomotoxicity and possible addition in the final formulations, which has been explored in this review. The role of Bt proteases in the processing of protoxins is relatively not well understood when compared to larval gut proteases. Furthermore, the two schools of thought on synergism or antagonism [9-13] have raised serious questions on retention of extracellular and intracellular proteases formed during Bt fermentation and subsequent carry-over to formulations or to separate them after fermentation. Thus, this review focuses on the state-of-the-art knowledge on: a) types of Bt proteases; b) production in conventional and alternative media; c) role in sporulation and ICP production; d) correlations between protease and cell count and entomotoxicity and; e) role of

intra and extracellular proteases in insect entomotoxicity to gain understanding of synergism and/or antagonism.

2. Types of proteases

Principally, the proteases are of two types, namely, exopeptidases (exoproteases/extracellular proteases) and endopeptidases (endoproteases/intracellular proteases) [14]. A more elaborate classification of proteases has been presented in Table 1. Bt produces metalloproteases, alkaline serine proteases and cysteine proteases which have been the subject of several biopesticide production studies [12,13,15-19].

3. Proteases production by Bt

3.1. Conventional media

Bt proteases could be constitutive or partially inducible in nature. They are normally extracellular produced during exponential phase and also comprise intracellular proteases released due to cell lysis during post-exponential growth phases. Extracellular protease production is strongly influenced by media components, namely, variation in C/N ratio, presence of easily metabolizable sugars, such as glucose [20], and metal ions [21]. Likewise, several environmental factors, such as, aeration, inoculum density, pH, temperature and fermentation time also affect the amount of proteases produced [22,23]. The protease production has been carried out by employing semi-continuous, fed-batch fermentations, and chemostat cultures [23]. Several other methods have been used for improving protease production from different microorganisms, such as, cell immobilization [24,25], aqueous two-phase (ATPase) systems [17,26], solid state fermentation [27-29] and biphasic growth systems [29]. Although some of the protease production methods relate to other *Bacillus* strains, yet there is a possibility of producing proteases from Bt strains by these methods.

Proteases during Bt fermentation are involved in spore and δ-endotoxin synthesis in three modes – 1) cell lysis and subsequent release of mature spore and crystal protein (also known as crystal toxin/delta-endotoxin/parasporal crystal protein) into the medium [30,31]; 2) making available proteinaceous components for subsequent formation of spores and crystal toxins [32,33] and; 3) expression of sporulation *spo* and crystal protein *cry* genes [34].

Furthermore, it has been proved that protease production was higher in a complex medium when compared to synthetic medium [35]. The extracellular proteases were secreted in the logarithmic growth phase to supply nitrogen and amino acids for the microorganism. Meanwhile, intracellular proteases were released in the medium during sporulation. The protease production by Bt on different media has been further compiled in Table 2 depicting the active pH range and the respective activity obtained. Thus, protease production during Bt growth and sporulation process is an interesting pursuit which needs to be understood in relation to its role in Bt fermentation and insecticidal action.

3.2. Alternative media (wastewater and wastewater sludge)

As discussed earlier, Bt releases proteases during the post-exponential and stationary phases to hydrolyse leftover/residual complex proteins in order to satisfy its nutritional needs, among other reasons. The hydrolytic activities of Bt are regulated by different induction levels depending on medium composition so that it grows and produces crystal protein, an important component of Bt entomotoxicity [35,36].

The protease activity (PA) profiles during Bt fermentation could be well understood from Figure 1 a and b. Protease activity was determined according to Kunitz [37] method. One international unit of enzyme activity (IU/mL, referred throughout the manuscript) was defined as the amount of enzyme which releases 1.0 µmole (181.0 µg) of tyrosine per min under assay conditions (37°C and pH 8.2). It was observed that temporal profiles of PA were similar in all raw materials with the increase in PA in the post-exponential phase (after 18 h of fermentation as seen in Figure 1b). An exception to the case was slaughterhouse wastewater where the PA was higher at 10 h of the fermentation time. This ambiguous behaviour could be due to lower complexity of the proteins. The starch industry wastewater; soyameal and primary wastewater sludge (higher carbon content, yet non-bioavailabile and/or limited carbon availability) showed lower PA which may be due to the repression of the enzyme production caused by higher carbohydrate concentration as reported by Zouari et al. [38]. Further, the signal transduction pathway defined by regulatory gene based DegS-DegU signaling system is involved in the control of the rates of synthesis of degradative enzymes, including extra- and intracellular protease in *Bacillus subtilis* [39,40]. The same signal transduction pathway may be involved in sensing limitations of carbon, nitrogen, or phosphate sources, which usually occur at the end of the exponential growth phase. This could trigger an adaptive response of the cell, which may react to nitrogen limitation and consequently raising the level of proteases

providing alternative nutrients. It would explain the increase in the rates of synthesis of proteases in rich media at the end of exponential growth phase. However, the Deg signal pathway and its likes may not be favored for the production of proteases resulting in lower PA in the case of starch industry wastewater; soyameal and primary wastewater sludge as they might have served as minimal medium with respect to nitrogen. Meanwhile, there is virtually no information available on the environmental signals involved in regulating toxin gene expression in *B. thuringiensis*. Since accumulation of crystal proteins coincides with sporulation, it is not known if the environmental signals which induce proteases secretion and sporulation, also trigger toxin synthesis [41]. Meanwhile, the PA increased with the complexity and suspended solids of the media as observed in the case for sludges. It is a known fact that complex proteins induce higher levels of PA into the medium [35].

Different Bt strains isolated from wastewater sludge [42] have been compared with commercial Bt var. *aizawai* (Bta) and Bt var. *kurstaki* (Btk) strains for the production of protease. The respective data of maximal protease activities (at 48 h) for isolated strains and commercial strains for soya and wastewater sludge are presented in Figures 2 a and b, respectively. When the data were analyzed for protease production in wastewater sludge (Figure 2b), the maximal protease activities were higher for all isolated Bt strains except INRS 8 and INRS 24 in comparison to soya medium (Figure 2a). On the contrary, the standard Bt strains (Bta and Btk) showed higher PA in soya medium. The higher PA in soya medium could be due to the fact that it was a nitrogen rich source for induction of proteases. On the other hand, sludge was relatively nitrogen deficient medium as well as contained complex nitrogen source resulting in lower protease production. Moreover, under nitrogen limiting condition, initiation of competent stage is not favored resulting in low protease production. Meanwhile, PA has been reported to be in the range 1.5 to 2.5 IU/ml for Bt fermentation of wastewater sludge in shake flasks; bench scale (15 L) and pilot scale fermenters (150 L) which has been attributed to the complexity and variability of this medium [43-45]. The composition of wastewater sludge could vary according to region, season and wastewater treatment technology [46]. Furthermore, irrespective of the growth medium, different Bt strains principally produced two types of proteases – neutral (pH 7) and alkaline (pH optimum between 8-11) with temperature optima of 40 and 50°C. Similar types of proteases were also produced by known Btk strain during fermentation of wastewater sludge [43]. Analogous characteristics of proteases produced on gruel-based media by Btk BNS3 were also

reported [35]. Thus, it is very likely that these neutral and alkaline proteases may affect the formulations of Bt fermented broth on storage (discussed later).

At this juncture, it is necessary to evaluate the role of Bt extracellular proteases having different pH and temperature optima, in synergism or antagonism of entomotoxicity (Tx) and finally the lethal or sub-lethal effects of delta-endotoxin.

3.3. Industrial applications of Bt proteases

As discussed earlier, several of protease enzymes of *Bacillus* sp. are commercially available and widely used in different industries namely, detergent, food, pharmaceutical, leather and silk [47]. This aspect has been less explored in the case of Bt despite the sporulation event being characterized by the synthesis of several proteins including proteases [48].

Early study of Li and Yousten [49] reported the production of metalloprotease by Bt var. *kurstaki* which showed optimum activity in the pH range 6.5 to 7.5. It was inhibited by chelating agents but not by a serine protease inhibitor and was thermal and salt stable. However, the activity was reported to be in the range 200 to 250 U/ml with less orientation towards industrial application. Later, Hotha and Banik [17] investigated the possibility of alkaline protease production by Bt H 14 in aqueous two-phase systems composed of polyethylene glycol X (X\9000, 6000,4000) and potassium phosphate. The protease activity was very high with a range of 1800-4000 IU/ml and showed promises of industrial application except for the costs of recovery and utilization of raw material used which could be an impeding factor in the commercial production. The interest in protease production was also later pursued by Zouari and Jaoua [35] who investigated the production and characterization of metalloproteases synthesized concomitantly with δ-endotoxin by Bt subsp. *kurstaki* BNS3 strain grown on gruel-based media. BNS3 proteases were shown to be neutral metalloproteinases, and salt and sodium dodecyl sulfate (SDS)-tolerant. The proteases were tolerant in the pH ranges of (7–10) and of temperatures (30–80°C). The authors proposed that the medium also contains spores and δ-endotoxin crystals (bioinsecticide) that may be separated from the proteases. Both of these products could be used for industrial applications. Likewise, the studies conducted on protease production by Bt var. *kurstaki* by using semi-synthetic soyameal and wastewater sludge medium demonstrated that protease activity was optimal in the pH range 7-11 and thermotolerant [43]. The authors proposed solid-liquid separation of proteases with the possibility of finding the enzymes in liquid fraction,

however, the separation process involving a complex medium like wastewater sludge would be a big bottleneck in the scale-up of the process.

Currently, proteases are major industrial enzymes, and constitute more than 65% of the world market [8]. However, the production of proteases by Bt with an acceptable activity/concentration level in the fermented broth for application remains a big challenge in the presence of other high performance *Bacillus* sp. Given the commercial success of the proteases, there is a possibility of looking into development of robust enzymes with desired properties for industrial processes through protein engineering and genetic manipulation which could be carried out in Bt in the foreseen future. Thus, Bt could draw great attention for its dual economic status – production of bioinsecticides to combat insect pests and proteases for use in industries.

4. Role of Bt proteases in spore maturation and ICP production

The sporulation event in Bt is characterized by the synthesis of several proteins including proteases [48]. Proteases related to sporulation were widely studied in *Bacillus subtilis* and to some extent in Bt [48]. Among them, the intracellular serine protease A (IspA) was especially well studied [50-52]. The IspA is responsible for most of the intracellular proteolytic activity in sporulating cells. In addition, the overall protease activity substantially increases during the post exponential growth phase after release of intracellular proteases through cell lysis into the surrounding medium [15,51,53] as presented in Figures 1a,1b and 2b. The formation of spore coats requires active synthesis of proteins. The synthesis of protein components occurs by utilizing oligopeptides and amino acids generated through the proteolytic cleavage of simple or complex proteins by extracellular proteases [54]. There is evidence that proteases are also involved in other sporulation processes. Firstly, serine proteases perform the post-translational modification of spore protein precursors. Secondly, proteases cleave the surface structures of fully sporulated cells, thereby promoting the release of spores into the medium. The sporulative events in sporulating bacteria occur in seven stages: preseptation (I), septation (II), engulfment (III), cortex formation (IV), spore coat formation (V), spore maturation (VI) and free spore formation through lysis of the mother cell (VII) [55]. Alkaline proteases from *B. intermedius* were secreted during the late stationary growth phase, which corresponded to stages V-VI of sporulation suggesting their role in spore formation [30]. Further, Balaban et al. [31] estimated β-galactosidase activity as a marker of cytoplasmic membrane

integrity and proved that the accumulation of the proteases (endopeptidases and subtilisin) in the medium was a result of their secretion and not of lysis of the cell envelope.

Proteases are also involved in sporulation gene expression. It is known that sporulation induces changes in the transcription factors such as, Spo0A and various Sigma factors (σ^K being the principal factor). Gene expression in the mother-cell compartment of the sporangium in *B. subtilis* is governed in part by the sporulation transcription factor σ^K, which brings about the formation of the major protective structures of cortex and coat [56]. The production of σ^K is controlled at three levels: a) chromosomal rearrangement that generates the σ^K-coding sequence (SigK); b) compartment-specific transcription of sigK, and; c) conversion of the inactive pro-protein product of SigK (pro-SigK) to active SigK [57,58]. As pro-SigK was a highly regulated transcription factor, it was not likely that this essential precursor protein was processed by a protease with no significant substrate specificity such as IspA. However, a delay in sporulation was observed in the IspA-deficient mutant [59]. Moreover, the *cry* genes have probably been superimposed on the sporulation process by the transfer of plasmids. Many *cry* genes are transcribed by both the mother cell forms of RNA polymerase containing SigE or SigK. Consequently, transcription begins at stage II of sporulation by the SigE form of RNA polymerase and continues into late sporulation by the σ^K form of the enzyme. This dual control ensures the prolonged synthesis and thus accumulation of large amounts of the ICPs/protoxins [34].

An immune inhibitor A (InhA) metalloprotease from Bt produced during stationary phase specifically cleaves antibacterial proteins produced by the insect host, suggesting that it may contribute to the overall virulence [60]. It is therefore likely that InhA transcription also requires sporulation-specific sigma factors or depends on the complex regulatory mechanisms that control late growth development in *Bacillus* species [61].

Hence, the extracellular proteases are required to hydrolyze the proteins present in the medium for growth/synthesis of new proteins for Bt cells. Likewise, if production of intracellular and extracellular proteases is altered, the sporulation may be affected. In fact, sporulation is an event marked by protein turnover as there is synthesis of cortex and spore coats interceded by intracellular proteases. If insufficient intracellular proteases are not produced, the sporulation will be affected drastically which will cause formation and release of immature spores into the medium. The

immature spores may not be fully active and hence may not contribute to synergism with other virulence factors for insecticidal activity, the ultimate goal of biopesticides. Further, it could have an impact on the formation of crystal proteins – a vital component of entomotoxicity which occurs concomitantly with stages I and II of sporulation. Moreover, immature spores may have no or less synergistic activity with ICPs affecting the overall entomotoxicity. Thus, proteases are important indicators of entomotoxicity for different Bt strains.

4.1. Correlation of protease activity (PA) with total cell (TC) count

The protease activity varies with cell count as it is also a direct determinant of Bt cell growth in a nitrogen-rich medium [35]. In a typical Bt fermentation, Bt consumes the easily biodegradable material to ensure growth in the early phase. So, there is a slow increase in PA during the exponential phase of growth and it increases further until stationary phase. Initial lower PA may also be a function of lower TC in the beginning of fermentation period so that PA may present a relation with TC. Nonetheless, if there is a presence of an initiation factor for competent stage (sporulation), for example, substrate limitation, it can bypass the cell density signal leading to initiation of competent stage. Meanwhile, no study is reported in literature on the effect of PA vs. TC trends. In this context, PA vs. TC profiles of different Bt fermented media are presented in Figure 3a. The PA followed an exponential relationship with TC count (0-30 h) for all media in concordance with earlier studies [44,45,62]. However, the values of proportionality constants were different for various media, so that any change in TC affected the PA which may be attributed to the medium simplicity or complexity. Irrespective of the type of medium, the PA was maximal during stationary phase. Thus, as cell concentration increased, the cells in the cultivation medium synthesized more extracellular proteases to hydrolyze the complex protein into amino acids for vegetative cell growth, eventually attenuating the competent (sporulation) stage. On the other hand, intracellular proteases will process factors such as pro-SigE and pro-Sigk leading to continuation of sporulation stage and formation of mature spores. Although protease production was related to spore production (proteases required to furnish different proteins during sporulation stages), still there was no mathematical relation between the two [62]. This is justified by the fact that Bt proteases are fermentation metabolites and are produced under nitrogen rich conditions, whereas spores are produced under nitrogen deficient conditions and other adverse pH and temperature conditions and are dormant cells of Bt. Further, Bt cell growth requires nitrogen source, however, once the cell density has reached the threshold level or nitrogen becomes limited, it will initiate the sporulation process and produces large amount of proteases

during the competent stage. In fact, production of protease determines the competent stage. Subsequently, sporulation will go through the late stages of sporulation, namely, spoII, spoIII, spoIV and others. Upon activation by phosphorylation, Spo0ACP becomes a DNA-binding activator for stage II gene transcription (*spoIIA*, *spoIIE* and *spoIIG*). Additionally, Spo also behaves as a repressor for *abrB*, a regulator of many post-exponential-phase functions (encodes a repressor that controls the expression of genes involved in starvation-induced processes such as sporulation and the production of antibiotics and degradative enzymes). Therefore, PA may not be consistent with spore counts and this particularly happens in the late spo mutant [40,61]. In the given context, PA may affect spore virulence, cortex formation, spore coat and maturation and eventually crystal protein formation, but its amount may not be consistent with spore counts and may be dependent on several other factors (discussed earlier).

It has been established that PA follows a slow increase in the initial 12 h followed by a gradual increase resulting in a peak at around 30 h irrespective of media composition, fermentation scale (shake flasks, bench and pilot scale) and different strains isolated from wastewater sludge [43-45,63]. In fact, *Bacillus* proteases, especially, the IspA were proved to be involved in the formation of spores with the turnover of intracellular proteins [59]. A decrease in PA was reported after 30 h of fermentation which could be a manifestation of nutrient limitation and denaturation of enzyme (auto-digestion of protease and proteolytic attack by other proteases such as intracellular protease released after cell lysis) [45]. At this stage, further research is required to verify whether the decrease of PA towards the end was a function of nutrient limitation and/or protease denaturation or perhaps both.

4.2. Correlation of entomotoxicity (Tx) with protease activity

It has been established that higher entomotoxicity could be achieved by several factors: a) higher maximum specific growth rate [38,64]; b) higher spore count [38,65]; c) higher nutrient availability by pre-treatment in case of complex media [44,66,67]; d) improved rheology [67-70] and; e) better spore maturation [34,66]. However, determination of Tx by the conventional insect bioassay method is highly dependent on insect physiology and diet composition and is laborious as well as costly, subject to estimation errors. Thus, the need was felt for a parameter which could act as indirect indicator of Tx. Recently, attempts have been made to correlate the Tx value with PA [62] so that PA could act as a rapid and indirect measurement of Tx.

The correlation of PA with Tx profiles for different media are presented in Figure 3b. The Tx evolution with PA was divided into two phases. In the 1st phase, PA increased proportionately with Tx value and attained its peak whereas in 2nd phase, Tx increased continually while PA started declining. The end of the first phase was noticed at approximately 30 h in a typical batch fermentation of 48 h, irrespective of the medium used to cultivate Bt. This correlation could be due to the fact that the crystal protein formation needed higher protein quantity that was probably furnished by hydrolysis of complex proteins (mediated by proteases) in the residual complex substrate (sludge, in this case). It was observed that about 80-90% of Tx was already achieved at peak protease activity (30 h) as reported earlier [44,45,63]. The residual increase in Tx in later stages (30-48h) may be due to probable prolongation of stages III and IV of sporulation (discussed earlier). The prolongation of stages III and IV of sporulation could result in enhanced assembly of crystal proteins and theiiuration so that when they are released during cell lysis, the final Tx was relatively higher. Apparently, individual *cry* genes on plasmids could be transcribed at different rates, resulting in unequal amounts of the protoxins in inclusions at different times [71,72]. The transcription differences may arise due to several factors: (a) differences in plasmid and thus *cry* gene copy number; (b) inherent differences in protoxin stability and/or the presence of factors (perhaps chaperones) which stabilize the protoxins for packaging; (iii) differing rates of transcription of *cry* genes [71] despite virtually identical overlapping promoter regions (apparently important for modulating the transcription of protoxin *cry* genes) in many cases. Further, the linearity in PA with Tx was also justified through the supernatant studies as discussed later (Figure 7).

Revisiting the data differently, the PA observed during early sporulation phase might have aided in protoxin hydrolysis so that the Bt toxin may directly enter the stage of receptor binding and pore formation (discussed later). This could cause skipping of proteolytic processing in the insect midgut averting the possibility of resistance (discussed later). It has been reported that resistance among other causative factors is also caused by degree, or rate, of activation of protoxin interceded by proteases [73]. This fact has not been ascertained through experimentation and therefore detailed experimental studies will be mandatory to provide the evidence, however, it always remains a possibility. Moreover, Kumar and Venkateswarlu [13] reported that a 69-kDa metalloprotease, produced at stage II that persisted until stage VI of sporulation, was involved in the generation of the active toxin. The latter had a different structure as compared to midgut digested Cry protein, lacking

an N-terminal of polypetides. Interestingly, the active toxin produced under these conditions was highly active against the cotton leafworm, *S. littoralis* species insensitive to toxin produced by exogenous proteases [13]. Since intracellular proteases seem to play an important role in the production of active toxins during Bt growth, under these conditions, there will be a parallel increase of free amino acid pool as reported by Reddy and Venkateswerlu [4]. This increase in amino acid pool is helpful during sporulation in spore coat and cortex formation (discussed earlier).

Another interesting observation was that PA and Tx increased for NH sludge by increasing suspended solids concentration from 10 to 25 g/L due to enhanced availability of nutrients from solids. However, NH sludge at higher solids concentration (NH-30) showed lower PA and Tx as the rheology became more complex. It might be due to lower nutrient availability stimulating lower protease secretion as well as reduced Tx. Nevertheless, NHT-30 showed lower PA and highest Tx (Figure 3b). Lower protease may be due to simplification of proteins during sludge treatment. High Tx could be due to increased availability of nutrients and amelioration in rheology causing better oxygen transfer and improved availability of nutrients as well as nutrient assimilation resulting in higher sporulation and production of entomotoxicity. However, it must be emphasized here that excessive aeration may result in partial inhibition of sporulation even in spite of increased entomotoxicity as observed by Sarrafzadeh and Navarro [74] during growth of *Bacillus thuringiensis* H14 fermentation on glucose medium. On the contrary, under O_2 limited conditions, δ-endotoxin and spore counts were found to decrease [75]. Hence, in case of higher solids concentration (NH-30), where viscosity was high (complex rheology), system was limited in oxygen transfer and hence lower sporulation and Tx value was observed. Moreover, O_2 must be continuously supplied if higher δ-endotoxin concentrations are to be reached and meticulous aeration in Bt fermentation plays an important role in formation of mature spores as well as δ-endotoxin.

Meanwhile, soyameal medium showed lower PA and lower Tx, which could be an effect of simplicity of the medium for lower proteases secretion. Moreover, the spores formed along with the crystal proteins in soya medium could be less toxic as compared to alternative wastewater sludge medium as reported by Barnabe [66]. Thus, PA played a major role in determining the Tx, in addition to the sporulation process discussed earlier.

In fact, there is a possibility that the active toxin generated by Bt intracellular proteases could be potentially beneficial in causing mortality of certain insect species (resistant ones). In this context, the pre-digestion of the Cry protein to active toxin during Bt fermentation (before feeding to larvae) may be beneficial in the sense that the active toxin can directly reach the midgut epithelium receptor site. Moreover, there is a possibility that the active toxins so generated might possess different characteristics [4] so that they can directly bind to the midgut epithelial receptors which further warrants extensive studies. This event would prevent chance of the Cry toxin to get overdigested by midgut proteases and may avert resistance development. Nevertheless, synergy of proteases linked (adsorbed) to Cry proteins cannot be ruled out so that the resultant entomotoxicity will be higher (discussed later in section 7). Thus, Bt proteases may offer potential advantages in overcoming the insect resistance to Bt in insects like *S. littoralis, P. xylostella* and others. In spite of the cited literature, it was not yet clear whether the presence of proteolytic enzymes during the course of the crystal formation was simply fortuitous or whether these proteases may play a definite role in the processing of crystal proteins.

5. General mode of action and synergy of Bt proteases with other virulence components

The Bt mode of action is a four-step process: solubilization, activation, binding, and pore formation and is a well researched subject [76-78]. The detailed mode of action is also illustrated in Figure 4 and the possible role of Bt proteases has been marked.

Bt also produces various virulence factors other than the crystal proteins, including, secreted insecticidal protein toxins (vegetative insecticidal proteins - Vips), hemolysins, enterotoxins, chitinases, proteases, phospholipases and others [29]. Besides the Cry proteins, cytolysins (Cyt toxins), smaller with non or less specific nature when compared to δ-endotoxin, which act by a different mechanism, are also found within the crystal. The Cyt1A proteins were known to synergize certain Bt toxins to increase Tx towards resistant species of a mosquito and a beetle [79,80]. The spore itself contributes to entomopathogenicity, often synergizing the activity of the crystal proteins as they germinate in the haemocoel and cause septicaemia [81]. There is a possibility that proteases may favour mode of action of Vips by degrading proteinaceous compounds that may interfere with their action and thus enhance entomotoxicity. There is no reported study on synergism of proteases with virulence factors, except for some fundamental studies on role of intracellular and extracellular proteases on insecticidal proteins (discussed later). In fact, there are broadly two types of proteases

that affect the specific entomotoxicity – endogenous (produced by Bt during growth and sporulation phase) and exogenous (insect midgut proteases produced during insecticidal activity in the midgut lumen of insects).

At present, it remains to be explored if the proteases already present in Bt fermented broths that end up in formulated products and subsequently play a pivotal role in the proteolytic processing of Cry protoxin or is it just the induced larval proteases secreted in the midgut. Some of the Bt proteases are associated with ICP and may cause proteolysis of the protoxins as stated earlier. This role has been a major objective of research as the ICPs could be affected by proteases in two divergent ways: (1) conversion of non-toxic protoxins to smaller insect-active toxins (explained later); (2) degradation of protoxins to small polypeptide fragments which do not have insecticidal activity and hence loss of insecticidal action.

5.1. Role of Bt intracellular proteases in insect mortality

The possible role of intracellular proteases in the mode of action of Bt has been a subject of interest to various researchers. Isolates of Bt var. *kurstaki* viz. LB1 and HD251, Bt var. *israelensis* (Bti) and Bt var. *berliner* strains have been reported to produce different levels of intracellular proteases [15,48]. Although the entomotoxicities of whole crystals, soluble crystal protein, and purified toxin from various strains were comparable; HD251 strain produced lower intracellular proteolytic activity than the LB1, Bti and Bt var. *berliner* strain. These studies suggested that production of intracellular proteases by different strains did not have any effect on the entomotoxicity. However, Venkateswerlu and Stotzky [82] found that there was generation of toxin when Bt var. *kurstaki* cells were incubated with 0.1 M 3-morpholinopropanesulfonic acid, pH 7.8, 0.5 M dithiothreitol and 1 M potassium thiocyanate. The bacterial or endogenous proteases were presumed to be responsible to cleave132 kDa protoxin to 66 kDa toxin under the conditions used by the authors for isolation of toxin and hence obviating the need for trypsin (exogenous protease from insect midgut).

Apart from the possible role of intracellular proteases in the biochemical events of spore formation, the involvement of metalloproteases of Bt var. *tenebrionis*, Bt var. *sphaericus* and Bti in proteolytic activation of protoxin has been proved by many other researchers [9-12,15,16,48,83-85]. These studies pointed out that the differences in protoxin accumulation during fermentation and

activated form of toxin does not possess specific Tx sequence. This would be disadvantageous as it will prevent bacteria from completing life cycle and preclude higher insecticidal potency.

Additionally, the intracellular proteases are also capable of degrading selectively attacin and ceprocin – two anti-bacterial proteins responsible for defence in the immune system of insects [60,86]. In the case of Btk HD-1, Kumar and Venkateswerlu [13] proved the key role of endogenous protease in effective killing of target insects. The study demonstrated that the toxin activated by Bt endogenous intracellular proteases was significantly active even against the naturally tolerant pest species, *Spodoptera littoralis.* The toxin thus pre-activated inside the cell, when finally ingested by the insect along with other virulence factors and spores, could aid in skipping the proteolytic processing step so that it could go to receptor binding site directly, causing insertion, osmosis and pore formation as illustrated in Figure 4. This possibility raised the fact that the proteolytic system of Bt was unique and effectively different from exogenous proteases with respect to the protoxin activation [13]. However, the pre-activated protoxin may not specifically bind to the receptors causing loss of specificity and possibly negligible insecticidal action which remains to be investigated.

In fact, Almond and Dean [87] examined the differences in protoxin accumulation in the cells among three different bacterial strains – *E. coli*, Bt and *B. subtilis* in which the recombinatorial chimeric protoxin genes were constructed and transformed. This study suggested that differences in intracellular proteolysis caused a switchover between different *cry* genes so that protoxin accumulation levels varied. Therefore, different polypeptides were obtained by incubation with enzymes from varied sources, resulting in a difference in entomotoxicity. This study further established that bacterial intracellular proteases contribute to insect mortality of otherwise resistant species. Meanwhile, genetic engineering and protease inhibitors have been reported as the possible modes to control the endogenous protease action [11,16,33] and hence the protoxin accumulation levels.

At this stage, the literature reports are conflicting on the possible role of intracellular proteases in Bt insecticidal action. This raises the question, "whether one needs to recover these intracellular proteases and add them to formulated product in order to enhance the Tx value"? The detailed studies at formulation level to ascertain their role in lethal or sub-lethal effects on larvae could give response

to earlier question. However, only a low percentage of intracellular proteases may actually reach the larvae as the viable cell count drops exponentially during different processing steps of Bt, namely, harvesting and formulation development, as seen in Figure 5. Thus, the only mode by which the intracellular proteases could reach the midgut is through the spores (if at all, they are adsorbed on them as Vips are known to be adsorbed on spores) and/or released as extracellular enzymes into the formulation broth. Interestingly, it could be interpreted that when the Bt spores enter into the blood stream (Figure 4, step "e"), the spores germinate and the released intracellular proteases (during cell lysis) may weaken the immune system leading to larval death. Moreover, proper spore germination is an important factor for enhancing Tx [88]. Another possibility was that the intracellular proteases could be released during cell lysis (in fermenter) along with toxin and spores and could reach the formulation and/or midgut. Therefore, the intracellular proteases could be available for the Tx effect along with spores and crystal toxins; provided they are not removed during centrifugation. Meanwhile, the intracellular proteases transferred to supernatant phase during centrifugation of broth could be concentrated by the ultrafiltration process and mixed with formulations. However, there is no reported study on the possible role of intracellular proteases once outside the cell.

5.2. Role of Bt extracellular proteases in insect mortality

Although extracellular proteases have not been found to play a dominant role in the entomotoxicity of Bt products, yet some studies point to the possible importance of them. Donovan et al. [11] partially deleted cloned neutral protease A (*nprA*) gene *in vitro*, and the deleted allele, designated *npr*A3, was used to construct an *npr*A3 strain (neutral protease A-deficient strain) of Bt. Growth and sporulation of the two strains were similar, although the extracellular proteolytic activity of the *npr*A3 strain was significantly less than the isogenic *nprA*1 strain. The *nprA3* strain produced ICPs that were more stable and sporulated cultures of the *nprA3* strain contained higher concentrations of full-length ICPs. The results indicated that crystal protein stability and yield may be improved by deletion of specific Bt extracellular proteases. Three reasons were proposed for the phenomenon: a) degradation-processing carried out by NprA may be of different type when compared to other proteases; b) in the absence of NprA, other proteases may be active in the protoxin processing and; c) proteolytic activity in the midgut may be actually sufficient for the crystal protein processing. Further, Tan and Donovan [33] investigated the extracellular proteolytic activities, and insecticidal activities of alkaline protease A (AprA)-deficient and neutral protease A (AprA–nprA)-deficient Bt strains in relation with ICP production. They found that Cry protein so produced showed

varying toxicity to diverse larvae with differences in quantities of the crystal proteins. This indicated that the AprA and NprA proteases affected only the concentration of crystal protein and influenced the insecticidal activity of Bt.

Thus, Bt intracellular proteases do influence the entomotoxicity by two modes – a) synergistic action in activation of protoxin to active toxin and; b) affecting defense system in the insect midgut. They may also cause resistance in insects towards crystal proteins. On the other hand, extracellular proteases (less studied counterpart) could affect only the concentration of the crystal protein and not the insecticidal activity of Bt. In fact, there is an ambiguity that presence of extracellular proteases may decrease the stability of ICP. Researchers are still actively pursuing research on action of Bt proteases for their probable role, but level of protease production may be one of the factors leading to virulence between different Bt strains. Furthermore, the commercial Bt formulations are normally at pH 4-5 so that the neutral extracellular protease activity may not be active at this pH range. Thus, doubting the importance of the neutral extracellular proteases, if at all they are released during Bt fermentation.

6. Mechanism of proteolysis of crystal proteins

Figure 6 presents processing of different Cry proteins. The mechanism of protoxin activation of different Cry proteins suggests the important role of Bt and larval midgut proteases. Thus, correct activation of a Bt δ-endotoxin is necessary for its specificity and insufficient processing or over digestion of a toxin may render it inactive or less specific.

7. Degradation of crystal protein by Bt extracellular proteases during formulation storage

It has been reported that the crystal proteins of Bt, when admixed with Bt alkaline proteases, during the solubilization caused gradual degradation of the ICPs to lower molecular weight fractions [83]. Preliminary inactivation of the proteases may help to better understand the protein composition (amino acid sequence) of the crystals formed by various strains of Bt. The extent of the proteolysis observed strongly depends on: a) the amount of the proteases in the crystal formed by a given strain, b) the conditions used for the protein solubilization and, c) the stability of the proteases against denaturing or inhibiting agents. Thus, the possibility of participation of the Bt proteases in the hydrolysis of crystal protein within the insect gut must be considered.

However, there is no literature available on the possible action of Bt extracellular or intracellular proteases during storage of Bt formulated samples. Hence, an experiment was carried out on Bt fermented non-hydrolyzed and hydrolyzed wastewater sludge samples with the corresponding extracellular PA (alkaline) and Tx values presented in Figures 7a and b, respectively. The PA, in this case, corresponded to intracellular proteases released into the surrounding fermented broth during cell lysis which now became extracellular proteases, true extracellular proteases and the intracellular proteases present on the spores and Cry proteins. However, in the event of difficulty of distinguishing between the intracellular and extracellular proteases, they will now be referred to as extracellular proteases. As seen in Figures 7 a and 7b, the PA for Bt fermented non-hydrolyzed and hydrolyzed sludge was 2.1 and 1.6 IU/ml, respectively which decreased in the pellet to 0.5 and 0.4 IU/ml, respectively. Further, the supernatant of each fermented broth that showed higher PA of 1.1 to 1.3 IU/ml was mixed with saline solution in different ratios. The corresponding Tx increased concomitantly with protease concentration in the supernatant (supernatant contains proteases along with other virulence factors). The observation suggested that Tx increased proportionately with PA. This further confirmed that the Bt extracellular proteases (alkaline, in this case) seem not to have degraded the Cry protein structure. In fact, the proteases would have synergized with other virulence factors (chitinases, phospholipases, Vips, and other unknowns) and also some residual Cry proteins or toxic fragments and free delta-endotoxin present in supernatant. Hence, supernatant which is a major niche of other virulence factors responsible for increase in entomotoxicity could be mixed with centrifuged pellets to achieve higher Tx [89,90]. Barnabe [66] also observed that the ratio, Tx/soluble proteins, after separating spores and crystal proteins from the Bt fermented broth (48 h) was 11.5-28 in sludge in contrast to semi-synthetic commercial medium, soya (1.5). Therefore, the mixing of supernatant with the pellet would yield highly entomotoxic products. The soluble proteins comprised various virulence factors (as enumerated earlier). Additionally, Bt extracellular proteases present might have contributed to protoxin activation so that the proteolytic processing step in larval midgut could be by-passed and the active toxin can directly reach the receptor binding and insertion step (Figure 4- step "d"). The active toxin so produced has been found to possess a thick core which may be difficult to be degraded by Bt extracellular proteases any further [91,92]. However, there is a possibility that degradation of Cry protein may render the protoxin inactive which may result in resistance and even lower overall Tx.

Further, Bt formulations of different media (semi-synthetic soyameal medium and non-conventional alternatives like wastewater and wastewater sludge) at pH 7.0 ± 0.1, when stored at -20°C for one month, showed no change in Tx ($P \leq 0.01$). However, the Bt formulations at pH 7.0 ± 0.1 after one month storage at 20°C resulted in a decrease in Tx by 20-25% ($P \leq 0.01$) which might be due to action of Bt extracellular proteases that are active at this pH (Table 2). Moreover, the fermented broth contains other virulence components – enzymes (chitinases and phospholipases), and other unknown components which could have been degraded or inactivated during storage and hence Tx decreased. In this context, further research should be conducted by using protease inhibitors like phenylmethanesulfonyl fluoride (PMSF, for serine proteases) and ethylenediamine tetraacetic acid (EDTA, for metalloproteases) to ascertain protease effect on Tx during storage.

Additionally, when fermented semi-synthetic soyameal medium; wastewater and wastewater sludge and their respective stable optimized Bt formulations at pH 4.0 ± 0.1 were stored at 20°C for one year, there was no change in Tx ($P \leq 0.01$) [89,90]. This behaviour could be due to the inactivation of extracellular proteases in this pH range.

In fact, further specific studies should be carried out by recovering the extracellular proteases by SDS-PAGE and incorporating the purified enzymes to enzyme free cells, spores and crystal proteins suspension to determine the exact role of different proteases on formulations.

8. Larval (midgut) proteases

Insect proteases are implicated in Bt toxin specificity, mode of action, insect adaptation to Bt and resistance development. Moreover, the pH of the midgut influences the PA of the midgut juice significantly, but there is no obvious effect of pH on the degradation of activated toxin. Table 3 enumerates the role of midgut proteases in Cry protein processing and resistance mechanisms. However, complete understanding of Cry toxin-resistance mechanisms requires further study and it is an interesting subject, not covered within the wider scope of this review.

9. Synopsis and research outlook

The Bt extracellular proteases are key players during sporulation as they hydrolyze proteins for formation of spore coats and cortex aiding in maturity of spores. The mature spores result in crystal protein formation and in fact, the entomotoxicity is linearly related to protease activity which makes it a valuable tool to identify and predict the entomotoxicity of a respective Bt growth substrate.

The endogenous (Bt) proteases studied so far had maximum activity at 40-50°C and pH ranging from 7-11 (majority serine alkaline and metalloprotease type) with the possibility of action during proteolytic processing in insect midgut. Furthermore, this review addresses the issue of protease production during Bt fermentation. This is to justify if there is any need to enhance the production of proteases as the literature (sparse) points to contradictions about the role of extracellular proteases at formulation level. However, the extracellular proteases could be characterized and recovered at product separation stage which might be used for other industrial purposes. There is a caveat that the exact role of extracellular proteases, namely, alkaline and neutral, is not precisely understood which would throw a volley of possibilities of either recovering them (if they synergize Tx) and/or rejecting them (if they antagonize Tx, that is rerouting for other uses). So, there is a requirement to study the role of different pH active proteases during Bt formulations under the field conditions.

On the other hand, the Bt intracellular proteases that are concomitantly produced during Bt fermentation are beneficial to weaken the immune response of the insects and arrest resistance towards Bt. Intracellular proteases could be carried to the larval midgut by concentrating the supernatant obtained after centrifugation by ultrafiltration step and further mixing the retentate with the centrifugate and different ingredients to make formulations. Meanwhile, all formulations are a mixture of spores and crystal proteins comprising other virulence factors adsorbed onto them or in the broth, and it is unclear as to how they reach the field? There is a possibility that the spores germinate in haemoceol after osmotic lysis and hence intracellular enzymes produced there could synergize Tx. Hence, more studies are required to address the issue of transport and role of Bt proteases (intra/extracellular) through formulations.

In fact, proteases (endogenous as well as exogenous) are an important component of Bt action on different insects. The endogenous proteases weaken the immune responses of the insects and also have synergistic effect on Bt mode of action until the pH of the applied formulations is in the acidic range. On the other hand, the exogenous proteases aid in overall processing of Bt protoxin and binding to specific receptors followed by pore formation, septicemia and ultimately death. A more rational view of the approach to be adopted to understand the role of Bt proteases based on different literature studies has been summed up in Figure 8. Further, comprehensive research is required to understand the inhibition or activation effect of endogenous proteases as Cry protein is nature

derived. In fact, over a series of evolutionary processes, the Cry protein has acquired tremendous complexity bringing in new challenges to Bt researchers. The understanding of insecticidal crystal protein toxicity biochemistry together with the progress in molecular biology would further aid in comprehending the toxicity mechanism of different Bt isolates.

10. Conclusions

Bt produces metalloproteases, alkaline serine and cysteine proteases. Proteases have been known to be produced in conventional semi-synthetic and alternative media with an increase in protease activity during the exponential and post-exponential phase. Bt proteases are also involved in sporulation and ICP production gene expression. The Bt extracellular proteases have been confirmed to be direct determinants of Bt growth and also serve as potential indicator of entomotoxicity. Proteases (endogenous as well as exogenous) play a vital role in the overall insecticidal activity of Bt strains against different insect orders. Bt proteases, in particular, intracellular proteases, have also been found to affect the preliminary hydrolysis of crystal proteins which has serious impact on the overall entomotoxicity of the concerned product. They concomitantly aid in destroying the defence mechanism of insects making even the resistant species more vulnerable. In fact, Bt extracellular proteases may selectively cleave the crystal protein in such a way that overdigestion is protected and activated toxin transferred directly to receptor site. Meanwhile, pH changes in the fermented broth affect the proteolytic activity which has a profound effect on the crystal protein stability and entomotoxicity. Further, the midgut proteases are a necessary pre-requisite for processing of protoxin to active toxin form and specific binding to different receptors on the brush border membrane vesicles of the insects. Thus, future research efforts must be concentrated to gain fundamental insights into the activation, degradation and resistance mechanism of different crystal proteins interceded by Bt proteases. Finally, there is a need to seriously address the issues of role of Bt intra and extracellular proteases at formulation level justifying field application.

NOMENCLATURE

AprA	Alkaline protease A
Bt	*Bacillus thuringiensis*
Bta	Bt var. *aizawai*
Bti	Bt var. *israelensis*
Bts	Bt var. *sphaericus*
Btt	Bt var. *tenebrionis*
DBM	Diamond back moth
Tx	Entomotoxicity
EDTA	Ethylene diamine tetraacetate
InhA	Immune inhibitor A
ICP	Insecticidal crystalline protein
IspA	Intracellular serine protease A
NprA	Neutral protease A
NH<30	Non-hydrolyzed sludge with less than 30 g/L suspended solids
NH-15	Non-hydrolyzed sludge at 15 g/L suspended solids
NH-20	Non-hydrolyzed sludge at 20 g/L suspended solids
NH-25	Non-hydrolyzed sludge at 25 g/L suspended solids
NH-30	Non-hydrolyzed sludge at 30 g/L suspended solids
NHT-30	Tween amended non-hydrolyzed sludge at 30 g/L suspended solids
PMSF	Phenylmethanesulfonyl fluoride
PA	Protease activity
SIW	Starch industry wastewater
TC	Total cell count
TH	Thermal hydrolysed sludge
Vips	Vegetative insecticidal proteins

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STR 202047); Canada Research Chair; University of Missouri, Columbia and U.S. EPA. The views and opinions expressed in this article are those of authors and should not be construed as opinions of the U.S. Environmental Protection Agency. Our sincere thanks to Mr. Khanh Dang Vu for giving valuable suggestions in this manuscript. The authors are also thankful to Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Services and

Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing Ph.D scholarship to Satinder K. Brar during the course of this research work.

References

[1] Hofte H, Whiteley HR. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 1989;53:242–255.

[2] Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean, DH. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 1998;62:775–806.

[3] Kumar NS, Venkateswerlu G. Endogenous protease activated 66-kDa toxin from *Bacillus thuringiensis* subsp. *kurstaki* active against *Spodoptera littoralis*. FEMS Microbiol Lett 1998;159:113-120.

[4] Reddy YC, Venkateswerlu G. Intracellular proteases of *Bacillus thuringiensis* subsp. *kurstaki* and a protease-deficient mutant Btk-q. Current Microbiol 2002;45:405-409.

[5] Outtrup H, Boyce COL. Microbial proteinases and biotechnology. In: Fogarty CT, Kelly K. (eds) Microbial enzymes and biotechnology. Elsevier, London; 1990; pp 227–254.

[6] Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 1998;62:597–635.

[7] Gupta R, Beg QK, Khan S, Chauhan B. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 2002;60:381–395.

[8] Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 2002;59:15–32.

[9] Kunitate A, Okamoto M, Ohmori I. Purification and characterization of a thermostable serine protease from *Bacillus thuringiensis*. Agric Biol Chem 1989;53:3251-3256.

[10] Dai SM, Gill SS. In vitro and in vivo proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases. Insect Biochem Molec Biol 1993;23:273-283.

[11] Donovan WP, Tan Y, Slaney AC. Cloning of the *nprA* gene for neutral protease A of *Bacillus thuringiensis* and effect of *in vivo* deletion of *nprA* on insecticidal crystal protein. Appl Environ Microbiol 1997;63:2311-2317.

[12] Kumar NS, Venkateswerlu G. Involvement of an endogenous metalloprotease in the activation of protoxin in *Bacillus thuringiensis* subsp. *kurstaki*. Biochem Molec Biol Intl 1997;42:901-908.

[13] Kumar NS, Venkateswerlu G. Intracellular proteases in sporulated *Bacillus thuringiensis* subsp. *kurstaki* and their role in protoxin activation. FEMS Microbiol Lett 1998b;166: 377-382.

[14] Hartley BS. Proteolytic enzymes. Annual Rev Biochem 1960;29:45–72.

[15] Andrews Jr. RE, Bibilos MM, Bulla Jr. LA. Protease activation of the entomocidal protoxin of *Bacillus thuringiensis* subsp. *kurstaki*. Appl Environ Microbiol 1985:737-742.

[16] Carroll J, Li J, Ellar DJ Proteolytic processing of a coleopteran- specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. Biochem J 1989;261:99–105.

[17] Hotha S, Banik RM. Production of alkaline protease by *Bacillus thuringiensis* H14 in aqueous two-phase systems. J Chem Tech Biotechnol 1997;69:5-10.

[18] Rukimini V, Reddy CY, Venkateswerlu G. *Bacillus thuringiensis* crystal delta-endotoxin : role of proteases in the conversion of protoxin to toxin. Biochimie 2000;82: 109-116.

[19] Donovan WP, Donovan JC, Engleman JT. Gene knockout demonstrates that vip3A contributes to the pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera exigua*. J Inv Pathol 2001;78:45-51.

[20] Beg QK, Saxena RK, Gupta R. De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. Process Biochem 2002 ;37:1103–1109.

[21] Varela H, Ferrari MD, Belobradjic L, Weyrauch R, Loperena ML Effect of medium composition on the production by a new *Bacillus subtilis* isolate of protease with promising unhairing activity. World J Microbiol Biotechnol 1996;12:643–645.

[22] Hameed A, Keshavarz T, Evans CS. Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. J Chem Technol Biotechnol 1999;74:5–8.

[23] Puri S, Beg QK, Gupta R. Optimization of alkaline protease production from *Bacillus* sp. using response surface methodology. Curr Microbiol 2002;44:286–290.

[24] Landau NS, Egorov NS, Gornova IB, Krasovskaya SB, Virnik AD. Immobilization of *Bacillus firmus* cells in cellulose triacetate fibers and films and their use for proteinase biosynthesis. Microbiol (USSR) 1995;64:530–536.

[25] Landau NS, Gornova IB, Gulikova OM, Egorov NS. Proteinase production by free and immobilized cells of *Bacillus firmus* 44b. Microbiol (USSR) 1997;66:310–315.

[26] Lee YH, Chang HN. Production of alkaline protease by *Bacillus licheniformis* in an aqueous two-phase system. J Ferment Bioeng 1990;69:89–92.

[27] Chakraborty R, Srinivasan M. Production of a thermostable alkaline protease by a new *Pseudomonas* sp. by solid substrate fermentation. J Microb Biotechnol 1993;8:7–16.

[28] George S, Raju V, Krishnan MRV, Subramanian TV, Jayaraman K. Production of protease by *Bacillus amyloliquefaciens* in solid-state fermentation and its application in the unhairing of hides and skins. Process Biochem 1995;30:457–462.

[29] Kaur S, Vohra RM, Kapoor M, Beg QK, Hoondal GS Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus* sp. P-2. World J Microbiol Biotechnol 2001;17:125–129.

[30] Balaban NP, Gabdrakhmanova LA, Sharipova MR, Mardanova AM, Sokolova EA, Rudenskaya GN, Leshchinskaya IB. Proteinases from *Bacillus intermedius* secreted in the late stages of sporulation. Medical Sci Monitor 2002;8(5):168-171.

[31] Balaban NP, Sharipova MR, Gabdrakhmanova LA, Mardanova AM, Tokmakova YS, Sokolova EA, Rudenskaya GN, Leshchinskaya IB. Synthesis and Secretion of Proteinases by *Bacillus intermedius* in the Late Stages of Sporulation. Microbiol 2003;72: 295–299.

[32] Salamitou S, Marchal M, Lereclus D. *Bacillus thuringiensis* : un pathogène facultatif. Annales de l'institut pasteur/actualité 1996;7:285-296.

[33] Tan Y, Donovan WP. Deletion of *aprA* and *nprA* genes for alkaline protease A and neutral protease A from *Bacillus thuringiensis* : effect on insecticidal crystal proteins. J Biotechnol 2000;84:67-72.

[34] Aronson A. Sporulation and δ -endotoxin synthesis by *Bacillus thuringiensis*. Cell Mol Life Sci 2002;59:417–425.

[35] Zouari N, Jaoua S. Production and characterization of metalloproteases synthesized concomitantly with δ-endotoxin by *Bacillus thuringiensis* subsp. *kurstaki* strain grown on gruel-based media. Enzyme Microbial Technol 1999;25:364–371.

[36] Kole MM, Draper I, Gerson DF. Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. J Chem Technol Biotechnol 1988;41:197–206.

[37] Kunitz, M. Crystalline soybean trypsin inhibitor. J Gen Physiol 1947;30: 291-310.

[38] Zouari N, Ali SBS, Jaoua S. Production of delta-endotoxins by *Bacillus thuringiensis* strains exhibiting various insecticidal activities towards Lepidoptera and diptera in gruel and fish meal media. Enzyme Microbial Technol 2002;31: 411–418.

[39] Kunst, F, Pascal, M, Lepesant-Kejzlarova, J, Lepesant, J-A, Billault, A, Dedonder, R. Pleiotropic mutations affecting sporulation conditions and the synthesis of extracellular enzymes in *Bacillus subtilis* 168. Biochimie 1974;56:1481-1489.

[40] Msadek, T, Kunst, F, Henner, D, Klier, A, Rapoport,G, Dedonder, R. Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in degS and degU. J Bacteriol, 1990: 824-834.

[41] Ghribi, D, Zouari, N, Trabelsi, H, Jaoua, S. Improvement of *Bacillus thuringiensis* delta-endotoxin production by overcome of carbon catabolite repression through adequate control of aeration. Enzyme and Microbial Technology 2006; doi:10.1016/j.enzmictec.2006.05.015.

[42] Mohammedi S, Subramanian B, Song Y, Tyagi RD, Valéro JR. Molecular screening of *Bacillus thuringiensis* strains from wastewater sludge for biopesticide production. Process Biochem 2006;41(4):829-835.

[43] Tyagi RD, Sikati Foko V, Barnabé S, Vidyarthi A, Valéro JR. Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using wastewater as a raw material. Water Sci Technol 2002;46:47-254.

[44] Yezza A, Tyagi RD, Valero JR, Surampalli, RY. Wastewater sludge pre-treatment for enhancing entomotoxicity produced by *Bacillus thuringiensis* var. *kurstaki*. World J Microbiol Biotechnol 2005;21:1165–1174.

[45] Yezza A, Tyagi RD, Valero JR, Surampalli, RY. Bioconversion of industrial wastewater and wastewater sludge into *Bacillus thuringiensis* based biopesticides in pilot fermenter. Biores Technol 2006;26:1850-1857.
[46] Tirado-Montiel ML, Tyagi RD, Valero JR. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. Water Res 2001;35(16):3807–3816.
[47] Gessesse, A, Hatti-Kaul, R, Gashe, BA, Mattiasson, B. Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enzyme and Microbial Technol 2003;32: 519–524.
[48] Bibilos M, Andrews RE Jr. Inhibition of *Bacillus thuringiensis* proteases and their effects on crystal toxin proteins and cell free translations. Can J Microbiol 1988;34:740–742.
[49] Li, E, Yousten, AA. Metalloprotease from *Bacillus thuringiensis*. Appl Microbiol 1975: 354-361.
[50] Koide Y, Nakamura A, Uozumi T, Beppu T. Cloning and sequencing of the intracellular serine protease gene of *Bacillus subtilis*. J Bacteriol 1986;67:110–116.
[51] Burnett TJ, Shankweiler GW, Hageman JH. Activation of intracellular serine proteinase in *Bacillus subtilis* cells during sporulation, J Bacteriol 1986;165:139–145.
[52] Sheehan SM, Switzer RL. Intracellular serine protease of *Bacillus subtilis* is formed in vivo as an unprocessed, active protease in stationary cells. J Bacteriol 1990;172:473–476.
[53] Sastry KJ, Srivastava OP, Millet J, FitzJames PC, Aronson AI. Characterization of *Bacillus subtilis* mutants with a temperature-sensitive intracellular protease. J Bacteriol 1983;153:511–519.
[54] Sharipova MR, Balaban NP, Gabdrakhmanova LA, Shilova MA, Kadyrova YM, Rudenskaya GN, and Leshchinskaya IB. Hydrolytic Enzymes and Sporulation in *Bacillus intermedius*. Microbiol 2002;71:420–424.
[55] Bulla LA, Bechtel DB, Kramer YI, Shethna YI, Aronson AI, Fitz-James PC. Ultra structure, physiology and biochemistry of *Bacillus thuringiensis*. CRC Critical Rev Microbiol 1980;8:174-204.
[56] Phillips ZE, Strauch MA. *Bacillus subtilis* sporulation and stationary phase gene expression. Cell Mol Life Sci 2002;59:392–402.
[57] Runder DZ, Losick R. A sporulation membrane protein tethers the pro-sigmaK processing enzyme to its inhibitor and dictates its subcellular localization. Genes Dev 2002;16: 1007–1018.
[58] Dong TC, Cutting SM. SpoIVB-mediated cleavage of Spo-IVFA could provide the intracellular signal to activate processing of pro-sigmaK in *Bacillus subtilis*. Mol Microbiol 2003;49:1425–1434.
[59] Chen, Fu-Chu, Shen, Li-Fen, Tsai, Ming-Chu, Chak, Kin-Fu. The IspA protease's involvement in the regulation of the sporulation process of *Bacillus thuringiensis* is revealed by proteomic analysis. Biochem Biophy Res Comm 2003;312:708–715.
[60] Dalhammar G, Steiner H. Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antibacterial proteins in insects. Eur J Biochem 1984;139(2):247-252.
[61] Grandvalet C, Gominet M, Lereclus, D. Identification of genes involved in the activation of the *Bacillus thuringiensis inhA* metalloprotease gene at the onset of sporulation. Microbiol 2001;147:1805–1813.
[62] Yezza A, Tyagi RD, Valero JR, Surampalli, RY. Correlation between entomotoxicity potency and protease activity produced by *Bacillus thuringiensis var. kurstaki* grown in wastewater sludge. Process Biochem 2006;41:794-780.
[63] Yezza A, Tyagi RD, Valero JR, Surampalli, RY. Production of *Bacillus thuringiensis* based biopesticides in batch and fed cultures using wastewater sludge as a raw material. J Ind Microbiol Biotechnol 2004;31:545-552.
[64] Lisansky SG, Quinlan RJ, Tassoni G. The *Bacillus thuringiensis* Production Handbook, CPL Press, Newbury;1993; pp.124.
[65] Vora D, Shethna YI. Enhanced growth, sporulation and toxin production by *Bacillus thuringiensis kurstaki* in oil seed meal extract media containing cystine. World J Microbiol Biotechnol 1999;15:747-749.
[66] Barnabé S. 2005. Hydrolyse et oxydation partielle des boues d'épuration comme substrat pour produire *Bacillus thuringiensis* HD-1. Ph.D thesis, Institut National de la Recherche Scientifique, Eau, Terre et Environnement, Université du Québec, Québec, Canada;2005; 255 p.
[67] Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. Sludge based *Bacillus thuringiensis* biopesticides: Viscosity Impacts. Water Res 2005;39:3001-3011.
[68] Morris ON, Converse V, Kanagaratnam P, Davies JS. Effect of cultural conditions on spore–crystal yield and toxicity of *Bacillus thuringiensis* subsp. *aizawai* (HD133). J Invertebr Pathol 1996;67:129–136.
[69] Rowe GE, Margaritis A, Wei N. Specific oxygen uptake rate variations during batch fermentation of *Bacillus thuringiensis* subspecies *kurstaki* HD-1. Biotechnol Prog 2003;19:1439-1443.

[70] Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges. Proc Biochem 2005;40: 2695–2705.

[71] Aronson A. I. The protoxin composition of *Bacillus thuringiensis* insecticidal inclusions affects solubility and toxicity. Appl Environ Microbiol 1995;61: 4057–4060.

[72] Masson L, Erlandson M, Puzstai-Carey M, Brousseau R, Juarez-Perez V, Frutos R. A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl Environ Microbiol 1998;64: 4782– 4788.

[73] Crickmore N. Using worms to better understand how *Bacillus thuringiensis* kills insects. TRENDS in Microbiol 2005;13(8):347-350.

[74] Sarrafzadeh, MH, Navarro, JM. The effect of oxygen on the sporulation, δ-endotoxin synthesis and toxicity of *Bacillus thuringiensis* H14. World J Microbiol Biotechnol 2006;22:305–310.

[75] Avignone-Rossa, C, Arcas, J, Mignone, C. *Bacillus thuringiensis* growth, sporulation and delta-endotoxin production in oxygen limited and non-limited cultures. World J Microbiol Biotechnol 1992;8: 301–304.

[76] Bravo A, Sánchez J, Kouskoura T, Crickmore N. N-terminal activation is an essential early step in the mechanism of action of the *Bacillus thuringiensis* Cry1Ac insecticidal toxin. J Biol Chem 2002;277(27):23985-23987.

[77] Porcar M, Juarez-Pérez V. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol Rev 2003;26:419-432.

[78] Whalon ME, Wingerd BA. Bt: mode of action and use. Arch. Insect Biochem Physiol 2003;54(4):200-211.

[79] Wirth MC, Georghiou GP, Federici BA. CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus.* Proc National Acad Sci United States of America 1997;94:10536–10540.

[80] Federici BA, Bauer LS. Cyt1Aa protein of *Bacillus thuringiensis* is toxic to the cottonwood leaf beetle, *Chrysomela scripta,* and suppresses high levels of resistance to Cry3Aa. Appl Environ Microbiol 1998;64:4368–4371.

[81] Johnson DE, Oppert B, McGaughey WH. Spore coat protein synergizes *Bacillus thuringiensis* crystal toxicity for the Indianmeal moth (*Plodia interpunctella*). Curr Microbiol 1998;36:278–282.

[82] Venkateswerlu G, Stotzky G. Simple method for the isolation of the antilepidopteran toxin from *Bacillus thuringiensis* subsp. *kurstaki*. Biotechnol Appl Biochem 1990;12: 245–251.

[83] Chestukhina GG, Zalunin IA, Kostina LI, Kotova TS, Kattrukha SP, Stepanov VM. Crystal-forming proteins of *Bacillus thuringiensis*. The limited hydrolysis by endogeneous proteinases as a cause of their apparent multiplicity. Biochem J 1980; 187(2):457-465.

[84] Bulla LA Jr, Kramer KJ, Cox DJ, Jones BL, Davidson LI, Lookhart GL. Purification and characterization of the entomocidal protoxin of *Bacillus thuringiensis*. J Biol Chem 1981; 256:3000-3004.

[85] Haider MZ, Knowles BH, Ellar DJ. Specificity of *Bacillus thuringiensis* var. colmeri insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur J Biochem 1986;156:531-540.

[86] Lovgren A, Zhang M, Engstrom A, Dalhammar G, Lamden R. Molecular characterization of immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis*. Mol Microbiol 1990;4:2137-2146.

[87] Almond BD, Dean DH. Intracellular proteolysis and limited diversity of the *Bacillus thuringiensis* CryIA family of the insecticidal crystal proteins. Biochem Biophy Res Comms 1994;201(2):788-794.

[88] Asano S, Ogiwara K, Indrasith LS, Takahashi M, Suzuki N, Hori H. Synergism of the spore on insecticidal activity of delta-endotoxin of *Bacillus thuringiensis* against the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae) is not observed at late stage in bioassay. Appl Entomol Zool 2000;35:583–590.

[89] Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY, Banerji, S. development of sludge based stable aqueous *Bacillus thuringiensis* formulations.Water Sci Technol 2004;50(9):229-236.

[90] Brar SK, Verma M, Tyagi RD, Valéro JR, Surampalli RY. Starch industry wastewater based stable *Bacillus thuringiensis* liquid formulations. J Econ Entomol 2005;98(6): 1890-1898.

[91] Li J, Carroll J, Ellar DJ. Crystal structures of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature 1991;353:815-821.

[92] Grochulski P, Masson L, Borisova S, Pusztai-Carey M, Schwartz JL, Brousseau R, Cygler M. *Bacillus thuringiensis* CryIA (a) insecticidal toxin: crystal structure and channel formation. J Mol Biol 1995;254:447-464.

[93] Felix F, Brouillet N. Purification and properties of two peptidases from brewer's yeast. Biochim Biophys Acta 1966;122:127–144.
[94] de Marco AC, Dick AJ. Aminopeptidase I activities in several microorganisms. Can J Biochem 1978;56:66–71.
[95] Lindberg RA, Eirich LD, Price JS, Wolfinbarger Jr. L, Drucker H. Alkaline protease from *Neurospora crassa*. J Biol Chem 1981;256:811–814.
[96] Hibbs MS, Hasty KA, Seyer JM, Kang AH, Mainardi CL. Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. J Biol Chem 1985;260:2493–2500.
[97] Barett AJ. Proteolytic enzymes: serine and cysteine peptidases. Methods Enzymol 1994;244:1–15.
[98] Barett AJ. Proteolytic enzymes: aspartic and metallopeptidases. Methods Enzymol 1995; 248:183.
[99] Reddy ST, Venkateswerlu G. Comparative analysis of intracellular proteases in sporulated *Bacillus thuringiensis* strains. Biotechnol Letts 1998;20(3):279-281.
[100] Zhang JH, Wang CZ, Qin JD. The interactions between soybean trypsin inhibitor and δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera* larva. J Invertebr Pathol 2000;75:259–266.
[101] Reddy ST, Kumar NS, Venkateswerlu G. Identification and purification of the 69-kDa intracellular protease involved in the proteolytic processing of the crystal N-endotoxin of *Bacillus thuringiensis* subsp. *tenebrionis*. FEMS Microbiol Letts 2000;183:63-66.
[102] Christeller JT, Laing WA, Markwick NP, Burgess EPJ. Midgut protease activities in 12 phytophagous lepidopteran larvae: dietary and protease inhibitor interactions. Insect Biochem Molec Biol 1992;22:735–746.
[103] Ogiwara K, Indrasith LS, Asano S, Hori H. Processing of δ-endotoxin from *Bacillus thuringiensis* subsp. *Kurstaki* HD-1 and HD-73 by gut juices of various insect larvae. J Invertebr Pathol 1992;60:121–126.
[104] Gringorten JL, Milne RE, Fast PG, Sohi SS, van Frankenhuyzen K. Suppression of *Bacillus thuringiensis* δ-endotoxin activity by low alkaline pH. J Invertebr Pathol 1992;60:47–52.
[105] Oppert B, Kramer KJ, Johnson D, Upton SJ, McGaughey WH. Luminal proteases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryIA(c) protoxin. Insect Biochem Molec Biol 1996;26:571–583.
[106] Oppert B, Kramer KJ, Beeman RW, Johnson D, McGaughey WH. Protease-mediated insect resistance to *Bacillus thuringiensis* toxins. J Biol Chem 1997;272:23473–23476.
[107] Pang ASD, Gringorten JL. Degradation of *Bacillus thuringiensis* δ-endotoxin in host insect gut juice. FEMS Microbiol Letts 1998;167:281-285.
[108] Shao Z, Cui Y, Liu X, Yi H, Ji J, Yu Z. Processing of δ-Endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors. J Invertebr Pathol 1998;72:73–81.
[109] Segura C, Guzman F, Patarroyo ME, Orduz S. Activation Pattern and Toxicity of the Cry11Bb1 toxin of *Bacillus thuringiensis* Subsp. *Medellin*. J Invertebr Pathol 2000;76: 56–62.
[110] Gomez I, Sanchez J, Miranda R, Bravo A, Soberon M. Cadherin-like receptor binding facilitates proteolytic cleavage of helix K-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. FEBS Letters 2002;513:242-246.
[111] Schernthaner JP, Milne RE, Kaplan H. Characterization of a novel insect digestive DNase with a highly alkaline pH optimum. Insect Biochem Mol Biol 2002;32:255–263.
[112] Bhattacharya M, Plantz BA, Swanson-Kobler JD, Nickerson KW. Non-enzymatic glycosylation of Lepidopteran-active *Bacillus thuringiensis* protein crystals. Appl Environ Microbiol 1993;59(8):2666-2672.
[113] Keller M, Sneh B, Strizhov N, Prudovsky E, Regev A, Koncz C, Schell J, Zilberstein A, Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC. Insect Biochem Molec Biol 1996;26:365–373.
[114] Oppert B. Protease interactions with *Bacillus thuringiensis* insecticidal toxins. Arch Insect Biochem Physiol 1999;42:1–12.
[115] Reeck G, Oppert B, Denton M, Kanost M, Baker J, Kramer K. Insect proteinases. In: Turk, V. (Ed.), Proteases. Birkhauser, Basel;1999.
[116] Huang F, Zhu KY, Buschman LL, Higgins RA, Oppert B. Comparison of midgut proteases in *Bacillus thuringiensis* susceptible and resistant European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). Pestic Biochem Physiol 1999;65:132–139.
[117] Sekar V, Held B, Tippett J, Amirhusin B, Robeff P, Wang K, Wilson HM. Biochemical and molecular characterization of the insecticidal fragment of CryV. Appl Environ Microbiol 1997;63(7):2798-2801.

[118] Sayyed AH, Haward R, Herrero S, Ferre J, Wright DJ. Genetic and biochemical approach for characterization of resistance to *Bacillus thuringiensis* toxin Cryl Ac in a field population of the diamondback moth, *Plutella xylostella*. Appl Environ Microbiol 2000;66:1509–1516.

[119] Barillas-Mury C, Wizel B, Han YS. Mosquito immune responses and malaria transmission: lessons from insect model systems and implications for vertebrate innate immunity and vaccine development. Insect Biochem Mol Biol 2000;30:429–442.

[120] Jiang H, Kanost MR. The clip-domain family of serine proteinases in arthropods. Insect Biochem Mol Biol 2000;30:95–105.

[121] Han YS, Thompson J, Kafatos FC, Barillas-Mury C. Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. EMBO J. 2000;19:6030–6040.

[122] de Maagd RA, Bravo A., Crickmore N. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genetics 2001;17:193–199.

[123] Herrero S, Oppert B, Ferre J. Different mechanisms of resistance to *Bacillus thuringiensis* toxins in the indianmeal moth. Appl Environ Microbiol 2001;67:1085–1089.

[124] Loseva O, Ibrahim M, Candas M, Noah Koller C, Bauer LS, Bulla Jr LA. Changes in protease activity and Cry3Aa toxin binding in the Colorado potato beetle: implications for insect resistance to *Bacillus thuringiensis* toxins. Insect Biochem Mol Biol 2002;32: 567–577.

[125] Ferre J, Van Rie J. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol 2002;47:501–533.

[126] Jurat-Fuentes JL, Gould FL, Adang MJ. Altered glycosylation of 63- and 68-kilodalton microvillar proteins in *Heliothis virescens* correlates with reduced Cryl toxin binding, decreased pore formation, and increased resistance to *Bacillus thuringiensis* Cryl toxins. Appl Environ Microbiol 2002;68:5711–5717.

[127] Mohan M, Gujar GT. Characterization and comparison of midgut proteases of *Bacillus thuringiensis* susceptible and resistant diamondback moth (Plutellidae: Lepidoptera). J Invertebr Pathol 2003;82:1–11.

[128] Rausell C, Garcia-Robles I, Sanchez J, Munoz-Garaya C, Martinez-Ramirez AC, Real MD, Bravo A. Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of *Leptinotarsa decemlineata* (Say). Biochimica et Biophysica Acta 2004;1660:99–105.

[129] Bah A, van Frankenhuyzen K, Brousseau R, Masson L. The *Bacillus thuringiensis* Cryl Aa toxin: effects of trypsin and chymotrypsin site mutations on toxicity and stability. J Invertebr Pathol 2004;85:120–127.

[130] Griffitts JS, Aroian RV. Many roads to resistance: how invertebrates adapt to Bt toxins. BioEssays 2005;27:614–624.

[131] Harnstadt C, Soares GC,Wilcox ER, Edwards DL. New strain of *Bacillus thuringiensis* with activity against coleopteran insects, Bio/Technol 1986;4:305–308.

[132] Lamontagne E. Caractérisation de nouvelles souches de *Bacillus thuringiensis* d'intérêt pour la production de biopesticides et d'enzymes par fermentation de boues d'épuration municipales. Master's thesis, Institut Nationale de la Recherche Scientifique-Eau, Terre et Environnement, Université du Québec, Canada ;2004.112 p.

[133] Paointara M, Angusthasombat C, Panyim S. The mosquito larvicidal activity of 130 kDa delta-endotoxin of *Bacillus thuringiensis var israelensis* residues in the 72 kDa amino terminal fragment. Biochem Biophys Res Commun 1988;153:294–300.

[134] Ward SE, Ellar DJ. Cloning and expression of two homologous genes of *Bacillus thuringiensis* subsp. *israelensis* which encode 130-kilodalton mosquitocidal proteins. J Bacteriol 1988;70:727–735.

[135] Angsuthanasombat C, Crickmore N, Ellar DJ. Comparison of *Bacillus thuringiensis* subsp. *israelensis* Cry IVA and Cry IVB cloned toxins reveals synergism in vivo, FEMS Microbiol Lett 1988;94:63–68.

Table 1 Classification of Proteases

Protease category	**Type**	**Sub-types**	**Microbial occurrence**	**Conditions for optimal activity**
Exopeptidases (site of action)				
Aminopeptidases	Majority intracellular	-	Bacteria and fungi	-
Carboxypeptidases	Extracellular and intracellular	serine carboxypeptidases, metallocarboxy peptidases, and cysteine carboxypeptidases	Bacteria and fungi	Variable
Endopeptidases (catalytic mechanism)				
Serine proteases	Extracellular and intracellular	Serine alkaline type – largest subgroup	Viruses, bacteria, and eukaryotes	Neutral and alkaline pH, with an optimum between pH 7 and 11. Broad substrate specificities including esterolytic and amidase activity.
Aspartic proteases	Extracellular and intracellular	Pepsin and rennin – like enzymes Acidic enzymes	Viruses, bacteria, and fungi	pH 3 to 4 and isoelectric points in the range of pH 3 to 4.5. Molecular masses - 30 to 45 kDa.
Cysteine/thiol proteases	Extracellular and intracellular	Papain-like; trypsin-like with preference for cleavage at the arginine residue; specific to glutamic acid; and others	Prokaryotes and eukaryotes	Normally, neutral pH But also, pH 4.9- 8.4
Metalloproteases	Extracellular and intracellular	Neutral; alkaline; *Myxobacter* I; and *Myxobacter* II	Prokaryotes and eukaryotes	pH 4.0- 10.0

References: [93-98]

Table 2 Different Bt production media for protease

Production medium	**Fermentation conditions**	**Bt strain**	**Protease concentration**	**Protease type**	**Specific comments**	**Reference**
Glucose yeast soya medium	pH : 7 Temperature : 30°C Incubation Time: 48h Batch fermentation	Bt subsp. *kurstaki*	0.017-0.22 absorbance units	Neutral	Optimal pH: 7; PA- absorbance units of trichloroacetic acid-soluble material, determined at 440 nm	[15]
Tryptose medium	pH : 7.2 Temperature : 30°C Incubation Time:56h Batch fermentation	Bt subsp. *kurstaki*; subsp. *tenebrionis*; subsp. *israelensis*	19-23 units/mg protein	-	One unit of protease is equivalent to the amount of the enzyme that produced an increase of 1.0 in A440 in 60 min under the experimental conditions	[99]
Gruel supplemented with nutrients	pH : 7 Temperature : 30°C Incubation Time: 72h Batch fermentation	Bt subsp. *kurstaki* BNS3	1280 IU/ml	Neutral metalloproteinases	Optimal pH: 7–10 Optimal temperature:30–70°C; One enzyme unit was defined as the amount of enzyme required to liberate 1 mg of tyrosine/min under the experimental conditions	[35]

Production medium	**Fermentation conditions**	**Bt strain**	**Protease concentration**	**Protease type**	**Specific comments**	**Reference**
Peptone beef medium	pH : 7 Temperature : 30°C Incubation Time: 24h Batch fermentation	Bt subsp. *kurstaki* HD-1	0.8-1.2 azocasein units/min/mg protein	Alkaline proteases	Optimal pH: 7–10; defined in the same way as Reddy et al. [99]	[100]
Nutrient growth medium	pH : 7 Temperature : 30°C Incubation Time: 24h Batch fermentation	Bt subsp. *tenebrionis*	11.76 U/mg protein	Metalloprotease	defined in the same way as Reddy et al. [99]	[101]
Gruel and fish meal	pH : 7 Temperature : 30°C Incubation Time: 72h Batch and Fed-batch fermentation	Bt subsp. *kurstaki*	695-2018 U/ml	Neutral metalloproteinases	U was defined as the amount of enzyme preparation required to liberate 1 µg of tyrosine from casein per min, under the experimental conditions	[38]
Wastewater sludge or biosolids	pH : 7 Temperature : 30°C Incubation Time: 48h Batch and Fed-batch fermentation	Bt subsp. *kurstaki* HD-1	1.2 IU/ml	Neutral and alkaline proteases	Optimal pH: 7–11 Optimal temperature: 30–50°C IU was defined as the amount of enzyme preparation required to liberate 1 µg of tyrosine from casein per min, under the experimental conditions	[43]

Table 3 Specific role of larval proteases and causes of resistance

Specific role of larval proteases	**References**
Larval proteases:- serine proteases, chymotrypsin, elastase, carboxypeptidase A and B, DNase and leucine amino peptidase	[10,83,85,102-111]
a) Primary cleavage of the protoxin to active toxin.	
b) Proteolytic cleavage of protoxin before receptor binding by active toxin promotes pre-pore oligomer formation as a pre-requisite for membrane insertion.	
c) Probable role in subsequent inactivation through excessive hydrolysis of protoxin.	
d) Innate responses in insect midgut by modulating signaling and amplification cascades that lead to the activation of specific defense mechanisms, such as pathogen recognition, melanization, coagulation and induction of antimicrobial peptides.	
Causes of resistance	
a) Altered binding of Cry toxins to receptors in the midgut.	[76,78,79,100,109,112-131]
b) Alterations in the proteolytic processing of the Cry toxin.	
c) Rapid regeneration of the damaged midgut epithelium which prevents septicemia.	
d) Reduced protoxin activation and increased toxin degradation.	
e) Glycosylation of the lysine amino groups during protoxin activation in larval midgut could alter the proteolytic cleavage pattern of these toxins.	
f) Changes in the profile of midgut proteases to situate the tissue to respond to toxin stress.	
g) Adaptive responses that can be genetically transmitted for survival advantage.	

List of Figures

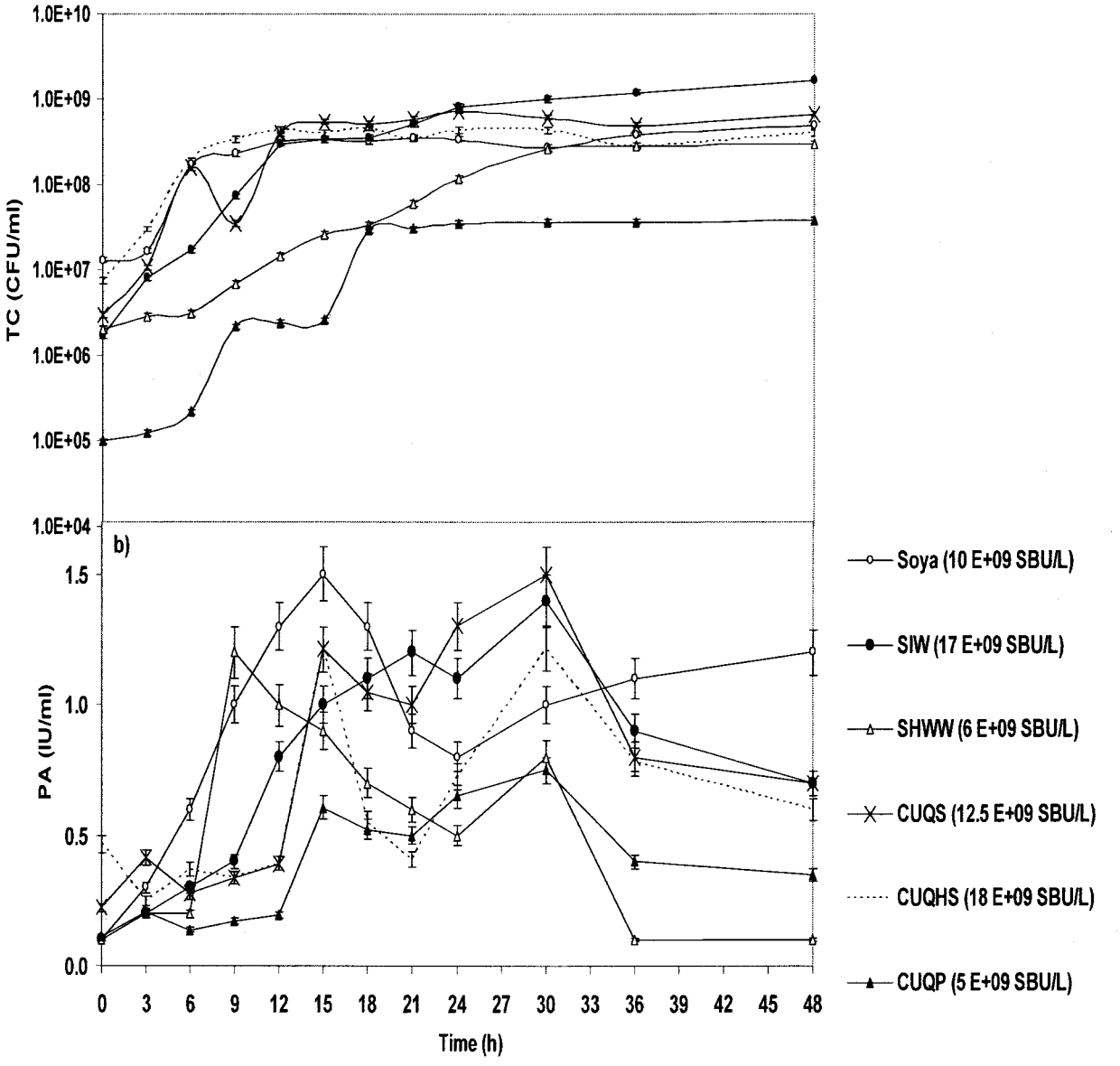


Fig. 1. a) Total cells (TC), and b) PA profile in different Bt fermented raw materials; SIW: starch industry wastewater; SHWW: slaughterhouse wastewater; CUQS: Communauté Urbaine de Québec sludge (secondary sludge from biofiltration process); CUQHS: CUQ hydrolyzed secondary sludge; and CUQP: CUQ primary sludge (sludge from physical-chemical treatment). BLS: Black Lake sludge (sequencing batch reactor sludge); JQS: Jonquière sludge (sludge from activated sludge process); CUQHOS: CUQ hydrolyzed oxidized secondary sludge; and, CUQM: CUQ mixed sludge (sludge from thickening tank) showed similar profiles and have not been presented due to clarity of data. Data in parentheses refers to maximum Tx obtained at 48 h. [132; Unpublished data].

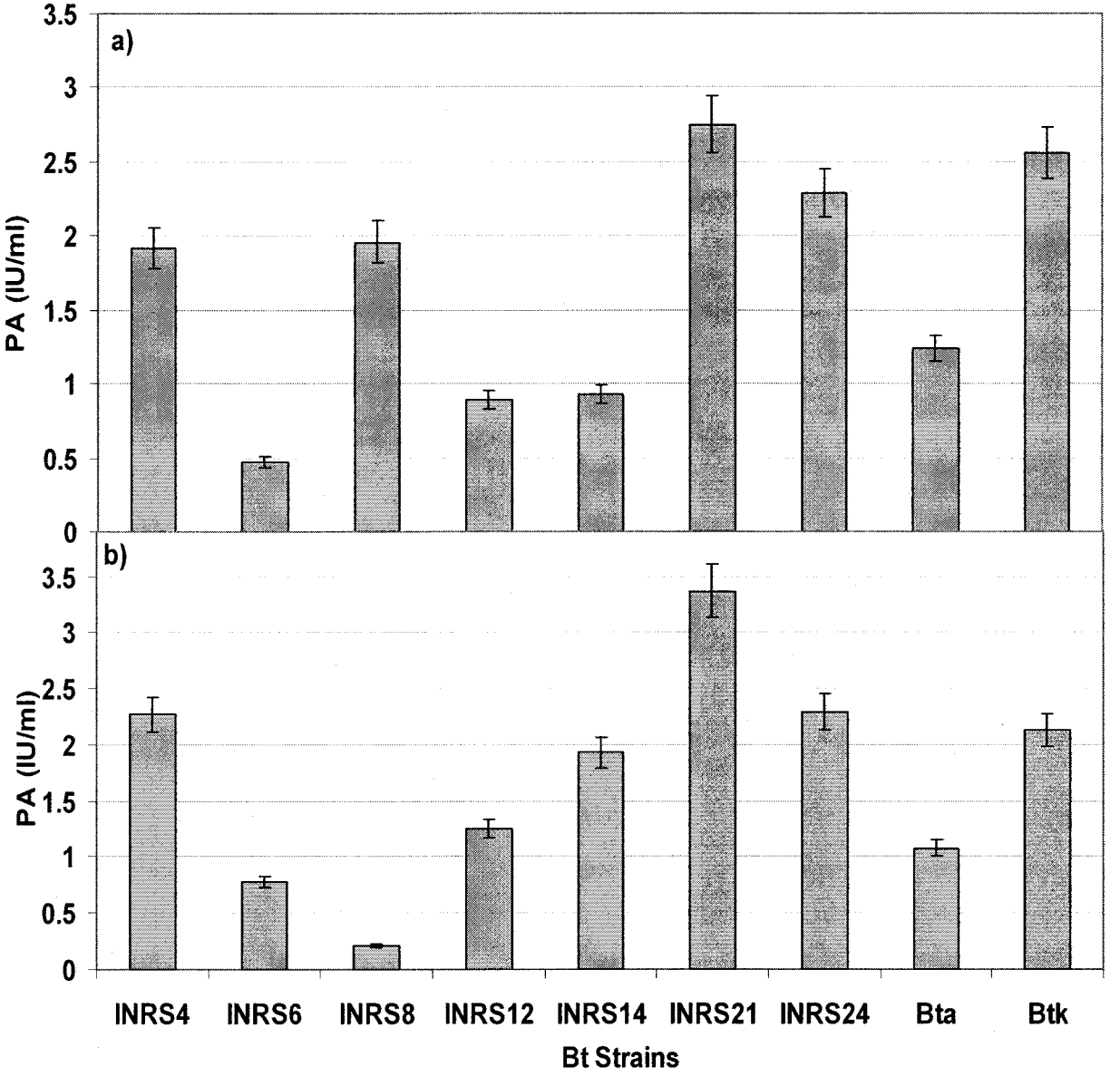


Fig. 2. Maximal protease activity during Bt fermentation (15 L fermenter) at 48 h for: a) soyameal (semi-synthetic commercial medium) with temperature optimum of 40-50°C and pH 6-11; and b) wastewater sludge temperature optimum of 50°C and pH 6-11. Data were computed from Lamontagne [132].

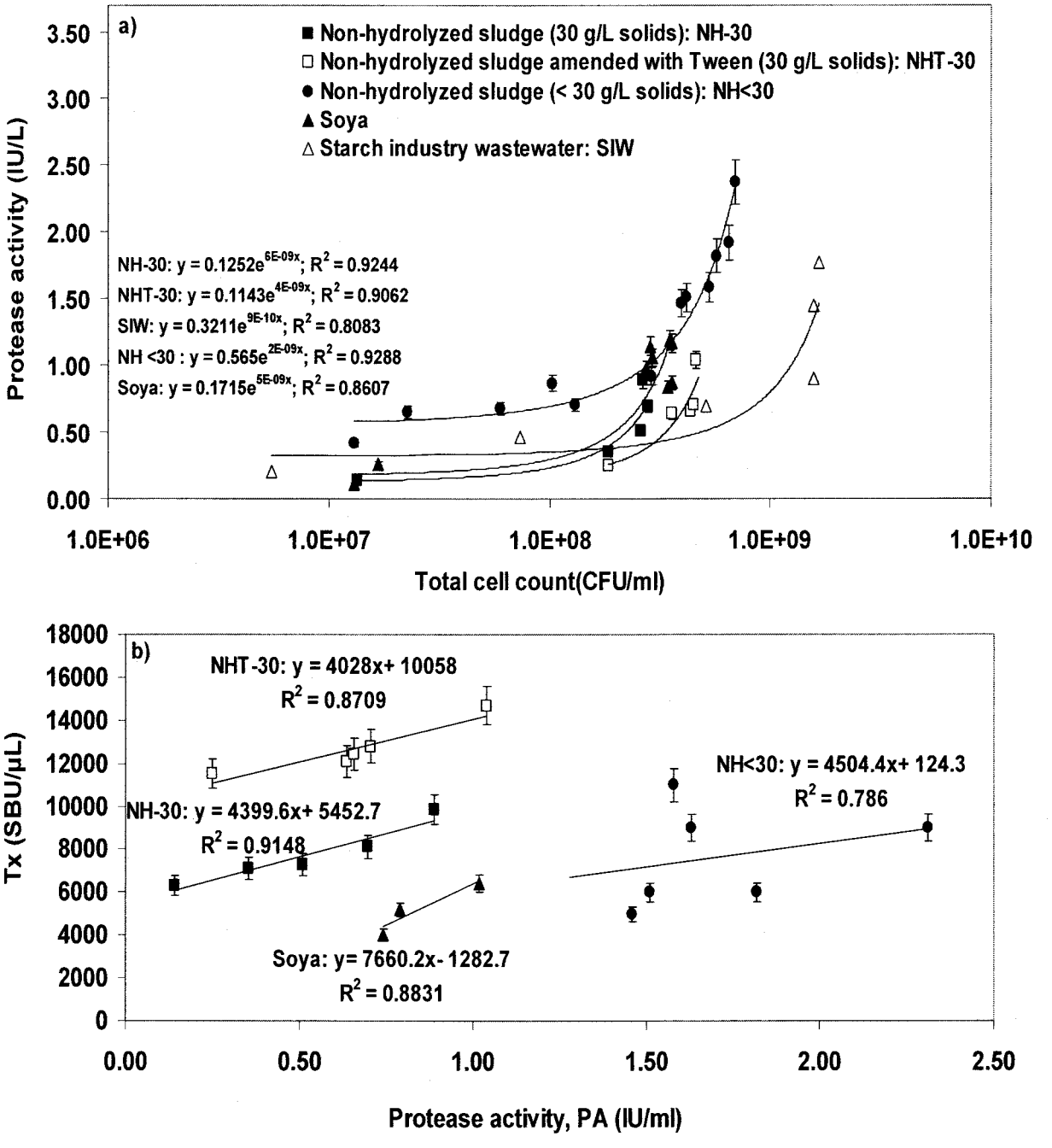


Fig. 3. Correlation of protease activity of different Bt production media with: a) Total cell count and; b) entomotoxicity. The data represented as NH<30 (raw sludge below 30 g/L suspended solids) have been re-analyzed from Yezza et al. [44,45,63] and comprises NH -15, NH-20, NH-25 and NH-25-150L data. The starch industry wastewater (SIW) data was re-analyzed from Brar et al. [67,70]. The number after the hyphen denotes the suspended solids concentration (g/L).

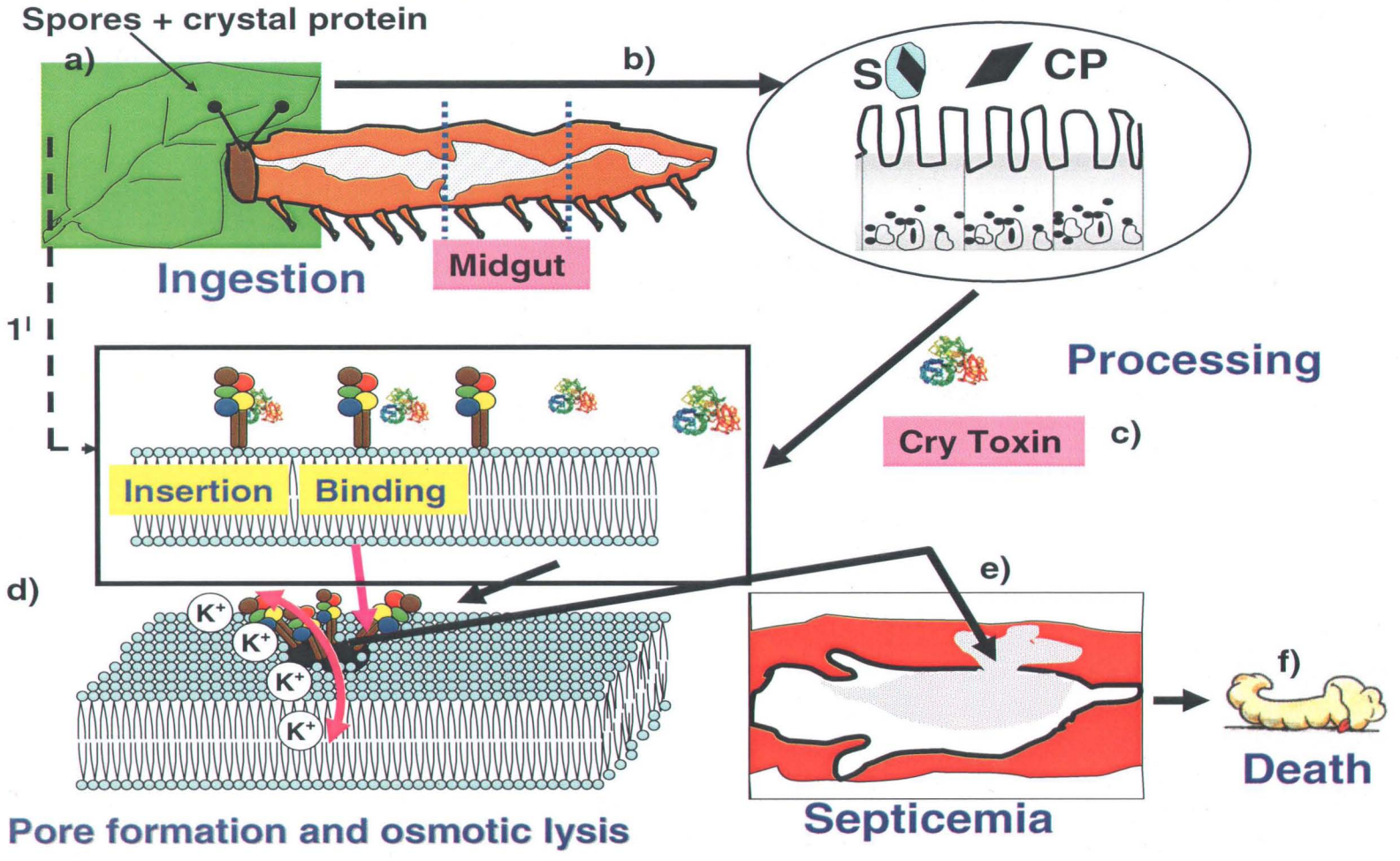


Fig. 4. Mode of action of Bt spores and crystal protein; a) Ingestion of spores and crystal toxin by larvae; b) Solubilization of Bt crystal protein in larval midgut; c) Proteolytic processing of Cry protoxins into active toxin, toxins bind to specific receptors on the epithelium and further insert into cell membrane; d) Cry toxins disturb ionic pump, cause pore formation resulting in osmotic lysis; and e) Midgut damage leading to feeding inhibition, septicemia by spores, and enterotoxicose by enteric bacteria and finally death; modified from Whalon and Wingerd [78]. 1^{I} pathway represents the pre-hydrolyzed protoxin so that steps "b" and "c" could be skipped and the pre-hydrolyzed Cry protein along with the spores will directly enter receptor binding stage.

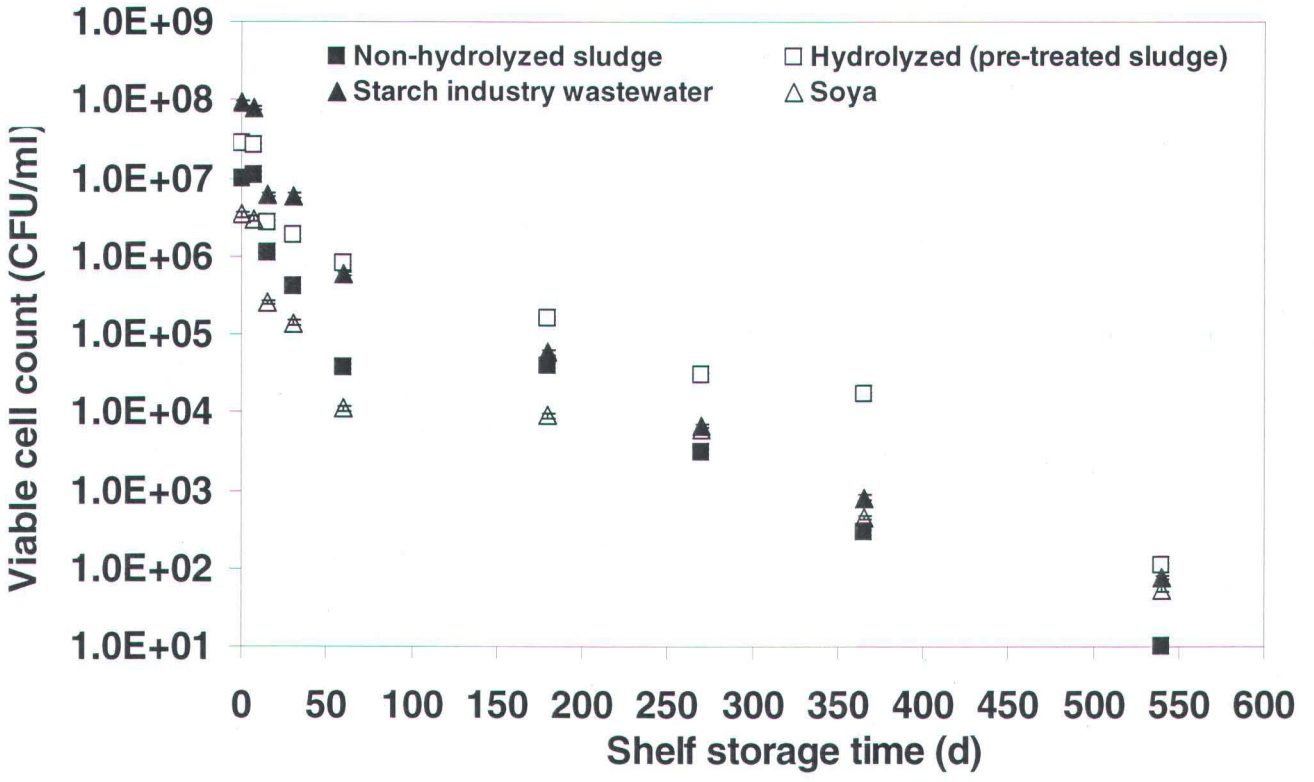


Fig. 5. Temporal profile of viable cells during shelf-storage of different Bt formulations.

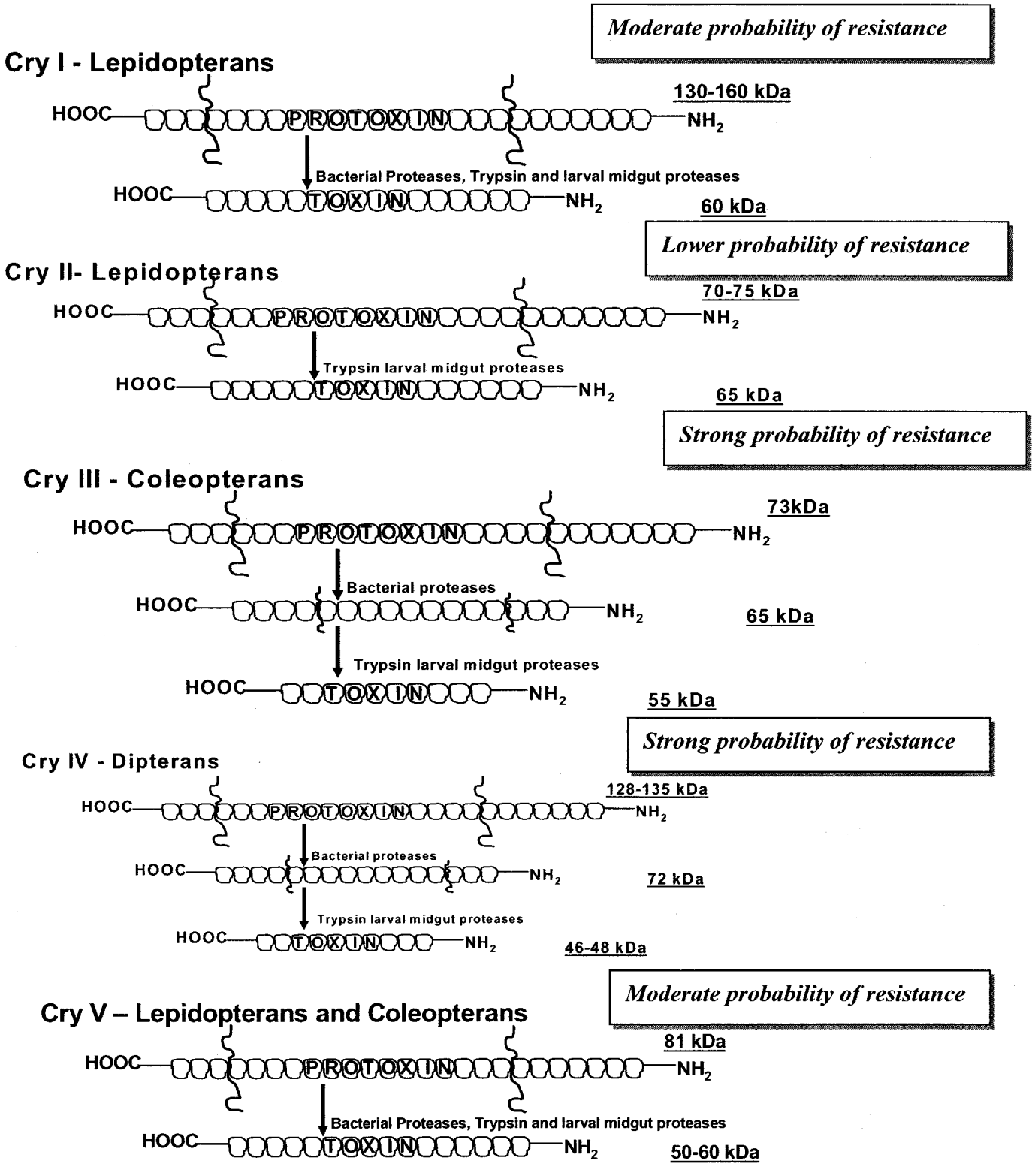


Fig. 6. Proteolytic processing schemes of Cry-I, Cry-II, Cry-III, Cry-IV and Cry-V toxins [1,16,109,117,122,131-135].

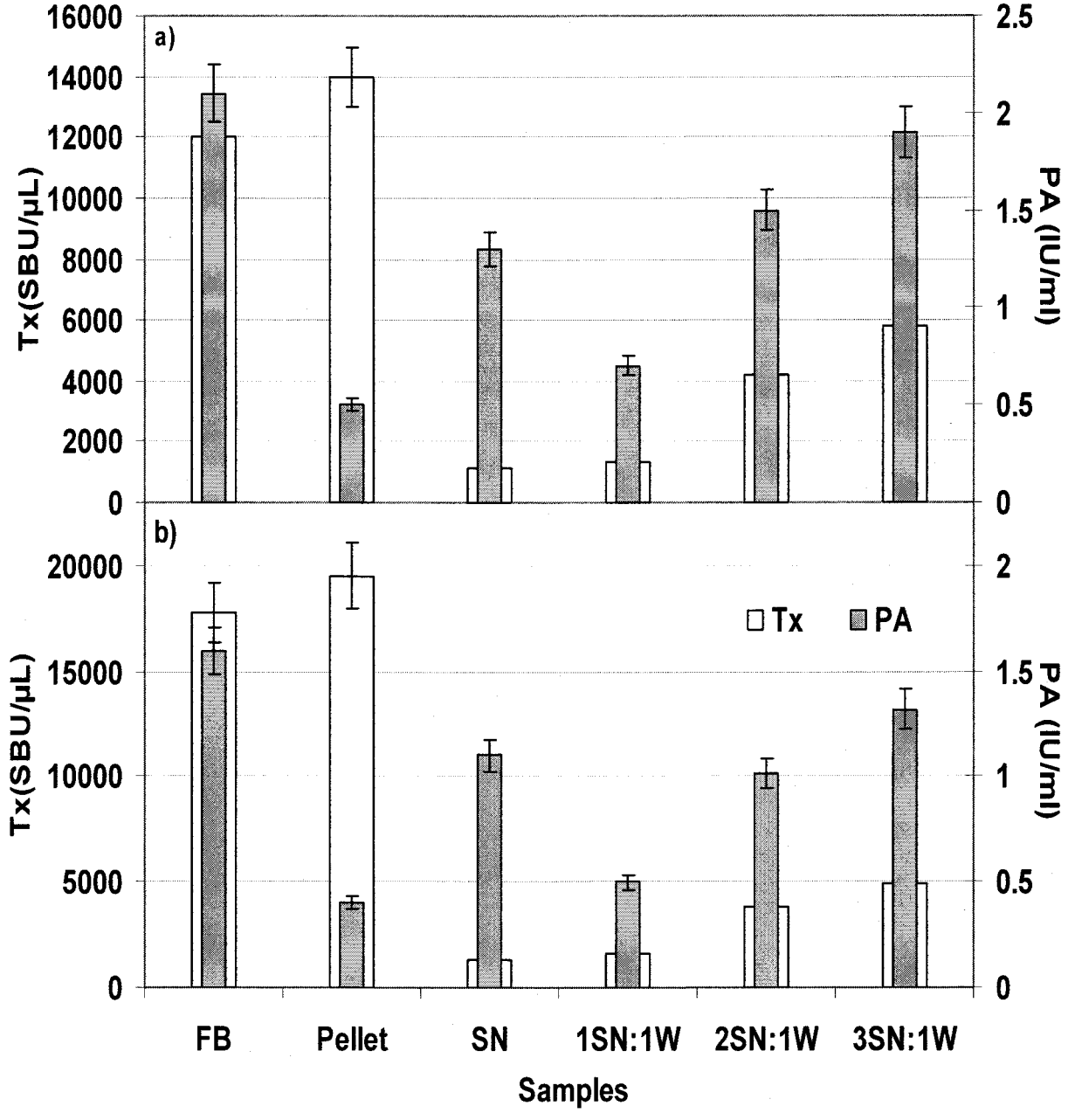


Fig. 7. Protease activity (PA) and entomotoxicity (Tx) of different samples of: a) Non-hydrolyzed sludge and; b) Hydrolyzed sludge; FB- Fermented broth (48h), Pellet- Centrifuged fermented broth (9000 g for 30 min), SN-supernatant, W-Saline solution (for dilution).

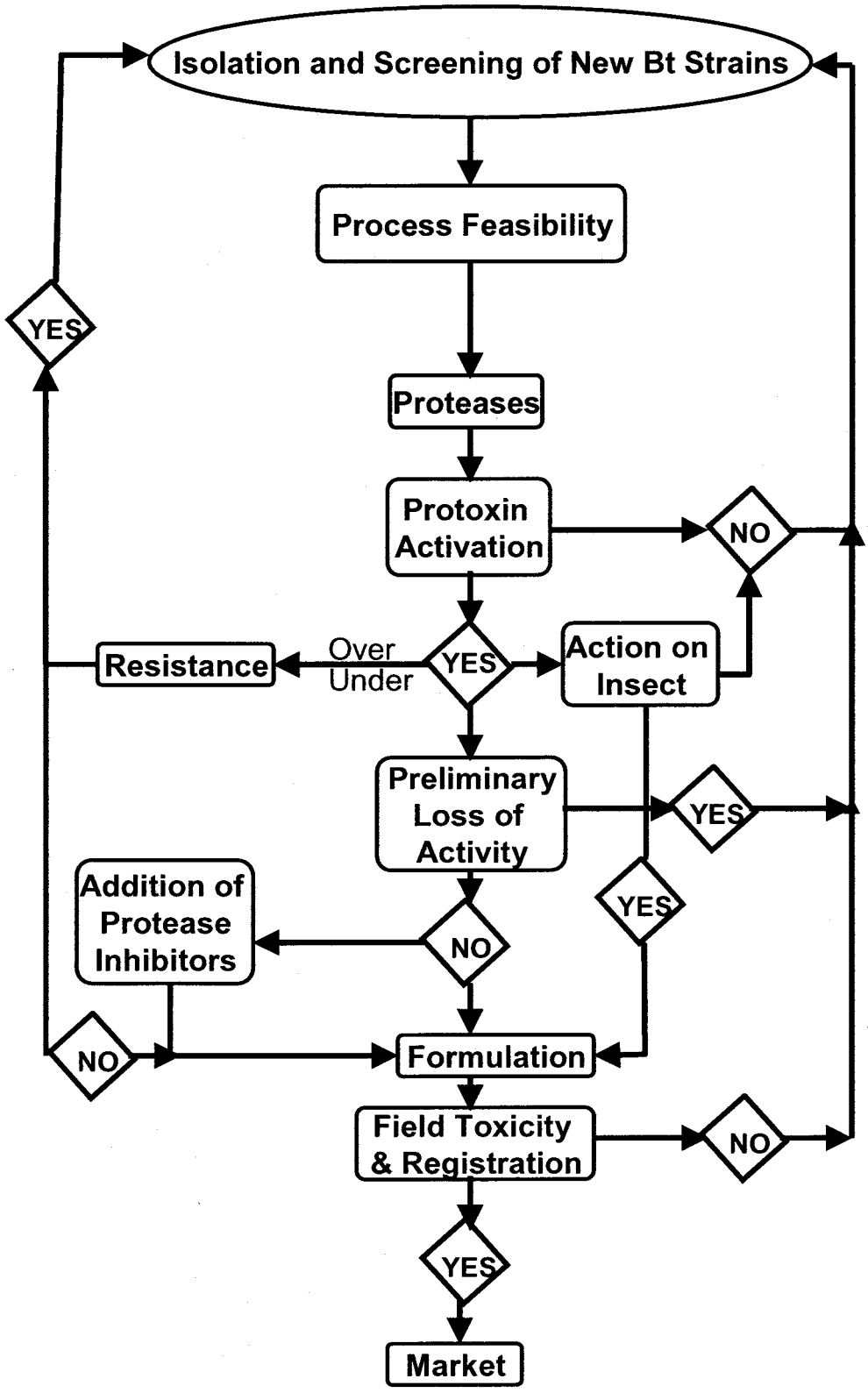


Fig. 8. Schematic algorithm of Bt biopesticide formulations development (with reference to Bt proteases)

Partie III

Screening of different adjuvants for wastewater/wastewater sludge based *Bacillus thuringiensis* formulations

Satinder K. Brar[1,2], M. Verma[1,2], R.D. Tyagi[1*], J.R. Valéro[1,2] and R.Y. Surampalli[3]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9
[2]Service canadien des forêts, Centre de foresterie des Laurentides, 1055 rue du PEPS, CP 3800, Sainte-Foy, Québec Canada, G1V 4C7
[3]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Journal of Economic Entomology (2006)

99(4) : 1065 - 1079

Évaluation des effets de différents adjuvants ajoutés dans les biopesticides à base des boues fermentées par *Bacillus thuringiensis*

Résumé

Différents additifs tels que des agents de suspension, des phagostimulants, des adhésifs, des agents antimicrobiens et des protecteurs contre les rayons ultra-violets (écran anti-UV) ont été ajoutés à des suspensions aqueuses de *Bacillus thuringiensis* (Bt) obtenues par fermentation en utilisant des boues non-hydrolysées (NH), des boues hydrolysées (H), des eaux usées de l'industrie de l'amidon (SIW), ou le milieu commercial à base de soya comme substrats. Les agents de suspension choisis (20% w/v) étaient le sorbitol, le monophosphate de sodium et le métabisulfite de sodium et ont permis d'atteindre une homogénéité de 74-92 %, de 69-85% et de 71-82%, respectivement. La mélasse à raison de 0,2% (p/v) augmente les propriétés adhésives de 84-90% pour tous les bouillons fermentés. Les phagostimulants (0,5% p/v) tels que le soya et la mélasse augmentent l'entomotoxicité (Tx) de 3-13% et de 7-13%, respectivement. Les acides sorbique et propionique ont démontré une action antimicrobienne élevée (0,5% p/v), indépendamment du milieu de fermentation. Le lignosulphonate de sodium, la mélasse et le rouge Congo sont de bons écrans anti-UV (0,2% p/v ; $P > 0,05$), mais provoquent des pertes en entomotoxicité de 3-5%, de 0.5-5% et de 2-16%, respectivement, après 8 heures d'exposition. Les suspensions de Bt avec ou sans écran d'UV avaient des demi-vies plus élevées par rapport à des bouillons fermentés non formulés, au milieu à base soya et à une formulation commerciale de Bt. Des suspensions de Bt obtenues avec NH, H ou SIW et contenant un écran d'UV présentaient une demi-vie de 1,3 à 1,5 fois plus élevée qu'une formulation commerciale de Bt ($P > 0,05$). Ces biopesticides à base de Bt obtenus par fermentation dans des milieux à base de boues d'épuration ou d'eaux usées de l'industrie de l'amidon offrent une meilleure résistance aux UV par rapport à une formulation commerciale.

Mots-clés: Additifs/adjuvants; agents antimicrobiens; *Bacillus thuringiensis*; biopesticide; formulations; UV; eaux usées/boues d'épuration.

ABSTRACT

Screening of different adjuvants, namely, suspending agents, phagostimulants, stickers, anti-microbial agents and UV screens to develop aqueous biopesticidal suspensions of *Bacillus thuringiensis* var. *kurstaki* HD-1 (Bt) fermented broths, specifically, non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater, and soya (commercial medium), were investigated. The selected suspending agents (20 % w/v) included – sorbitol, sodium monophosphate and sodium metabisulfite with corresponding suspendibility of 74-92 %, 69-85 % and 71-82 %, respectively. Molasses (0.2 % w/v) increased adherence by 84-90 % for all fermented broths. The optimal phagostimulants (0.5 % w/v), namely, soya and molasses caused entomotoxicity increase of 3-13 % and 7-13 %, respectively. Sorbic and propionic acids showed high anti-microbial action (0.5 % w/v), irrespective of fermentation medium. Sodium lignosulphonate, molasses and Congo red with corresponding entomotoxicity losses of 3-5 %, 0.5-5 % and 2-16 %, respectively as UV screens (0.2 % w/v; $P > 0.05$). The Bt formulations when exposed to UV radiation, showed higher half-lives (with and without UV screens) than the fermented broths or semi–synthetic soya medium and commercial Bt formulation. UV screen amended non – hydrolyzed; hydrolyzed and starch industry wastewater formulations showed 1.3-1.5 folds higher half-life than commercial Bt formulation ($P > 0.05$). Thus, the recommended formulation will comprise sorbitol, sodium monophosphate, sodium metabisulphite (suspending agents); molasses, soya flour (phagostimulants); molasses and skimmed milk powder (rainfasteners); sorbic and propionic acids (anti-microbial agents) and sodium lignosulphate; molasses and Congo red (UV screens). These waste based Bt formulations will offer better UV resistance in comparison to commercial formulation.

KEYWORDS Adjuvants; anti-microbial; *Bacillus thuringiensis*; biopesticide; formulations; UV; wastewater; sludge.

INTRODUCTION

CHEMICAL PESTICIDE USAGE in agriculture, forestry and health sector has caused immense damage to the environment which has led to an increasing demand for microbial biopesticides, with the market expected to exceed $500 million in 2006 (Eagan 2002). Microbial biopesticides comprise bacteria, fungi, algae, nematodes and viruses. Bacterial biopesticides have been utilized more frequently in pest management programmes than biopesticides based on other microorganisms. Globally, *Bacillus thuringiensis* represents 95 % of the total microbial biopesticide market and has 1 % of the total pesticide market. The expectation is that this percentage will keep on increasing over time (Bishop 2002).

There are several factors limiting successful development of *Bacillus thuringiensis* (Bt) biopesticides - in particular the need for cheaper raw materials and formulations. Wastewater and wastewater sludge are inexpensive raw materials that can be used for biopesticide production (Vidyarthi et al. 2002; Tirado-Montiel et al. 2003; Yezza et al. 2004, 2005; Brar et al. 2004 a, b; Barnabe et al. 2005). The cost of biopesticide production by using wastewater/wastewater sludge will be lower than semi-synthetic media currently used in commercial practice because wastewater sludge posses following advantages: replacement of synthetic medium by wastewater sludge (zero/negative cost raw material) (Sachdeva et al. 2000; Vidyarthi et al. 2002); higher entomotoxicity (higher toxicity of crystal protein) even at low cell/spore concentration when compared to soyameal medium (Yezza et al. 2004, 2005; Barnabe et al. 2005). Furthermore, reutilization of wastewater/wastewater sludge is a social, economical and eco-friendly approach.

Earlier studies aimed at Bt process optimization with no detailed investigation of formulation development. The choice of different adjuvants, namely, suspending agents (better suspensions during storage and spray application); phagostimulants (feeding stimulants); stickers (adherents for rainfastness); anti-microbial agents (microbial contamination inhibitors) and UV screens (UV protectors) depends on the final formulation, i.e., aqueous or solvent/oil-based, non-aqueous form (Burges 1998).

The phagostimulant (gustatory stimulation) studies began with sugar based derivatives viz. lactose and sucrose (Bartlet et al. 1990). With the progress in understanding of the actual and specific action of phagostimulants, newer options of amino acids, starch, ascorbic acid and nature derived cucurbitacins and garbanzo beans were explored (Bartlet et al. 1994; Gillespie et al. 1994; Lopez Jr. et al. 1994). As the phagostimulant interest soared, some commercial ones like Coax, Entice, Gusto Konsume, Mo-Bait were introduced to increase feeding responses of pests in the field (Lopez Jr and Lingren 1994; Farrar Jr and Ridgway 1995).

Fermented biopesticidal broths are highly susceptible to foreign microorganisms due to their biodegradable characteristics (live microbial cells, toxin crystals and spores). This necessitates lowering of the pH and addition of anti-microbial agents to inhibit the growth of various microorganisms (Burges 1998). Occassionally, microflora like yeast and mold, fecal cocci/enterococci coliforms such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi* are likely to infest these broths (Lisansky et al. 1993; Burges 1998). Therefore, International Union of Pure and Applied Chemistry (IUPAC) has established maximal allowable limits of these probable contaminants in formulations (Quinlan 1990).

Another problem with Bt formulations is the loss of residual entomotoxicity (Tx) on exposure to UV under field conditions (Cohen et al. 1991). Several approaches on UV protection of Bt formulations included the use of oil-soluble sunscreens with oil-carriers, oil-water emulsions (Burges 1998), water-soluble or suspendable absorbers or blockers with water carriers (Shasha et al. 1998), or encapsulation (e.g., insoluble starch) with a water carrier (McGuire et al. 1996; Behle et al. 1997; Tamez-Guerra et al. 2000). Other studies included incorporation of various UV screens like Congo Red, folic acid, molasses, lignin, alginate, cellulose, shellac yeast, p-amino benzoic acid with mixed results (Shapiro 1989; Dunkle and Shasha 1989; McGuire et al. 1996; Behle et al. 1997a, b; Ragaei 1998; Wirtz et al. 1999). Certain fluorescent brighteners, especially, compounds of stilbene type have been also found to enhance biological activity up to 1000-fold and protecting Bt from UV exposure (Shapiro et al. 1992). However, external addition of UV screens and/or modification of formulation matrix are expensive methods. This cost could be reduced if the fermentation medium possessed inherent characteristics that provided UV resistance when compared to commercial formulations.

This study focuses on screening of different formulation adjuvants (suspending agents; phagostimulants; stickers; anti-microbial agents and UV screens) for wastewater/wastewater sludge-based Bt liquid suspensions to assist in development of stable formulations for field application. Furthermore, the study investigates the effect of UV on entomotoxicity (Tx) of Bt fermented broths and formulations (with and without UV screens) of starch industry wastewater, wastewater sludge (raw and hydrolyzed) and soya (semi-synthetic commercial medium).

Materials and Methods

***Bacillus thuringiensis* Production Media.** Three different media were utilized for Bt growth: a) conventional semi-synthetic soybean medium (control) (Lisansky et al. 1993) that comprised of (in 1 liter water): soybean meal, 15 g; glucose, 5 g; starch, 5 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.3 g; $FeSO_4.7H_2O$, 0.02 g; $ZnSO_4.7H_2O$, 0.02 g; $CaCO_3$, 1 g; b) secondary wastewater sludge (raw or non-hydrolyzed, NH sludge) from Communauté Urbaine du Québec wastewater treatment plant,

Québec (complete medium without addition of external ingredients) and; c) starch industry wastewater (SIW, complete medium) from ADM-Ogilvie (Candiac, Québec, Canada). The wastewater sludge and SIW were utilized with a minimum delay (within 1 week of sampling) for fermentation as long-term storage at even 4°C would lead to deterioration (slow endogenous respiration of microbes). The sludge sampled at different time periods of the year (incorporates seasonal, day and night, and other possible variations) did not vary much in terms of entomotoxicity at optimal sludge solids concentration (Yezza et al. 2005).

Characterization of Wastewater Sludge/Starch Industry Wastewater. Different characteristics of raw wastewater sludge /starch industry wastewater as presented in Table 1 were determined according to standard methods (APHA, AWWA, WPCF 1998).

Solids Amendment and Pre-Treatment Procedure. The sludge was concentrated from approximately 1.7 % to 5 % (w/v) suspended solids by gravity settling followed by centrifugation of the settled sludge at 7650 g for 15 min at 20 ± 1 °C in a Sorvall RC 5C plus Macrocentrifuge. The sludge supernatant was stored in the refrigerator at 4 ± 1°C and used to dilute the sludge samples as required. The concentrated sludge was then homogenized using a Waring blender for optimal suspended solids concentration of 25 g/liter (Vidyarthi et al. 2002).

For steam hydrolysis (to enhance nutrient availability in non-hydrolyzed sludge), 5 % w/v solids concentration sludge (considering 1.67 times dilution by steam; empirically obtained factor) was transferred into a custom-made mechanical steam hydrolyzer with working volume of 10 liters (stainless steel, SS 316L, EBR Quebec, Canada). The sludge was hydrolyzed by sequential alkaline (pH 10.25 ±0.1) and thermal pre-treatment at 140 ± 1°C for 30 minutes at a pressure of ≈40 psig (Barnabé et al. 2005). The pH of the sludge for Bt growth after pre-treatment was re-adjusted to 7.0 ±0.1 by using 2N H_2SO_4. The non-hydrolyzed and hydrolyzed sludge was referred to as NH and H sludge, each at 25 g/ liter and 30 g/ liter suspended solids concentration, respectively.

Bacterial Strain. *Bacillus thuringiensis* var. *kurstaki* HD-1 (ATCC 33679) was used in this research. The culture conditions and maintenance are described elsewhere (Vidyarthi et al. 2002).

Inoculation and Fermentation. Preliminary inoculation and acclimatization of Bt in shake flasks was carried out according to the method utilized by Vidyarthi et al. (2002). Fermentation was carried out in two stirred tank 15-liter fermenters (Biogénie, Québec, Canada) with 10 liter working volume. The fermenter was equipped with accessories and computer coupled programmable logic controller (iFix 3.5 software, Intellution, GE Fanuc Inc., USA) for dissolved oxygen, pH, anti-foam, impeller

speed, aeration rate and temperature as per details given earlier (Brar et al. 2005). The fermentation was carried out in two independent batches. Further, the results reported were average of the two batches as no significant difference (paired *t*-test, $P > 0.2$) between the two fermented broths based on entomotoxicity was observed.

Bioassay. Bioassays were conducted using the diet incorporation method (Beegle 1990). The entomotoxicity was evaluated by bioassays using eastern spruce budworm larvae (*Choristoneura fumiferana* (Clemens), Lepidoptera: Tortricidae) in second instar, provided by Natural Resources Canada (Sault Ste-Marie, Ontario, Canada). The larvae were raised on an artificial diet for 7 d to obtain the third and fourth instar (L3-L4) larvae. In this technique, 1.5 ml of appropriately diluted Bt samples of fermented broths were incorporated into 30 ml of molten agar based diet (at 60 ± 1°C), the composition of the artificial diet is described elsewhere (Tirado-Montiel et al. 2001). Afterwards, the mixture was distributed in aliquots of 1 ml in twenty 15 x 45 mm glass vials (VWR Canlab, Canada) with perforated plastic caps. For each sample, at least three dilutions were used, and hence sixty glass vials were used for each sample.

Sixty vials containing 1 ml artificial diet (C1) were used as a control and another control contained sterilized non-hydrolyzed sludge/hydrolyzed sludge/starch industry wastewater/soya medium (C2). One L3-L4 larva was placed into each vial and allowed to feed *ad libitum* for 7 d at 25 ± 1°C. Mortality was monitored after 7 d. If mortality in control vials was higher than 10 %, the sample was repeated. The Tx of sample preparations was obtained by comparing the relative mortality (percentages) of spruce budworm larvae compared with the mortality induced by Foray 76B and expressed as relative spruce budworm units (SBU/µl) and calculated using Equation 1.

$$\left\{\frac{\dfrac{\%\text{ mortality in sample }(10^{-3})}{\%\text{ standard mortality }(10^{-3})}+\dfrac{\%\text{ mortality in sample }(10^{-4})}{\%\text{ standard mortality }(10^{-4})}+\dfrac{\%\text{ mortality in sample }(10^{-5})}{\%\text{ standard mortality }(10^{-5})}}{3}\right\}\text{X }19950\ \text{IU}/\mu\text{L} = \text{Tx (SBU}/\mu\text{L)} \quad (1)$$

Foray 76 B contained spores and crystals of Bt var. *kurstaki* at a potency of 20.1 x 10^9 IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni*) (Hübner). On comparison of Tx of Bt fermented sludge samples, it was found that SBU reported in this study was 20-25 % higher than IU. Data were analyzed with one way analysis of variance (ANOVA) for significant test (Chatfield, 1983). The standard deviation for Tx measurement was 8–10 %.

Harvesting Techniques. *Post-fermentation Step.* Temperature of fermented broths (non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and soya) was gradually lowered from 30 ± 1 to 25 ± 1°C. Individually sterilized 4M H_2SO_4 and 4M NaH_2PO_4 were mixed in 1:1 volume ratio and the mixture was added to fermented broths via automated peristaltic pumps

integrated into the fermentation set-up to lower the pH from 7 ± 0.1 to 4.5 ± 0.1. If 4M NaH_2PO_4 was used singly for pH adjustment, it was observed that the volume required was ten times more than the mixture mentioned earlier as well as there was a loss of ≈ 45 % Tx (initial Tx of fermented broth at 12000 SBU/µL gave Tx of 7000 SBU/µL after pH adjustment) during measurement due to dilution. Later, the broth at pH 4.5 ± 0.1 was collected aseptically in sterile high density polyethylene (HDPE) containers (12 liter capacity, VWR Canlab, Canada), was sealed with Parafilm™ and stored in a freezing chamber (DuPont, Illinois, MI, USA) at -20°C until used for formulation studies.

Pre-formulation Step. The frozen acidified broth (non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and soya – from step above) was freeze thawed by holding the container in water at 30 ± 1°C for a day. In order to compare the results of different acidified fermented broth formulations at 19.5 x 10^9 SBU/l, the fermented broths (after thawing) with initial Tx values of 9.54, 12.7, 16.5 and 18.9 x 10^9 SBU/l for soya, non-hydrolyzed sludge, starch industry wastewater and hydrolyzed sludge, respectively needed to be concentrated to higher Tx values. Thus, the acidified fermented broths were centrifuged in sterilized 500 ml HDPE bottles (Nalgene, Fisher Labs, Ontario, Canada) at 9682 g at 20 ± 1°C. The concentrated broths of hydrolyzed sludge and starch industry wastewater at approximately 70 g/liter solids concentration yielded an approximate entomotoxicity of 25.4 x 10^9 SBU/l and non-hydrolyzed sludge and soya at 120 g/liter solids gave a Tx of 24.6 and 18.8 x 10^9 SBU/l, respectively. The estimation of Tx at this stage was essential for further studies on adjuvant screening.

Screening/Selection Tests. Specific adjuvants with known characteristics/property and recommended concentrations were selected from literature (Lisansky et al. 1993; Burges 1998, Bishop 2002). The screening/selection criteria of each adjuvant was based on specific characteristic/property (e.g. suspendibility – suspension; phagostimulant – feeding action (increase in entomotoxicity); stickers/adherents – rainfastness; anti-microbial agents – percent contamination and UV screens – percent viable spore and entomotoxicity losses) as listed in protocol (Table 2).

Preparation of Samples (Suspending agents/Phagostimulants/Stickers). Each selected adjuvant was tested individually (one-at-a-time basis) as per the concentrations given in Table 2 for their specific property by amending the concentrated fermented broth (from pre-formulation step, described above) in 125 ml HDPE bottles (100 ml formulation). The adjuvant amended concentrated broth was further fortified with 0.5 % w/v propionic acid to eliminate contamination followed by addition of 0.5 % w/v sodium monophosphate as a buffer to maintain pH (as addition of propionic acid and individual adjuvants may alter the pH). Further, the volume of individual samples thus obtained after amendment with respective suspending/phagostimulating/sticking agents was made up

with supernatant of centrifuged broth to attain final Tx of 19.5 x 10^9 SBU/l approximately equivalent to industry standard Foray 76B (Abbott Laboratories, Chicago, IL, USA). The adjuvant/additives composition (mentioned above and in Table 2) was the final concentration found in each adjuvant amended formulation (19.5 x 10^9 SBU/l) of respective broths. The screening agents comprised five replicates of each adjuvant in each category (class of adjuvant, namely, suspending agents, rainfasteners, phagostimulants). Thirty (6 adjuvants x 5 replicates) adjuvants were used for each treatment. The value of 5 replicates was treated as the experimental unit. Data were subjected to one way analysis of variance (ANOVA) (Chatfield 1983). Therefore, 5 (replicates of each adjuvant) x 6 (adjuvants) = 30 samples were tested for each adjuvant category resulting in 30 x 3 (categories) = 90 total samples. Each screened preparation also included a control (fermented centrifuged broth) without adjuvants at pH 4.5 ± 0.1. The bottles were stored at 20 ± 1°C for 24 hours.

Assessment of Screening/Selection Criteria. *Suspendibility Tests.* The tests were used to adjudge the suspension properties of formulations during shelf storage. Each suspending agent formulation (50 ml) was suspended in 100 ml deionized water (turbidity free Milli-Q water). The sample was compared with a well mixed suspension of industry standard Foray 76B, Abbott Laboratories, Chicago, IL) as control. The suspended sample was well dispersed by shaking in a separating funnel several times and then letting it stand undisturbed for 30 min. Afterwards, 20 ml of the sample was withdrawn from the bottom to determine the amount of settled solids (dry weight basis, concentration in g/L). The supernatant from the top was utilized to determine the apparent turbidity (Nephelometric Turbidity Units, NTU) as optical density using UV-Visible spectrophotometer (Varian Cary 100 Bio, Mississauga, Ontario, Canada) at λ_{max} of 450 nm (APHA, AWWA, WPCF 1998). The suspendibility of formulations was defined as per Equation 2,

$$\text{Suspendibility} = \frac{\text{Turbidity (NTU)}}{\text{Total solids (g/L)} - \text{Settled solids (g/L)}} \qquad (2)$$

The percentage relative suspendibility (Equation 3) was calculated, based on industry standard Foray 76B (control).

$$\text{Percentage Relative Suspendibility} = \frac{\left(\frac{\text{Turbidity}}{\text{Total solids} - \text{Settled solids}}\right)_{\text{sample}}}{\left(\frac{\text{Turbidity}}{\text{Total solids} - \text{Settled solids}}\right)_{\text{control}}} \times 100 \qquad (3)$$

Percentage standard deviation for suspendibility measurement was 2-5 %.

Rainfastness Tests. A simple bench top modified glass slide (Surgipath pre-cleaned ground edge blood smear slides 25.4 mm x 76.2 mm x 645.16 mm, Frankfurt, Germany) assay method was developed instead of the conventional simulated rainfall in a greenhouse. Pre-cleaned and pre-weighed glass microscopic slides were used for the purpose.

One ml sticker formulation sample was uniformly spread on these slides and it was allowed to air dry (for quick drying, kept inside a laminar flow chamber). The slides were held approximately at 2 cm under a stream of distilled water released from a burette (high precision 100 ml burette, VWR Canlab, Toronto, Ontario, Canada). About 40 ml of distilled water was poured from the burette at a rate of 20 ml/min over the slide, which was continually moved back and forth using a jack mounted slide. Slides were air dried, and the procedure was repeated for three wash-dry cycles. Slides were reweighed to determine the suspension losses as per Equation 4 and denoted as: Percent sticking =

$$100 - \left[\frac{\{\text{Before rain dry weight (sample + slide)}\} - \{\text{After rain dry weight (sample + slide)}\}}{\{\text{Before rain dry weight (sample + slide)}\} - \{\text{weight of slide)}\}} \right] \quad (4)$$

Five slides were prepared for each formulation and control (without stickers/adherents). This method was a minor modification of already reported protocol (McGuire and Shasha 1992). Standard deviation of loss in weight measurements for calculation of percent sticking was 10-12 %.

Phagostimulant Tests. The phagostimulant action was estimated by increase in Tx of each phagostimulant-amended formulation in comparison to unamended sample determined by bioassay tests (discussed earlier).

Preparation of Samples for Anti-microbial/UV Tests. The concentrated broths (~ 7 % or 12 % w/v solids; obtained from pre-formulation step) with respective Tx (mentioned earlier) were amended with basic adjuvants (in % w/v) – glycerol (2 %, humectant), Tween–80 (0.2 %, surfactant), Triton X–100 (0.1 %, surfactant) as reported by Lisansky et al. (1993), and sorbitol (21 %, pre-screened suspending agent). This suspension was further used to screen anti–microbial agents and UV blockers and investigate half-life. The final anti-microbial agent/UV amended broth was stoichiometrically made up with supernatant to attain Tx of 19.5 x 10^9 SBU/l as described earlier. The anti-microbial agent/UV formulations comprised five replicates in each category of adjuvant. Thirty (6 adjuvant x 5 replicates) adjuvants were used for each treatment. The value of 5 replicates was treated as the experimental unit. Data were again subjected to one way analysis of variance (ANOVA) (Chatfield 1983). Therefore, 5 (replicates of each adjuvant) x 6 (adjuvants) = 30 samples were performed for each screening agent resulting in 30 x 2 (categories) = 60 total samples. Each screened preparation comprised a control-I at pH 4.5 ± 0.1 for each formulation (raw, fermented centrifuged broth) without screened agents. Likewise, an additional control-II for molasses at 0.2 % w/v, unexposed to UV radiation was set-up to account for the phagostimulant effect, if any.

Microbiological Purity Testing. The above stated suspension was amended with 5 (replicates of each adjuvant) x 6 (anti-microbial agents) as stated in Table 2 and was stored in HDPE bottles at

room temperature (20 ± 1°C). Samples were drawn at a period of 1, 7, 15, 30 and 60 d for microbial contamination. Each sample was appropriately diluted in sterile saline solution (0.85 % NaCl) to detect microbial contamination on selective agars using protocol given in Table 3. Various nutrient media were purchased from EM Science (Merck KGaA, Darmstadt, Germany).

Sunlight Exposure/UV Inactivation Tests. The suspensions (preparation method described earlier) with UV blockers were transferred in cylindrical HDPE sample vials (1.5 cm in diameter, 10 ml capacity, VWR Canlab, Ontario, Canada). The sample vials (each containing 1 ml of sample) were placed at a 20 cm distance from the UV radiation source as seen in the laboratory set-up (Fig. 1). A 15 W UV-A tube (emission maxima at 366 nm) and a 15 W UV-B tube (emission maxima at 312 nm) (Transilluminator, UVP®, San Gabriel, CA, USA) provided specific UV radiation. These tubes were mounted parallel to each other in a customized set-up with aluminium sheet covering the entire interior of the compartment so as to reflect the stray radiation (Fig. 1). The combination of UV-B/UV-A tubes produced 4788 x 10^{-8} W/cm^2, which emitted 1403 x 10^{-8} W/cm^2 of UV-B radiation (=280-320 nm), 2952.5 x 10^{-8} W/cm^2 of UV-A radiation (= 320-400 nm) and 421.9 x 10^{-8} W/cm^2 of visible light (= 400-800 nm) as per details provided by VWR-Canada. UV agents were screened by exposing samples for 8h in laboratory set-up. The absorbance spectra of different UV screens and NH and H fermented sludge were scanned by using UV-Visible spectrophotometer (Varian Cary 100 Bio, Mississauga, Ontario, Canada).

For half-life studies, Bt suspensions or formulations (duplicate samples) were set-up as follows: (a) Fermented broths were exposed to light for different time intervals of 0, 1, 6, 10, 16, 28, 32 and 52 h and, (b) formulations (obtained from different fermented media) were exposed to 0, 5, 10, 20, 30, 45, 90, 300 and 1000 h. Half-life ($T_{0.5}$ –time required for Bt formulation to lose half of its Tx by UV action) was calculated from residual Tx versus exposure time plots. Eight h in laboratory under these UV conditions was considered equivalent to 1-day field exposure to the complete UV spectrum. This assumption was in concordance with McGuire et al. 1996 who used Sun Test apparatus to test solar stability of Bt formulations against *P. xylostella*. The control samples (without UV protection agents) were wrapped with thick aluminium sheet and stored at ambient temperature (20 ± 1°C) for same period of exposure. Samples were taken at regular intervals for viable spores and Tx determination. The UV exposure regimens were repeated three times and a standard deviation of 8-12 % for Tx and VS measurement was observed.

Viable spore Count. Procedure specified in Vidyarthi et al. (2002) was utilized for viable spore count with a small modification of subjecting samples to heat treatment ("heat shock") in a silicone bath (Thermo-Lift, Buchler Instruments, New Jersey, USA) at 80 ±1°C for 10 min and then cooling

on an ice bath for 5 min before plating on tryptic soya agar. The standard deviation for viable spore measurement was 8.0 %.

Results and Discussion

Suspending Agents. The percentage suspendibility (suspendibility response) for different screened suspending agents is shown in Fig. 2a. Sorbitol (D 1), sodium monophosphate (D 3) and sodium metabisulphite (D 4) showed better suspendibility for all formulations (Fig. 2a). The suspendibility of the three suspending agents ranged between 68-92 % with significant difference (F = 3.29; df = 5, 24; $P < 0.05$). These results agreed with the work of other researchers who have pointed out the role of sorbitol in better suspensions in food industry and biopesticidal sprays (Smirnoff and Juneau 1982; Petersen et al. 2004). Addition of sorbitol produced well dispersed suspensions as it would carry out hydrophilic stabilization of proteins and provide cryoprotection of the fermented broths (all broths in this study are highly proteinaceous in nature). The cryoprotection property of sorbitol (Petersen et al. 2004) would aid further in better stability of Bt formulations during storage at lower temperatures, which are commonly encountered in many parts of Canada and USA. The low temperatures refer to the storage temperatures as when the biopesticides will be stored in warehouse facilities, there is a possibility of encountering low temperatures which may affect the properties of formulation. Further, the unused/partially used biopesticide barrels are often left in the open sites which may affect biopesticide stability.

Furthermore, sodium monophosphate finds widespread use as a dispersing agent in pesticidal sprays and acts as buffering agent (WHO 1999). This will also aid in maintenance of pH during shelf storage and further retain the physical consistency of formulations. Although, sodium metabisulfite has been reported as a preservative (WHO 1999), but at the same time it can stabilize suspensions as established in this study. So, sodium metabisulfite could be employed for its properties of suspension as well as preservative (anti-microbial agent). However, there will be difference in concentrations at which it can serve as a suspending agent (20 % w/v) or preservative (0.2 to 0.5 % w/v), but all the same, suspending agent concentrations can also provide anti-microbial properties (saving on formulation economy). Similarly, sodium monophosphate offered dual advantages - buffering and structuring of suspensions.

Phagostimulants. The phagostimulants were screened depending on the feeding response of spruce budworm larvae on formulations (determined by relative Tx increase when compared to control) as presented in Fig. 2b. The soya flour (P 5) showed increase in Tx by 13-14 % (F = 2.84; df = 5, 24; $P < 0.05$) for all formulations, except fermented H sludge when compared to control (no phagostimulants). However, molasses (P 3) showed 9 % increase in Tx for H sludge formulations as well as 13 % increase for NH sludge formulations (F = 2.97; df = 5, 24; $P < 0.05$). The maximum

phagostimulant action was based on increase in Tx obtained for each phagostimulant treatment. Soya flour showed higher Tx increase for most of the formulations probably due to the feeding preference of larvae because of granular consistency of soya, known to possess high phagostimulant response (Castillejos et al. 2002). It is possible that the spruce budworm larvae have selective taste for soya formulations. Furthermore, molasses provided phagostimulant response in non-hydrolyzed and hydrolyzed sludge formulations selectively due to the enhancement of odor and sugar content of these formulations.

Stickers/Rainfasteners. The screening of various stickers based on percent sticking property is presented in Fig. 2c. It was observed that molasses (R 1) increased adherence/rainfastness by 84-90 % of all media based Bt formulations, except soya formulations. In fact, molasses has been shown to have good sticking property and are widely used in various biopesticidal formulations (Burges 1998). Similarly, skimmed milk powder (R 4) increased sticking by 93 % and 64 % for fermented NH sludge and soya formulations, respectively in comparison to formulations not amended with stickers and the values were significantly different ($F = 4.63$; df = 5, 24; $P < 0.05$) between each sticker treatment. The stickiness (resistance to wash-off) of the molasses was a result of sugar content as any sugar on drying (here, glass slide simulated as leaf of the tree) will lend the characteristic sticking property as it forms a cohesive bond with the glass slide which will aid in adherence and probable stickiness during rainfall. Furthermore, polymeric chain of amino acids in molasses cross-links to form a contiguous, three-dimensional molecular network in solution. This network traps the water within to form a firm, flow-resistant structure (Xu et al. 2005) which may be difficult to wash off by rain. In fact, gelling of molasses has been found to cause pumping problems from storage tanks for use in animal feed. Likewise, skimmed milk powder based formulation when air dried during field application would lend adherence to the formulation (probably due to presence of casein protein molecules-polypeptide chains) preventing wash-out.

The screening of different formulation adjuvants would aid in further quantification for use in development of stable formulations. Further, it will be more cost-effective to utilize multifunctional adjuvants, for example, molasses, which possess sticking, phagostimulation and UV resistance properties (discussed later).

Anti-microbial agents. Screening results of different anti-microbial agents for non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and soya formulations are presented in Table 4 and the corresponding profiles after a period of two months are given in Fig. 3. There was no contamination for first 7 d, however, after a period of 7 d, a contamination was detected in some of the anti-microbial agent treated samples as illustrated in Table 4. This contamination could be due to the adjuvants added and the ambient air in the amendment room which might have become

contaminated over the 7d period. Meanwhile, the room where manipulations were made was free of contamination as tested through exposure of agar plates from 0 to 5d. Thus, the contamination would have set on 7th day. Furthermore, other possible sources of contamination – fermented broth (not contaminated on day 1, Table 4) and sampling (use of sterile equipments) were totally ruled out. It was evident that soya suspensions showed no contamination with any of the agents except for sodium metabisulfite (AM - 3), citric acid (AM - 4) and lactic (AM - 5) acids and the results were significantly different ($F = 3.79$; df = 5, 24; $P < 0.05$) for each anti-microbial treatment. Moreover, the best anti-microbial agents for all liquid suspensions were sorbic (AM - 1) and propionic acid (AM - 6) which controlled the contamination by 10^2 to 10^6 times in comparison to other AMAs. This could be due to pK_a (negative logarithm of dissociation constant) of sorbic acid (4.8) and propionic acid (4.87) being in the working pH range of 4.5 ± 0.1 leading to more effective form (dissociated) with an ability to permeate into microbial cells causing maximum anti-microbial activity in comparison to sodium metabisulfite (pK_a = 1.89). In fact, Tyagi et al. (1998) have reported that organic acids in the dissociated form were more toxic in inhibiting bacterial growth. Likewise, metals in ionic form have been found to be toxic to Bt metabolism (Entwistle et al. 1993).

Sorbic acid has been shown to inhibit Gram positive and negative, catalase positive and negative, aerobes and anaerobes, and thermophilic, mesophillic and psychotropic bacteria (Bracey et al. 1998). This could have also resulted in inhibition of Bt cells, but periodic Tx measurements yielded stable results (data not reported), as deduced from Brar et al. (2004b). Thus, development of formulated product of different fermentation broths without addition of anti-microbial agents is impractical and may lead to serious contamination problems degrading the product, reducing its effectiveness and integrity and hence shortening the overall shelf-life. Propionic (AM - 6) and sorbic (AM - 1) acid salts (weak organic acids) gave better performance than other anti-microbial agents owing to their well-defined and established mechanism of growth inhibition and excellent safety records (Bracey et al. 1998). The growth inhibition could be due to inhibition of essential metabolic reactions and accumulation of toxic anions (Piper et al. 2001).

Thus, the best anti-microbial agents were propionic and sorbic acids with ability to act on a wide spectrum of contaminants. More studies need to be carried out on quantitative optimization of these anti–microbial agents.

UV Screens. The UV blockers screening results (8 h exposure) are shown in Figs. 2d and 2e and corresponding UV absorption spectra scans of screened agents as well as those of media used for Bt formulation are presented in Fig. 4. Semi-synthetic soya medium and starch industry wastewater did not show any absorbance whereas fermented non-hydrolyzed and hydrolyzed sludge showed average absorbance of 10 and 2, respectively. It was seen that the package formulations containing each of

sodium lignosulfonate (UV 1); molasses (UV 2) and Congo red (UV 3) served as universally good UV screens with the exception of p-amino benzoic acid (PABA, UV 5) which was good for starch industry wastewater formulations based on significant difference between all UV treatments (F = 4.48; df = 5, 24; $P < 0.05$). Moreover, Tx losses were reported as: control -I (without UV screens, exposed to UV radiation) - 70-80 % Tx losses (depending on broth); control -II (with 0.2 % w/v molasses, without UV exposure) – 2 % Tx increase which was negligible, and sample (with UV screens, exposed to UV radiation) - 10-15 % Tx losses. Furthermore, molasses at 0.2 % w/v is used as UV screen agent that will distinguish it from its action as a phagostimulant at ≥ 0.5 % w/v (Burges 1998).

Despite all the screening agents possessing their wavelength spectrum in the range of UV-A (320-400 nm) and UV-B (280-390 nm) as seen in Fig. 4, only sodium lignosulfonate (UV 1) and molasses (UV 2) gave higher average absorbance of 10 and 3.5, respectively, over a broad range of spectrum, especially for sodium lignosulfonate. Although Congo red gave lower average absorbance of 0.09, yet the absorbance was spread over a wide wavelength range from 200 to 400 nm which provided better UV protection. Moreover, the persistent nature of Congo red (synthetic azo dye) with a property to remain inert under field conditions and provide enhanced UV resistance. On the other hand, folic acid (UV absorbance - 0.7) has a tendency to get degraded rapidly by heat, cold, and exposure to light, including sunlight which would finally affect its overall phostabilization properties.

Half-life studies. The half-lives of entomotoxicity (Tx) potential of different Bt fermented broths and formulations (with and without UV screens) is presented in Fig. 5. The residual Tx (percent Tx remaining after UV exposure) data of different fermented broths and package formulations fitted fairly well into first order rate model (R^2 from 0.8 to 0.93 and first order rate constants; k = 0.1735, 0.1118, 0.2629 and 0.5835 d^{-1} for non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and soya fermented broths and k = 0.0622, 0.0729, 0.0768 and 0.2423 d^{-1} for non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and soya formulations, respectively) with truncation at 6.5 d (fermented broths); 50 d (package formulations with UV screen) and 125 d (package formulations without UV screen). The difference in UV resistance between Bt fermented broths and package formulations could be due to two reasons – a) stabilization of the broth by adjuvants/additives and, b) centrifugation of the broth resulting in higher suspended solids concentration leading to less exposure of spores, δ-endotoxin and other virulence factors like chitinases, vegetative insecticidal proteins, phospholipases and cytolytic toxins. The virulence factors, spore and crystal toxin were probably embedded inside the concentrated solids rather than being dispersed free in the supernatant.

Spore count has been used to calculate $T_{0.5}$ as a measure of UV resistance by many researchers (Cohen et al. 1991; Burges 1998; Bishop 2002). However, spore count cannot be an accurate representation of Tx as reported in our earlier work (Yezza et al. 2005). Therefore, in this study, half-lives were calculated on the basis of residual Tx as it would provide direct indication of UV inactivation and the results for UV treatments were significantly different ($F = 3.67$; df = 5, 24; $P < 0.05$). Herman et al. (2002) also advocated that it was more precise if calculations of $T_{0.5}$ were based on residual Tx.

Fermented broths. The half lives (Fig. 5) and $T_{0.9}$ (time required for Bt formulation to lose 90 % of its Tx by action of UV) of different fermented broths, followed the order: H > NH > SIW > soya and were found to be (in d) as 6.2 > 3.99 > 2.63 > 1.19 and 20.59 > 13.27 > 8.76 > 3.95, respectively. $T_{0.5}$ of hydrolyzed sludge was higher probably due to lower concentration of protease in the medium (protease of 0.8 IU/ml for H sludge vis-à-vis 2.4 IU/ml for NH) which resulted in lower inactivation of crystal protein during storage and hence better residual entomotoxicity. Proteases are well known to cause degradation of crystal proteins and act as inhibitors of potency of Bt formulations (Burges 1998). On the contrary, although protease activity of fermented non-hydrolyzed sludge was higher than starch industry wastewater and soya and yet $T_{0.5}$ of non-hydrolyzed sludge was higher than starch industry wastewater and soya. This was probably due to overriding factor of relatively bigger floc size of non-hydrolyzed sludge and hydrolyzed sludge (particle size of Bt fermented broths: non-hydrolyzed sludge = 35 μm; hydrolyzed sludge = 25 μm; starch industry wastewater = 6 μm and soya = 3 μm) which formed a sheath around spores, protected the crystal protein and other virulence factors against UV effect. Lower particle size in case of soya and starch industry wastewater did not provide the advantage of UV resistance (Brar et al. 2004a).

Formulations (without UV screens). When the formulated package products (without UV screen) were exposed to UV, half-lives of Tx were higher relative to fermented broths (Fig. 5). This was due to the fact that the protease activity was not observed in formulations at acidic pH (4.5 ± 0.1) where proteases were not active. $T_{0.5}$ (in d) was found to be in the order: non-hydrolyzed sludge > hydrolyzed sludge > starch industry wastewater > soya as 11.14 > 9.51 > 9.02 > 2.8. The non-hydrolyzed sludge, hydrolyzed sludge and starch industry wastewater based formulations showed higher residual entomotoxicity relative to soya formulations. Half-life of conventional soya based Bt formulations in field conditions usually ranged from 16 h to 2 d (Ragaei 1998). Moreover, when UV spectra of non-sterilized non-hydrolyzed sludge and hydrolyzed sludge sludge were taken (Fig. 4), absorbance was very high (10) for non-hydrolyzed sludge (wavelength range 200-400 nm) and 2 for hydrolyzed sludge (wavelength range 200-300 nm). Furthermore, domestic sludge has been reported to possess components with chromophoric compounds or auxochromes (majority of fulvic, humic and hymathomelanic acids) with absorbance at 350 nm (Manka et al. 1974). These components in the

concentrated suspensions (more cations) could have acted as natural UV screens in non-hydrolyzed sludge and hydrolyzed sludge based formulations. Meanwhile, the UV resistance could be further enhanced by addition of UV screens.

Formulations (with UV screens). When molasses (0.2 % w/v) was chosen as a selective UV screen (Fig. 5), the UV resistance (half-life in d) followed the order: starch industry wastewater > non-hydrolyzed sludge > hydrolyzed sludge > Foray 76B > soya (10.9 > 10.72 > 9.54 > 7.23 > 6.43). $T_{0.5}$ was relatively higher ($P > 0.05$) for starch industry wastewater and soya, whereas for hydrolyzed sludge, $T_{0.5}$ was equal and for non-hydrolyzed sludge, it was slightly lower due to unknown reasons. Interestingly, the half-lives of alternative media based formulations, namely, non-hydrolyzed sludge, hydrolyzed sludge and starch industry wastewater were higher than the UV agent amended commercial Foray 76B formulations ($T_{0.5}$ = 7.23 d, Fig. 5).

Thus, this study will be instrumental in setting a base for probable additives/adjuvants recipe for non-conventional wastewater/wastewater sludge based Bt formulations. The best combination of additives recommended are sorbitol, sodium monophosphate, sodium metabisulphite (suspending agents); molasses, soya flour (phagostimulants); molasses and skimmed milk powder (rainfasteners); sorbic and propionic acids (anti-microbial agents) and sodium lignosulphate; molasses and Congo Red (UV resistance screens). Furthermore, this study showed that the residual entomotoxicity was higher for non-hydrolyzed sludge, hydrolyzed sludge and starch industry wastewater based formulations after exposure to UV (200-400 nm) in comparison to conventional soya based control and commercial Bt formulation. Higher half-lives for wastewater/wastewater sludge based Bt formulations adds newer Bt products which may have higher field efficacy thus enhancing marketability and gamut of biopesticides. The total solids concentration in final formulated product (with Tx of 19.5 x 10^9 SBU/l) for non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and soya was 22, 19.5, 19.6 and 21.1 % w/v, respectively with corresponding viscosity of 100, 50, 15 and 20 cP, respectively. These viscosity values were lower than Foray 76B (600 cP) enhancing their compatibility with spray equipments during field application.

Acknowledgments

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. The views and opinions expressed in this article are those of authors. We are also thankful to Natural Sciences and Engineering Research Council of Canada, Canadian Forestry Service and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing scholarship to Satinder K. Brar.

References

APHA, AWWA, WPCF. 1998. Standard Methods for Examination of Water and Wastewaters. (Clesceri, L.S., A.E. Greenberg, and A.D. Eaton, eds.), 20th edn. American Public Health Association, Washington, DC, USA.

Barnabé, S., M. Verma, R. D. Tyagi, and J. R. Valéro. 2005. Culture media for increasing biopesticide producing microorganism's entomotoxicity, methods of producing same, biopesticide producing microorganisms so produced and method of using same. US patent International application PCT/CA2005/000235.

Bartelt, R. J., M. R. McGuire, and D. A. Black. 1990. Feeding stimulant for the corn borer (Lepidoptera: Pyralidae): additives to a starch based formulation for *Bacillus thuringiensis*. Environ. Entomol. 19(1): 182–189.

Bartlet, E., D. Parsons, I. H. Williams, and S. J. Clark. 1994. The influence of glucosinolates and sugars on feeding by the cabbage stem flea beetle, *Psylliodes chrysocephala.* Entomol. Exp. Appl. 73: 77–83.

Beegle, C. C. 1990. Bioassay methods for quantification of *Bacillus thuringiensis* δ-endotoxin, Analytical Chemistry of *Bacillus thuringiensis*, pp.14-21. In L.A. Hickle and W.L. Fitch (eds.), Analytical Chemistry of *Bacillus thuringiensis*. American Chemical Society, USA.

Behle, R. W., M. R. McGuire, and B. S. Shasha. 1997a. Effects of sunlight and simulated rain on the residual activity of *Bacillus thuringiensis* formulations. J. Econ. Entomol. 90: 1506-1516.

Behle, R. W., M. R. McGuire, R. L. Gillespie, and B. S. Shasha. 1997b. Effects of alkaline gluten on the insecticidal activity of *Bacillus thuringiensis*. J. Econ. Entomol. 90: 354–360.

Bishop, A. 2002. *Bacillus thuringiensis* insecticides, pp. 160–175. In R. Berkley (ed.), Applications and systems of *Bacillus* and relatives. Blackwell Science Inc., Malden, MA.

Bracey, D., C. D. Holyoak, and P. J. Coote. 1998. Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? J. Appl. Microbiol. 85: 1056-1066.

Brar, S. K., M. Verma, R. D. Tyagi, J. R. Valéro, R. Y. Surampalli, and S. Banerji. 2004a. Comparative rheology and particle size analysis of various types of *Bacillus thuringiensis* fermented sludges. J. Residuals Sci. Technol. 1: 231-237.

Brar, S. K., M. Verma, R. D. Tyagi, J. R. Valéro, R. Y. Surampalli, and S. Banerji. 2004b. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations.Water, Sci. Technol. 50: 229-236.

Brar, S. K., M. Verma, R. D. Tyagi, J. R. Valéro, and R. Y. Surampalli. 2005. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges. Process Biochem. 40: 2695–2705.

Burges, H. D. 1998. Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments. Kluwer Academic Publishers, Dordrecht, Netherlands.

Castillejos, V., J. Trujillo, L. D. Ortega, J. A. Santizo, J. Cisneros, D. I. Penagos, J. Valle, and T. Williams. 2002. Granular phagostimulant nucleopolyhedrovirus formulations for control of *Spodoptera frugiperda* in maize. Biol. Control 24: 300–310.

Chatfield, C. 1983. The design and analysis of experiments: 1. Comparative Experiments. pp. 224-256. In Statistics for Technology. 3rd edition. Chapman and Hall Inc., Bristol, U.K.

Cohen, E., H. Rozen, T. Joseph, S. Braun, and L. Margulies. 1991. Photoprotection of *Bacillus thuringiensis* var. *kurstaki* from ultraviolet irradiation. J. Invertebr. Pathol. 57: 343-351.

Dunkle, R. L., and B. S. Shasha. 1989. Response of starch encapsulated *Bacillus thuringiensis* containing UV screens to sunlight. Environ. Entomol.18: 1035–1041.

Eagan, K. 2002. Demand for pesticides on the rise. Grounds Maintenance. PRIMEDIA Business Magazines & Media Inc.

Entwistle, P. F., Cory, J. S., Bailey, M. J., and S. Higgs (eds.) 1993. *Bacillus thuringiensis,* An environmental biopesticide: Theory and practice. New York: John Wiley & Sons.

Farrar Jr, R. R., and R. L. Ridgway. 1995. Enhancement of activity of *Bacillus thuringiensis* Berliner against four Lepidopterous insect pests by nutrient based phagostimulants. J. Entomol. Sci. 30: 29–42.

Gillespie, R. L., M. R. McGuire, and B. S. Shasha. 1994. Palatability of flour granular formulations to European corn borer larvae (Lepidoptera: Pyralidae). J. Econ. Entomol. 87: 452–457.

Herman, R. A., J. D. Wolt, and W. R. Halliday. 2002. Rapid degradation of the Cry1F insecticidal protein in soil. J. Agric. Food Chem. 50: 7076-7078.

Lisansky, S. G., R. J. Quinlan, and G. Tassoni. 1993. The *Bacillus thuringiensis* Production Handbook. CPL Press, Newbury, pp.124.

Lopez Jr, J.D., T. N. Shaver, and P. D. Lingren. 1994. Evaluation of feeding stimulants for adult *Helicoverpa zea*, pp. 920–924. In Proceedings, Beltwide Cotton Production and Research Conferences, National Cotton Council, Memphis, TN.

Lopez Jr, J. D., and P. D. Lingren. 1994. Feeding response of adult *Helicoverpa zea* (Lepidoptera: Noctuidae) to commercial phagostimulants. J. Econ. Entomol. 87: 1653–1658.

Manka, J., M. Rebhun, A. Mandelbaum, and A. Bortinger. 1974. Characterization of organics in secondary effluents. Environ. Sci. Technol. 8: 1017-1020.

McGuire, M. R., and B. S. Shasha. 1992. Adherent starch granules for encapsulation of insect control agents. J. Econ. Entomol. 85: 1425-1433.

McGuire, M. R., B. S. Shasha, C. E. Eastman, and H. Oloumi-Sadeghi. 1996. Starch and flour based sprayable formulations- effect on rainfastness and solar stability of *Bacillus thuringiensis*. J. Econ. Entomol. 89: 863-869.

Petersen, S. B., V. P. Jonson, P. Fojan, R. Wimmer, and S. Pedersen. 2004. Sorbitol prevents the self-aggregation of unfolded lysozyme leading to and up to 13°C stabilisation of the folded form. J. Biotechnol. 114: 269–278.

Piper, P., C. O. Calderon, and K. Hatzixanthis. 2001. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiol. 147: 2635-2642.

Quinlan, R. J. 1990. Registration requirements and safety considerations for microbial pest control agents in the European Economic Community, pp 11-18. In M. Laird, L.A. Lacey, and E.W. Davidson (eds.), Safety of microbial pesticides. CRC Press, Boca Raton, Florida.

Ragaei, M. 1998. Laboratory evaluation of shellac as ultraviolet screen for the *Bacillus thuringiensis* var. *entomocidus* against *Spodoptera littoralis* larvae. Anz. Schädlingskunde, Pflanzenschutz,Umweltschutz 71: 132-134.

Sachdeva, V., R. D. Tyagi, and J. R.Valéro. 2000. Production of biopesticides as a novel method of wastewater sludge utilization/ disposal. Water Sci. Technol. 42: 211–216.

Shapiro, M. 1989. Congo red as an ultraviolet protectant for the gypsy moth (Lepidoptera: Lymantriidae) nuclear polyhedrosis virus. J. Econ. Entomol. 82: 548–550.

Shapiro, M., E. Dougherty, and J. J. Hamm. 1992. Compositions and methods for biocontrol using fluorescent brighteners. United States Patent 5,124,149.

Shasha, B. S., M. R. McGuire, and R. W. Behle. 1998. Lignin-based pest control formulations. US Patent 5, 75, 0467.

Smirnoff, W. A., and A. Juneau. 1982. Physical analysis of the dispersion of *Bacillus thuringiensis* against spruce budworm. Can. Serv. Can. For. Centre Rech For. Laurentides, Ste-Foy, Quebec, Report LAU-X-55.

Tamez-Guerra, P., M. R. McGuire, R. W. Behle, B. S. Shasha, and L. J. Galan-Wong. 2000. Assessment of microencapsulated formulations for improved residual activity of *Bacillus thuringiensis*. J. Econ. Entomol. 93: 219–225.

Tirado-Montiel M. L., R. D. Tyagi, J. R. Valéro, and R. Y. Surampalli. 2003. Production biopesticides using wastewater sludge as a raw material – Effect of process parameters. Water Sci. Technol. 48 (8): 239-246.

Tyagi, R. D., D. Kluepfel, and D. Couillard. 1998. Bioconversion of cheese whey into organic acids. In A.M. Martin (ed.), Bioconversion of waste materials into industrial products, pp 313-333. Elsevier Science Publishers Ltd., London.

Vidyarthi, A. S., R. D. Tyagi, J. R. Valéro, and R. Y. Surampalli. 2002. Studies on the production of *B-thuringiensis* based biopesticides using wastewater sludge as a raw material. Water Res. 36 (19): 4850-4860.

WHO Food Additives Series: 5. 1974. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers and thickening agents. Joint FAO/WHO Expert Committee on Food Additives 25 June - 4 July 1973, Geneva, Switzerland.

WHO Food Additives Series: 42. 1999. International Programme on Chemical Safety. Safety evaluation of certain food additives. Prepared by the Fifty-first meeting of the Joint FAO/WHOExpert Committee on Food Additives (JECFA), Geneva, Switzerland.

Wirtz, K. R., S. E. Lebo, and M. E. Sanford. 1999. Method for providing a stable protective coating for UV sensitive pesticides. United States Patent 5,939,089.

Xu, Y., S. Barringer, and V. Alvarez. 2005. Cause and prevention of cane molasses gelling. J. Food Sci. 70(8): C461-464.

Yezza, A., R. D. Tyagi, J. R. Valéro, R. Y. Surampalli, and J. Smith. 2004. Scale-up of biopesticide production processes using wastewater sludge as a raw material. J. Ind. Microbiol. Biotechnol. 31(12): 545 - 552.

Yezza, A., R. D. Tyagi, J. R. Valéro, and R. Y. Surampalli. 2005. Production of *Bacillus thuringiensis* based biopesticides in batch and fed-batch cultures using wastewater sludge as a raw material. J. Chem. Technol. Biotechnol. 80: 502-510.

Table 1. Wastewater sludge and starch industry wastewater (SIW) characteristics

Parameter (s)	**Wastewater sludge ± SE**		**SIW± SE**	
Total solids (g/liter)	24	± 0.84	17.4	±0.2
Total volatile solids (g/liter)	18.8	±0.16	14.4	±0.1
Suspended solids (g/liter)	16.7	±0.15	2.3	±0.15
Volatile suspended solids (g/liter)	14.3	±1.4	2.3	±0.4
pH	5.4	±0.1	3.0	±0.1
Concentration (mg/kg of total solids)				
Total Carbon	287307	±4678	649854	±6435
Total Nitrogen	43683	±236.6	43648	±1711
Total Phosphorus	8901	±131	33858	±2134
$N\text{-}NH_3$	986	±189.4	111.2	±48.4
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$	16.7	±1.11	5	±1.2
$P\text{-}PO_4^{3-}$	5238	±247	14981	±2215
Al	5394	±333	56889	±1422
Ca	13248	±368.4	12008	±126
Cd	2.49	±1.12	ND	
Cr	27	±1.12	1.1	±0.06
Cu	388	±136	326.5	±159.5
Fe	12391	±592	8061.9	±758.6
K	1086	±346	22584	±3432
Pb	27.6	±5.3	3	±1.8
S	4327	±621	2288.3	±61.7
Zn	321	±192	238.1	±86.2
Na	1255	±331	2196.7	±225
Ni	9.42	±3.61	ND	

ND – Not detectable; SE – Standard error; SE was obtained by analyzing three samples

Table 2. Screening and selection of different adjuvants/additives for Bt formulations

Suspending agents[†] (20 % w/v)	**Phagostimulants[†] (0.5 % w/v)**	**Stickers/Adherents[†] (0.2 % w/v)**	**Anti - microbial agents[†] (0.5 % w/v)**	**UV Screens[†] (0.2 % w/v)**
Suspendibility	Tx increase	Sticking	Contamination	VS and Tx losses
Sorbitol (D1)[a,b,c,d]	Glucose (P1)	Molasses (R1)[a,b,c]	Sorbic acid (AM-1) [a,b,c,d]	Sodium lignosulphonate (UV1) [a,b,c,d]
Sucrose (D2)	Sucrose (P2)	Ghatti gum (R2)	Methyl para benzoate (AM-2)[d]	Molasses (UV2) [a,b,c,d]
Sodium monophosphate (D3) [a,b,c,d]	Molasses (P3)[a,b]	Carboxy methyl cellulose (R3)	Sodium metabisulfite (AM-3)	Congo Red (UV3) [a,b,c,d]
Sodium metabisulphite (D4) [a,b,c,d]	Oatmeal (P4)[d]	Skimmed milk powder (R4)[a,d]	Citric acid (AM-4)	Folic acid (UV4)
Sodium silicate (D5)	Soya flour (P5)[a,c,d]	Xanthan gum (R5)	Lactic acid (AM-5)	p-Amino benzoic acid (PABA)- (UV5)[c]
Veegum (regular grade) (D6)	Corn meal (P6)[a]	Polyvinylpyrrolidone (R6)	Propionic acid (AM-6) [a,b,c,d]	Benzilidine sulphonic acid (BSA)- (UV6)

D-Dispersant; P-Phagostimulant; R-Rainfasteners; AM-Anti-microbial; UV-Ultra-violet; 1...6-number code of specific adjuvant
Shaded row represents the screening criteria for each adjuvant/additive
a – Non-hydrolyzed (NH) formulations; **b** –Hydrolyzed (H) formulations; **c** – Starch industry wastewater (SIW) formulations; **d** – soya formulations
The adjuvants with superscript a,b,c,d represent > 50 % suspendibility; >10 % increase in Tx; >50 % sticking; no microbial contaminant and <50 % VS and Tx losses.
[†] Each adjuvant was tested independently ("one-at-a-time-basis")

Table 3. Microbiological purity test schedule

Microorganisms	Medium	Environmental Conditions	Incubation Time	Inference Test	*Upper limits of concentration (CFU/ml)
Yeast & Mold	Malt Extract Agar	pH = 3.5; T = 30 ± 1°C	3 - 5 d	Colonial morphology (dumb bell shaped under 100X magnification)	<100
Fecal Streptococci	Slanetz & Bartley Agar	Temp = 37 ± 1°C	48 h	Pink / Dark red with white border	$<1 \times 10^4$
Coliforms/ Fecal Coliforms	Lauryl sulfate broth/ MFC Agar /m- Endo Agar	Temp = 44.5 ± 1°C	24 h	Pink or blue colonies ("chain-like'' under the microscope)	<10
Staphylococcus aureus	TSB ; plating on Baird Parker Agar + 5 % Egg Tellurite	Temp = 35 - 37 ± 1°C	48 h (check at 24 & 46 h)	Gram stain positive colonies (black and glossy) – resemble "bunch of grapes" under the microscope	<1
Salmonella and Shigella	Lactose Broth 35°C for 24-48 h and then Selenite (10ml) & Tetrathionate Broth (10ml) and testing growth on Brilliant green phenol red lactose sucrose agar and bismuth sulfite agar	Temp = 35 ± 1°C	18 - 24 h	Blackening with sheen (rod shaped without spores or crystal protein and characteristic flagella under the microscope) are *Salmonella* colonies and green, moist, flat and transparent colonies represent *Shigella* presence	<1/10

(References: Quinlan 1990; Lisansky et al. 1993; Burges 1998; Bishop 2000)
According to IUPAC recommendation, upper limit of concentration for microbial contaminants should be expressed in CFU/g and CFU/ml for solid and liquid formulations, respectively.

Table 4. Screening results of different anti-microbial agents

	Yeast and Mold				***Enterococcus***				**Coliforms**				***Escherichia coli***				***Salmonella typhae***				***Staphylococcus sp.***			
T=7 d	NH	H	SIW	Soya	NH	H	SIW	Soya	NH	H	SIW	Soya	NH	H	SIW	Soya	NH	H	SIW	Soya	NH	H	SIW	Soya
AM-1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AM-2	7	8	37	nd	31	nd	62	nd	47	44	13	nd	34	8	2	1	1	1	3	4	nd	1	nd	nd
AM-3	55	34	31	28	28	33	43	33	63	33	38	22	46	4	4	7	1	2	5	2	1	1	1	nd
AM-4	108	31	nd	26	39	45	45	22	55	19	101	26	71	9	11	3	4	4	6	3	1	1	2	3
AM-5	132	34	61	38	46	51	12	25	68	nd	nd	45	4	8	3	5	1	5	7	1	1	1	1	2
AM-6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Control	143	115	213	331	136	68	141	126	114	125	134	158	210	223	231	251	208	134	84	67	107	208	117	109
T = 15 d																								
AM-1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AM-2	10	21	69	nd	89	3	121	nd	189	105	34	1	71	12	15	3	2	1	8	9	nd	1	nd	nd
AM-3	84	95	59	58	68	67	83	95	198	39	86	68	83	15	18	15	1	2	11	7	1	1	2	nd
AM-4	9.1*	96	4	67	84	87	91	73	123	27	134	81	151	23	38	8	6	7	17	8	1	1	3	7
AM-5	9.2*	1*	1.6*	75	93	101	68	100	158	1	248	136	9	28	59	9	2	11	21	3	1	2	3	4
AM-6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Control	21*	2.9*	6.8*	9.6*	221	320	410	362	289	333	326	357	458	485	512	563	326	456	305	308	258	269	321	269
T = 30 d																								
AM-1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AM-2	2*	1*	2.3*	nd	100	8	186	nd	335	188	87	3	138	35	65	3	3	6	10	9	3	18	5	nd
AM-3	.89*	5*	3.4*	3*	105	105	211	185	638	168	163	131	151	39	69	43	28	28	38	18	7	35	25	3
AM-4	1.2*	3*	2.7*	2*	164	94	203	238	453	210	209	236	259	68	82	53	59	64	57	29	43	43	36	27
AM-5	3.1*	4*	2.4*	1*	191	135	204	325	381	18	364	343	45	77	94	47	86	63	68	38	28	44	51	33
AM-6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
**Control	6.5	0.68	1.2	1.6	2	4.5	6.1	4.9	3.1	6.5	8.3	7.9	7.4	7.6	1.3	1.9	1.1	1.5	2.3	1.9	3.2	4.5	6.2	4.7

Microbial concentrations are reported in CFU/ml (colony forming units) and are average of 05 replicates ($P < 0.05$). At T = 1 day, the concentration was non–detectable (nd) for all anti–microbial agents for all fermented broths; nd – non–detectable; The control (without anti-microbial agents) was not considered further on the basis of range of contamination observed; * Actual value = value x 10^2 CFU/ml; ** Actual value = value x 10^3 CFU/ml in the row

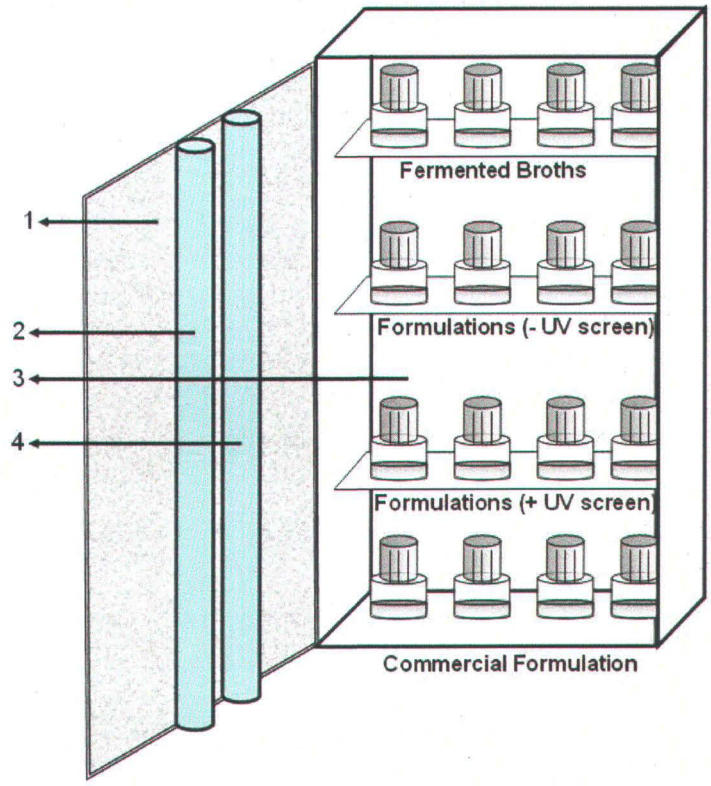


Fig. 1. Schematic of set-up of UV Inactivation studies (1-reflecting surface; 2- UV-A tube; 3 – reflecting aluminium sheet and 4- UV-B tube)

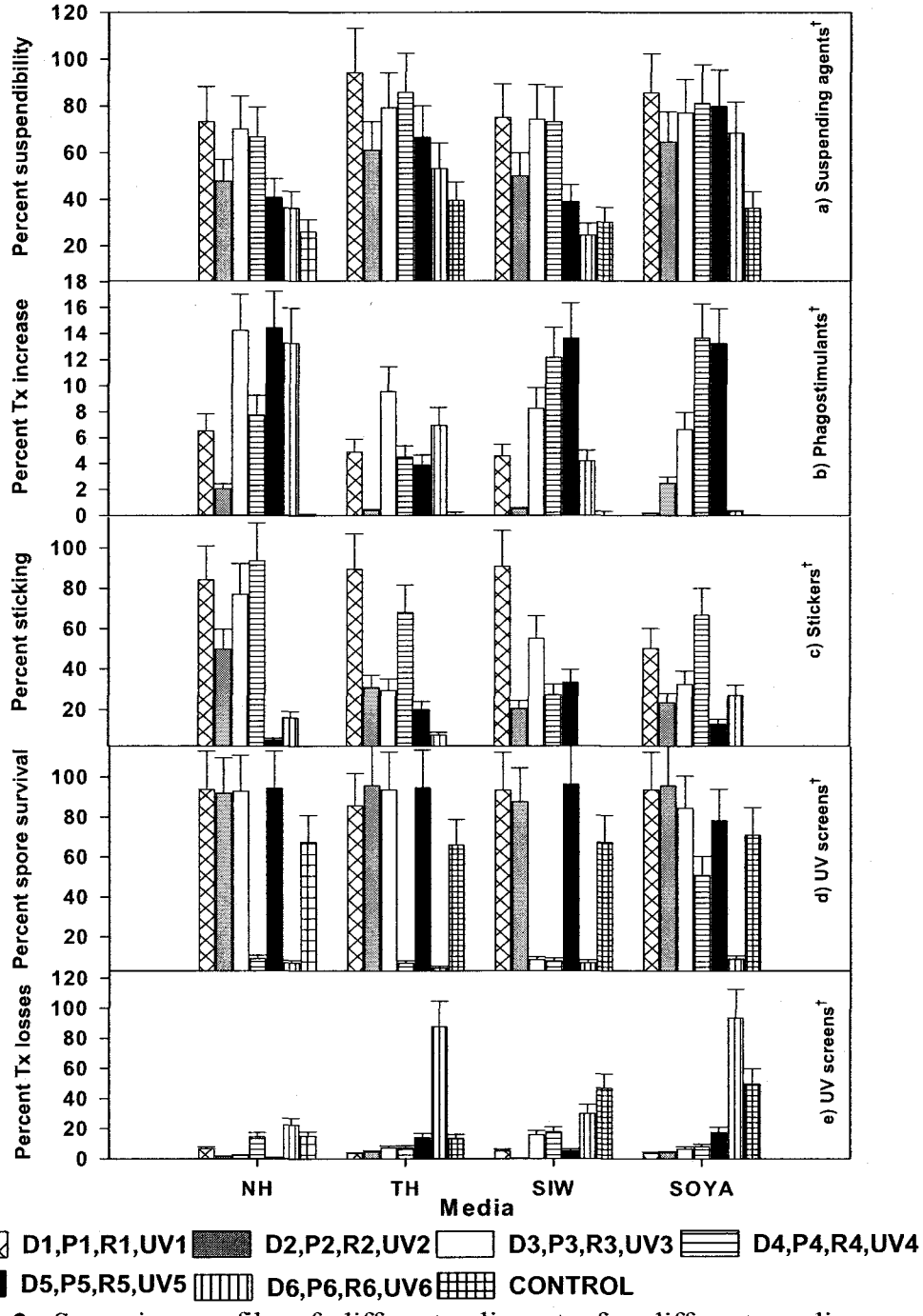


Fig. 2. Screening profile of different adjuvants for different media, namely, non-hydrolyzed sludge (NH); hydrolyzed sludge (H); starch industry wastewater (SIW) and soya: a) suspending agents; b) phagostimulants; c) stickers; d) and e) UV screens (UV1 to UV5). Error bars represent standard deviations. Control refers to sample without adjuvants. All symbols D_x, P_x, R_x, UV_x (x=1, 2, 3, 4, 5, 6) are defined in Table 2. † Each adjuvant was tested independently ("one-at-a-time-basis").

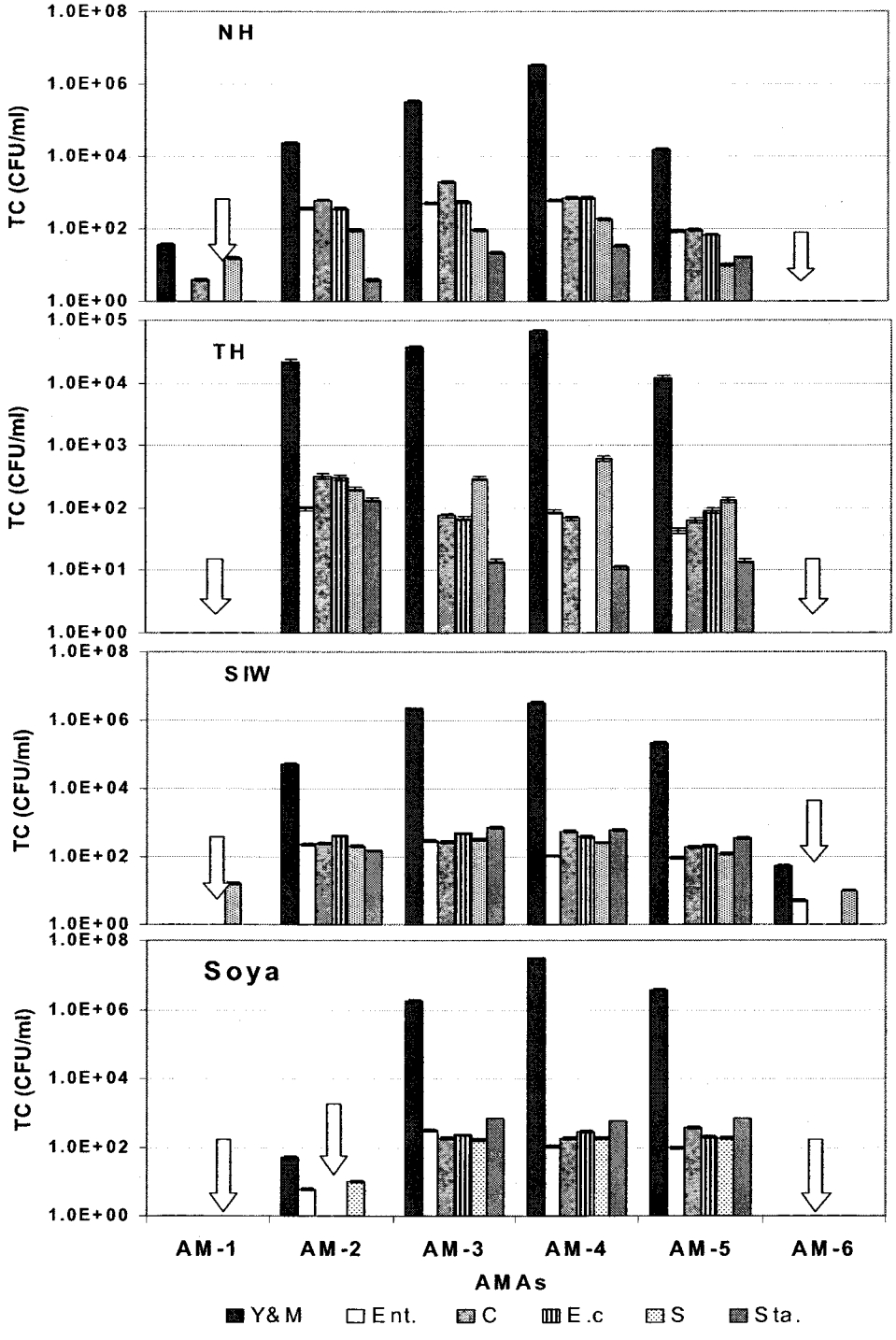


Fig. 3. Screening of different anti-microbial agents (AMAs) for non–hydrolyzed sludge (NH); hydrolyzed sludge (TH); starch industry wastewater (SIW) and soyameal formulations (period – two months) ; (Y&M – yeast and mold, Ent – *Enterococcus*, C – Coliforms, E.c – *Escherichia coli*, S- *Salmonella*, Sta.- *Staphylococcus,* TC–total cell count). Error bars indicate standard deviations. The arrows indicate screened AMAs with higher AMA action for different formulations than other AMAs.

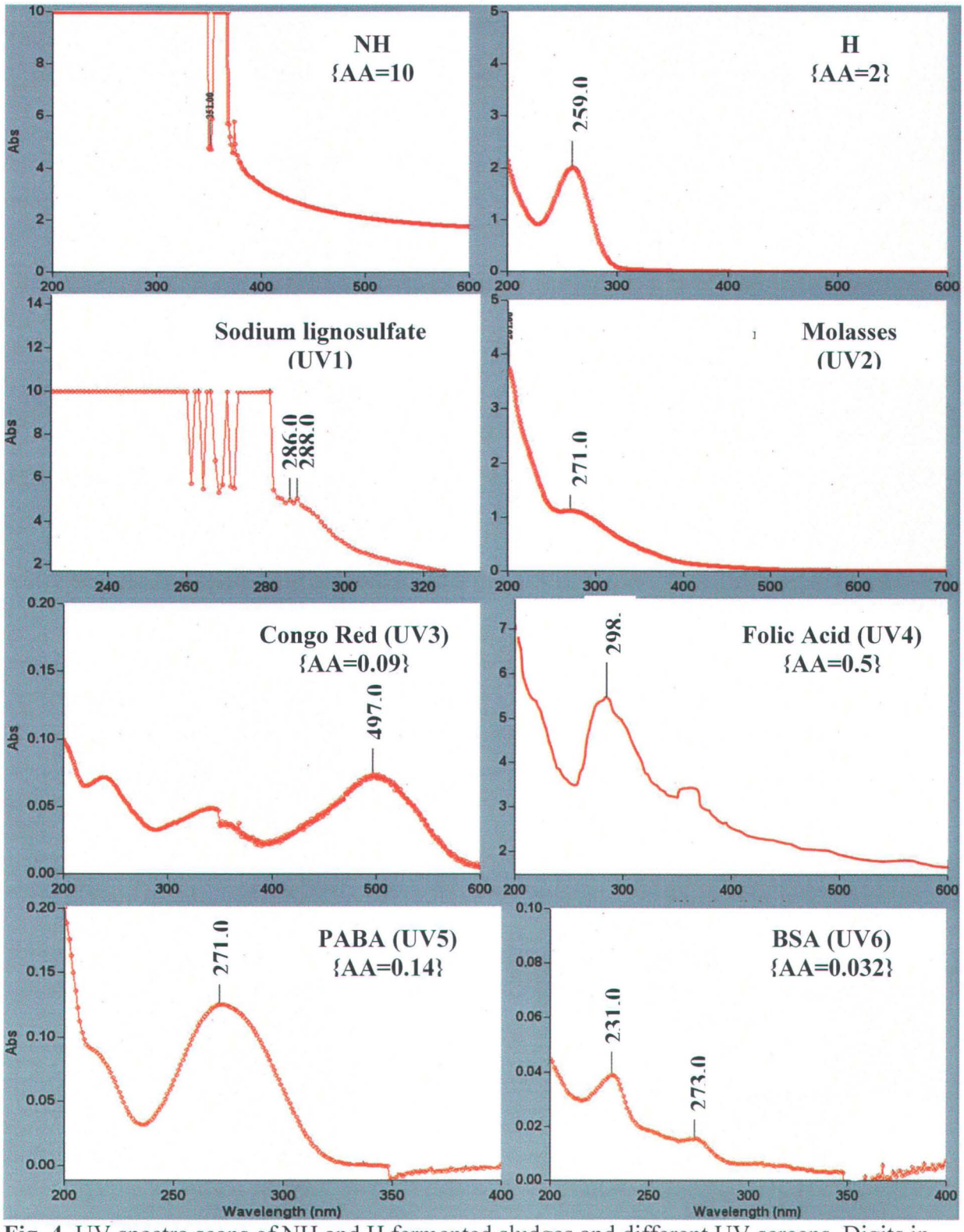


Fig. 4. UV spectra scans of NH and H fermented sludges and different UV screens. Digits in parentheses represent average absorbance (AA).

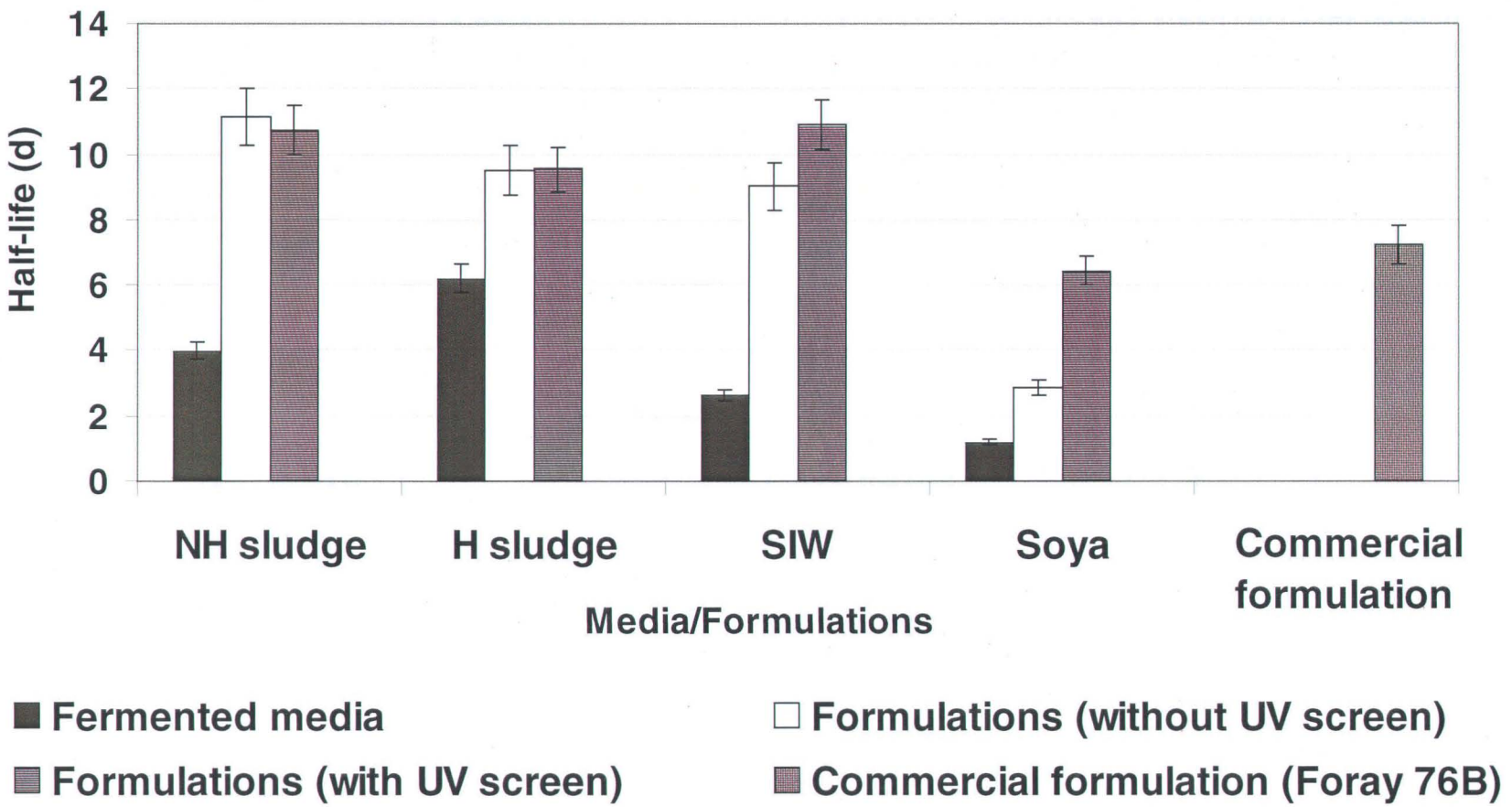


Fig. 5. Half-lives of entomotoxicity potential (Tx) for different fermented media (control) and formulations when exposed to UV radiation; based on pooled data. Error bars represent standard deviations.

Partie IV

Development of Sludge based Stable Aqueous *Bacillus thuringiensis* Formulations

S.K. Brar[1], M. Verma[1], R.D. Tyagi[1], J.R. Valéro[1], R.Y. Surampalli[2] and S. Banerji[3]

[1]INRS-ETE, Université Du Québec 2700 rue Einstein, Sainte- Foy Québec, G1V 4C7, Canada
[2]U.S. EPA, P.O. Box 172141, Kansas City, Kansas 66117
[3] University of Missouri - Columbia, Columbia, USA, MO 65211

Water, Science and Technology (2004)
50(9):229-236

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. et Banerji, S.K. (2004). Development of sludge based stable aqueous *Bacillus thuringiensis* formulations. *Water Sci. Technol.* 50(9): 229-236.
http://www.iwaponline.com/wst/05009/wst050090229.htm

Partie V

Starch Industry Wastewater based Stable *Bacillus thuringiensis* Liquid Formulations

Satinder K. Brar[1], M. Verma[1], R. D. Tyagi[1*], J. R. Valéro[1] and R. Y. Surampalli[2]

[1]INRS-ETE, Université Du Québec 2700 rue Einstein, Sainte- Foy Québec, G1V 4C7, Canada
[2]U.S. EPA, P.O. Box 172141, Kansas City, Kansas 66117

Journal of Economic Entomology (2005)
98(6):1890-1898

Suspensions aqueuses stables à partir des eaux usées de l'industrie de l'amidon fermentées par *Bacillus thuringiensis*

Résumé

Des suspensions aqueuses de *Bacillus thuringiensis* (Bt) ont été développées à partir de bouillons fermentés d'eaux usées de l'industrie de l'amidon (SIW) et une comparaison a été faite avec un milieu de référence à base de soya. La stabilité a été évaluée durant une année. La stabilité d'entreposage a été examinée en étudiant divers paramètres physico-chimiques (viscosité, tailles des particules, corrosion et homogénéité) et biologiques (contamination microbienne, viabilité des spores et potentiel entomotoxique) et ce, à différents niveaux de températures et de pH. Trois agents de suspension, soit le sorbitol, le monophosphate du sodium et le métabisulfite du sodium, ont été ajoutés à différentes concentrations aux bouillons fermentés. La combinaison de sorbitol et de monophosphate du sodium dans un rapport de 3:1 a donné les meilleurs résultats comme agent de suspension pour les deux formulations. La fermentation des SIW a permis d'augmenter le nombre de cellules et de spores viables de 10 et 4 fois, respectivement, et l'entomotoxicité de 1,7 fois par rapport aux valeurs obtenues avec le milieu à base de soya. Cependant, les deux formulations se détérioraient à pH 6 et 6,5 et à des températures de 40 et 50 °C. Par ailleurs, il n'y avait aucun signe de corrosion ou de contamination microbienne dans les deux types de formulations.

Mots-clés: *Bacillus thuringiensis*, biopesticide, formulation liquide, durée de conservation, soya, eaux usées de l'industrie de l'amidon.

ABSTRACT

Liquid formulations were developed from *Bacillus thuringiensis* (Bt) fermented broths of starch industry wastewater (SIW) and of soya. Stability studies were carried out for one year. Storage stability was tested by studying various physical/chemical (viscosity, particle size, corrosion and suspendibility) and biological (microbial contamination, viable spores and entomotoxicity) parameters at different pH levels and temperatures. Three different suspending agents, including sorbitol, sodium monophosphate and sodium metabisulfite, were added to fermented broth in different concentrations. Sorbitol and sodium monophosphate in the ratio 3:1 was the best suspending agent combination for both formulations. Starch industry wastewater fermentation yielded cell and viable spore counts 10- and 4-fold greater than those from soya medium, respectively, and a 1.7-fold increase in entomotoxicity. However, both formulations started deteriorating at pH 6 and 6.5 and temperatures 40 and 50°C. There were no signs of corrosion and microbial contamination in both types of formulations.

KEYWORDS: *Bacillus thuringiensis*; biopesticide; liquid formulation; shelf-life; soya; starch industry wastewater

INTRODUCTION

Infestation by pests in agriculture, forestry and public health sectors has been traditionally controlled by chemical pesticides, some of which have been currently replaced by eco-friendly biopesticides (Jarvis 2001). *Bacillus thuringiensis* (Bt) based biopesticides occupy a major share of the biopesticide market. There are numerous factors controlling the success of a biopesticide, including yield during production, stability of formulation and ease of application (Lisansky 1993, Burges 1998). Production of Bt biopesticides has moved from the use of synthetic to semi-synthetic and recently, to the use of non-conventional waste materials like wastewater/wastewater sludge (Lisansky et al. 1993, Vidyarthi et al. 2002, Tirado-Montiel et al. 2001, 2003).

Although production economy has been greatly improved, formulation cost continues to limit use of Bt biopesticides. Principal features that dictate the viability of a formulation include extended shelf life (storage stability), ease of application, higher field efficacy, synergy with pre-existing application equipment, and economy (Navon 2000, van Frankenhuyzen 2000). Despite many advances in Bt formulation research to overcome various drawbacks and introduction of microencapsulations and microgranules, much remains to be accomplished (Dunkle and Shasha 1989, McGuire and Shasha 1992, Behle et al. 1997, Shasha et al. 1998, Tamez-Guerra et al. 2000; Teera-Arunsiri et al. 2003, Martin 2004).

Production of Bt biopesticides from wastewater/wastewater sludge has been investigated extensively with respect to process optimization, pilot scale and formulation feasibility studies (Sachdeva et al. 2000, Vidyarthi et al. 2002, Tirado-Montiel et al. 2003, Barnabé 2004, Yezza et al. 2004a,b, Brar et al. 2004). Further, feasibility studies on formulation were carried out on Bt fermented wastewater sludge broth and were based on shake flask fermentation. Meanwhile, there has been use of other raw materials for Bt production including starch industry wastewater (SIW) which produces higher entomotoxicity (Tx, measure of entomopathogenic potency) yield and constant composition compared with wastewater sludge (Yezza et al. 2004a). The formulations development of SIW Bt fermented broth will have different process steps of and may respond differently to storage stability. Moreover, establishment of optimal composition of Bt formulation for SIW could lead to new commercial products. Bt liquid suspensions are the most widely used formulations in forestry (Burges 1998). Hence, the present study is an attempt to explore the Bt fermentation of SIW in bench scale fermenter and develop liquid suspensions of fermented SIW.

The shelf life stability (pH, temperature) of different formulations was also investigated. The Bt fermented SIW formulations were also compared with conventional soya formulations (control) by analyzing different physical-biological test parameters.

Materials and Methods

Bacterial Strain. *Bacillus thuringiensis* var. *kurstaki* HD-1 (ATCC 33679) was used in this study. The culture conditions, maintenance, inoculum production, and fermentation (biopesticide production) procedure are described elsewhere (Vidyarthi et al. 2002).

Bt Production Media. Two different media were utilized for Bt growth: Conventional medium of soybean meal that comprised (g/l): soybean meal, 15.0; glucose, 5.0; starch, 5.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.02; $CaCO_3$, 1.0 and starch industry wastewater (SIW) from ADM-Ogilvie (Candiac, Québec, Canada). The SIW was immediately utilized for fermentation as long term storage at even 4°C would lead to decay (slow degradation of starch).

Characterization of SIW. Total solids (TS), total volatile solids (TVS), suspended solids (SS) and volatile suspended solids (VSS); ammonia nitrogen ($N\text{-}NH_4^+$); pH; total Kjeldahl nitrogen (TKN); total phosphorus; metals concentration (Al, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Na, Ni, Pb and Zn) were determined according to standard methods (APHA, AWWA, WPCF 1998). Characteristics of SIW are presented in Table 1.

Fermenter Details. Fermentation was carried out in a bioreactor (15 liter, Biogenie Inc., Quebec, Canada) equipped with accessories and programmable logic control (inerfaced with iFix 3.5 software, Intellution, GE Fanuc Inc., USA) for dissolved oxygen, pH, anti-foam, impeller speed, aeration rate and temperature. Dimensions of the fermenter, wastewater charging and sterilization procedure are described elsewhere (Brar et al. 2005). The volumetric oxygen transfer coefficient (k_La) was measured by dynamic gassing out method (Aiba et al. 1973). This method comprises shut-down and starting of aeration in sequence during each sampling period. Consequent decrease and increase in dissolved oxygen (D.O.) was used to evaluate k_La by performing mass balance on D.O.

Downstream processing

Post-fermentation Procedure. Temperature of fermented broths (SIW and soya) was gradually lowered from 30 ± 1 to 20 ± 1°C. Individually sterilized 4M H_2SO_4 and 4M NaH_2PO_4 were aseptically mixed in volume ratio 1:1 and utilized to lower the pH of fermented broth from 7 ± 0.1 to 4.5 ± 0.1 by addition in small increments via automated peristaltic pumps integrated with the fermenter set-up. Later, the broth was collected aseptically in sterile HDPE containers (12 liter

capacity, VWR Canlab, Canada), sealed with Parafilm™ and stored in a freezing chamber (DuPont, USA) at -20°C until use for formulation.

Pre-formulation step. The frozen acidified broth (SIW and soya) was thawed in a water bath at room temperature. It was centrifuged aseptically in 500 ml HDPE bottles (Nalgene, USA) at 9682 g at 20 ± 1°C in a Sorvall RC 5C plus Macrocentrifuge (DuPont, USA). The concentrated solids (≈ 7-10 %w/v) obtained by centrifugation were further diluted with supernatant and additives as per subsequent protocol.

Storage Stability Experiment. The concentrated broth sample of known Tx (obtained by centrifugation) was further amended with different concentrations of adjuvants/additives (Table 2). The slurry thus obtained was blended in a Waring Blender. Five different series of formulations were prepared for soya and SIW named as Sy-1, Sy-2, Sy-3, Sy-4, Sy-5 and S-1, S-2, S-3, S-4 and S-5, respectively, by adding different concentrations of dispersing/suspending agents (Table 2) to the above slurry. Subsequently, the volume of the slurry was made up with the supernatant of the centrifuged fermented sludge so as to attain an entomotoxicity (Tx) approximately equivalent to Foray 76B (Abbott Laboratories, Chicago, IL, USA). The pH stability studies were carried out at 4.0 ± 0.1, 4.5 ± 0.1, 5.0 ± 0.1, 6.0 ± 0.1 and 6.5 ± 0.1 by adjusting the pH with 2N NaOH and H_2SO_4 and stored at room temperature (20 ± 1 °C) in HDPE bottles (150 ml capacity, VWR Canlab, Canada) for one year.

Similarly, temperature storage studies were carried out at various temperatures of 4 ± 1, 10 ± 1, 20 ± 1, 30 ± 1, 40 ± 1 and 50 ± 1°C with pre-adjusted pH 5.0 ± 0.1. Samples were drawn at regular intervals (0, 3, 7, 10, 15, 30, 60, 90, 120, 150, 180 and 365 d) for determination of physical (viscosity, particle size, suspendibility and corrosion) and biological characteristics (microbial contamination, viable spore and entomotoxicity).

Physical/Chemical and Biological Analysis. Viscosity and particle size were measured as per the method reported by Brar et al. (2005). A standard deviation of 8 and 10% was observed for viscosity and particle size measurements respectively. Iron and aluminum strips (10 cm x 5 cm) were immersed in different formulations at different pH overnight and visible signs of corrosion, if any, were noted. Bt fermented SIW could be prone to contamination due to the presence of residual starch and other nutrients which may create favorable conditions for undesired microbial growth. Thus, microbial contamination was tested as per the protocol reported by Lisansky et al. (1993).

Suspendibility. Suspendibility of aqueous flow formulations was tested by an indigenously designed method. Each formulation (50 ml) was suspended in 100 ml deionized water (ultrapure

Milli-Q water). The sample was compared with a well mixed suspension of industry standard (Abbott Labs., Chicago) as control. The suspended sample was well dispersed by shaking in a separating funnel several times and then letting it stand undisturbed for 30 minutes. Afterwards, 20 ml of the sample was withdrawn from the bottom to determine the amount of settled solids and supernatant from the top was utilized to determine the apparent turbidity in terms of optical density (O.D.) using a UV-Visible spectrophotometer (Varian Cary 100 Bio, USA) at λ_{max} of 450 nm (APHA, AWWA, WPCF 1998). The suspendibility of samples was defined as per equation (1) given below,

$$Suspendibility = \frac{Turbidity}{Total\ solids - Settled\ solids} \quad (1)$$

The percentage relative suspendibility (equation 2) was calculated, based upon Foray 76B.

$$Percentage\ \ Relative\ Suspendibility = \frac{\left(\frac{Turbidity}{Total\ solids - Settled\ solids}\right)_{sample}}{\left(\frac{Turbidity}{Total\ solids - Settled\ solids}\right)_{control}} \times 100 \quad (2)$$

Percentage standard deviation for suspendibility measurement was 5%.

Total Cell (TC) and Viable Spore (VS) Count. The procedure specified in Vidyarthi et al. (2002) was utilized for TC and VS count. Respective samples were heat treated ("heat shock") in a silicone oil bath (Thermo-Lift, Buchler Instruments, USA) at 80 ±1°C for 10 min and then cooled on an ice bath for 5 min before plating on tryptic soya agar. Plates were incubated for 16h and plates containing 30-300 colonies were counted. Percentage of spore survival was reported as 100 (N/N_0), where N and N_0 (CFU/ml) represent number of spores of each formulation after 1 year and before storage, respectively. Replicates were performed and data of three dilutions of the samples were analyzed with one way analysis of variance (ANOVA) to test significant difference at $P = 0.05$ (Chatfield, 1983). The standard deviation for cell and spore count was 7.0 and 8.0 %, respectively.

Bioassays. The entomotoxicity (Tx) was evaluated by bioassays using eastern spruce budworm larvae (SB) (*Choristoneura fumiferana* (Clemens), Lepidoptera: Tortricidae) in second instar, provided by Natural Resources Canada (Sault Ste-Marie, Ontario, Canada). The larvae were raised on an artificial diet for 7 days to obtain the third and fourth instar (L3-L4) larvae. The bioassays were conducted using the diet incorporation method (Beegle 1990). In this technique, 1.5 ml of appropriately diluted Bt samples of fermented SIW/soya were incorporated into 30 ml of molten agar based diet (at 60 ± 1°C), the composition of the artificial diet is described elsewhere (Tirado-Montiel et al. 2001). Afterwards, the mixture was distributed in aliquots of 1 ml in twenty 15 x 45 mm glass vials (VWR Canlab, Canada) with perforated plastic caps. For each sample, at least three dilutions were used, and hence sixty glass vials were used for each sample.

Sixty vials containing 1 ml artificial diet (C1) were used as a control and another control contained sterilized SIW/soya medium (C2). One L3-L4 larva was placed into each vial and allowed to feed ad libitum for 7 days at 25 ± 1°C. Mortality was monitored after 7 days. If mortality in control vials was higher than 10%, the experiment was repeated. The Tx of sample preparations was obtained by comparing the relative mortality (percentages) of SB larvae responsible for the destruction of conifers compared with the mortality induced by Foray 76B and expressed as relative spruce budworm units (SBU/µl). Foray 76 B contained spores and crystals of Bt var. *kurstaki* at a potency of 20.1 x 10^9 IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni*) (Hübner). On comparison of Tx of Bt fermented sludge samples, it was found that SBU reported in this study was 20-25 % higher than IU. Data were analyzed with one way analysis of variance (ANOVA) for significant test (Chatfield, 1983). The standard deviation for Tx measurement was 8–10 %.

Results and Discussion

Starch Industry Wastewater (SIW) Fermentation. Bt growth profile for SIW fermentation incorporating operational (agitation rate, air flow rate and dissolved oxygen (DO) concentration) and process performance (TC and VS counts, viscosity, particle size and density) parameters is illustrated in Figs. 1 a and b, respectively. The DO ranged from 50 to 85% with the agitation rate ranging from 250 to 400 rpm and air flow rate from 2.5 to 3.5 LPM. As is evident from Fig. 1a, DO varied (3 – 15 h) during active exponential phase of fermentation and later the variations leveled off during stationary phase until 48h.

TC increased from 1 x 10^7 to 1.67 x 10^9 CFU/ml until 21 h and then remained almost constant ($P > 0.05$) (Fig.1b). The corresponding ANOVA factors for TC and VS are given in Table 3. Similarly, VS increased from 6.5 x 10^5 to 8 x 10^8 CFU/ml up to 18h and attained constancy at 21 h (8.1 x 10^8 CFU/ml, $P > 0.05$). These values of TC and VS were 10 and 4-fold higher than soya where maximum specific growth rate (μ_{max}) was 0.31 with Tx of 8.78 and 9.54 x 10^9 SBU/L ($P > 0.05$) at 36 and 48h, respectively. The resulting Tx was 15.87 and 17.1 x 10^9 SBU/L ($P > 0.05$) at 36 and 48h, respectively and μ_{max} of 0.36 h^{-1}. Despite higher VS, percentage of sporulation was 48%, which could be due to excess carbon and nitrogen that resulted in accumulation of different metabolites and hence regulating spore-crystal formation mechanism (Alves et al. 1997). The increase in entomotoxicity relative to soya could be due to the fact that there was higher VS and also probably action of other virulent factors like enzyme systems and vegetative insecticidal proteins known to play a vital role in potency of the product (Prieto-Samsonov et al. 1997).

Density remained almost constant from 0.9865 to 1.03 g/ml all through the fermentation. Viscosity decreased until 9h of fermentation and then there was a monotonic increase with a hump at 21h when TC concentration practically stabilized. It would have been expected that viscosity should increase with TC, but this was not the case as viscosity was also a factor of changes occurring in SIW. Active generation of enzymes during exponential growth phase resulted in decrease in floc (loose aggregate mass of flocculated particles suspended in bulk water phase) sizes and hence lower resistance to flow. The physical regime was also altered by continuous manipulation of agitation and aeration rate in order to maintain DO. Similar peak in viscosity was observed towards the end of exponential phase in raw and hydrolyzed wastewater sludge Bt fermentation (Brar et al. 2005). Moreover, fermented SIW showed thixotropic and pseudoplastic behavior with corresponding consistency and flow behavior index of 1.46 $mPa.s^n$ and 1.04 respectively. The particle size showed a continuous decline until 48h of fermentation which was primarily controlled by physical parameters like agitation in the fermenter (Brar et al. 2005).

At the beginning of fermentation, k_La was higher at 160 h^{-1} and DO was almost 98% resulting in efficient oxygen transfer due to lower viscosity. The k_La is dependent on many factors, where viscosity of the liquid phase plays a dominant role (Kawase and Hashimoto 1996). The k_La reached a maximum of 235 h^{-1} at 12h which could be a function of the continuous increase in TC and DO requiring fewer adjustments in aeration and agitation rates. Eventually, k_La started decreasing with small fluctuations which could be a function of variable rheology (defined as change in physical form and flow behavior of broth influenced by viscosity and particle size) during fermentation. Most microorganisms produce extracellular polymeric substances (EPS) in the presence of excess carbohydrates (Lee et al. 1997) which is possible in the case of SIW fermentation resulting in rheology variations. There is a possibility of slight variation in Tx values during Bt fermentation of SIW. In fact, studies are being carried out in our laboratory to test the effect of variability of SIW composition on Bt fermentation.

Soya fermentation has not been discussed in detail as the results have been reported elsewhere (Barnabé 2004). The soya medium was more consistent showing fewer fluctuations in k_La.

Selection of optimal formulations. Five different formulations of Bt fermented soya and SIW were developed and were individually studied for physical (viscosity, particle size, suspendibility) and biological (Tx and VS) stability at different pH and temperature conditions over a period of one year. Further, the optimal formulation was based on quantitative judgment analysis (based on quantitative changes in physical and biological parameters over 1 year and noting the frequency of significant change, if any). Thus, the results presented henceforth discuss relatively most stable formulations.

Soya formulations. Figs. 2a, 2b and 2c illustrate the prominent features (particle size, viscosity and Tx) of Sy-3 formulations (optimal) of soya at different pH and temperatures. A study involving five different combinations of suspending agents was carried out to screen the best choice of suspending agents which form an important component of any biopesticidal suspension (Carroll 2001). Other formulations were ruled out on the basis of quantitative judgment analysis (discussed earlier). It is a known fact that an optimal biopesticide formulation is the one that possesses higher biological efficacy with compatible physical characteristics which aid in extended shelf-life and better application (Lisansky et al. 1993; Burges 1998).

Even the optimal Sy-3 formulation behaved differently when subjected to pH and temperature variability over a period of 365d. Viscosity decreased slightly from 15.7 to 14 cP at different pH (Fig. 2a). Moreover, the particle size also decreased slightly from 6 to 5 μm and the decrease was higher at higher pH. Considering the isoelectric point of bacteria to be pH 2 to 4 (Tenney and Stumm 1965), it was clear that soya particles would carry increasingly negative charges if the pH was increased above the isoelectric point. Therefore, at pH 6 and 6.5, the floc surface will be increasingly negatively charged. Thus, increased similar charges in the floc structure caused repulsion and hence expansion of floc matrix. Further, this floc may be subject to the activity of enzymes, especially proteases produced during Bt fermentation which is active at these pHs (Tyagi et al. 2001). This will cause degradation of floc matrix and hence influence the particle size to a great extent which also showed a similar declining profile (Fig. 2a). At lower pH, these issues may not arise and hence most of the formulations are developed at lower pH which may be particularly maintained by buffers (Burges 1998). At this juncture, role of sodium monophosphate as a buffer too cannot be denied making it a multipurpose adjuvant which would dictate economy of the formulation. Meanwhile, it would be justified to mention the role of other adjuvants: propionic and sorbic acid acting as anti-microbial agents; glycerol as humectant and anti-evaporant; Tween 80 and Triton X -100 as surfactants and wetting agents, respectively.

As the temperature increased from 4 to 50°C, there was a decrease in viscosity (15 to 13 cP) and particle size with a much steeper decrease (6 to 3 μm) after 30d storage (Figure 2b). At higher temperatures, certain inter and intra particle interactions such as hydrogen bonding and Van der Waals forces would be minimized leading to the decrease in viscosity.

There was no growth of pathogenic microorganisms (*Salmonella*, *Staphylococccus*, yeast and mould, total and fecal coliforms and *Enterococcus*) at any pH and temperature.

Similarly, Tx at pH 6.5 and temperatures 30 and 50°C decreased gradually from 15,800 to 13,000 SBU/μL ($P < 0.05$) (Fig. 2c). Lowering of Tx could be an outcome of degradation of crystal protein

by the action of protease enzymes at pH 6.5 and higher temperatures as Bt proteases are also known to be thermophilic (Burges 1998; Tyagi et al. 2001). Further, at higher temperature, spore degeneration and crystal protein inactivation will also take place on long term storage (Rhodes 1993, Burges 1998).

Although percent spore survival (45 – 87 %, $P > 0.05$) (Fig. 3a) was not high for Sy-3, it was determined to be the best formulation tested considering physical stability and ultimate losses in Tx. Formulation Sy-3 also showed higher percent suspendibility ranging from 75 to 88 % at different pH and 73-82 % at various temperatures (Fig. 3b). Thus, the percent spore survival decreased and percent suspendibility increased with increase in pH and temperature. No apparent corrosion of Al and Fe strips was observed at any pH after overnight storage, confirming compatibility with loading tanks. Loading containers used for formulations are frequently made up of Fe or Al which may be prone to corrosion if acidic suspensions are contained in them (Burges 1998).

SIW formulations. The physical performance of S-3 formulation in terms of change in viscosity (18 to 15 cP) and particle size (3 to 1 μm) with different pH (Fig. 4a) was excellent over a time period of 365d confirming good structuring of the formulation. However, the profiles were slightly altered with temperature (Fig. 4b) and there was a decrease in particle size (3 to 1.4 μm) and viscosity (18 to 10 cP); more so at 40 and 50°C. These changes were apparent after 10 days of storage, possibly due to the thermal effects as stated above. Similarly, Tx at pH 6.5 and temperatures 30 and 50°C decreased slightly from 19,000 to 18,800 SBU/μL (Fig. 4c), but after 60 d a gradual decrease to 18,000 SBU/ μL was significant ($P < 0.05$). Probable factors accounting for this phenomenon have been already discussed.

Fig. 5a shows the percent spore survival profile for different formulations of SIW. S-3 showed better profile at different pH and temperatures. Irrespective of the formulations, spore survival decreased with increase in pH (99 to 86 %) or temperature (93 to 82 %). This decrease may be due to the accentuating effect at both extremes of pH and temperature as discussed earlier (protease pH effect and thermal effect). Likewise, percent spore survival (99 %) (Fig. 5a) was significantly higher at pH 4 than 6.5 ($P < 0.05$), possibly again due to the deleterious effects of proteases at pH 6-6.5 (active pH for protease activity) as reported by Tyagi et al (2001). The formulation also started breaking up in terms of viscosity and particle size at this pH with analogous decreasing profile with temperature variations (Fig. 4b). Moreover, percent suspendibility increased with temperature and pH (Fig. 5b). Suspendibility is a physical phenomenon also determined by particle size. The suspendibility increased as particle size decreased with temperature (better dispersion and poor settling). The increase in suspendibility with pH could be due to the increase in negative charges on the particles resulting in repulsion and hence improved suspendibility. There was no apparent

corrosion of Al and Fe strips at any pH, confirming compatibility with metallic tanks. There were no signs of microbial contamination as in the case of soya formulations.

It was noteworthy that there was evidence of sedimentation of SIW formulation during 12 months storage period. However, the formulations remained in active suspension for more than 6h. The liquid suspension seemed to be increasingly thicker at bottom and required gentle stirring by glass rod to dislodge the settled materials. However, any shortcoming arising from slow sedimentation could be obviated if the preparation was thoroughly shaken to mix before dilution with water and/or directly without dilution for field application. Six hours of suspension would give enough time for aircraft loading and spraying aerially onto forests (Rhodes 1993).

The formulation based on Bt fermented SIW would be an interesting commercial product. As could be inferred from this study, it performed better in terms of shelf stability (1 year) relative to commercial soya formulations which start degenerating on an average in 30d.

It would be relevant to mention salient advantageous features of SIW formulations over soya: SIW availability would be cost-effective; biopesticide production could be integrated into the starch industry in a resourceful manner; production of a "value-added product" from waste; sequestration of carbon resulting in mitigation of greenhouse gases and hence resulting in an eco-friendly product in an environmentally benign way. Further, there are some positive speculations of the product – presence of residual starch in abundance at the end of Bt fermentation can aid in self-encapsulation leading to UV protection and also provide stickiness helpful in rainfastness, two delimiting factors for commercialization of any Bt biopesticide. This will be extensively investigated in the future course of studies.

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, Canada Research Chair) for financial support. The views and opinions expressed in this article are those of authors. We are also thankful to Natural Sciences and Engineering Research Council of Canada and Canadian Forestry Service for providing Ph.D. postgraduate scholarship to Satinder K. Brar.

References Cited

Aiba, S., Humphrey, A.E., and Millis, N.F. 1973. Biochemical engineering, 2nd ed. Academic Press, New York, US.

Alves L.F.A., Alves S.B., Pereira R.M., and Capalbo D.M.F. 1997. Production of *Bacillus thuringiensis Berliner* var. *kurstaki* grown in alternative media. Biocontrol Science and Technology 7: 377-383.

APHA, AWWA, WPCF. 1998. Standard Methods for Examination of Water and Wastewaters. 20th edn. (Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. eds.), American Public Health Association, Washington, DC, USA.

Barnabé, S. 2004. Hydrolyse et oxydation partielle des boues d'épuration comme substrat pour produire *Bacillus thuringiensis* HD-1. Ph.D thesis, Institut National de la Recherche Scientifique, Eau, Terre et Environnement, Université du Québec, Québec, Canada, pp 63-70.

Beegle, C.C. 1990. Bioassay methods for quantification of *Bacillus thuringiensis* δ-endotoxin, Analytical Chemistry of *Bacillus thuringiensis*, In: Hickle L.A. and Fitch W.L. (eds.) Analytical Chemistry of *Bacillus thuringiensis*, American Chemical Society, pp.14-21.

Behle, R.W., McGuire, M.R., and Shasha, B.S. 1997. Effects of sunlight and simulated rain on the residual activity of *Bacillus thuringiensis* formulations. J. Econ. Entomol. 90: 1506-1516.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., and Banerji, S. 2004. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations.Water, Sci. Technol. 50 (9): 229-236.

Brar, S.K., Verma, M., Barnabé, S., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. 2005. Impact of Tween 80 during *Bacillus thuringiensis* fermentation of wastewater sludges. Process Biochemistry 40: 2695–2705.

Burges, H.D. (ed.) 1998. *Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments*, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Carroll, B. 2001. Selecting the right adjuvant. Helena Chemical Company's Guide to Adjuvant. http://www.helenachemical.com/proprietary/products/adjuvants.

Chatfield, C. 1983. The design and analysis of experiments: 1. Comparative Experiments. pp. 224-256. In: Statistics for Technology. 3rd edition, Chapman and Hall Inc., Bristol, U.K.

Dunkle, R.L., and Shasha, B.S. 1989. Response of starch encapsulated *Bacillus thuringiensis* containing UV screens to sunlight. Environ. Entomol. 18: 1035-1041.

Jarvis, P. 2001. Biopesticides: trends and opportunities, Agrow Reports, PJB Publications, www.pjbpubs.com/agrow, August, 98 pp.

Kawase, Y., and Hashimoto, N. 1996. Gas hold-up and oxygen transfer in three phase external-loop airlift bioreactors: non-Newtonian fermentation broths. J. Chem. Tech. Biotechnol. 65: 325–334.

Lisansky, S.G., Quinlan, R.J., and Tassoni, G. 1993. The *Bacillus thuringiensis* Production Handbook, CPL Press, Newbury, pp.124.

Lee I. Y., Seo W. T., Kim G. J., Kim M. K., Ahn S. G., Kwon G. S., and Park Y. H. 1997. Optimization of fermentation conditions for production of exopolysaccharide by *Bacillus polymyxa*. Bioprocess Biosys. Eng. 16(2): 71-75.

Martin, P.A.W. 2004. A stilbene optical brightener can enhance bacterial pathogenicity to gypsy moth (Lepidoptera: Lymantriidae) and Colorado potato beetle (Coleoptera: Chrysomelidae). Biocontrol Sci. Technol. 14(4): 375–383.

McGuire, M.R., and Shasha, B.S. 1992. Adherent starch granules for encapsulation of insect control agents. J. Econ. Entomol. 85(4): 1425-1433.

Navon, A. 2000. *Bacillus thuringiensis* insecticides in crop protection – reality and prospects. Crop Protection 19: 669-676.

Prieto-Samsonov, D.L., Vazquez-Padron, R.I., Ayra-Pardo, C., Gonzalez-Cabrera, J., and de la Riva, G.A. 1997. *Bacillus thuringiensis*: from biodiversity to biotechnology. J. Ind. Microbiol. Biotechnol. 19: 202–219.

Rhodes, D.J. 1993. Formulation of Biological Control Agents, in Exploitation of Microorganisms (ed. D.G. Jones), Chapman and Hall, London, pp 411-439.

Sachdeva, V., Tyagi, R.D., and Valéro, J.R. 2000. Production of biopesticides as a novel method of wastewater sludge utilization/disposal. Water Sci. Technol. 42: 211-216.

Shasha, B.S., McGuire, M.R., and Behle, R.W. 1998. Lignin-based pest control formulations. US Patent 5 75 0467.

Tamez-Guerra, P., McGuire, M.R., Behle, R.W., Shasha, B.S., and Galan-Wong, L.J. 2000. Assessment of microencapsulated formulations for improved residual activity of *Bacillus thuringiensis*. J. Econ. Entomol. 93 (2): 219-225.

Teera-Arunsiri, A., Suphantharika, M., and Ketunuti, U. 2003. Preparation of spray-dried wettable powder formulations of *Bacillus thuringiensis* based biopesticides, J. Econ. Entomol. 96 (2): 292-299.

Tirado-Montiel, M.L., Tyagi, R.D., and Valéro, J.R. 2001. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. Water Res. 35 (16): 3807–3816.

Tirado-Montiel M.L., Tyagi R.D., Valéro J.R., and Surampalli R.Y. 2003. Production biopesticides using wastewater sludge as a raw material – Effect of process parameters. Water Sci. Technol. 48 (8): 239-246.

Tenney, M.W., and Stumm, W. 1965. Chemical flocculation of microorganisms in biological waste treatment. JWPCF 37 (10): 1370-1388.

Tyagi, R.D., Foko, V.S., Barnabé, S., Vidyarthi, A.S., and Valéro, J.R. 2001. Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using sewage sludge as raw material. Water Sci. Technol. 46 (10): 247-254.

van Frankenhuyzen, K. 2000. Application of Bt in forestry. In: Entomopathogenic Bacteria: from Laboratory to Field Application. Francois, J., Delecluse, A., Nielsen-LeRoux, C. (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. 2002. Studies on the production of *B-thuringiensis* based biopesticides using wastewater sludge as a raw material. Water Res. 36 (19): 4850-4860.

Yezza, A., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., and Smith, J. 2004a. Scale-up of biopesticide production processes using wastewater sludge as a raw material. J. Ind. Microbiol. Biotechnol. DOI 10.1007/s10295-004-0176-z.

Yezza, A., Tyagi, R.D., Valéro, J.R., and Surampalli, R.Y. 2005. Production of *Bacillus thuringiensis* based biopesticides in batch and fed-batch cultures using wastewater sludge as a raw material. J. Chem. Technol. Biotechnol. 80: 502-510.

Table 1. Composition of starch industry wastewater

Parameter (s)	**Concentration ± SE (mg/kg, unless stated)**
TS (g/l)	17.4±0.1
TVS (g/l)	14.4±0.1
SS (g/l)	2.3±0.11
VSS (g/l)	2.3±0.4
pH	3.0±0.1
Total carbon	649854±435
Total nitrogen	43648±1811
Total phosphorus	33858±2234
$N\text{-}NH_3$	111.2±48.4
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$	5±1.20
$P\text{-}PO_4^{3-}$	14981±2215
S	2288.3±61.7
Al	56889±1422
Ca	12008±126
Cd	-
Cr	1.1±0.06
Cu	326.5±159.5
Fe	8061.9±758.6
K	22584±3432
Pb	3.0±1.8
Zn	238.1±86.2
Na	2196.7±225
Ni	-
As	-

N.B.: The mg/kg conversion corresponds to weight of metals per unit weight of TS.

Table 2. Formulation recipe chart (concentrations in % w/v, unless otherwise stated)

No.	Basic Additives/Adjuvants		Concentration
1.	Propionic acid		0.5
2.	Sorbic acid		0.4
3.	Glycerol		2
4.	Tween-80		0.2
5.	Triton X-100		0.1
6.	Broth (%v/v)	soya	47.2
		SIW	57.4
7.	supernatant		to make up
8.	Dispersing/suspending agents		Variation

Type of formulation	Sorbitol	Sodium monophosphate	Sodium metabisulfite	Final Total Solids Concentration
S1/Sy1	21	0	0	14.3
S2/Sy2	18	3	0	13.6
S3/Sy3	15	5	0	14.1
S4/Sy4	11	5	5	14.7
S5/Sy5	9	7	5	15.3

Table 3. List of ANOVA factors

Figure	**Mean**	***F***	***df*; *df****	***P***
1b	$1.67x10^9$(TC at 48 h)	1.399	2 ; 6	> 0.05
1b	$8x10^8$(VS at 48 h)	3.712	2 ; 6	> 0.05

df : degree of freedom, between groups

*df**: degree of freedom, within group

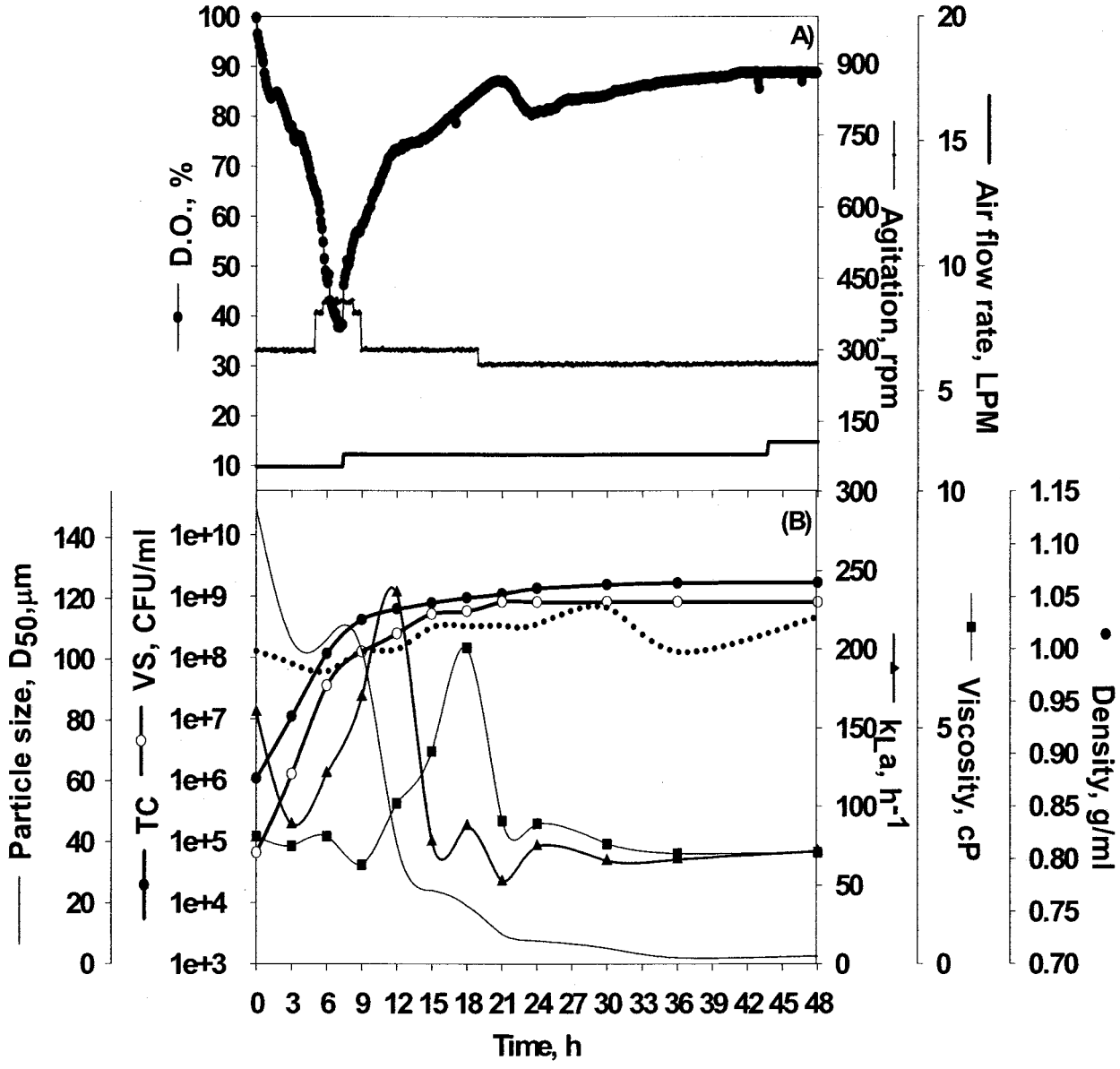


Fig. 1. Bt Growth profile in SIW. (A) Operational parameters. (B) Process performance parameters.

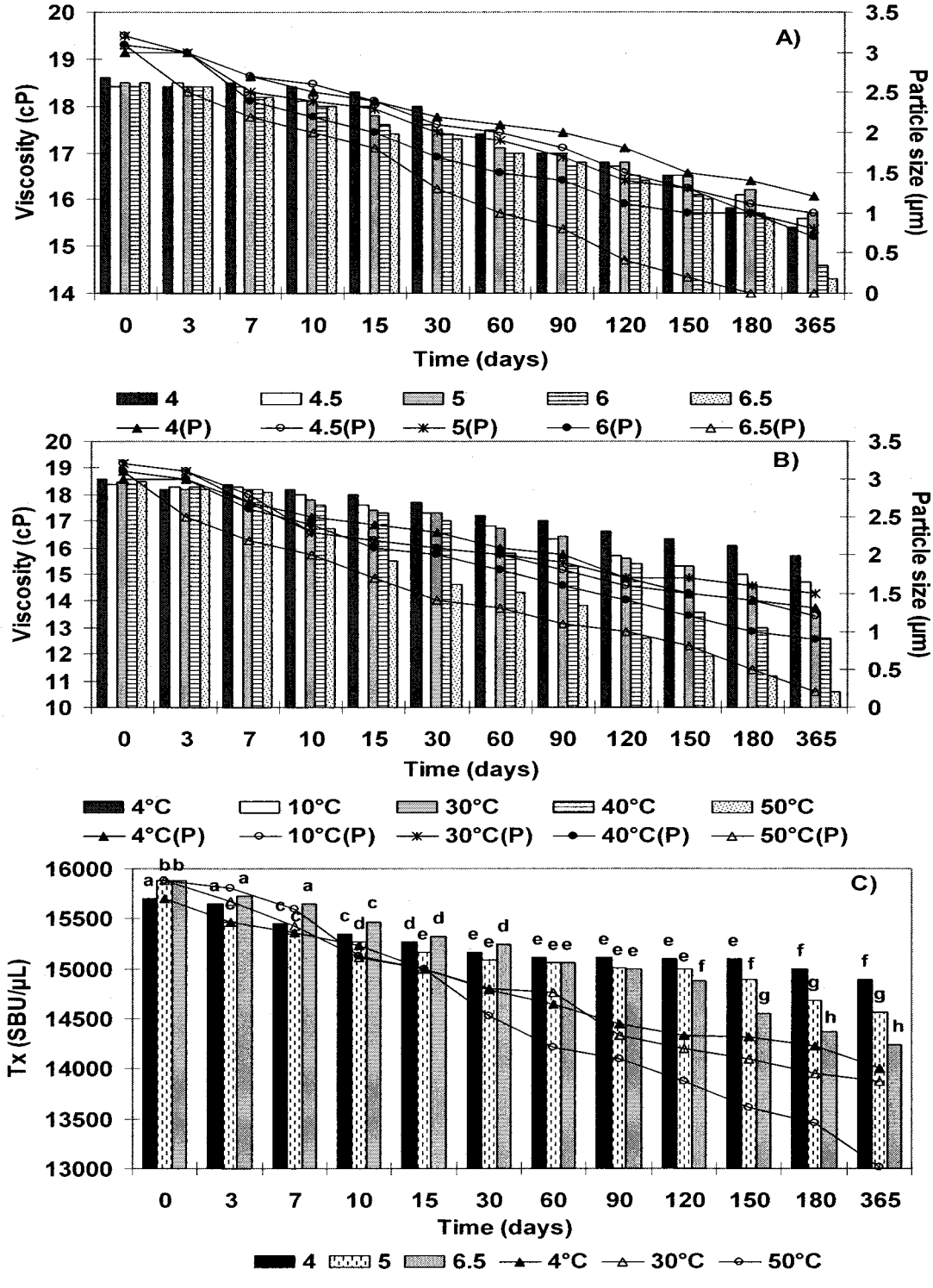


Fig. 2. Optimized formulation of soya (Sy-3). (A) Rheological profile at different pH. (B) Rheological profile at different temperatures. (C) Biological efficacy (P, particle size); bars shown with different letters are significantly different at $P \leq 0.05$. Error bars for standard deviation in graphs have been omitted for clarity. Points and lines designated "P" correspond to the particle sizes on the right axis in 2 a and 2b.

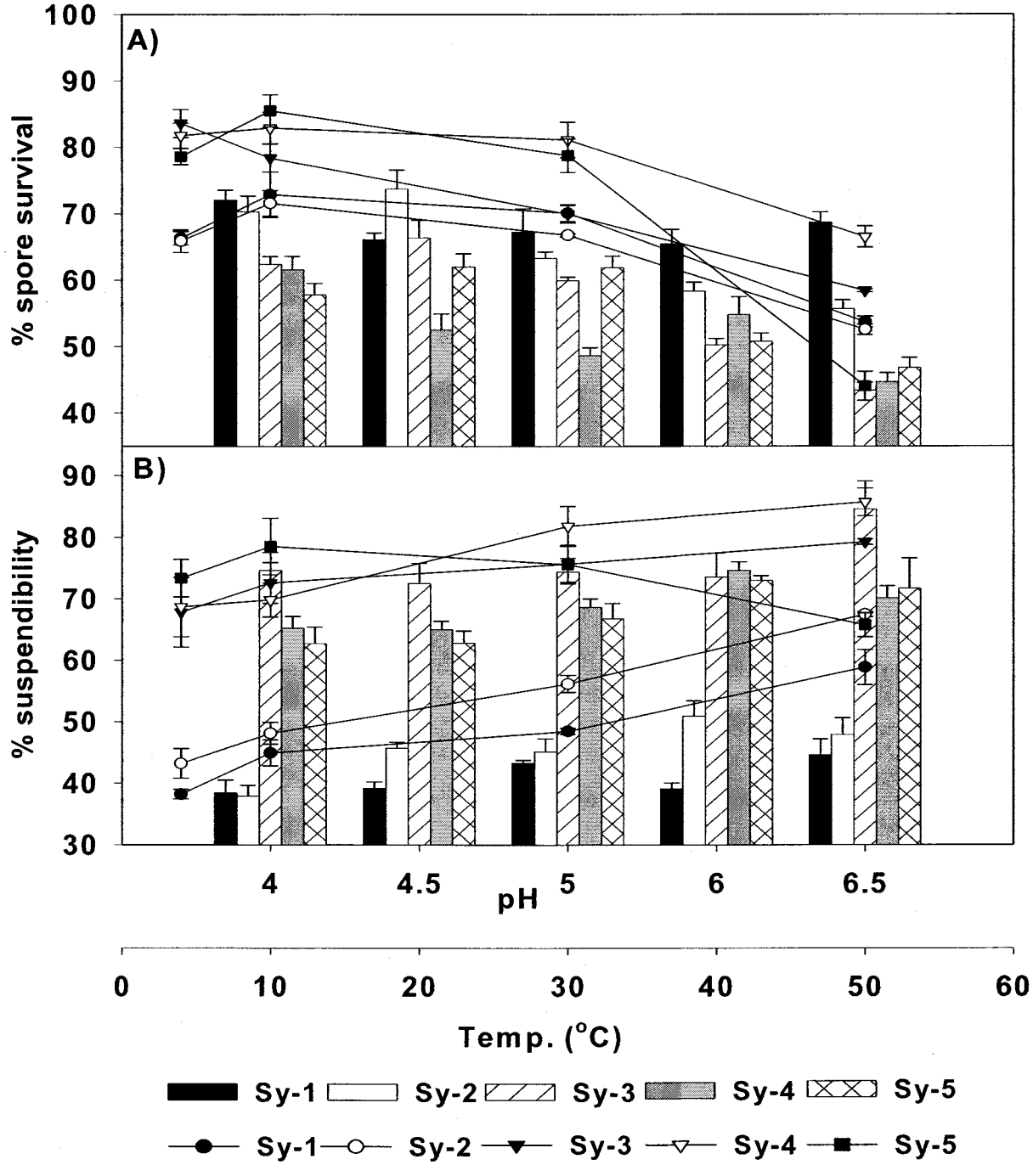


Fig. 3. Stability profile of soya formulations at different pH and temperatures (Time = 365 d). (A) Percentage of spore survival. (B) Percentage of suspendibility (T, temperature). "T" indicates the effect of temperature on the two variables, while the bars indicate effects of pH. Error bars represent standard deviations.

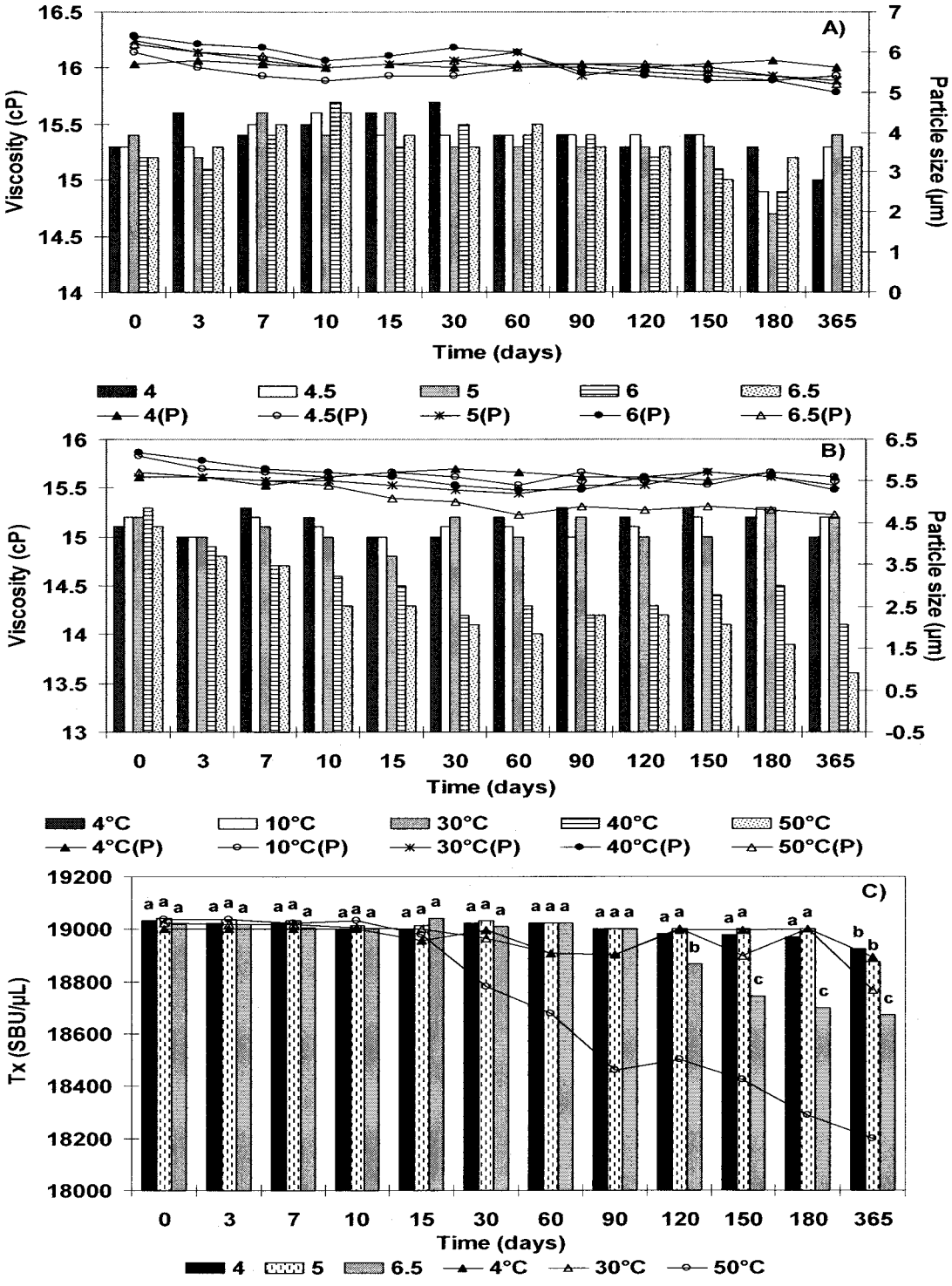


Fig. 4. Optimized formulation of SIW (S-3). (A) Rheological profile at different pH. (B) Rheological profile at different temperatures. (C) Biological efficacy (P, particle size); bars shown with different letters are significantly different at $P \leq 0.05$. Error bars for standard deviation in graphs have been omitted for clarity. Points and lines designated "P" correspond to the particle sizes on the right axis in 4 a and 4b.

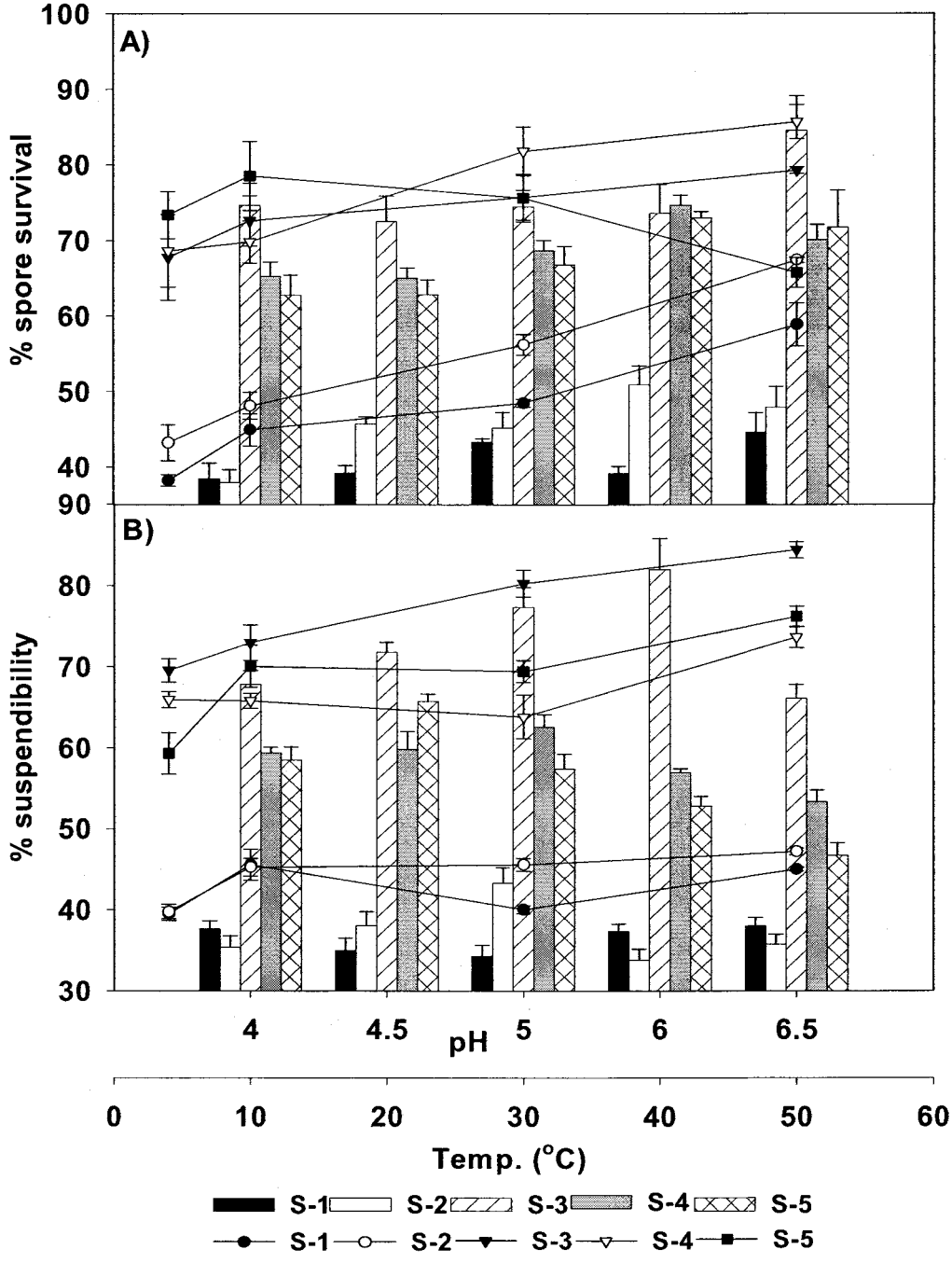


Fig. 5. Stability profile of SIW formulations at different pH and temperatures (Time = 365 d). (A) Percentage of spore survival. (B) Percentage of suspendibility (T, temperature). "T" indicates the effect of temperature on the two variables, while the bars indicate effects of pH. Error bars represent standard deviations.

Partie VI

Bacillus thuringiensis Fermentation of Hydrolyzed Sludge - Rheology and Formulation Studies

Satinder K. Brar[a], M. Verma[a], R. D. Tyagi[a*], J.R. Valéro[a], R.Y. Surampalli[b]

[a]INRS-ETE, Université du Québec, 490, de la Couronne, Québec, Canada G1K 9A9

[b]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

Chemosphere (2007)
67: 674–683

Fermentation des boues hydrolysées par *Bacillus thuringiensis* : Études de la rhéologie et du développement de suspension

Résumé

La rhéologie de la fermentation des boues hydrolysées par *Bacillus thuringiensis* (Bt) a été étudiée en bioréacteur. Des suspensions aqueuses stables ont été développées et optimisées avec une étude réalisée sur deux années consécutives. Cette étude comportait une évaluation de divers paramètres physico-chimiques (viscosité, tailles de particules, corrosion et homogénéité) et biologiques (contamination microbienne, viabilité des spores et potentiel insecticide) à différents niveaux de pH et de températures. Les boues hydrolysées ont présenté un comportement non Newtonien et pseudoplastique pendant la fermentation avec une niveau de confiance de 90 à 96% selon les modèles de Casson, de puissance et d'IPC. Des valeurs plus élevées des indices de consistance et d'écoulement pendant la croissance exponentielle et la phase stationnaire peuvent affecter les procédés en aval. Les suspensions aqueuses stables respectaient la loi de puissance. Le sorbitol, le monophosphate du sodium et le métabisulfite du sodium à des proportions de 2,2:1:1 comme agents de suspension ont permis d'obtenir un potentiel de suspension se situant entre 69 et 94%. La formulation stable (FH-4) comprenant du sorbitol, du monophosphate de sodium et du métabisulfite de sodium se détériorait à pH 6 et 6,5 et à des températures de 40 et 50 °C, sans signe de corrosion ou de contamination microbienne. La viscosité de la suspension FH-4 diminuait avec le taux de cisaillement, ce qui est favorable à la manipulation et pulvérisation de l'insecticide.

Mots-clés: *Bacillus thuringiensis*; biopesticide; boues hydrolysées; formulation liquide; rhéologie; durée de conservation

ABSTRACT

Rheology of *Bacillus thuringiensis* (Bt) fermentation of hydrolyzed sludge was investigated in bench scale fermenter. Stable liquid formulations were developed and optimized for two year based studies comprising various physical/chemical (viscosity, particle size, corrosion and suspendibility) and biological (microbial contamination, viable spores and entomotoxicity) parameters at different pH and temperatures. The hydrolyzed sludge depicted non-Newtonian and pseudoplastic behaviour during fermentation with 90 to 96% confidence of fits into Casson, Power and IPC paste models. Higher values of consistency and flow index during exponential growth and stationary phase, respectively affected downstream processing. The power law was also followed by stable formulations. Sorbitol, sodium monophosphate and sodium metabisulfite (2.2:1:1) as suspending agents produced suspendibility ranging from 69 to 94%. The stable formulation (FH-4) comprising sorbitol, sodium monophosphate and sodium metabisulfite deteriorated at pH 6, 6.5 and temperatures, 40 and 50°C, with no signs of corrosion and microbial contamination. The viscosity of FH-4 formulations decreased with shear rate which could help improving handling and consequent spraying.

Keywords: *Bacillus thuringiensis*; biopesticide; hydrolyzed sludge; liquid formulation; rheology; shelf-life

1. Introduction

Bacillus thuringiensis (Bt) based biopesticides are conventionally used options for pest control in agriculture, forestry and public health sectors (Burges, 1998). The conventional production of Bt in semi-synthetic medium has been recently replaced with non-conventional wastes like wastewater/wastewater sludge (Lisansky et al., 1993; Vidyarthi et al., 2002; Yezza et al., 2004). Additionally, in order to achieve higher entomotoxicity (biopesticidal potential), hydrolysis (pre-treatment) of wastewater sludge has been investigated to increase nutrient availability (Barnabe et al., 2005).

Rheology, during fermentation, affects oxygen transfer and hence entomotoxicity, likewise downstream processing (centrifugation) for recovery of products. The fermented non-hydrolyzed wastewater sludge depicted pseudoplastic and thixotropic behaviour as established earlier (Brar et al., 2005a). The rheology of fermented microbial broth is a net effect of formation of different metabolites, increase in biomass and degradation of growth medium components causing mass and heat transfer problems resulting in decreased productivity (Hwang et al., 2004). Changing the fermentation medium from non-hydrolyzed to hydrolyzed sludge would also shift the rheological behaviour. Further, these problems are compounded during scale-up due to medium complexity and generally lower agitation intensities. Thus, the rheological profiles during Bt fermentation of hydrolyzed sludge need to be explored to better understand the fermentation process.

Despite the availability of alternative economical raw materials producing higher final entomotoxicity, the formulation cost still eludes the broader application of biopesticides. Moreover, rheological considerations are important for the selection of different adjuvants required to obtain requisite properties (higher shelf-life, suspendibility, rainfastness, UV resistance, higher field efficacy, compatibility with pre-existing application equipment, and cost-effectiveness) of final Bt formulations (Burges, 1998). Formulation feasibility studies have been carried out using Bt fermented non-hydrolyzed wastewater sludge (Brar et al., 2004). Meanwhile, hydrolyzed sludge offered various advantages – higher entomotoxicity (Barnabe et al., 2005; Brar et al., 2005b); low viscosity (Brar et al., 2005b); possible lower production cost by incorporating sterilization in hydrolysis step (Barnabe et al., 2005); lower centrifugation time to recover targeted entomotoxicity in the pellet (Brar et al., 2006a); higher half-life (UV protection) enhancing efficacy during field

application (Brar et al., 2006b) and possibly lower particle size causing synergy with conventional application equipments. At this stage, it becomes imperative to investigate the rheology effects on development of stable hydrolyzed sludge formulations.

Hence, the present study will focus on following objectives: a) rheology of hydrolyzed sludge fermentation and formulation; b) shelf-life stability (pH, temperature) of different formulations by analyzing various physical/chemical-biological test parameters.

2. Materials and Methods

2.1. Upstream processing

2.1.1. Sludge procurement, amendment and characterization

The secondary wastewater sludge was obtained from Communauté Urbaine du Québec wastewater treatment plant at Ste-Foy. The sludge was concentrated from approximately 1.6% to 5% (w v^{-1}) suspended solids (SS) by gravity settling followed by centrifugation at 7650 g for 15 min at 20 ± 1 °C. The sludge supernatant stored at 4 ± 1°C was used to dilute the sludge as required. The concentrated and homogenized sludge (Waring blender) was hydrolyzed by direct steam injection in a custom made hydrolyzer at 140 ± 1 °C for 30 min (Barnabé et al., 2005). A dilution factor of 1.67 (empirically obtained) due to steam condensation was incorporated requiring an initial SS of 50 g L^{-1} to achieve a final SS of 30 g L^{-1}. Hydrolyzed sludge was henceforth, referred to as TH-30. The sludge characteristics (APHA, 1998) are given in Table 1.

2.1.2 Bacterial strain and fermentation

Bt var. *kurstaki* HD-1 (ATCC 33679) was used with culture conditions, maintenance and inoculum production described elsewhere (Vidyarthi et al., 2002).

Fermentation was carried out in a bioreactor at pH 7 ± 0.1 and 30 ± 1 °C (10 L working volume, Biogenie Inc., Quebec, Canada) equipped with accessories and programmable logic control (interfaced with iFix 3.5 software, Intellution, GE Fanuc Inc., USA) for dissolved oxygen, pH, anti-foam, impeller speed, aeration rate and temperature. Fermenter dimensions, wastewater charging and sterilization procedure are described elsewhere (Brar et al., 2005b).

2.2. Downstream processing

2.2.1. Post-fermentation and pre-formulation step

The pH of TH-30 fermented broth was lowered *in-situ* from 7 ± 0.1 to 4.5 ± 0.1 (Brar et al., 2006b). The fermented broth (entomotoxicity denoted as "Tx", 19 x 10^9 SBU L^{-1}, spruce budworm units/l) was centrifuged aseptically in order to compare with industrial standard equivalent of 19.5 x 10^9 IU L^{-1}. The concentrated hydrolyzed sludge broth at 70 g L^{-1} solids yielded an approximate entomotoxicity of 26.4 x 10^9 SBU L^{-1}.

2.2.2. Storage stability tests

The concentrated broth of known Tx was further amended with basic adjuvants/additives (Table 2). The basic adjuvants served following purposes: propionic and sorbic acid - anti-microbial agents; glycerol - humectant and anti-evaporant; Tween-80 and Triton X-100 - surfactants and wetting agents, respectively. Furthermore, the basic formulations were supplemented with different concentrations of specific adjuvants (suspending agents, sorbitol, sodium monophosphate and sodium metabisulfite, Burges, 1998). The amendments resulted in five different series of TH-30 formulations, designated as FH-1 through FH-5 (Table 2) and were blended as slurry in a Waring Blender.

Further, the slurry was diluted with supernatant of centrifuged broth to achieve final Tx of 19.5 x 10^9 SBU L^{-1}. The pH stability studies were carried out at 4.0, 4.5, 5.0, 6.0 and 6.5 ± 0.1 by adjusting the pH with 2N NaOH and/or H_2SO_4 and stored at 20 ± 1 °C for two years. Similarly, temperature storage studies were carried out at 4, 10, 20, 30, 40 and 50 ± 1°C at pre-adjusted pH 5.0 ± 0.1. Samples were drawn at regular intervals (0, 3, 7, 10, 15, 30, 60, 90, 120, 150, 180, 365, 545 and 730 d) to determine physical (viscosity, particle size, suspendibility and corrosion) and biological (microbial contamination, viable spores and entomotoxicity) parameters.

2.3. Physical/chemical and biological analysis

Rheological properties of fermented hydrolyzed sludge were determined by using a rotational viscometer Brookefield DV II PRO+ (Brookfield Engineering Laboratories, Inc., Stoughton, MA, USA) equipped with Rheocalc32 software (for rheological models). Three different spindles, namely, SC-34, L2 and ultra-low centipoise adapter were used with a sample cup volume of 18 ml/50 ml (spindle dependent). Time dependent profile was studied at low shear rate (7.34 s^{-1}) and viscosity at each sampling point was measured at 36.71 s^{-1}. The shear rate behaviour was determined from 0.1 to

200 s^{-1}. All measurements were done at 25 ± 1°C with a measurement time lag of 1 min between consecutive shear rates. Viscosity was referred to "apparent viscosity", unless stated otherwise.

Particle size analysis was carried out by LASER diffraction method as reported earlier (Brar et al., 2005b). A standard deviation of 8 and 10% was observed for viscosity and particle size measurements, respectively.

Suspendibility was determined by comparing ratio of supernatant turbidity and weight of settled solids after 30 min settling by using the procedure established earlier (Brar et al., 2006b). The standard deviation for suspendibility measurement was 5%.

Corrosion was tested by immersing iron and aluminum strips (10 x 5 cm^2) overnight in various formulations at different pHs. The pH was measured by simple glass electrode (Delta 320 Mettler Toledo, NJ, USA). Further, microbial contamination was tested as per the protocol reported by Lisansky et al. (1993) as fermentation was prone to contamination.

Total cell (TC) and viable spore (VS) count was analyzed according to earlier methods (Vidyarthi et al., 2002). Three replicates were performed and data analyzed with one way analysis of variance (ANOVA) to test significant difference at $P = 0.05$. The standard deviation for cell and spore count was 7.0 and 8.0%, respectively.

The bioassay was performed to determine entomotoxicity by using diet incorporation method as described in details in earlier studies (Brar et al., 2005b; 2006b; Yezza et al., 2004). The SBU reported in this study was 25-30% higher than international units with standard deviation of 8–10%.

3. Results and discussion

3.1. Rheology of hydrolyzed sludge (TH-30)

Figures 1a and 1b illustrate rheogram and shear rate behavior, respectively of TH-30 at different fermentation times with various rheological models presented in Table 3.

The rheograms (Figs. 1a and 1b) all through fermentation showed non-linear relationship (strong non-Newtonian behaviour) and viscosity vs. shear rate curves showed pseudoplastic (viscosity decreased with shear rate) behaviour. Interestingly, the shear stress increased from 0 to 15 h with higher values from 9 to 15 h possibly due to the resistance offered by actively growing Bt cells.

However, the shear stress decreased during stationary phase (21 - 48 h) probably due to cell lysis; release of spores and other virulence factors (namely, enzymes, phospholipases, proteases, chitinases; vegetative insecticidal proteins and cytolytic proteins) in the fermented broth. Spores, crystal proteins and the virulence factors synergize to contribute to Tx (Hansen and Salamitou, 2000). The microbial fermentation broths show diverse rheology changes from initial Newtonian to pseudoplastic behaviour during exponential phase (Hwang et al., 2004). The pseudoplasticity will have a significant effect on mass and heat transfer in the fermenter as shear rates will be higher near the impeller and low elsewhere.

Table 3 also showed that TH-30 during fermentation followed various rheological models with varying degree of confidence of fits. The TH-30 sludge strongly followed Bingham plastic model during the exponential phase (0 – 21 h, Table 3 and Fig. 2) whereas a poor fit was observed during stationary phase (30 – 48 h). Bingham model is a simple rheological model that relates shear stress and shear rate and quantifies yield stress and high-shear viscosity. The Bingham law is represented mathematically by Equation 1:

$$\tau = \tau_o + \eta D \qquad (1)$$

The effects of yield stress on flow are somewhat similar to those of pseudoplasticity but are much more pronounced. For example, the Bingham plastic behaviour of TH-30 until 24 h of fermentation suggested that higher shear stress will be required to overcome the heterogeneity of fermentation medium as reflected in higher agitation rates required (Brar et al., 2005b). The lower yield stress of fermented broth during stationary phase may be contributed by two factors- larger proportion of cells converted to spores and hence less resistance (shear thinning); and extracellular polymeric substances (EPS) possibly formed during the exponential phase (cellular growth) were broken due to weaker interactions during stationary phase, which remains to be ascertained. In fact, Bt was identified as a principal EPS producing bacterial strain in marine environment (Kwon et al., 2002).

Meanwhile, Casson and NCA-CMA Casson (modified Casson) model was followed with good confidence of fits (82 to 96%) throughout the fermentation. Casson model quantifies yield stress and high shear viscosity, a model which incorporates features of the power law and the Bingham plastic equations. The mathematical representation of Casson law is given as:

$$\sqrt{\tau} = \sqrt{\tau_o} + \sqrt{\eta D} \qquad (2)$$

Likewise, NCA-CMA Casson model has been derived from the standard set forth by the National Confectioners Association (NCA) and the Chocolate Manufacturers Association (CMA). Although based on the original Casson equation, this implementation has been tailored by the NCA and CMA

specifically to applications involving chocolate type behaviour. The NCA-CMA model is mathematically represented by Equation 3:

$$(1+a)\sqrt{\tau} = 2\sqrt{\tau_o} + (1+a)\sqrt{\eta D} \quad (3)$$

Considering Casson law relation, plastic viscosity increased from 0 - 12 h and later decreased with again a sudden peak at 30 h due to unknown reasons. The decrease in plastic viscosity and yield stress towards the end of fermentation suggested that the fermented broth would require lower shear stress to maintain flow. The yield stress affected flow in a similar manner as pseudoplasticity with more pronounced effects. This will aid in pump design for centrifugation facilities where cell debris aggregates are identified as being reversibly de-aggregated by low shear stresses which might exist in the separation zone of the disc space of a disc stack centrifuge (Maybury et al., 2000). Furthermore, when the fermented centrifuged broth is carried over for formulations, the flowability will be maintained and different adjuvants can be amended homogeneously (as discussed later). Additionally, during field application, the flowability could aid in the spray application to achieve desired droplet spectrum and efficient spray which is a function of viscosity.

3.2. Fermentation and Power law behaviour

The Bt fermentation and power law behaviour profiles of hydrolyzed sludge are illustrated in Fig. 2. Power and IPC paste (extension of power model) models were followed with moderate fits in the first 6 h of growth (Table 3). However, power law was followed with good fits (79 to 96%) from 9 to 48 h of fermentation (Table 3). The power law was used to define Bt fermentation as it has been very widely utilized to determine the rheology of several microbial fermentation broths (Hwang et al., 2004). Power law model is a useful rheological model that describes the relationship between viscosity or shear stress and shear rate over the range of shear rates where shear thinning occurs in a non-Newtonian fluid. It quantifies overall viscosity range and degree of deviation from Newtonian behaviour and is well represented by Equation 4:

$$\tau = K D^n \quad (4)$$

Akin to power law, IPC paste model is intended to calculate the shear sensitivity factor and the 10 RPM viscosity value of pastes. The law is primarily used in the solder paste industry, thus the name IPC (Institute for Interconnecting and Packaging Electronic Circuits) and is mathematically denoted as:

$$\eta = K R^n \quad (5)$$

It was seen in Fig. 2 that the TC and VS concentration increased during 0 to 24 h, from 2.4 x 10^4 to 1.1 x 10^6 CFU m^{-3} and 0.9 to 5.8 x 10^5 CFU m^{-3}, respectively ($P \leq 0.05$). Likewise, the consistency index (K, measure of thickness of the fermented broth) and the flow index (n) also varied. The corresponding ANOVA factors for TC and VS are also given in Table 4. During 0 - 3 h of fermentation, viscosity decreased and later attained a hump at 12 h as seen in Fig. 2 in agreement with earlier studies (Brar et al., 2005b). Likewise, "K" decreased from 0 to 3 h which may be due to mechanical force (agitation) that led to floc disruption and hence thinning of medium. Floc, in this case, refers to the aggregates of biomass present in hydrolyzed sludge. However, "K" increased from 0 to 12 h with a peak at 12 h (Fig. 2) which may be due to: a) increase in medium consistency with Bt cell growth; b) addition of anti-foam during active growth phase (0 – 12 h, note dissolved oxygen values, Fig. 2) causing a peak and c) increase in the phase volume (volume of suspended material/volume of continuous phase) which has been reported to increase "K" in fermented broths (Hwang et al., 2004).

Interestingly, "K" decreased towards the end of fermentation and "n" increased continuously attaining constancy between 30 to 36 h of fermentation. In fact, "n" followed exponential profile ($n = 0.2373e^{0.0245t}$; $R^2 = 0.92$, Fig. 2) so that increase in fermentation period resulted in a broth with higher flowability. Likewise, a correlation between "K" and "n" followed exponential profile ($K = 468.5e^{-4.4584n}$; $R^2 = 0.91$) implying that increase in flowability (broth thinness) will decrease the consistency (broth thickening). The correlation suggested ease of downstream processing (centrifugation) which was also established in our previous studies where the centrifugation time to achieve certain degree of separation (77% entomotoxicity recovery) was lower for hydrolyzed Bt fermented sludge when compared to other fermented broths (Brar et al., 2006a).

As "n" tends to 1, the shear-thinning properties normally increase so as to reach Newtonian behavior. In contrast, TH-30 during fermentation exhibited a marked shear-thinning response ($n \leq 0.6$, Fig. 2). Furthermore, a gradual increase in "n" from 0.2 to 0.6 (Fig. 2) also confirmed shear rate as a function of Bt growth (as discussed earlier, viscosity decreased with shear rate). Meanwhile, in concentrated microbial suspensions, the increase in viscosity with cell concentration is less pronounced due to increase in shear rate (Hwang et al., 2004). The viscosity of FH-4 stable formulation (discussed later) was found to decrease with shear rate (Fig. 1b) showing relatively less non-Newtonian behavior (Fig. 1a). This is necessary for practical applications because marked

differences in viscosity may be expected at different operative shear rates during pouring, mixing, pumping, and spraying which will consequently affect the droplet size spectrum and hence performance as biopesticides. In fact, viscosity has also been found to affect synergy with spray equipment and field application of Bt biopesticides (Burges, 1998).

3.3. Optimization of TH-30 formulations

Five different formulations of Bt fermented TH-30 were developed (FH-1 to FH-5) and analyzed for various physical (viscosity, particle size, suspendibility, corrosion) and biological (Tx and VS) stability parameters at different pHs and temperatures over a period of two years. Further, the optimal formulation was selected by adopting a quantitative judgement analysis approach. The quantitative judgement referred to selection of formulations (optimal and stable) which showed no significant difference ($P > 0.05$) in the physical and biological parameters over two years of shelf storage. If any significant change ($P \leq 0.05$) occurred, the formulations were rejected. However, the most important criterion for selection of the optimal formulation was the entomotoxicity. The viscosity, particle size and Tx profiles of FH-1, FH-2, FH-3 and FH-5 at different pHs and temperatures showed significant decline after 30 d period ($P \leq 0.05$, results unreported). Moreover, percent suspendibility and spore survival (Table 5) decreased significantly ($P \leq 0.05$) over the storage period (730 d). Thus, FH-4 comprising sorbitol, sodium monophosphate and sodium metabisulfite (2.2:1:1) was found to be the optimal recipe with nominal variations (discussed later).

3.4. Stable liquid formulation (FH-4)

3.4.1. Rheology, physical and biological parameters

The rheology of stable FH-4 formulation was investigated by studying different mathematical models as seen in Table 3. The results suggested that with further increase in solids concentration of the formulation, the viscosity will increase as per exponents of the power law and IPC paste equations. Further, the consistency index was lower and flow behaviour index was higher than 48 h fermented broth (Table 3). Hence, the pseudoplasticity of FH-4 formulation was lower and hence the decrease in viscosity with shear rate was tending more towards Newtonian behaviour. This is desired for the flowability of a formulation so as to achieve better droplet spectrum and thus, field application. The FH-4 formulation (Figs. 3a, 3b and 3c) performed differently during pH and

temperature storage studies for 730 d. Viscosity and particle size did not vary significantly ($P > 0.05$) over the selected pH range except for a decrease in particle size (10 μm units) and viscosity (10^{-2} kg m^{-1} s^{-1}) after 90 d storage at pHs 6 and 6.5 (Fig. 3a). Considering the isoelectric point of bacteria from pH 2 to 4 (Tenney and Stumm, 1965), it was clear that sludge particles would carry increasingly negative charges at pH 6 and 6.5. Thus, increased similar charges in the floc structure caused repulsion and hence expansion of floc matrix. Further, the floc may be acted upon by enzymes, especially, proteases produced during Bt fermentation which were active at these pHs (Tyagi et al., 2001). This would have caused degradation of floc matrix and hence influenced the particle size which also showed a similar declining profile after 90 d storage (Fig. 3a). At lower pH, these issues may not arise and hence most of the formulations are developed at these pHs maintained by buffers (Burges, 1998).

The effect of temperature on viscosity and particle size profiles (viscosity; 57×10^{-3} to 22×10^{-3} kg m^{-1} s^{-1} and particle size; 34 to 24 μm) showed a decline at 40 and 50 °C after 30 d storage (Fig. 3b). There was a possibility that at higher temperatures, shear-induced interactions increased which resulted in floc structure break-up causing a particle size decline after 120 d. Similarly, certain inter and intra particle interactions such as hydrogen bonding and van der Waals forces would have probably decreased causing a change in the orientation of EPS flocs leading to the viscosity decrease.

The Tx at pH 6 and 6.5 and temperatures 30 and 50 °C decreased gradually from 19550 to 16550 SBU μL^{-1} ($P < 0.05$) (Fig. 3c). The Tx was lowered probably due to degradation of crystal protein by protease at pH 6 and 6.5 and higher temperatures as Bt proteases were reported to be thermophilic (Tyagi et al., 2001) as well as weakening of spore cortex at temperature extremes (Burges, 1998). There may also be consequent effect on other virulence factors and thus decreasing the effective Tx.

Although the spore survival (77–88%, $P < 0.05$) (Table 5) was not so high for FH-4, yet it was selected as optimal formulation based on physical and Tx stability. FH-4 formulation showed higher suspendibility ranging from 69 to 94% at different pHs and 62-86% at various temperatures (Table 5). Thus, the percent spore survival decreased and suspendibility increased with increase in pH. This suggested that at higher pH, the floc interactions were repulsive leading to enlarged floc structure and possible attack by proteases (discussed earlier) causing an increased in suspendibility.

3.4.2. Contamination, corrosion and caking

There was no growth of pathogenic microorganisms (*Salmonella*, *Staphylococccus*, yeast and mould, total and fecal coliforms and *Enterococcus*) at any pH and temperature. Likewise, no corrosion was observed in the tested pH range through 24 h. Corrosion plays an important role in Bt sprays as often the loading containers are made up of aluminium or iron (Burges, 1998).

No sedimentation and caking of FH-4 formulation was observed during 24 months storage. Lower sedimentation and caking are desirable for shelf stability as well as application of formulations (Burges, 1998). Additionally, when particle size profile of FH-4 (stable hydrolyzed sludge formulation) was plotted against commercial Bt product, Foray 76B (Fig. 4), maximum volume percent of particles of FH-4 formulation overlapped with Foray and smaller percentage fell outside the range, yet below the standard norm (recommended particle size $\leq$ 25μm). This will result in better synergy with application equipment easing eventual field application. Thus, the stable Bt fermented hydrolyzed sludge formulations will form a strong proponent of the Bt biopesticides repertoire increasing their marketability.

Furthermore, the optimal combination of sodium monophosphate (buffer), sorbitol (suspending agent) and sodium metabisulfite (suspending as well as anti-microbial agent) is being explored to reduce the utilization of propionic/sorbic acid as anti-microbial agent which will reduce the overall cost of the formulations. Interestingly, preliminary studies, where propionic and sorbic acid concentration was decreased from 0.5% and 0.4% w v^{-1} to 0.3 and 0.2% w v^{-1}, respectively showed no microbial contamination perhaps aided by anti-microbial action of sodium metabisulfite.

4. Conclusions

The rheology study of hydrolyzed sludge during Bt fermentation and development of stable formulations thereof led to following conclusions:

1. During fermentation, hydrolyzed sludge showed non-Newtonian pseudoplastic behaviour.
2. The rheological models during fermentation and formulation showed varying degree of confidence of fits.

3. The power law constants, consistency index increased with a peak at 12 h and later decreased, whereas, the flow behaviour index increased continuously and attained constancy during stationary phase and followed exponential law.
4. The stable formulation followed power law and the formulation pseudoplasticity decreased as compared to Bt fermented broth, enhancing the flow properties of former.
5. The stable formulation comprising sorbitol, sodium monophosphate and sodium metabisulfite approached Newtonian behaviour, but viscosity decreased with shear rate.
6. A cocktail of suspending agents, namely, sorbitol, sodium monophosphate and sodium metabisulfite in the ratio 2.2:1:1 was optimal and suspendibility ranged from 69 to 94%.
7. The stable formulation showed consistent physical and biological profiles at pHs 4 to 4.5 and 4 to 30 °C.

Acknowledgements

The authors sincerely thank the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STP235071, and Canada Research Chair) for financial support. We are also thankful to NSERC, CFS and SOPFIM for providing scholarship to Satinder K. Brar.

List of Abbreviations

ANOVA	Analysis of variance
Bt	*Bacillus thuringiensis*
EPS	Extra cellular polymeric substances
F	Fisher-Snedecor distribution (continuous probability distribution)
SBU	Spruce budworm units
SD	Standard deviation
SS	Suspended solids (g L^{-1})
TC	Total cell count (10^{-3} CFU m^{-3})
TH-30	Hydrolyzed sludge at solids concentration of 30 g L^{-1}
TS	Total solids (g L^{-1})
TVS	Total volatile solids (g L^{-1})
Tx	Entomotoxicity (SBU μL^{-1})
VS	Viable spore count (10^{-3} CFU m^{-3})
VSS	Volatile suspended solids (g L^{-1})

Nomenclature

η_{10}	10 rpm viscosity (10^{-3} kg m^{-1} s^{-1})
K	Consistency index (10^{-3} kg m^{-1} s^{-n})
K_m	Consistency multiplier
n	Flow behaviour index
η	Plastic viscosity and/or viscosity (10^{-3} kg m^{-1} s^{-1})

a	Ratio of spindle radius and inner cup radius
R	Rotational speed (rpm)
D	Shear rate (s^{-1})
n_s	Shear sensitivity factor
τ	Shear stress (10^{-1} kg m^{-1} s^{-2})
τ_0	Yield stress (10^{-1} kg m^{-1} s^{-2})

REFERENCES

APHA, AWWA, WPCF, 1998. Standard Methods for Examination of Water and Wastewaters. 20th edn. (Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. eds.), American Public Health Association, Washington, DC, USA.

Barnabé, S., Verma, M., Tyagi, R.D., Valéro, J.R., 2005. Culture Media for Increasing Biopesticide Producing Microorganism's Entomotoxicity, Methods of Producing Same, Biopesticide Producing Microorganisms so Produced and Method of Using Same. US patent International application PCT/CA2005/000235.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Banerji, S., 2004. Development of sludge based stable aqueous Bacillus thuringiensis formulations.Water Sci. Technol. 50, 229-236.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., 2005a. Sludge based Bacillus thuringiensis biopesticides: Viscosity Impacts. Water Res. 39, 3001-3011.

Brar, S.K., Verma, M., Barnabé, S., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., 2005b. Impact of Tween-80 during Bacillus thuringiensis fermentation of wastewater sludges. Process Biochem. 40, 2695–2705.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., 2006a. Efficient centrifugal recovery of Bacillus thuringiensis biopesticides from fermented wastewater and wastewater sludge. Water Res. 40, 1310-1320.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., 2006b. Screening of different adjuvants for wastewater/wastewater sludge based Bacillus thuringiensis formulations. J. Economic Entomol. 99, 1065-1079.

Burges, H.D. (Ed.) 1998. Formulation of Microbial Biopesticides: Beneficial organisms, Nematodes and Seed Treatments. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Hansen, B.M., Salamitou, S., 2000. Virulence of Bacillus thuringiensis. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds.). Entomopathogenic Bacteria: From Laboratory to Field Application. Kluwer Academic Publishers, Dordrecht, The Netherlands pp. 41–64.

Hwang, H.J., Kim, S.W., Xu, C.P., Choi, J.W., Yun, J.W., 2004. Morphological and rheological properties of the three different species of basidiomycetes Phellinus in submerged cultures. J. Appl. Microbiol. 96, 1296–1305.

Kwon, K.K.L., Hyun, S.J., Sung, Y.Y., Joung, H.L., Jung, H.L., Hong, K., 2002. Isolation and identification of biofilm-forming marine bacteria on glass surfaces in Dae-Ho Dike, Korea. J. Microbiol. 260-266.

Lisansky, S.G., Quinlan, R.J., Tassoni, G., 1993. The Bacillus thuringiensis Production Handbook. CPL Press, Newbury, pp. 124.

Maybury, J.P., Hoare, M., Dunnill, P., 2000. The use of laboratory centrifugation studies to predict performance of industrial machines: studies of shear-insensitive and shear-sensitive materials. Biotechnol. Bioeng. 67, 265-273.

MENV, 2004. Guide sur la valorisation des matières résiduelles fertilisantes: Critères de références et normes réglementaires. Direction du Milieu Rural, Environnement Québec, Canada, pp. 138.

Tenney, M.W., Stumm, W., 1965. Chemical flocculation of microorganisms in biological waste treatment. J. Water Pollution Cont. Fed. 37, 1370–1388.

Tyagi, R.D., Foko, V.S., Barnabé, S., Vidyarthi, A.S., Valéro, J.R. 2001. Simultaneous production of biopesticide and alkaline proteases by Bacillus thuringiensis using sewage sludge as raw material. Water Sci. Technol. 46, 247-254.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., 2002. Studies on the production of Bacillus thuringiensis based biopesticides using wastewater sludge as a raw material. Water Res. 36, 4850-4860.

Yezza, A., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Smith, J., 2004. Scale-up of biopesticide production processes using wastewater sludge as a raw material. J. Ind. Microbiol. Biotechnol. 31, 545-552.

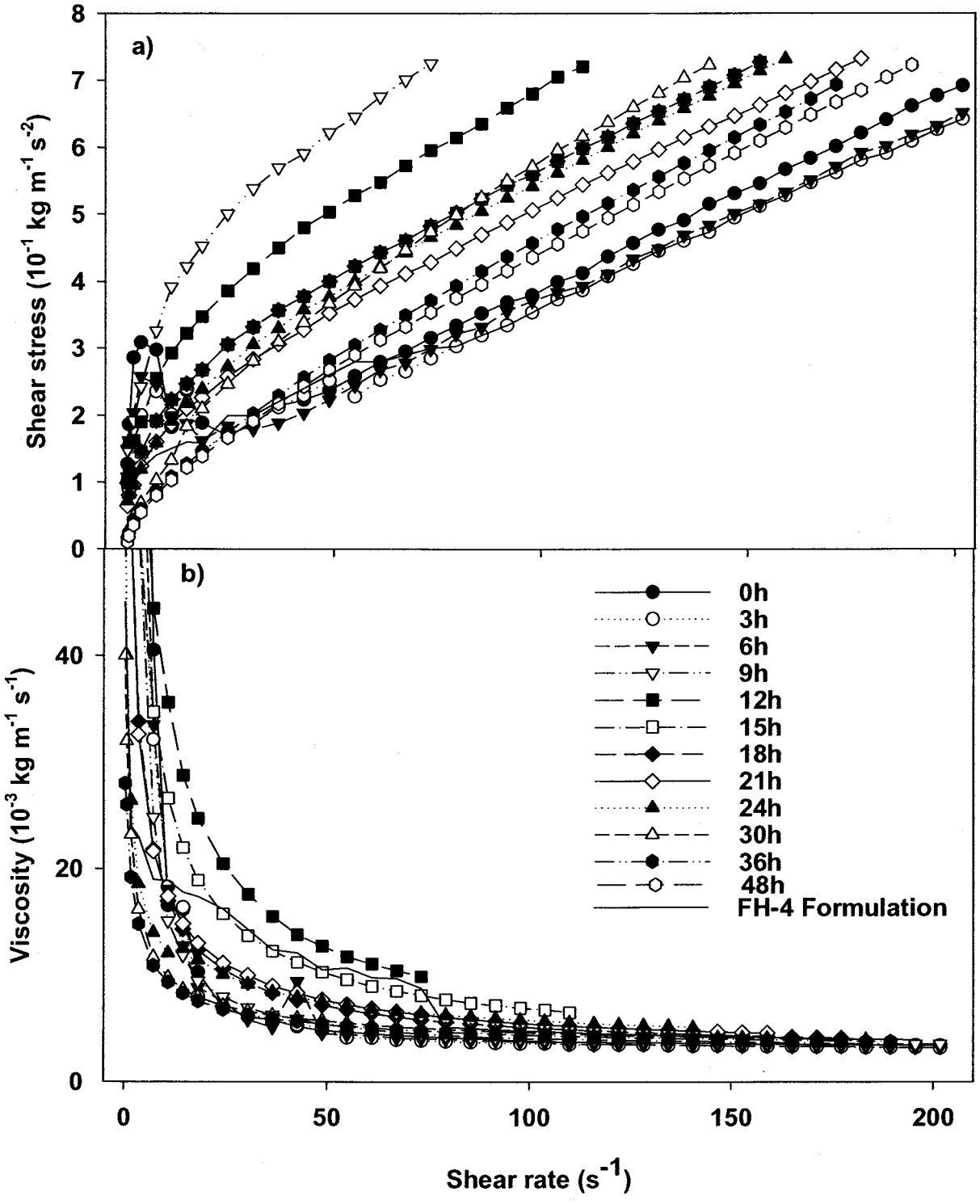


Fig. 1. Rheograms of hydrolyzed sludge during fermentation and formulation: a) shear stress profile and; b) viscosity profile.

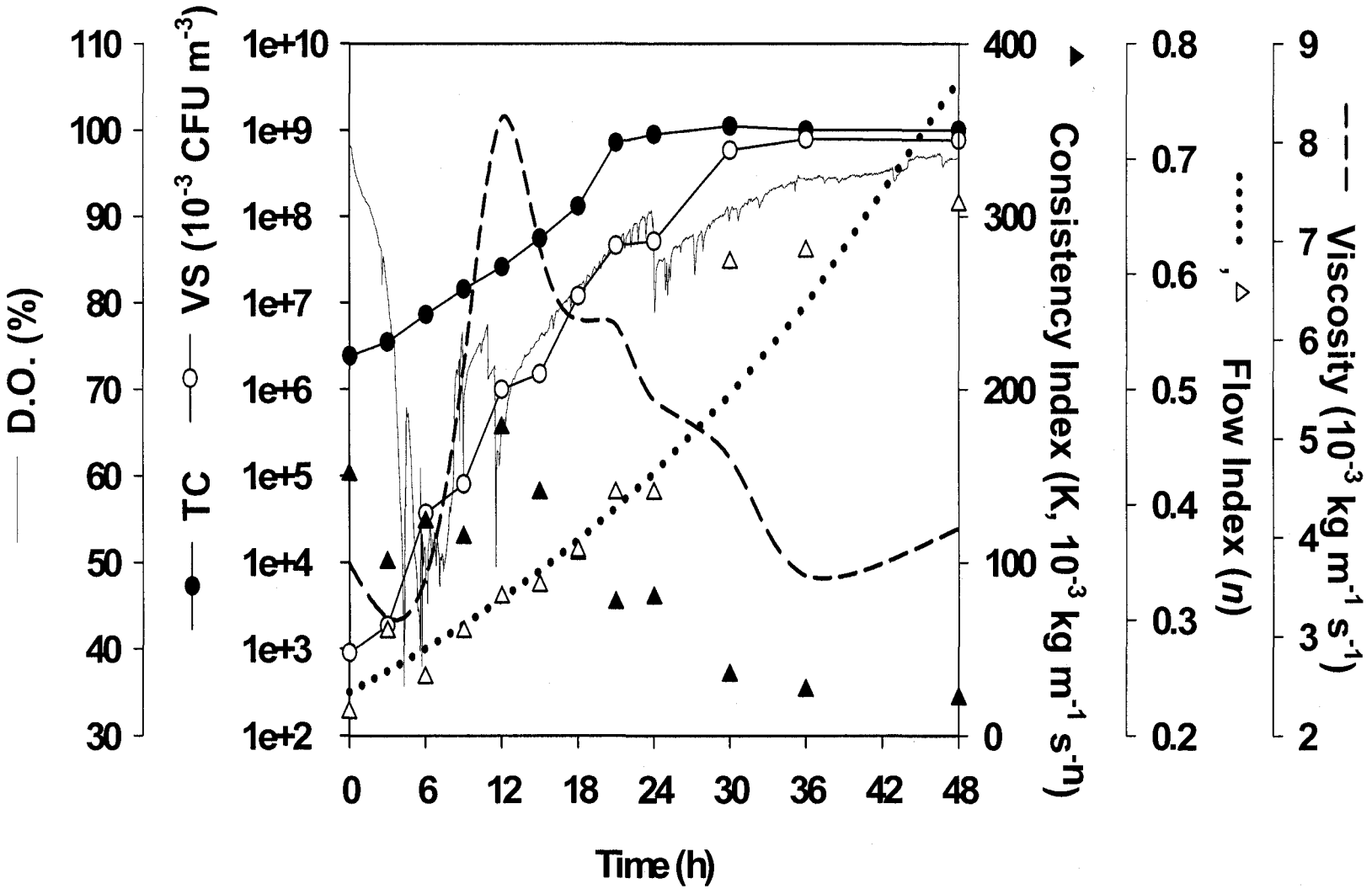


Fig. 2. Power law behaviour of hydrolyzed sludge and its correlation with Bt growth (15 L fermenter).

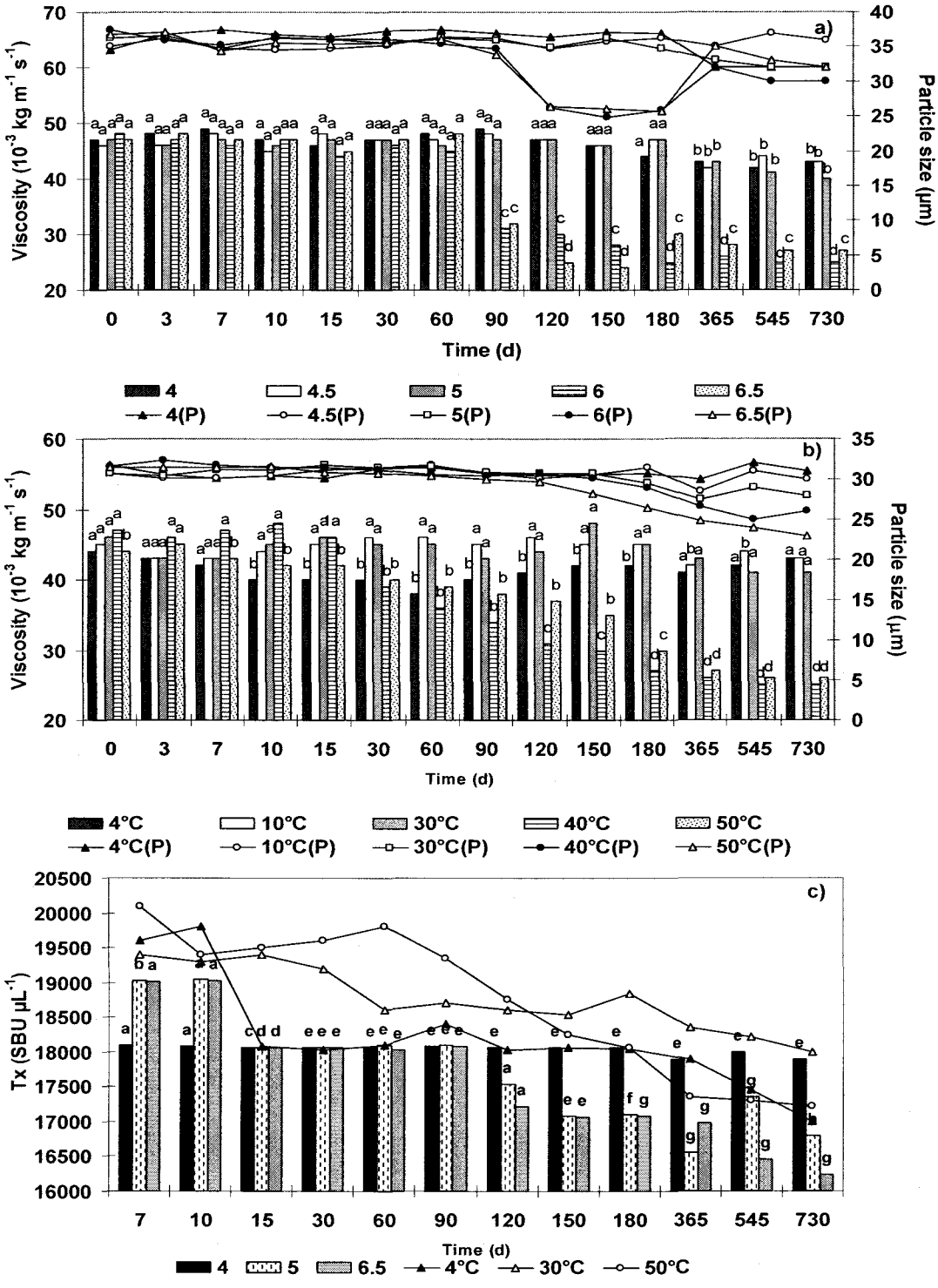


Fig. 3. Stability profiles of TH-30 formulation (FH-4); (a) Rheological profile at different pHs; (b) rheological profile at different temperatures; and (c) Biological efficacy (P, particle size); bars shown with different letters are significantly different at $P \leq 0.05$. Error bars for standard deviation in graphs have been omitted for clarity. Points and lines designated "P" correspond to the particle sizes on the right axis in Fig. 3a and 3b.

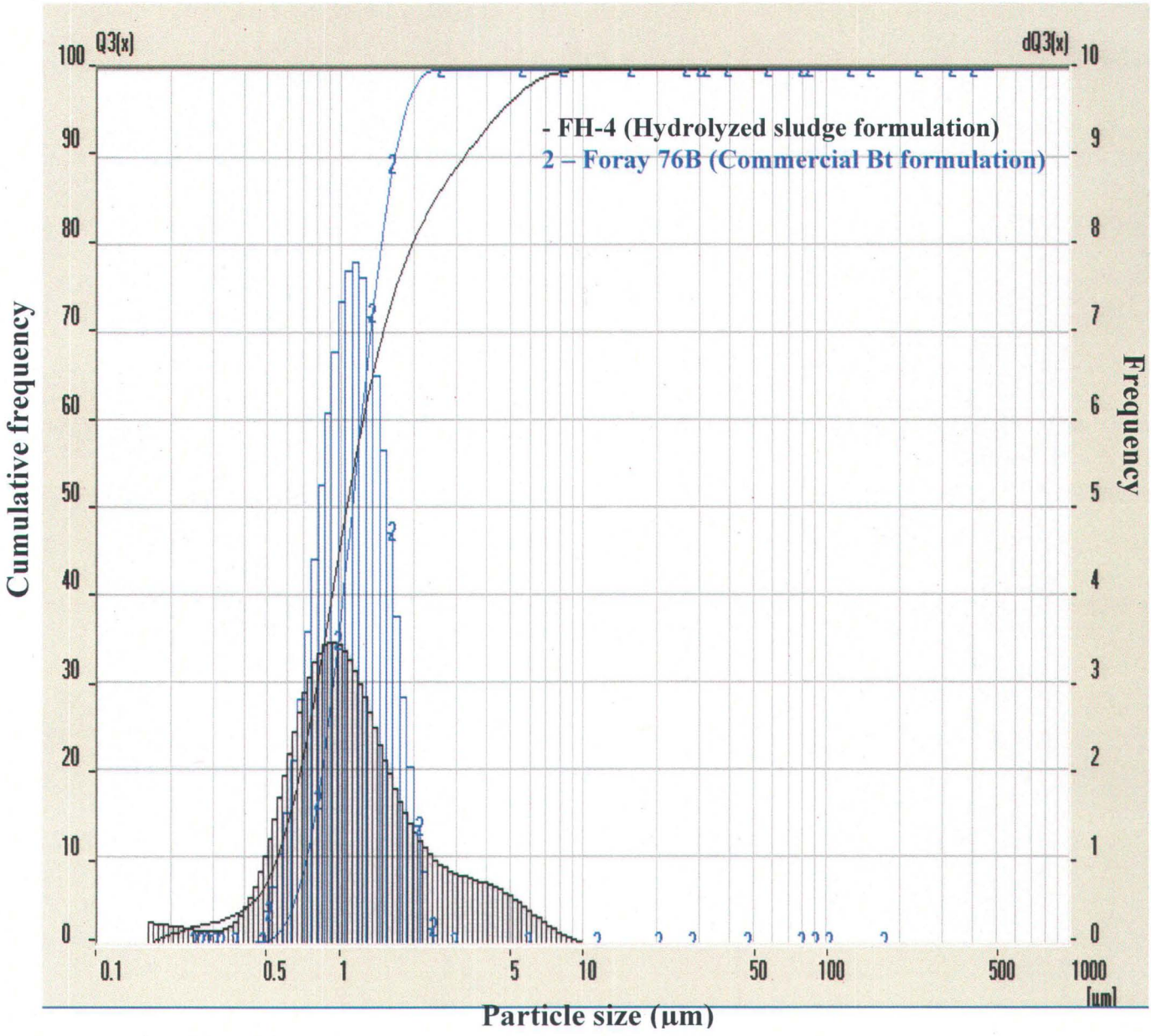


Fig. 4. Particle size profile of stable hydrolyzed sludge formulation and commercial Bt formulation (Foray 76B).

Table 1.

Composition of hydrolyzed wastewater sludge

Parameter (s)	**Concentration ± SE (mg kg^{-1} of TS, unless otherwise stated)**
TS (g L^{-1})	53.5±0.1
TVS (g L^{-1})	28.4±0.1
SS (g L^{-1})	44±0.23
VSS (g L^{-1})	2.3±0.4
pH	8.5±0.1
Total carbon	418977±785
Total nitrogen	39989±2211
Total phosphorus	15702±1734
NH_3-N	549±73.4
NO_2^--N, NO_3^--N	21.9±2.30
PO_4^{3-}- P	8004±3335
S	5978±332.3
Al	13978±2522
Ca	23697±336
Cd	0.3±0.1 (3)
Cr	71±23.6 (210)
Cu	300±161.5 (100)
Fe	8061.9±758.6
K	23.3±4.2
Pb	4.1±1.9 (150)
Zn	395.8±100.2 (500)
Na	15287±1021
Ni	5.1±2.2 (62)
As	n.d. (13)

N.B.: The mg kg^{-1} conversion corresponds to weight of metals per unit weight of TS (dry basis).

n.d. – not detected

Values in parentheses represent the levels recommended by Ministry of Environment, Quebec, Canada (MENV, 2004).

Table 2.

Formulation recipe chart (concentrations in % w v^{-1}, unless stated otherwise)

No.	**Basic Additives/Adjuvants**[†]			**Concentration**
1	Propionic acid			0.5
2	Sorbic acid			0.4
3	Glycerol			2
4	Tween-80			0.2
5	Triton X-100			0.1
6	TH Broth (% v v^{-1})			67.3
7	supernatant			to make up
8	Dispersing/suspending agents			Variation
	Specific Adjuvants [††]			
Formulations	Sorbitol	Sodium monophosphate	Sodium metabisulfite	Final Total Solids Concentration
FH-1	21	0	0	14.3
FH-2	18	3	0	13.6
FH-3	15	5	0	14.1
FH-4	**11**	**5**	**5**	**14.7**
FH-5	9	7	5	15.3

[†] Basic adjuvants were added to all formulations

[††] Specific adjuvants were added to respective formulations to study their effect during shelf storage

Table 3. Different rheological model fits of Bt fermented hydrolyzed sludge and stable formulation (FH-4)

Time (h) →	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**	**36**	**48**	**FH-4**
Bingham Law													
Plastic Viscosity (η, 10^{-3} kg m^{-1} s^{-1})	2.44	2.46	2.38	2.82	7.4	5.06	3.84	3.49	3.91	4.82	3.8	3.6	-
Yield Stress (τ_0, 10^{-1} kg m^{-1} s^{-2})	0.17	0.13	0.15	0.14	0.25	0.21	0.18	0.15	0.15	0.09	0.07	0.07	-
Confidence of fit (%)	87.7	90.4	89.7	95.8	82.8	85.8	88	86.5	85.6	67.3	74.2	68.5	-
Casson law													
Plastic Viscosity (η, 10^{-3} kg m^{-1} s^{-1})	0.85	1.07	0.93	1.27	3.32	2.37	1.97	2	2.25	3.64	2.92	2.84	1.9
Yield Stress (τ_0, 10^{-1} kg m^{-1} s^{-2})	0.13	0.09	0.11	0.09	0.15	0.13	0.1	0.07	0.07	0.03	0.02	0.02	0
Confidence of fit (%)	82.5	87	85.3	89.8	91.4	93.9	96.1	95.7	95.2	88.9	92.6	90.6	71.0
NCA/CMA Casson													
Plastic Viscosity (η, 10^{-3} kg m^{-1} s^{-1})	0.85	1.07	0.93	1.27	3.32	2.37	1.97	2	2.25	3.64	2.92	2.84	1.89
Yield Stress (τ_0, 10^{-1} kg m^{-1} s^{-2})	0.12	0.08	0.1	0.08	0.14	0.12	0.09	0.06	0.07	0.02	0.02	0.02	0
Confidence of fit (%)	82.5	87	85.3	89.8	91.4	93.9	96.1	95.7	95.2	88.9	92.6	90.6	71.0
Power law													
Consistency index (K, 10^{-3} kg m^{-1} s^{-n})	150.6	100	123.2	114.4	177.7	140.1	105.2	76.6	79.6	34.6	26	21.3	18.5
Flow behaviour index (n)	0.22	0.29	0.25	0.29	0.32	0.33	0.36	0.41	0.41	0.61	0.62	0.66	0.84
Confidence of fit (%)	73.6	79.5	76.9	78.6	96.9	94.8	91.9	92.5	92.3	96.8	95.2	96.3	93.6
IPC Paste													
Shear sensitivity factor (n_s)	21.3	17	19	19.1	32.5	26.3	21.2	17.6	18.4	13	10.1	9.12	5.6
10 rpm viscosity (η_{10})	0.78	0.71	0.75	0.71	0.68	0.67	0.64	0.59	0.59	0.39	0.38	0.34	0.24
Confidence of fit (%)	73.6	79.5	76.9	78.6	96.9	94.8	91.9	92.5	92.3	96.8	95.2	96.3	91.6

Shaded cells represent lower confidence of fits.

Table 4.

List of ANOVA factors for Bt fermentation of hydrolyzed sludge

Figure	Mean (10^{-3} CFU m^{-3})	F	df ; df*	P
2	1.03 x 10^{9} (TC at 48 h)	1.643	2 ; 6	> 0.05
2	7.68 x 10^{8} (VS at 48 h)	0.879	2 ; 6	> 0.05

df : degree of freedom, between groups

*df**: degree of freedom, within group

Table 5.

Effect of different specific additives/adjuvants on percent spore survival and suspendibility of different formulations

	Percent spore survival				
pH	FH-1	FH-2	FH-3	FH-4	FH-5
4	79 a	79 a	84 b	87 bd	77 a
4.5	71 ac	77 a	80 b	88 bd	80 ab
5	84 b	84 b	90 d	84 b	86 b
6	75 a	81 b	87 bd	81 b	88 b
6.5	66 c	77 a	82 b	77 a	86 b
Temperature (°C)					
4	90 d	81 a	82 b	82 b	80 ab
10	89 b	94 d	84 b	85b	76 a
30	88 b	92d	88 bd	78 a	77 a
50	77 a	88 b	74 ac	83 b	61 c
	Percent suspendibility				
pH	FH-1	FH-2	FH-3	FH-4	FH-5
4	65 ± 5.6a	51 ± 5.6d	54 ± 6.2b	69 ± 6.3ac	68 ± 7.1ac
4.5	54 ± 7.4b	63 ± 7.3a	64 ± 5.3a	76 ± 5.2c	69 ± 6.4ac
5	55 ± 3.5b	65 ± 4.7a	66 ± 5.1a	82 ± 4.3e	81 ± 7.3e
6	72 ± 5.1ac	71 ± 7.6ac	62 ± 2.8a	85 ± 3.7e	82 ± 4.3e
6.5	71 ± 5.6ac	73 ± 4.2ac	63 ± 4.2a	90 ± 6.4f	84 ± 7.8e
Temperature (°C)					
4	48 ± 4.4d	49 ± 6.4bd	54 ± 3.4b	62 ± 2.3a	45 ± 6.3d
10	49 ± 5.2bd	54 ± 4.3b	56 ± 6.5b	68 ± 3.4c	59 ± 5.9b
30	44 ± 6.7d	57 ± 4.5b	57 ± 6.3b	86 ± 4.6e	62 ± 7.7a
50	64 ± 3.9a	68 ± 5.6ac	59 ± 7.3b	80 ± 5.3e	84 ± 8.1e

Values within a column followed by different letters are significantly different at $P \leq 0.05$.

Mean ± SD, means (n = 3). Refer to Table 2 for formulation composition.

CHAPITRE 7.

ÉTUDES TECHNICO-ÉCONOMIQUES

Partie I

Techno-economic Analysis of *Bacillus thuringiensis* Biopesticides Production from Wastewater and Wastewater Sludge

Satinder K. Brar[1], M. Verma[1], R.D. Tyagi[1*], J.R. Valéro[1], R.Y. Surampalli[2]

[1]Institut National de la Recherche Scientifique (INRS -ETE), Université du Québec, 490, de la Couronne, Québec, Canada G1K 9A9

[2]US Environmental Protection Agency, P.O. Box 17-2141, Kansas City, USA, KS 66117

(**P.S**.: This chapter is an extracted version of the comprehensive report submitted at INRS)

Analyse technico-économique de la production de biopesticides à la base de *Bacillus thuringiensis* à partir des eaux usées et boues d'épuration

Résumé

L'analyse technico-économique du procédé de production de biopesticides à base de Bt par fermentation de boues d'épuration et d'eaux usées a pris en considération les étapes de la – fermentation, de la récolte de l'entomotoxicité par centrifugation ou ultrafiltration et de la formulation. Pour les intrants du procédé de fermentation, l'analyse technico-économique a considéré les boues d'épuration brutes, les boues hydrolysées, les eaux usées de l'industrie de l'amidon et, pour la comparaison, le milieu commercial semi-synthétique à base de soya. L'analyse technico-économique est basée sur des résultats expérimentaux obtenus à partir de différentes configurations du procédé. L'analyse a porté sur cinq scénarios applicables à chacun des intrants : (1) la récolte du bouillon (entomotoxicité) par centrifugation; (2) la récolte du bouillon par centrifugation et ultrafiltration; (3) des formulations sèches récoltées par centrifugation; (4) des formulations sèches récoltées par centrifugation et ultrafiltration; et; (5) des formulations liquides avec ajout de mélasse comme phagostimulant. L'analyse des boues d'épuration brutes a inclus deux scénarios additionnels : (1) la fermentation de type <<Fed-batch>> (27% de l'augmentation d'entomotoxicité); et (2) l'addition de Tween-80 comme agent tensio-actif pendant la fermentation pour améliorer le transfert d'oxygène, et ainsi augmenter l'entomotoxicité par 26,6%.

Les calculs de coût ont été effectués à l'aide du chiffrier Excel.

Les coûts estimés de production sont présentés sous la forme d'un ratio « $/entomotoxicité nette ». L'entomotoxicité nette est exprimée en milliards d'unités internationales par litre du produit (MUI/L). Les coûts du procédé complet de production ont été estimés en considérant l'investissement, la consommation de matières premières et les frais d'exploitation. L'analyse a mis en évidence que, pour les scénarios étudiés, la production de biopesticides à partir des eaux usées et de boues d'épuration est économiquement viable. C'est le scénario des boues d'épuration hydrolysées avec ajout de mélasse qui s'est avéré le moins coûteux avec un coût

de production de $ 0,228 Can/MUI. L'utilisation de la mélasse comme un multi-adjuvant (additif multifonctionnel) a permis d'augmenter l'entomotoxicité de 13%. L'ajout de Tween-80 pendant la fermentation des boues brutes s'est avéré encore plus intéressant en raison des meilleures conditions de fermentation avec un coût de produit de $ 0,256 Can/MUI. Le calcul des taux de rendement de l'investissement établis sur la base d'une période de remboursement de 10 ans a démontré que le milieu soya était en tête de liste avec 79,1 %. Le seuil de rentabilité pour un prix de vente de $0,33 Can/MUI est atteint pour une production annuelle de 2×10^7 MUI en utilisant des les eaux usées et des boues d'épuration comme matières premières alternatives et de 3×10^7 MUI/an pour le milieu soya. Pour une usine ayant une capacité de production annuelle de 7×10^7 MUI, les frais d'exploitation (exprimé en $Can/MUI) étaient de 30 à 45 % inférieurs à une usine d'une capacité de production annuelle de $3,5 \times 10^7$ MUI. Les coûts de production sont peu influencés par la distance entre l'usine de production et la source des eaux usées ou des boues d'épuration. L'analyse technico-économique fait ressortir que l'utilisation des eaux usées et des boues d'épuration comme matières premières pour la production de biopesticides à base de Bt est une option commerciale prometteuse.

Mots-clés : *Bacillus thuringiensis*, soya, analyse technico-économique, eaux usées, boues d'épuration

Abstract

The study comprised a techno-economic analysis of alternative growth substrates, namely, raw wastewater sludge; hydrolyzed wastewater sludge (pre-treated); starch industry wastewater for *Bacillus thuringiensis* var. *kurstaki* HD-1 (Bt) biopesticides production in comparison with semi-synthetic commercial soyameal medium. The analysis was based on experimental results obtained with different process configurations (principally, batch type) considered as process scenarios. The universal principal scenarios included, liquid formulation (harvesting of fermented broth by centrifugation); liquid formulation (harvesting by centrifugation and ultrafiltration); dry formulations (harvesting by centrifugation); dry formulations (harvesting by centrifugation and ultrafiltration) and liquid formulations where molasses was added at the formulation stage as a phagostimulant (feeding stimulant, increased the biopesticidal potential, entomotoxicity by 13 %). Furthermore, the raw wastewater sludge included two additional scenarios of fed-batch fermentation (27 % increase in entomotoxicity) and addition of Tween-80 as a surfactant during fermentation to improve the substrate rheology for higher oxygen transfer and consequent entomotoxicity increase by 26.6 %. The estimated product costs (calculated via Excel program) were calculated on net entomotoxicity (billions international units per litre, BIU/L) obtained after formulation development. The hydrolyzed wastewater sludge gave the lowest product cost of $Can 0.228/BIU when molasses was amended as a phagostimulant. Likewise, the addition of Tween-80 during fermentation of raw wastewater sludge was the best process condition at product cost of $Can 0.256/BIU. The discounted cash flow return rate (measure of profitability) for a payback period of 10 years was highest for soyameal medium (79.1 %); however, the profitability was also not lower for alternative raw materials as the value has to be higher than 40 % to justify the economics of the process. The break-even point for unit selling price of $0.33 Can/BIU was reached at production scale of 2 x 10^7 BIU/year for alternative raw materials (wastewater and wastewater sludge) when compared to 3 x 10^7 BIU/year for soyameal medium. The analysis showed that within the range of parameters experimented and studied, the biopesticides production from wastewater and, more particularly, wastewater sludge is economically viable. Thus, use of wastewater and wastewater sludge as Bt biopesticide production raw materials is more promising option from environmental as well as commercial point of view.

Keywords: *Bacillus thuringiensis*; Soyameal; Techno-economics; Wastewater; Wastewater sludge

Introduction

Insect pests have been plaguing the agriculture and forests from time immemorial which results in damage worth several billion dollars. Chemical pesticides with their potential damage to environment, risks to humans and destroying useful insects has stimulated interest in "second generation" of products referred to as biopesticides with growing market (Eagan, 2002). Microorganisms commonly used as biopesticides include fungi, bacteria, viruses, protozoa and other microbial products. These formulated products are normally cost intensive. At this crux, wastewater and wastewater sludge which is omnipresent can be utilized for production of *Bacillus thuringiensis* (Bt) based biopesticides. In fact, various studies have been already carried out on isolation and identification of new Bt strains; process optimization; enhancement of entomotoxicity through pre-treatment and testing at laboratory and pilot scale fermenters; rheology and its effects on fermentation, downstream processing and formulation development (Lachhab et al., 2001; Tirado-Montiel et al., 2001, 2003; Tyagi et al., 2001; Vidyarthi et al., 2000, 2001, 2002; Brar et al., 2004, 2005a,b, 2006a,b; Yezza et al., 2004, 2005 a,b,c, 2006 a,b; Barnabé et al., 2005; Mohammedi et al., 2006). However, to evolve and establish Bt-INRS process as an integrated technology marketing needs to be explored. The marketability and extensive use of Bt based biopesticides is a function of production as well as formulation costs (Lisansky, 1993; Burges, 1998). Earlier techno-economic analysis carried out on soymeal-corn steep liquor medium established 50 % of cost incurred by formulation ingredients (Rowe and Margaritis, 2004). However, this study stand alone cannot predict the economical analysis of other raw material based biopesticides production and comprises following drawbacks:

1. The entomotoxicity of the broth is taken to be very low at 0.61, 1.21and 1.73 BIU/L for batch, low density and high density fed-batch fermentation, respectively.
2. The operation period assumption was typically higher at 330 d than the recommended value (Peters and Timmerhaus, 1980).
3. The fermenter size was very big which could have been otherwise divided into multiple fermenters.
4. Discounted cash flow rate of return (DCFRR), an important parameter which addresses the risk encountered by a typical process technology was not calculated which is the heart of techno-economic analysis (Peters and Timmerhaus, 1980; Ulrich, 1984).
5. The sole harvesting process was assumed to be carried out by centrifugation.
6. The input data required for costing was based on old publications based on results with higher spore and biomass concentration as indicators of higher entomotoxicity. In fact, spore concentrations cannot give the true picture of entomotoxicity of the fermentation broths. One of the references had reported comparison of entomotoxicity to 16000 IU/mg

standard formulation which has become obsolete in the current scenario. The Bt biopesticides production has undergone tremendous change in terms of search for alternative economical raw materials, optimization of process parameters, higher performance strains and entomotoxicity measurements. Hence, some of the interpretations may be over or underestimated.

In the present ordeal to produce economical biopesticides, wastewater and wastewater sludge were used as raw materials which put a question on the future economical repercussions of the alternative technology (for simplicity named, "Bt-INRS process"). Thus, this leads us to the main themes addressed in this article:

- Can the wastewater and wastewater based biopesticide technology become a competitive alternative to conventional soya based technology and which developments and research is necessary to make it more cost-effective?
- Can the wastewater and wastewater based biopesticide technology provide an economically viable opportunity for waste management, suitable for future scale-up and development of biopesticides thereof?

Thus, a detailed techno-economic analysis of Bt-INRS process is presented herein with possible process scenarios; projected cost simulations and break-even curves entailing future ramifications of the alternative technology based on our research derived from fermentation at 15 and 150 L fermenter scale.

Materials and Methods

All prices stated are in Canadian dollars ($Can), taken as equivalent to $ 1.12 (dated 18 May, 2006) for purposes of conversion, unless noted.

General Process Details

Process analysis and economic evaluation of the production of Bt biopesticides for different process scenarios was performed using EXCEL program. The baseline biopesticides production plant capacity was assumed to be 3×10^7 BIU/year based on stand-alone plant in southern Ontario, Canada (Rowe and Margaritis, 2004). The batch and fed-batch fermentation along with dry and liquid formulations were explicitly modeled along with modeling of recovery by centrifugation and ultrafiltration. The principal substrates used for Bt fermentation were – non-hydrolyzed sludge (NH), hydrolyzed sludge (TH), starch industry wastewater (SIW) and soyameal (conventional semi-synthetic medium) along with

different process scenarios as provided in Table 1. A generic flow sheet showing major unit operations for the Bt-INRS process is also illustrated in Figure 1. Although several items of equipment are not included in the flow sheet, their cost was taken into account in the economic analysis (as discussed later).

Upstream processing steps

Transportation of the substrates viz. wastewater sludge (WWS) and/or SIW was assumed to be carried from the wastewater treatment/industrial site to the plant site. If WWS/SIW was considered to be transported from remote source, the maximum distance was assumedly fixed to be 25 km as the radius beyond this distance is not essential and/or preferred for biopesticides production. Subsequently, the substrates were pumped by using a centrifugal pump (P_{c1}) into the storage tank (T-1) which was made up of stainless steel as the substrates are normally at acidic pH (pH=3-3.5 and 5-6.5 for SIW and WWS, respectively). There are numerous valves (number as V_n, n = 1,2....etc.) in the process diagram to control flow of various streams.

Later, a centrifugal pump (P_{c2}) was used to pump the stored raw material (specifically, WWS as it needs to be concentrated) to disc vane centrifuge (DVC-1) where the total solids (10-13 g/L) were concentrated to the optimal suspended solids (25 g/L) concentration as established in earlier studies (Lacchab et al., 2001). The centrifuge was assumed to work continuously. Meanwhile, for hydrolyzed sludge, the raw sludge was concentrated to 45 g/L suspended solids (discussed later). The concentrated sludge (45 g/L) was adjusted to pH 10.25±0.1 (by using 4N NaOH). The WWS (a part of it) was pumped by gear pump (GP-1) directly to pre-fermenter (PF-1) for acclimatization and other to fermenter (F-1). Centrifuge was not considered for soya and SIW as substrates. Details of the raw materials required for each step of production have been discussed in the report with total cost as given in Table 2. Meanwhile, the soya raw materials will be directly transferred to the media tank (MeT-1) where it will be mixed with the process water to make a suspension. Likewise, SIW will be directly pumped using the centrifugal pump (P_{c2}) into the pre-fermenter (PF-1) or fermenter (F-1) as desired. Acid and alkali tanks and pumps have not been shown in the process diagram, but were considered for cost analysis as stated earlier.

The concentrated WWS/ raw SIW was directly transferred to pre-fermenter (for acclimatization, PF-1) and fermenter (F-1) for the fermentation process. The hydrolysis of raw sludge to yield TH sludge was carried out at optimal conditions: 140 ± 1°C for 30 min at

a pressure of 276 kPa (Barnabe et al. 2005) *in-situ* in F-1. Consequently, the decreased pH (8±0.5) was re-adjusted to pH 7.0 ± 0.1 with 4N H_2SO_4 and suspended solids were re-adjusted to 30 g/L. The fermentation facility comprised air compressor (AC-1) for supply of air into the fermenter and steam generation facility included demineralization plant (DMP-1), gas fuelled boiler (GFB-1) and cooling tower (CT-1). There were anti-foam tanks and pumps which have not been shown in the process diagram. Meanwhile, soyameal synthetic medium was kept mixed with an impeller motor (IM-1) in a media tank (MeT) for later use in pre-pre-fermenter (PPF-1) for inoculum preparation (2 %v/v). The sterilization of the respective alternate media, namely, NH, TH sludge and SIW was carried out at 121±0.1°C for 30 min at 15 psig in the fermenter (F-1) after adjusting the pH to 7.0 ± 0.1 by addition of 4N NaOH. However, the soyameal medium was sterilized at 121±0.1°C for 15 min at 15 psig. A part of the pre-inoculum was eventually transferred to pre-fermenter (PF-1) where sterilized raw WWS/SIW will be used to prepare the acclimatized inoculum for subsequent Bt fermentation. The fermentation was considered to be carried out for a total period of 36-38 h (Batch time (h) = medium pumping (1 h) + safety checks (1 h) + sterilization & cooling (8 h) + inoculation (1 h) + (cleaning + wait time) (1+8 h) + fermentation time (36-38 h) = 1 + 1 + 8 + 1 + 8 + 19 + 36-38 = 56-58 h) for a batch scenario (Vidyarthi et al., 2002; Brar et al., 2005a). The fermentation period was 48 h (fed-batch time (h) = medium pumping (1 h) + safety checks (1 h) + sterilization & cooling (8 h) + inoculation (1 h) + (cleaning + wait time) (1+8 h) + fermentation time (48 h) = 1 + 1 + 8 + 1 + 8 + 19 + 36-38 = 56-58 h; intermittent feeding – 2 L at 30 h and 3 L at 48 h)) for fed-batch fermentation (Yezza et al., 2005 a). The entomotoxicity (Tx) of various fermented broths for different fermented broths for batch and fed-batch process is also presented in Table 2.

Downstream processing

After fermentation is complete, the pH of the fermented broth was lowered *in-situ* from 7±0.1 to 4±0.1 with 6M H_2SO_4 (50 ml of H_2SO_4 was approximately used up per 10 L of the fermented broth) inside the fermenter (Brar et al., 2006a). The Bt fermented broths (NH/TH/SIW/soya) were harvested by the use of disc-vane centrifuge (DVC-2) from where the centrifugate slurry was pumped via a gear pump (GP-2) to the formulation mixing tank (MT-1) which comprised an impeller motor (IM-5) for mixing. The commercial centrifuge was sized for RCF of 12000 g for 10 min residence time with 70 % recovery of the entire fermented broth in 2 h based on discussion with a representative from Westfalia Inc. and our earlier studies (Brar et al., 2006a). Meanwhile, the supernatant directly flowed into the ultrafiltration system (UF-1) by gravity where it was concentrated to a retentate which was mixed in a defined proportion based on detailed mass balance carried out. The UF system

was supposed to operate with a membrane of MWCO = 5 kDa. Various optimal parameters used were as follows: transmembrane pressure = 10.33 kPa and feed flux rate = 900 L/h/m^2 as established in our study (Adjalle et al., 2006). The percent Tx recovery based on our experimental work was in the 82-92 % range and the percent used in the calculations here was 90 %.The permeate from the UF system was sent to the wastewater treatment facilities and/or else also used as process water. Different adjuvants (as given in Table 3) for each fermented broth were mixed along with the centrifugate in the mixing tank for liquid formulations. The end-product was directly packed as a liquid formulation product and transported to the market. The concentrated centrifuged broth was mixed with basic ingredients, namely, potassium sorbate, Tween-80, Triton X-100 and additionally with talc powder at 9.8 % w/v with optimal process conditions derived from earlier studies (Lisansky (1993; Teera-Arunsiri et al., 2003). The adjuvant mixed paste was pumped through a gear pump (GP-3) into the ball mill (BM-1) to carry out wet grinding of the mixed paste to attain a specific particle size (standard norm, < 25 μm). The ground paste was later pumped by a gear pump (GP-4) into the spray dryer (SD-1). The final product was packed in bags as dry formulation and was finally transported via a conveyor (Con) to the market.

Economic Analysis

Economic analysis was carried out according to the standard economic protocols as given in Ulrich (1984) and Peters and Timmerhaus (1980), unless stated otherwise. The plant capacity was drawn from a pre-existing biopesticide production plant in southern Ontario as stated earlier. The plant was assumed to operate 24 h/d, 300 days per year, resulting in 126 batches (for all scenarios) and 107 (for fed-batch) per annum with fermentation cycle of 56-58 h and 67 h for batch and fed-batch process, respectively. The base case was taken to employ batch fermentation yielding different net Tx as given in Table 2, resulting in aqueous flowable formulations for use in forestry sold at an average price of $ Can 0.33/BIU. Raw material costs for different ingredients like soyameal medium composition, acid and bases, anti-foam, adjuvant added during formulations were derived from different vendors and only a total of this raw material cost is presented in Table 2. Raw material usage was calculated as kg/d and then the corresponding cost was evaluated as $Can/year.

The capital cost of different equipments and profitability analyzes was based on production of different formulations. The calculations for process equipment design, number of identical units, installed cost, total capital investment, operating costs, revenue and overall profitability (DCFRR – discounted cash flow rate of return) were carried out in EXCEL program. The total capital cost for each of the process scenarios is given in Table 2 with further break-up of each equipment and number of units in Table 4.

Total Capital Cost

Equipment costs were, in general, obtained from Ulrich (1984) and Peters and Timmerhaus (1960), using a Chemical Engineering Plant Cost Index of 473. Both references give log-log graphs of purchase cost vs. unit size from which the equations of linear segments of the appropriate plots were estimated, generally over size ranges and coded to allow calculation of purchase cost as a function of unit size. Pumps and pipes are designed with a safety/overdesign factor of 10-20% (Peters and Timmerhaus, 1980). The capital costs included, total investment, residual value of equipments, depreciation, inventory management and rolling funds. The capital costs comprised, direct costs, namely, purchased equipment costs, purchased equipment-installation costs, instrumentation and controls, piping, electrical equipment and materials, buildings (including services), service facilities, and land and also indirect costs, constituting, engineering and supervision, construction expenses, contractor's fee and contingency (Peters and Timmerhaus, 1980; Ulrich, 1984). For use with process-equipment estimates and chemical-plant investment estimates, the Marshall and Swift equipment cost indices and the Chemical Engineering plant cost indices were considered for the year 2006 (Chemical Market Reporter, 2006). All equipments in contact with aqueous liquids was costed on the basis of 316 stainless steel, unless stated otherwise.

Based on net Tx obtained (derived from mass balance) for different process scenarios, sizing and units of each equipment were carried out in EXCEL program by including design equation of individual equipments (sourced from Peters and Timmerhaus, 1980; Perry et al., 1984). The cumulative figure resulting from this procedure was purchase cost of the equipments. To this value was added, 47 % of purchased-equipment installation cost, 18 % of instrumentation and controls, 66 % of piping (installed), 11 % of electrical (installed), 18 % of building (including services), land (purchase required) at 6 %. Furthermore, the values were summed up with indirect costs that comprised: 33 % of engineering and supervision, 41 % of construction expenses, 21 % of contractor's fee and 42 % of contingency. The sum total of these values resulted in total fixed-capital investment. Further, the total fixed capital-investment was 85 % of total capital investment for a process scenario confirming 15 % of working capital. This type of preliminary capital cost estimate, based on a detailed process flow sheet and approximate mass balances is estimated to be accurate to ± 30 % limits (Peters and Timmerhaus, 1980; Ulrich, 1984). The capital cost estimates for various equipments along with detailed specifications have been presented in Table 3.

Total Product Cost

Total product cost comprised manufacturing costs and general expenses. They included three classification of costs; 1) fixed charges, 2) direct production costs, and 3) plant-overhead costs. Raw material usage and costs were summed up. Operating labor consisted of four operators on each of three shifts, each costed at $Can 40 836 per annum (36838 – salary + 3998 – social welfare and insurance allowance, for Quebec). Direct and supervisory clerical labour was assumed to be 10% of the working labour. Individual costs were then calculated for utilities - steam generation ($Can 0.0051 per kg of steam), electricity ($0.05 per kWh), cooling and process water, demineralized water (for steam), refrigerated water, and compressed air. Maintenance and repairs were assumed to be 2% of fixed-capital investment; operating supplies were 10% of the cost of maintenance and repairs; laboratory charges were 10 % of operating labour. Fixed charges included – fixed-capital investment, depreciation which was 10% of the fixed-capital investment for machinery; local taxes (0.6% of fixed-capital investment); insurance (1 % of fixed-capital investment). Plant-overhead costs were assumed to be sum of 15 % of (cost of operating labour, 10 % of cost of operating labour and 2% of fixed-capital investment). General expenses included – administration labour at sum of 15% of the cost for operating labor, supervision and maintenance, or 2 % of total product cost; distribution and selling (20% of total product cost); research and development (30 % of each sales dollar) and; financing (10% of total-capital investment). Detailed cost calculations showed costs for different waste streams disposal to be negligible. Total product cost for various process scenarios is presented in Table 3.

Profitability Analysis

The profitability analysis was carried out by performing discounted cash flow rate of return (DCFRR) simulations in EXCEL program based on a trial-and-error procedure to establish a rate of return (break-even point) which could be applied to yearly cash flow so that original investment reduced to zero during the entire project life (Ulrich, 1984). The calculation was performed for a payback period of 10 years assuming that $\geq$ 40% DCFRR justified the economics of the process with negligible risks (Peters and Timmerhaus, 1980). Payback period was estimated by Equation 1:

$$\text{Payback period including interest} = \frac{\text{depreciable fixed} - \text{capital investment} + \text{interest on total capital investment during estimated service life}}{(\text{avg profit/yr} + \text{avg depreciation/yr})_{\text{as constant annuity}}} \quad (1)$$

Current Canadian federal tax rate was taken to be 32%.

Detailed DCFRR simulations were performed as follows: a) the DCFRR was designated as "i"; b) the cumulative of product of discount factor ($f_d = \frac{1}{(1+i)^n}$) and net cash income ($A_{NCI}=A_I+A_{BD}+A_A+A_{NNP}$) was calculated; and c) iterations were performed with respect to "i" so that $A_{NCI} \rightarrow 0$.

Results and Discussion

Total Capital Cost

Table 2 presents total capital investment for each of the substrates with various process scenarios and the break-up for each of the equipments is given in Table 4 with number of units and specification ranges for each piece of equipment. For a stand-alone plant manufacturing liquid formulations, total capital cost was approximately $ Can 21 million for NH-Tween -80; $ Can 18 million for TH-Molasses; $Can 20 million for SIW-Molasses and $ Can 20 million for soya-Molasses, the best scenarios of each of the substrates. Direct costs {purchased-equipment delivered cost, purchased-equipment installation cost, instrumentation and controls, electricity (installed), piping (installed), buildings (including services) and, land (purchase required); indirect costs (engineering and supervision, construction expenses, contractor's fee and contingency) and; working capital were 56, 29 and 15%, respectively for NH-Tween, TH-Molasses, SIW-Molasses and Soya-Molasses. Major items of equipment included, a fermenter of nearly 125 m^3, two medium sized disc-stack centrifuges; 10 ultrafiltration units, a spray dryer (not required for aqueous flowable formulations) and, utilities (air compressor unit, gas fuelled boiler, chilling unit, cooling tower) accounting collectively for 25.2 % of total capital investment.

Production Cost Analysis

Raw material costs for different process scenarios are presented in Table 2. It was found that TH-UFDF scenario showed the least raw material costs. Sludge transportation costs as given by Boileau & Associates (1989) comes out to be $Can 58 per ton at 3 tons per day over a distance of 25 km for a thickened sludge at 22% solids incorporating the costs of diesel and natural gas prevalent currently into a model developed at INRS (Modéle Métix). With the solids from 2.5 to 3 %, the transportation costs for sludge would come down to $Can 8 / ton when $Can 58/ton was considered as a standard incorporating the costs of transportation - handling, loading, unloading, and gas expenditure. In fact, according to the transport tariff established by different truck owners as validated by Transports Quebec was also found to be approximately $ Can 9/tonne over a distance of 25-30 km (costs regulated until 29 March, 2007).

Best Scenarios

Figure 2 represents cost distribution break-even pie charts for best scenarios. It was established that WWS scenarios, namely, NH-Tween and TH-Molasses showed the least contribution by formulation raw materials at approximately 7 %. On the contrary, SIW and soya-Molasses scenarios showed approximately 29 % contribution by formulation adjuvants. However, raw material-substrate cost contribution was higher at 16 % exclusively in the case of soyameal medium due to the requirement of different medium components.

Figure 3 shows unit production cost vs. annual production rates for the best scenarios (NH-Tween 80; TH-Molasses; SIW-Molasses and Soya-Molasses) for each of the raw materials at different simulated production scales. The Figure 3 also shows a line corresponding to the assumed selling price of $0.33 per BIU (personal communication, J.R.Valéro).

It was seen from the Figure 3 that regardless of the type of raw material for Bt fermentation, the production cost decreased sharply as the production scale was increased from 1 x 10^6 BIU/year to 1 x 10^7 BIU/year. Later, the production cost did not decrease much as the production scale was increased. It was also seen that increasing formulation entomotoxicity from 14.3 BIU/L (Soya) to 16.3 BIU/L (SIW) showed a strong effect on lowering the unit production cost, while a further increase to 16.5 BIU/L (TH sludge) only had a slight effect on the production cost. The production scale at the break-even point for the assumed selling price of $Can 0.33/BIU occurred at approximately 2 x 10^7 BIU/year for NH sludge, TH sludge and SIW and at 3 x 10^7 BIU/year for soyameal medium. This established the fact that the unit selling price for the alternative materials, i.e., NH, TH sludge and SIW will have great impact on the production scale at which the break-even point will be reached.

These results were comparable to those obtained by Rowe and Margaritis (2003) who explored the fermentation of soyameal-corn steep liquor medium for batch, low density and high density fed-batch fermentation. They reported the production scale of 2 x 10^7 BIU/year for low and high density fed-batch fermentation. Interestingly, the batch fermentation break-even point reported by Rowe and Margaritis (2003) was 6 x 10^7 BIU/year which was very high in comparison to the one reported in our case as we have considered all batch scenarios. This difference could have arisen due to the net Tx difference which was almost 2-folds (Table 2) as compared to the one reported in this study (1.21 to 1.73 BIU/L). The lower net Tx or as referred to as "broth potency" (it appeared to be a misnomer) by the authors was based on some data derived from Lisansky et al. (1993) which would just change the capital-dependent operating costs. However, nowadays, the trend is to achieve maximum kill with

minimum dosage (maximum efficacy per droplet) and in this context, a study carried out in Quebec, Canada established 30 BIU/ha (BIU, billion international unit) as the optimal dosage at 1.5 L/ha spray volume by using a high potency (20 BIU/L) product (Bauce et al., 2004). Thus, the production cost estimates in our study are more realistic as compared to theirs as these are based on the actual experimental results in our laboratory.
The output from detailed manufacturing cost analysis carried out for the best case scenarios (NH-Tween80; TH-Molasses; SIW-Molasses and Soya-Molasses) for the batch fermentation for different production scales are presented in Table 5.

It was observed from Table 5 that at all the three scales (7.5 x 10^6 BIU/year; 3 x 10^7 BIU/year and 6 x 10^7 BIU/year), TH sludge and SIW as raw materials gave significantly lower direct production and utilities costs than NH sludge and soya as well as TH sludge gave somewhat lower fixed charges and general expenses dictated by the development of enhanced potency products. The lowest overall manufacturing/production costs, whatever the production scale may be, occurred for the TH sludge alternative although the direct production costs were slightly lower for SIW.

As observed in Figure 3 and Table 5, production scale had a large impact on the total per-unit operating cost ($Can/BIU), which at 3 x 10^7 BIU/year is 30-45 % of that at 7.5 x 10^6 BIU/year. Amounts in all categories of operating costs were substantially reduced at higher scale, with the category showing the least reduction depending on the type of raw material used for Bt fermentation and also the best scenario conditions that is the productivity in terms of higher entomotoxicity. Thus, for batch fermentation of different raw materials (NH sludge, TH sludge, SIW and soya), the reduction in the operating costs was more for utilities followed by direct production costs followed by fixed charges and plant overhead costs and general expenses.

Comparison of Techno-Feasibility of Different Process Scenarios

When the techno-feasibility of different batch (until, specified otherwise) fermentation scenarios, namely, four different substrates, NH sludge, TH sludge, SIW and soyameal – scenario-I: LS; scenario-II: UFLS; scenario-III: UFDF; scenario-IV: DF; scenario-V-Molasses; scenario-VI-Fed-batch and scenario-VII: Tween-80 was considered, it was found that the use of molasses as a phagostimulant, sticker and UV screen was found to be the best case for all scenarios and more particularly, NH sludge showed the best scenario with the use of Tween-80 as a surfactant.

It was found that TH sludge came out to be the best raw material for Bt fermentation despite the hydrolysis process involved which incurred additional equipment costs. However, utilization of fermenter as a hydrolyzer minimizes the cost of equipment and hence, production costs. It was observed that at the base production scale of 3 x 10^7 BIU/year, the direct production costs for SIW was the lowest at $Can 0.064/BIU (Table 2, 5, Figure 4) which may be due to the lower need of acid and base for pH adjustment as well as lower centrifugation costs (as centrifugation was not required at the preliminary stage of medium amendment). Meanwhile, the DCFRR results showed that soyameal medium gave the highest DCFRR of 79.1 % in a payback period of 10 years which could be due to the reduction in fixed charges (no need of centrifugation in the upstream processing and lower steam requirements as sterilization time before fermentation would be 15 min compared to 30 min for other alternative materials as well as lower quantity of adjuvant in formulations). The DCFRR (%) followed the order: Soya (79.1) > TH sludge (63.27) > NH sludge (61.1) ≥ SIW (60.03).

However, despite the higher profitability for soyameal medium, the TH sludge comes out to be a winner with lower product price of $Can 0.228/BIU when compared to other raw materials for Bt fermentation. In terms of the product entomotoxicity, 66B product was obtained for TH sludge and on the contrary 69B was obtained for NH sludge, this is far from the actual commercial product, yet much higher when compared to the control scenario of soyameal medium (54B, highest possible net Tx) as a semi-synthetic conventional medium.

The rate of return results have been plotted for different raw materials for Bt production process in Figure 4. The plots showed that irrespective of the raw materials used for Bt fermentation, as net Tx increased, the product cost reduced and the DCFRR (rate of return) increased. Thus, net Tx of the formulated product was the principal determinant in calculating the product cost for different scenarios of various raw materials as it dictated the potency of the final formulation. Meanwhile, higher DCFRR in soya as compared to TH sludge despite lower net Tx was as a result of non-requirement of certain equipments in medium amendment (centrifugation, discussed earlier).

However, when different raw materials were compared amongst each other, under the best-estimate sale price and moderate production scale assumptions, much appears to be gained by employing batch fermentation of hydrolyzed wastewater sludge (TH sludge scenario) as opposed to other raw materials.

Conversely, there is little incentive to employ ultrafiltration to recover the lost Tx in the supernatant during centrifugation so as to push the Tx towards higher values. In fact, the equipment cost needs to be seriously considered (perhaps a cost-benefit analysis could be done) before adopting this option for a full-scale plant. Moreover, the fed-batch process seems to push the net Tx towards higher value as well as reducing the product cost as seen in NH sludge (studies in our laboratory have been only carried out on this alternative). Thus, fed-batch needs to be explored as a sustainable and Tx boosting option for SIW and TH sludge.

The recent trend in Bt biopesticides for forestry sector is decreasing (Dr. Valéro, CFL, Quebec, personal communication). However, the market in public health sector for Bt var. *israelensis* is increasing which comprises about 25 % of the total global usage to prevent the spread of diseases such as West Nile virus, dengue fever, and malaria and thus the calculation methods developed during this study could be further employed/extrapolated for the cost calculations of the other bacterial species. A Bt biopesticides production facility will normally be suited to produce other fermentation based products like *Rhizobium* biofertlizers, *Trichoderma* based bioherbicides, biodegradable plastics, biocoagulants, proteases, antibiotics and various probiotics which will justify the moderate production capacity of 2 x 10^7 to 3 x 10^7 BIU/year. Further, the capital and product cost calculations developed in this study can be extended to the techno-economics of other fermentation technologies to develop value-added products from wastewater/wastewater sludge.

Conclusions and Recommendations

The detailed calculations of techno-economic feasibility for the Bt production process carried out in Excel program have clearly established the possibility of production of Bt based biopesticides from alternative raw materials, namely, non-hydrolyzed sludge, hydrolyzed sludge and starch industry wastewater when compared to semi-synthetic commercial medium. The "ALTV" process comprising wastewater and wastewater sludge as substrates showed cheaper alternative for Bt production of biopesticides. The process can be easily extrapolated to the production of other Bt subspecies like Bt var. *israelensis* and Bt var. *tenebrionis*.

Following conclusions can be drawn from the cost-economics study:

1. The entomotoxicity after fermentation, consequent recovery during the harvesting step (centrifugation and ultrafiltration) and adjuvant addition leading to formulation stability and increase in net Tx after formulations are the three most important factors controlling the Bt production at an industrial scale. These factors direct the total

volume of the raw material or substrate to be fermented which in turn governs the size of fermentors as well as the auxiliary units.

2. The major cost regulating components of the Bt production process is additional link-up of the equipments like ultrafiltration vis-à-vis the recovery of entomotoxicity. Meanwhile, the air compressor and the fermenter single handedly govern the final production cost of the product by affecting both the equipment cost and the operational expenses.
3. The hydrolyzed sludge gave the lowest product cost of $Can 0.228/BIU.
4. The use of molasses as a multi-adjuvant, for phagostimulation, sticking and UV resistance lowered the total product cost by approximately 25 % for all substrates.
5. The addition of Tween-80 in the non-hydrolyzed sludge as a surfactant modified the sludge rheology lowered the product cost to $Can 0.256/BIU when compared to other Bt fermentation substrates.
6. The discounted cash flow return rate (measure of profitability) was highest for soyameal medium (79.1 %); however, the profitability was also not lower for alternative raw materials as the value has to be higher than 40 % to justify the economics of the process (Peters and Timmerhaus, 1980).
7. The break-even point for unit selling price of $0.33 Can/BIU was reached at production scale of 2×10^7 BIU/year for alternative raw materials (wastewater and wastewater sludge) when compared to 3×10^7 BIU/year for soyameal medium.
8. The increase of production scale from 3×10^7 BIU/year to 6×10^7 BIU/year reduced drastically the overall operating costs in terms of $Can/BIU which was again lower for hydrolyzed sludge as a Bt fermentation medium.
9. The production scale had a large impact on the total per-unit operating cost ($Can/BIU), which at 3×10^7 BIU/year was 30-45 % of that at 7.5×10^6 BIU/year.
10. For batch fermentation of different raw materials (non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and soya), the production costs were affected more by the change in direct production costs and utilities in comparison to other parameters.
11. The equipment cost calculations (capital cost parameters) also demonstrated lower cost input for hydrolyzed sludge.
12. The scenario with ultrafiltration as an additional unit operation for recovery of entomotoxicity did not enhance the overall entomotoxicity of the final Bt formulations and in fact turned out to be a cost intensive scenario for both liquid and dry formulations which could be further investigated for further directions.

13. The dry formulations do produce the same net entomotoxicity as liquid formulations, however, the cost of spray drying as a unit operation makes the entire process more costly with higher overall operating costs.
14. Thus, liquid suspensions supplemented with molasses as a multi-adjuvant came out to be the best scenario for batch fermentation of hydrolyzed sludge at a base scenario of 3×10^7 BIU/year with formulation entomotoxicity of 16.52 BIU/L (62.4B).
15. The production costs showed negligible changes for the remote site location of the Bt biopesticides production plant even when transportation cost was included (within a distance of 25 km).
16. The risk factor in the entire Bt biopesticide production process from alternative raw materials was almost negligible and thus, the process was cost-effective.
17. Thus, use of alternative raw materials as Bt biopesticide production raw materials is more promising option from environmental as well as commercial point of view.
18. The province of Quebec is the right choice in Canada to produce sludge based Bt by virtue of its low electricity tariffs as well as close proximity to major Bt (forestry and public health sector) markets such as Quebec itself, New Brunswick, New York and Ontario.

Thus, the aforediscussed conclusions take us to following recommendations and future outlook in research on use of alternative materials as raw materials for Bt biopesticides production:

1. The production cost of the wastewater sludge scenarios can be lowered by omitting the need for centrifugation and directly using the dewatered sludge as a fermentation medium. Although dewatered sludge has been found to give lower Tx, still it could be supplemented with some nutrients so that it can serve as a raw material for Bt biopesticides production.
2. It is recommended to set up the biopesticides production plant in the same facility as the raw material production, this will save transportation expenses or, at least it must be located closer to the wastewater/wastewater sludge generation facility.
3. The Tx can be increased by addition of Tween-80 which also needs to be looked into as Tween addition during fermentation can help in saving on the costs during formulation stage.
4. There is a need to increase the spore concentration during fermentation of especially starch industry wastewater by at least one log units which could be carried out by testing fed-batch strategy or addition of soluble starch.

5. There is also a need to test the supplementation of chitin in starch industry wastewater fermentation which could increase the entomotoxicity by increasing the crystal protein toxicity. There is no need to do this in wastewater sludge scenario as the wastewater sludge does contain inherent source of chitin (dead fungal cell walls) and thus, chitinases, important virulence factor that synergize Tx is produced *in-situ*.
6. The volume of the supernatant lost in the centrifugation process is higher in starch industry wastewater (8L when compared to 6L in hydrolyzed sludge for a 10L of final fermented broth), efforts need to be concentrated on the possible recovery of higher Tx lost in the supernatant either through use of filter aids or differential centrifugation. Meanwhile, the solids at the upstream level for starch industry wastewater could be concentrated (by ultrafiltration) so as to enhance the nutrient content which is embedded in the suspended solids; this could also improve the recovery during downstream processing.
7. Fed-batch fermentation studies must also be carried out for hydrolyzed wastewater sludge which can substantially reduce the product cost.
8. Although ultrafiltration process aids in recovery of entomotoxicity, yet addition of this process in a large scale plant would warrant higher cost finally increasing the selling price of the final formulation product.
9. It is possible that the ultrafiltration process recovered suspension can be used as such as Bt formulations for the low quantity requirement sectors like, agriculture and gardens.
10. The best way to reduce the product cost is to carry out research on multi-purpose adjuvants, for example, molasses, which can serve as a sticker, phagostimulant as well as a UV screen.
11. The use of sorbitol as an expensive suspending agent in Bt formulations can be drastically used by utilizing alternatives such as carboxy methyl cellulose in much lower quantities and cost as well as further quantitative optimization studies on the same would yield better results and lower cost of formulations.
12. There is a need to investigate cheaper ingredients/adjuvants which can replace the present cocktail of adjuvants in Bt formulations.
13. There is a need to explore advanced formulations like microencapsulations where the Bt spores and crystal proteins can be protected in the suspended solids core of wastewater sludge by simple physico-chemical reaction of binding and encapsulation and the cost reduced by means of use of various adjuvants.
14. Dry formulations like wettable powders must be investigated as they offer dual advantages of techno-feasibility as well as ease of transportation and handling.

15. Finally, field application of biopesticides developed from alternative materials need to be carried out to establish the potential sustainability, registration and marketability of the alternative raw material based Bt biopesticides.

LIST OF ABBREVIATIONS

AC	Air compressor
A_{NCI}	Net cash income ($ Can)
A_I	Net income ($ Can)
A_{BD}	Book value depreciation ($ Can)
A_A	Allowances ($ Can)
A_{NNP}	Net profit after taxes ($ Can)
BSBU	Billion spruce budworm units
BIU	Billion international units
BM	Ball mill
Con	Conveyer
CEPCI	Chemical engineering plant cost index
CER	Currency exchange rate
CT	Cooling tower
DCFRR	Discounted cash flow rate of return ($)
DM	Demineralization
DF	Dry formulation
DVC	Disc vane centrifuge
F	Fermenter
f_d	Discounted interest (%)
GFB	Gas fuelled boiler
GP	Gear pump
IM	Impeller motor
IU	International units
i	Interest rate
kDa	Kilo Dalton
LS	Liquid suspension
MSI	Marshall and Swift equipment cost index
MOC	Material of construction
MeT	Media tank
MT	Mixing tank
MWCO	Molecular weight cut-off
n	Project life-time (years)

NH	Non-hydrolyzed sludge
P_c	Centrifugal pump
PF	Pre-fermenter
PPF	Pre-pre-fermenter
SD	Spray dryer
SIW	Starch industry wastewater
T	Storage tank
TH	Thermal alkaline hydrolyzed sludge
Tx	Entomotoxicity (SBU/L)
Tx_i	Initial entomotoxicity (SBU/L or BIU/L)
Tx_c	Entomotoxicity in the centrifugate (SBU/L or BIU/L)
Tx_{for}	Entomotoxicity of formulation (BIU/L)
UF	Ultrafiltration
V	Valves
WW	Wastewater
WWS	Wastewater sludge

Acknowledgements

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Grants A4984, STR 202047); Canada Research Chair; University of Missouri, Columbia and U.S. EPA). The views and opinions expressed in this article are those of authors and should not be construed as opinions of the U.S. Environmental Protection Agency. The authors are also thankful to Natural Sciences and Engineering Research Council of Canada, Canada Forestry Services and Société de protection des forêts contre les insectes et maladies (SOPFIM) for providing scholarship to Satinder K. Brar during the course of this research work. The authors are sincerely thankful to Isabelle Bernier and other vendors who provided the price for different equipments and chemicals.

References

Adjalle, K.D., Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006. Ultrafiltration recovery of entomotoxicity from *Bacillus thuringiensis* fermented wastewater and wastewater sludge. *Water Research* (submitted).

Barnabé, S., Verma, M., Tyagi, R.D. and Valéro, J.R. 2005. Culture media for increasing biopesticide producing microorganism's entomotoxicity, methods of producing same, biopesticide producing microorganisms so produced and method of using same. US patent International application PCT/CA2005/000235.

Boileau and Associes Inc. 1989. Etude des couts d'immobilisation et d'exploitation concernant les techniques de traitement des boues et modes d'elimination finale – volet I. Consortium de financement pour la realisation d'un projet de recherche et de demonstration sur les techniques de la valorisation agricole des boues de station d'epuration. Rapport integral 480-87-04. Projet : 000-4-11-04-71-13.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. and S. Banerji. 2004. Development of sludge based stable aqueous *Bacillus thuringiensis* formulations.*Water, Science and Technology*, 50 (9), 229-236.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005a. Sludge based *Bacillus thuringiensis* biopesticides: Viscosity Impacts. *Water Research*, 39, 3001-3011.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005b. Starch industry wastewater based stable *Bacillus thuringiensis* liquid formulations. *Journal of Economic Entomology*, 98(6), 1890 - 1898.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006a. Efficient centrifugal recovery of *Bacillus thuringiensis* biopesticides from fermented wastewater and wastewater sludge. *Water Research*, 40, 1310-1320.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2006b. Screening of different adjuvants for wastewater/wastewater sludge based *Bacillus thuringiensis* formulations. *Journal of Economic Entomology*, 99(4), 1065-1079.

Burges, H.D. (ed.) 1998. *Formulation of Microbial Biopesticides: Beneficial organisms, nematodes and seed treatments*, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Chemical Market Reporter. 2006, April 24. New York: Schnell Publishing.

Eagan, K. 2002. Demand for pesticides on the rise. The Freedonia Group, Inc. PRIMEDIA Business Magazines & Media Inc. NY, USA.

Lachhab, K., Tyagi, R.D., Valéro, J.R., 2001. Production of *Bacillus thuringiensis* biopesticides using wastewater sludge as a raw material: effect of inoculum and sludge solids concentration. *Process Biochemistry*, 37(2), 197–208.

Lisansky SG, Quinlan RJ, Tassoni G. 1993. The *Bacillus thuringiensis* production handbook. Newbury: CPL Press, p. 124.

Mohammedi, S., Subramanian, B., Song, Y., Tyagi, R.D. and Valéro, J.R. 2006. Molecular screening of *Bacillus thuringiensis* strains from wastewater sludge for biopesticide production. *Process Biochemistry*, 41(4), p. 829-835.

Perry, R.H., Green, D.W. and Maloney, J.O. 1984. Perry's Chemical Engineer's Handbook, 6th edition, McGraw Hill Inc., New York, NY, U.S.A.

Peters, M. S., and Timmerhaus, K. D. 1980. Plant design and economics for chemical engineers. 3rd edition, McGraw Hill Book Co., Toronto, Canada, 973 pp.

Rowe, G.E. and Margaritis, A. 2004. Bioprocess design and economic analysis for the commercial production of environmentally friendly bioinsecticides from *Bacillus thuringiensis* HD-1 *kurstaki*. *Biotechnology and Bioengineering*, 86(4):377–88.

Teera-Arunsiri A, Suphantharika M, Ketunuti U. 2003. Preparation of spray-dried wettable powder formulations of *Bacillus thuringiensis*-based biopesticides. *Journal of Economic Entomology*, 96(2):292–299.

Tirado-Montiel, M.L., Tyagi, R.D., Valéro, J.R., 2001. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. *Water Research,* 35 (16), 3807–3816.

Tirado-Montiel M.L., Tyagi R.D., Valéro J.R. and Surampalli R.Y. 2003. Production biopesticides using wastewater sludge as a raw material – Effect of process parameters. *Water Science and Technology*, 48 (8): 239-246.

Tyagi, R.D., Foko, V.S., Barnabé, S., Vidyarthi, A.S., Valéro, J.R. 2001. Simultaneous production of biopesticide and alkaline proteases by *Bacillus thuringiensis* using sewage sludge as raw material. *Water Science and Technology*, 46 (10), 247–254.

Ulrich GD. 1984. A guide to chemical engineering process design and economics. New York: Wiley. 472 p.

Valéro, J.R. 2006. Canadian Forestry Service, Laurentides Forestry Centre, Sainte-Foy, Quebec (personal communication).

Vidyarthi, A.S., Desrosiers, M., Tyagi, R.D., Valéro, J.R. 2000. Foam control in biopesticide production from sewage sludge. *Journal of Industrial Microbiology and Biotechnology*, 25, p. 86–92.

Vidyarthi, A.S., 2001. Effect of surface active agents on the production of biopesticides using wastewater sludge as a raw material. *Water Science and Technology*, 44 (10), p. 253–259.

Vidyarthi, A.S., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y. 2002. Studies on the production of *B-thuringiensis* based biopesticides using wastewater sludge as a raw material. *Water Res.* 36 (19), 4850–4860.

Yezza, A., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y., Smith, J. 2004. Scale-up of biopesticide production processes using wastewater sludge as a raw material. *Journal of Industrial Microbiology and Biotechnology*, 31, 545-552.

Yezza, A., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. 2005a. Production of *Bacillus thuringiensis* based biopesticides in batch and fed cultures using wastewater sludge as a raw material. *Journal of Chemical Technology and Biotechnology*, 80, p. 502-510.

Yezza, A., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. 2005b. Wastewater sludge pre-treatment for enhancing entomotoxicity produced by *Bacillus thuringiensis* var. *kurstaki*. *World Journal of Microbiology and Biotechnology*, 21, p. 1165–1174.

Yezza, A., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. 2005c. Influence of pH control agents on entomotoxicity potency of *Bacillus thuringiensis* using different raw materials. *World Journal of Microbiology and Biotechnology*, 00, p. 1–10.

Yezza, A., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. 2006a. Correlation between entomotoxicity potency and protease activity produced by *Bacillus thuringiensis var. kurstaki* grown in wastewater sludge. *Process Biochemistry*, 41, p. 794-780.

Yezza, A., Tyagi, R.D., Valéro, J.R. Surampalli, R.Y. 2006b. Bioconversion of industrial wastewater and wastewater sludge into *Bacillus thuringiensis* based biopesticides in pilot fermentor. *Bioresource Technology*, 26, p. 1850-1857.

Table 1. Different scenarios for equipment design and cost-estimation of the Bt process (scenarios applicable to all raw materials, unless stated otherwise)

Scenario (s)	Description	Code	Details
I	Liquid formulation/suspension	LS	Batch process comprising conventional steps of raw material amendment, fermentation, **harvesting by centrifugation** and formulation (aqueous) development comprising optimal concentration of adjuvants for each raw material as presented in Table 3.
II	Liquid formulation with recovery step of ultrafiltration	UFLS[†]	Batch process comprising conventional steps of raw material amendment, fermentation, **harvesting** by centrifugation and **ultrafiltration** and formulation (aqueous) development comprising optimal concentration of adjuvants for each raw material as presented in Table 3.
III	Dry formulation with recovery step of ultrafiltration	UFDF[†]	Batch process comprising conventional steps of raw material amendment, fermentation, **harvesting** by centrifugation and **ultrafiltration** and **formulation (dry)** development comprising basic adjuvants, namely, potassium sorbate, Tween-80, Triton X-100 and talc powder as filler (Lisansky et al., 1993)
IV	Dry formulation without ultrafiltration	DF	Batch process comprising conventional steps of raw material amendment, fermentation, **harvesting** by **centrifugation** and **formulation (dry)** development comprising basic adjuvants, namely, potassium sorbate, Tween-80, Triton X-100 and talc powder as filler (Lisansky et al., 1993)
V	Molasses as phagostimulant in formulation	molasses	Batch process comprising conventional steps of LS scenario (I) with **supplementation of molasses** (0.2 % w/v) at formulation step which increased Tx of formulations by 13 % and acted as a multi-adjuvant, phagostimulant, sticker and UV screen as reported by Brar et al. (2006b)
VI	Fed-batch process (**applicable only to NH sludge**)	fed-batch	**Fed-batch strategy** has been reported to increase Tx from 13 BSBU/L to 18 BSBU/L almost equivalent to TH sludge (Yezza et al., 2005a), the subsequent steps involved **harvesting by centrifugation** and formulation (aqueous) development comprising optimal concentration of adjuvants as presented in Table 3.
VII	Tween fortification during fermentation (**applicable only to NH sludge**)	Tween-80	**Tween-80 addition** during NH sludge fermentation has been found to increase the Tx by 26.6 % by improving rheology (Brar et al., 2005a) and the increased Tx after fermentation affected the net Tx and the subsequent steps comprised **harvesting by centrifugation** and formulation (aqueous) development comprising optimal concentration of adjuvants as presented in Table 3.

[†] The UFLS and UFDF scenarios were based on following assumptions: 1).Based on preliminary results of a mixture of 2 x centrifugate: 1 x supernatant, the mixture gave approximately 5.8 % increase in Tx of the mixture and; 2) After adjuvant addition, in scenario-1, total dilution may be assumed as 1.13 x (folds), the change in Tx after formulation may be calculated on the basis of $Tx_{scenario\text{-}I} \times Tx_{scenario\text{-}II}/Tx_{centrifugate}$. The preliminary results were drawn from Adjalle et al. (2006).

Table 2. Total cost of raw materials, capital investment, product cost and DCFRR of different substrates of Bt-INRS process

Scenario (s)	Raw material cost ($Can/year)[†]	Total capital investment ($ Can 000)	Annual total product cost ($Can/year)	Net Tx (BIU/L)[††]	Total product cost, $ Can/ BIU (Unit price)[†††]	DCFRR (%)[††††]
NH						
NH-LS	1783438	21492	8232536	2.88 (55B)	0.274	63.08
NH-UFLS	1610626	26837	9352735	3.04 (58B)	0.311	58.13
NH-UFDF	179685	36416	9822422	3.04 (58B)	0.327	46.55
NH-DF	189667	31075	8608736	2.88 (55B)	0.287	47.64
NH-Fed-batch	1890764	22755	8774800	3.044 (58.2B)	0.292	62.47
NH-Molasses	1535725	21169	7954211	3.221 (61B)	0.265	62.01
NH-Tween-80	1392594	20846	7700526	3.646 (69B)	0.256	61.11
TH						
TH-LS	1652138	18427	7221960	5.848 (55B)	0.241	64.32
TH-UFLS	1560856	23817	8355069	6.19 (66B)	0.279	58.44
TH-UFDF	155345	33411	8854661	6.190 (58.5B)	0.296	45.85
TH-DF	164430	28021	7624888	5.848 (55B)	0.254	46.91
TH-Molasses	1468062	17861	6868632	6.608 (62.4B)	0.228	63.27
SIW						
SIW-LS	1307780	20977	7714862	2.44 (55B)	0.258	60.87
SIW-UFLS	1227301	26153	8804954	2.6 (58B)	0.293	56.39
SIW-UFDF	205270	35747	9755519	2.6 (58B)	0.325	47.02
SIW-DF	218730	30571	8586613	2.44 (55B)	0.287	48.21
SIW-Molasses	1164291	20479	7411759	2.757(61.3B)	0.248	60.03
Soya						
Soya-LS	2476355	21799	10455028	2.772 (48B)	0.357	81.53
Soya-UFLS	2342818	26749	11437821	2.93 (50.3B)	0.389	71.97
Soya-UFDF	1152483	36317	12190461	2.93 (50.3B)	0.415	57.75
Soya-DF	1152484	30859	10922146	2.93 (50.3B)	0.372	60.81
Soya-Molasses	2199510	20799	9799766	3.132 (54B)	0.335	79.07

[†] Total raw material cost = raw materials for fermentation and formulation

[††] Net Tx (BIU/L) = = $Tx_{formulation}$ x $V_{formulation}$/ L of medium; respective values in parentheses represent the final Tx of the formulation reported as BIU/ U.S. gallon which is represented as, "B".

[†††] The Canadian price was calculated at an exchange rate of $US 1 = $Can1.12 as on 18th May, 2006.

[††††] DCFRR has been calculated over a payback period of 10 years.

Shaded cells represent best scenarios with lower total product cost

Table 3. Formulation recipe chart (concentrations in % w/v, unless stated otherwise)

No.	Basic Additives/Adjuvants[†]	Concentration
1.	Propionic acid	0.5
2.	Sorbic acid	0.4
3.	Glycerol	2
4.	Tween-80	0.2
5.	Triton X-100	0.1
6.	Concentrated fermented broth, NH/TH/SIW/Soya (% v/v)	54/67.3/57.4/47.2
7.	supernatant	to make up
8.	Dispersing/suspending agents	Variation

	Specific adjuvants [††]			
Formulations	Sorbitol	Sodium monophosphate	Sodium metabisulfite	Final Total Solids Concentration
1	21	0	0	14.3
2	18	3	0	13.6
3[‡‡‡†††]	15	5	0	14.1
4[‡‡††]	11	5	5	14.7
5[‡†]	9	7	5	15.3

[†] Basic adjuvants were added to all formulations

[††] Specific adjuvants were added to respective formulations to study their effect during shelf storage

[‡] NH fermented sludge formulation

[‡‡] TH fermented sludge formulation

[‡‡‡] SIW/soya fermented formulation

Table 4. Equipment details and capital investment of different process scenarios for Bt-INRS process

Item description	Unit Value $ Can 000[†]	Specification range[††]	Number of each equipment (s)																						
			NH							TH					SIW					Soya				
			NH-LS	NH-UFLS	NH-UFDF	NH-DF	NH-Fed batch	NH-Molasses	NH-Tween 80	TH-LS	TH-UFLS	TH-UFDF	TH-DF	TH-Molasses	SIW-LS	SIW-UFLS	SIW-UFDF	SIW-DF	SIW-Molasses	Soya-LS	Soya-UFLS	Soya-UFDF	Soya-DF	Soya-Molasses
Unit-100: Medium handling, storage and concentration																									
101: Centrifugal pump (P_{c1} & P_{c2})	10292.8	50-75 cu.m@100psi	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
102: Medium storage tank (T-1)	37800	58-150 cu.m	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
103: Disc centrifuge (DVC-1)	514624.3	36-72 cu.m/h, 0-5 kW	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Unit-200: Medium Preparation Utilities																									
201:Media mixing tank (Me T-1)	256.48	12.0 cu.m, 15 kW	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
202: De-mineralization plant (DMP-1)	50453.76	0.002 cu.m/s	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
203: Cooling tower (CT-1)	252266.6	0.1 cu.m/s	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
204: Steam generator (GFB-1)	169827.8	9000-11500 kg steam per h @ 121°C & 7000-8000 kg steam per h @ 140°C	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Item description	Unit Value $ Can 000[†]	Specification range[††]	Number of each equipment (s)																						
			NH							TH					SIW					Soya				
			NH-LS	NH-UFLS	NH-UFDF	NH-DF	NH-Fed batch	NH-Molasses	NH-Tween 80	TH-LS	TH-UFLS	TH-UFDF	TH-DF	TH-Molasses	SIW-LS	SIW-UFLS	SIW-UFDF	SIW-DF	SIW-Molasses	Soya-LS	Soya-UFLS	Soya-UFDF	Soya-DF	Soya-Molasses
Section-300: Fermentation																									
301: Air compressor (AC-1)	167254	800-1000 scfm, 75 kW	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2
302: Fermenter (F-1)	1597847	51-139 cu.m, 15-81 kW	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
303: Pre-fermenter (PF-1)	266167	1.02 cu.m, 2-5 kW	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
304: Pre pre-fermenter (PPF-1)	62235	0.022-0.056 cu.m, 1 kW	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
305: Alkali storage tank	16128	1.5 cu.m	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
306: Alkali pump	7719	6.8 cu.m per h	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
307: Acid storage tank	16128	1.5 cu.m	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
308: Acid pump	7719	6.8 cu.m per h	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
309: Anti-foam tank	16128	1.5 cu.m	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
310: Anti-foam pump	7719	6.8 cu.m per h	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Item description	Unit Value $ Can 000†	Specification range††	Number of each equipment (s)																					
			NH							TH					SIW					Soya				
			NH-LS	NH-UFLS	NH-UFDF	NH-DF	NH-Fed batch	NH-Molasses	NH-Tween 80	TH-LS	TH-UFLS	TH-UFDF	TH-DF	TH-Molasses	SIW-LS	SIW-UFLS	SIW-UFDF	SIW-DF	SIW-Molasses	Soya-LS	Soya-UFLS	Soya-UFDF	Soya-DF	Soya-Molasses
Section-400: Harvesting and product recovery																								
401: Disc-vane centrifuge (DVC-2)	514624	36-72 cu.m/h, 5 kW	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
402: Ultrafiltration (UF-1)	115593	180 LPH, 93 kW	0	10	10	0	0	0	0	0	10	10	0	0	0	10	10	0	0	0	10	10	0	0
403: Gear pump (GP-2)	11580	100 kW	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Subtotal																								
Section-500: Formulation and packaging																								
501: Gear pump (GP-3)	11580	40-75 cu.m/h, 100 kW	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
502: Mixing tank (MT-1)	129696	12-29 cu.m, 15 kW	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
503: Ball mill (BM-1 & BM-2)	308717	10.0 ton/h	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
504: Spray dryer (SD-1)	2018134	20 cu.m	0	0	1	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	1	1	0

Table 5. Annual production costs for best scenario of different substrates for Bt production process at different production scales (X: 7.5 x 10^6 BIU/year; Y: 3 x 10^7 BIU/year; Z: 6 x 10^7 BIU/year); all values in $Can/BIU*

	NH sludge			TH sludge			SIW			Soya		
Production scale →	X	Y	Z	X	Y	Z	X	Y	Z	X	Y	Z
Direct production costs (including raw materials)†	0.132	0.072	0.062	0.122	0.072	0.063	0.124	0.064	0.054	0.162	0.110	0.092
Utilities††	0.088	0.022	0.011	0.073	0.018	0.009	0.096	0.024	0.012	0.264	0.066	0.032
Fixed charges + plant overhead costs†††	0.321	0.081	0.040	0.274	0.068	0.035	0.321	0.080	0.039	0.307	0.076	0.038
General expenses††††	0.321	0.082	0.043	0.274	0.071	0.037	0.320	0.081	0.041	0.315	0.082	0.043
Total*	**0.864**	**0.256**	**0.156**	**0.744**	**0.228**	**0.143**	**0.851**	**0.248**	**0.147**	**1.048**	**0.335**	**0.206**

† - Comprises raw materials, operating labor and direct supervisory and clerical labor

†† - Comprises steam, electricity, process water, wastewater treatment, maintenance and repairs, operating supplies, laboratory charges, patents and royalties

††† - Comprises depreciation, local taxes, insurance and rent

†††† - comprises administration, distribution and selling, research and development and financing (interest)

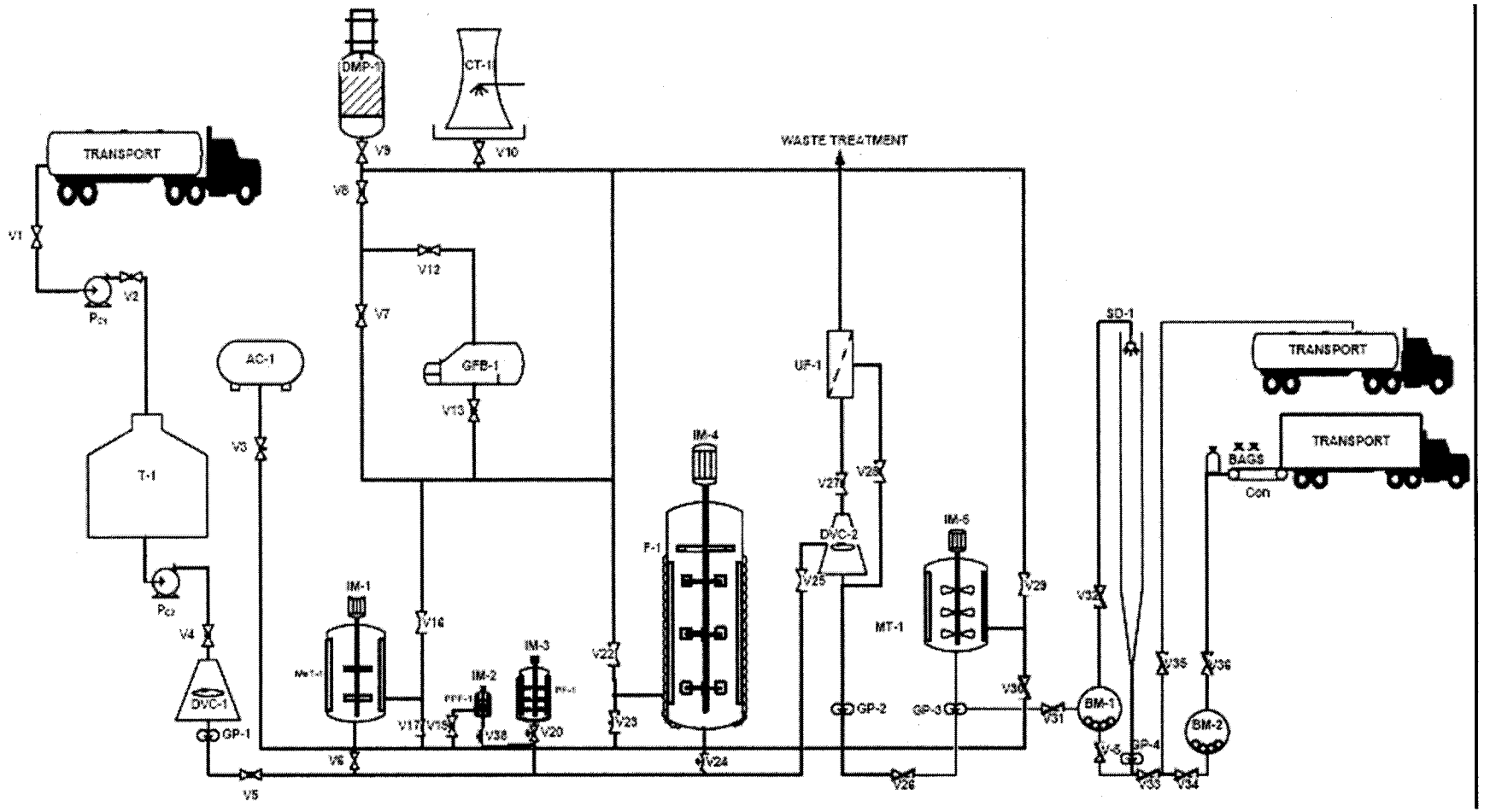


Figure 1. Flow diagram of "Bt-INRS" production process (includes all possibilities, namely, scenario with centrifugation and ultrafiltration as well as dry and liquid formulations). Acid, alkali and anti-foam addition tanks are not shown for the simplicity of the schematic, but they have been included in the equipment design and costs.

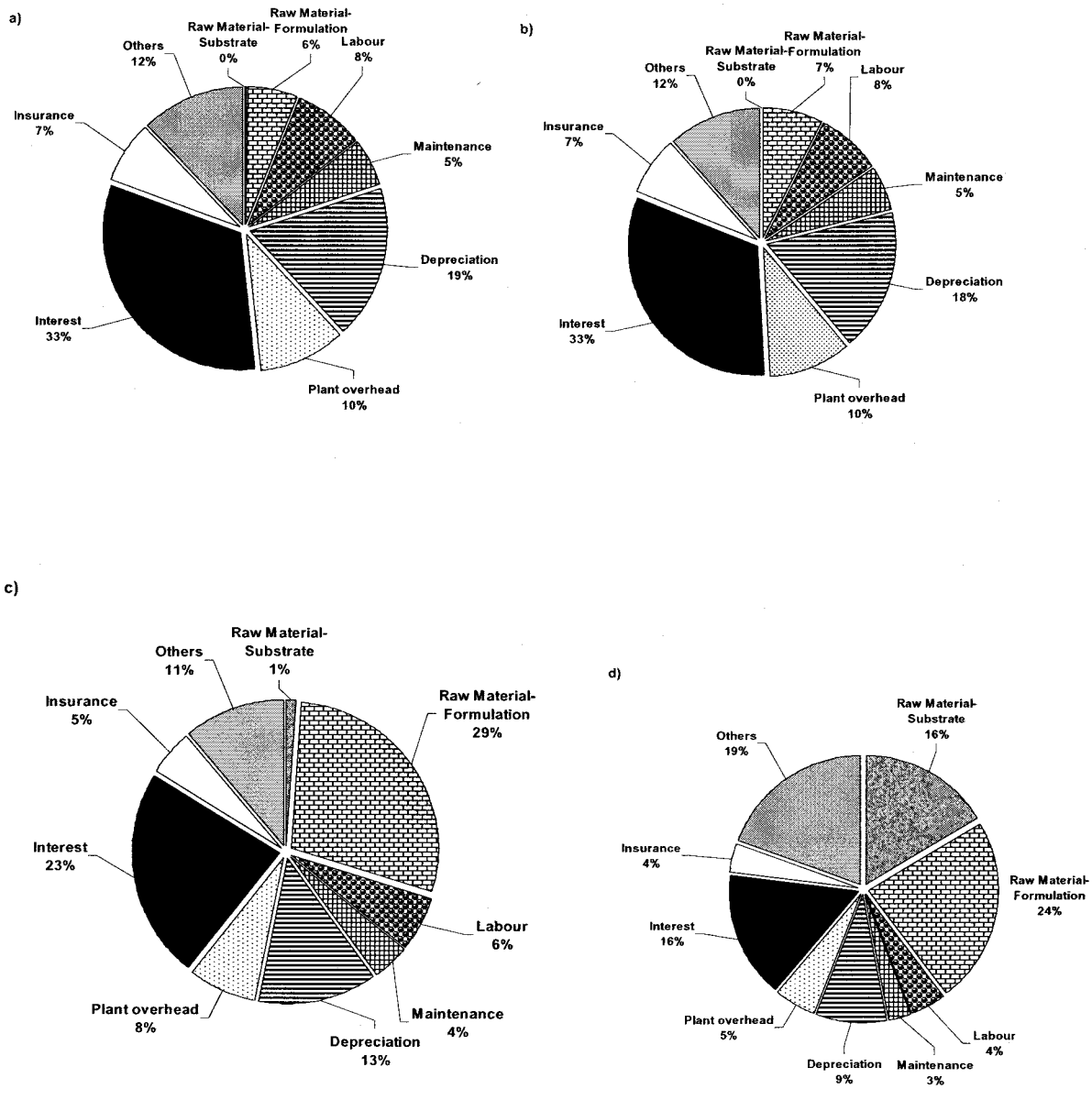


Figure 2. Production cost distribution break-even pie charts for best process scenarios of different substrates; a) NH-Tween 80; b) TH-Molasses; c) SIW-Molasses and; d) Soya-Molasses

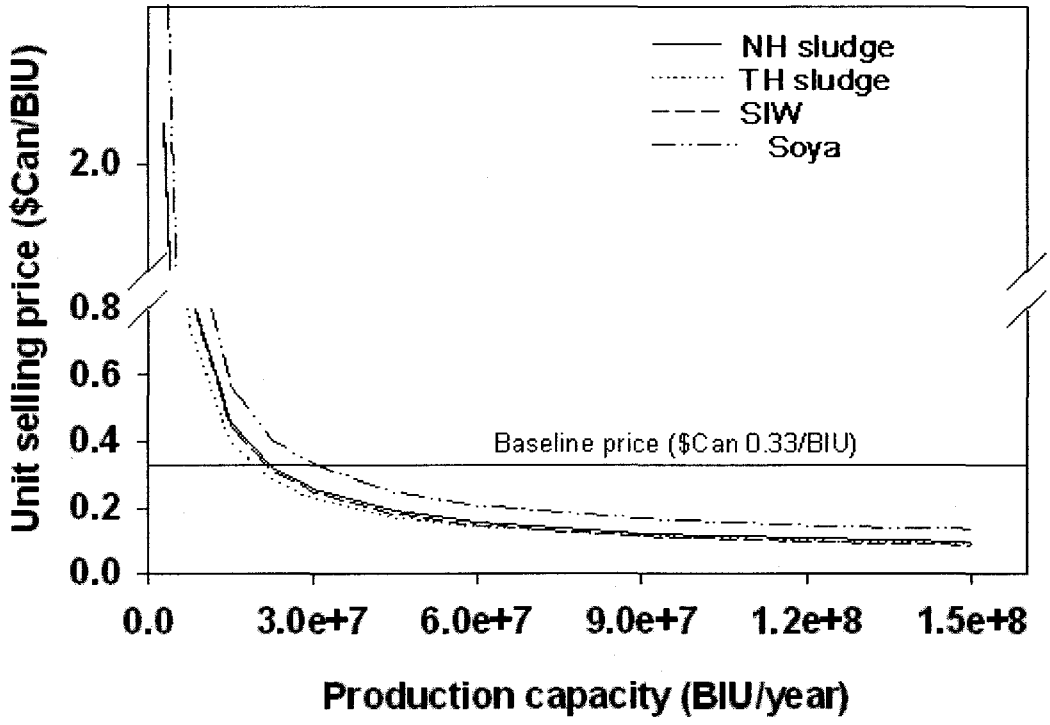


Figure 3. Total per unit production cost vs. production scale for best scenarios: NH sludge (NH-Tween 80); TH sludge (TH-molasses); SIW (SIW-Molasses) and Soyameal (Soya-Molasses) media fermentation

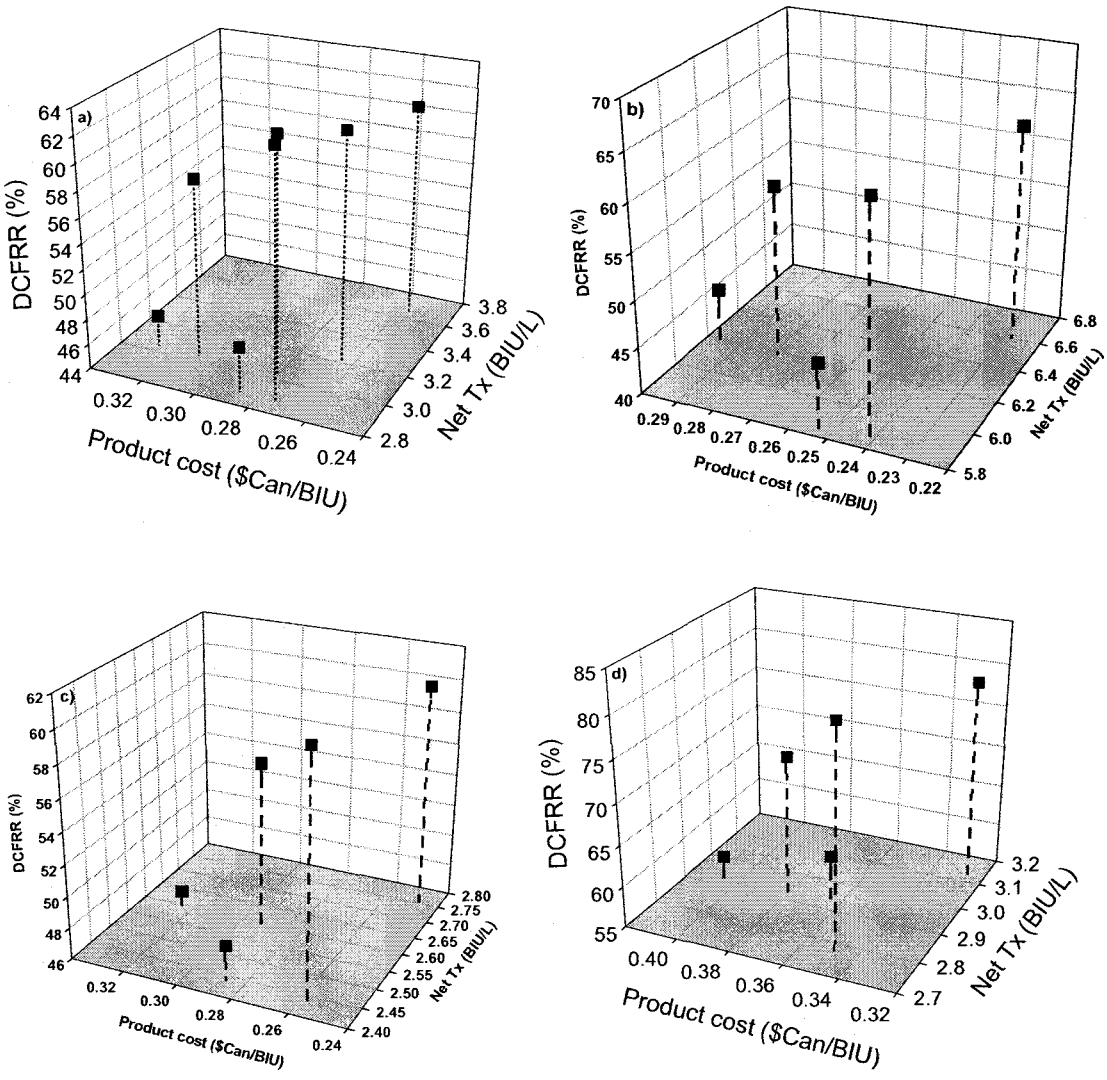


Figure 4. Rate of return on capital invested in batch fermentation by using different raw materials vs. product selling price and net Tx obtained after formulation development for different scenarios: a) NH sludge; b) TH sludge; c) SIW and; Soyameal.

CHAPITRE 8.

CONCLUSIONS ET RECOMMANDATIONS

8.1. Conclusions

La recherche effectuée a démontré que le développement des biopesticides à base de *Bacillus thuringiensis* (Bt) en utilisant des eaux usées et des boues d'épuration comme matières premières alternatives pour la fermentation est une technologie intéressante, rentable, efficace et écologique. Cette technologie présente divers avantages par rapport aux biopesticides de Bt produits à partir de milieux semi-synthétiques conventionnels:

1. Les eaux usées ou des boues d'épuration sont disponibles en tout temps à travers le monde et ont un coût nul et même négatif, ce qui réduit les coûts de production de 30 à 40%. L'utilisation de ces matières premières alternatives est donc une option économique dans le secteur de la production de biopesticides à base de Bt en comparaison avec le milieu semi-synthétique normalement utilisé.
2. Lorsque les biopesticides à base d'eaux usées ou de boues fermentées par Bt seront pulvérisés sur le terrain, il y aura une biofertilisation des sols en combinaison avec le contrôle d'insectes nuisibles.
3. Le produit final obtenu après la fermentation des eaux usées/boues d'épuration par Bt a un potentiel insecticide plus élevé.
4. Les eaux usées et les boues d'épuration possèdent quelques facteurs intrinsèques qui en font un milieu de fermentation s'adaptant bien à la production et à la formulation commerciale de biopesticides : des composantes s'agglomérant en flocs et favorisant la centrifugation ; des polymères agissant comme agents adhésifs ; des chromophores fournissant la résistance contre les UV et des tampons maintenant le pH au niveau désiré. Ces caractéristiques fournissent aux boues des avantages additionnels par rapport aux milieux conventionnels, tant dans le traitement en aval que dans le développement de formulation.

Cette recherche a permis de développer une stratégie efficace pour le développement des biopesticides de Bt, comprenant la fermentation, le traitement en aval et le développement de formulation afin qu'ils puissent atteindre le marché. L'étude technico-économique réalisée en a fait la démonstration. Cette recherche a aussi mis au point de nouvelles méthodes pour augmenter l'entomotoxicité et développer des formulations stables.

L'augmentation de l'entomotoxicité est un paramètre essentiel de l'action insecticide et peut se réaliser par une étude rigoureuse de la rhéologie du procédé de fermentation. Le

prétraitement et l'utilisation des modificateurs de surface comme le Tween-80 améliorent la rhéologie du bouillon, ce qui permet un meilleur transfert de l'oxygène, une assimilation améliorée des nutriments par Bt et une augmentation subséquente de l'entomotoxicité. Les chitinases, un facteur important de virulence produit par Bt qui est connu pour accroître par synergie l'entomotoxicité, ont été identifiées et caractérisées pour la première fois dans les eaux usées et les boues d'épuration fermentées par Bt. Le rôle des protéases dans les formulations a été passée en revue et des expériences ont été entreprises pour comprendre leur rôle. Des formulations stables et aqueuses ont été développées avec une durée approximative de conservation de deux ans par rapport au soya de 1½ an.

Ainsi, les conclusions spécifiques suivantes sont émises avec les résultats obtenus pendant les différentes études:

1. Les boues fermentées (primaires, secondaires et mélangées) présentaient des caractéristiques pseudoplastiques et thixotropiques. Les relations entre la viscosité et la concentration en solides suivent une loi exponentielle pour tous les types de boues fermentées. L'hydrolyse a réduit la viscosité des boues secondaires et mixtes et a fourni de meilleurs substrats de croissance pour la production et la formulation de biopesticides de Bt. La rhéologie (viscosité et taille de particule) a été influencée par trois facteurs, soit la concentration en solides, le type de boues et le type de procédé de traitement. La caractérisation rhéologique joue donc un rôle très important dans le choix du substrat de croissance approprié et également du bouillon fermenté pour la production de produits à valeur ajoutée à partir des boues. D'ailleurs, les résultats en fioles ont permis de prévoir les tendances de la viscosité dans le bioréacteur.

2. La distribution de la taille des particules était de type log-normale bimodale. La taille moyenne des particules a été réduite de presque que 50% suite à une hydrolyse thermo-alcaline. Basé sur la loi de Stoke, la corrélation entre la taille des particules et l'indice volumétrique des boues (vélocité de sédimentation), les boues non hydrolysées fermentées par Bt pourraient être récoltées à faible force centrifuge. Parallèlement, les boues hydrolysées fermentées ont donné de meilleures suspensions liquides (augmentation de la dispersion) et s'adaptent beaucoup mieux avec les équipements usuels de pulvérisation de biopesticides.

3. L'ajout de Tween-80 aux boues non hydrolysées à 25 g/L de solides a permis d'atteindre des valeurs plus élevées de k_La, de concentrations en cellules et en spores, d'entomotoxicité, de taux de croissance spécifique maximal et de taux de consommation d'oxygène par rapport aux boues non hydrolysées sans ajout. À l'opposé, une formation de mousse intense a été observée pour les boues hydrolysées (contenant 30 g/L concentration en solides) en raison de l'action combinée de l'agent tensio-actif Tween 80 et la présence de peptides simplifiés. Toutefois, l'entomotoxicité n'a pas sensiblement changé par rapport aux boues hydrolysées sans ajout. L'addition de Tween-80 à des boues non hydrolysées contenant davantage de solides (30 g/L) a accru le k_La, les concentrations en cellules et en spores, la Tx, le taux de croissance spécifique maximal et le taux de consommation d'oxygène en comparaison avec les boues sans ajout.

4. Les chitinases, un facteur important de virulence par synergie, étaient présentes dans les eaux usées et les boues d'épuration fermentées par Bt. Les eaux usées d'amidon et les boues secondaires fermentées ont permis la production de chitinases avec des pics d'activité plus élevés à pH 4 et à 50°C. Les chitinases étaient stables pendant 14 jours à la température ambiante et à pH 4 après que les échantillons aient été modifiés avec différents adjuvants utilisés pour les formulations de Bt. La présence de chitinases a augmenté l'entomotoxicité des différents bouillons fermentés (boues non hydrolysées > boues hydrolysées > eaux usées d'amidon) et montré leur effet synergique.

5. La faisabilité du traitement en aval par centrifugation a été démontrée. La récupération de l'entomotoxicité (Tx) était élevée pour les milieux à base d'eaux usées et de boues relativement au milieu semi-synthétique à base de soya, sans addition de tous les additifs, contribuant globalement aux économies sur les procédés en aval. Le débit d'alimentation vers la centrifugeuse et les calculs de la vélocité de sédimentation basés sur l'efficacité de récupération de Tx ont permis les calculs du facteur Sigma. Pour une efficacité de récupération de Tx donnée, il a été établi que la consommation en électricité pour une centrifugeuse commerciale à 9000 x *g* et une usine de capacité donnée était: soya > boues non hydrolysées > eaux usées d'amidon > boues hydrolysées.

6. Les essais de différents adjuvants étaient essentiels afin de préparer un mélange de base pour les formulations basées sur les eaux usées et les boues d'épuration fermentées par Bt. La combinaison recommandée est présentée au Tableau 8.1. Les demi-vies pour les formulations de Bt basées sur des eaux usées/boues d'épuration en contact avec les rayons d'UV étaient plus élevées par rapport aux formulations à base du milieu semi-synthétique. Cela suggère que les produits de Bt basées sur des eaux usées ou des boues fermentées auront une efficacité plus élevée sur le terrain.

Tableau 8.1 Concentration optimale des différents adjuvants pour les diverses formulations.

Type de formulation	Agents de suspension	Phagostimulants† (0,5% p/v)	Agents collants / Adhérents† (0,2% p/v)	Agents anti-microbiens† (0,5% p/v)	Protecteurs UV † (0,2% p/v)
Boues non hydrolysées	Sorbitol- 9%; monophosphate de sodium – 7%; metabisulfite de sodium -5%	Mélasses; farine de soya; farine de maïs	Mélasses; Poudre de lait écrémé	Acide sorbique; Acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo
Boues hydrolysées	Sorbitol- 11%; monophosphate de sodium – 5%; metabisulfite de sodium -5%	Mélasses	Mélasses	Acide sorbique; Acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo
Eaux usées d'amidon	Sorbitol- 15%; monophosphate de sodium – 5%	Farine de soya	Mélasses	Acide sorbique; Acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo; acide p-Amino benzoïque
Soya (milieu semi-synthétique)	Sorbitol- 15%; monophosphate de sodium – 5%	Avoine; farine de soya	Poudre de lait écrémé	Acide sorbique; Methyl para benzoate; Acide propionique	Lignosul-phonate de sodium; mélasses; rouge de Congo

† Ces adjuvants ont été seulement testés aux concentrations indiquées.

7. Il est possible de produire des formulations aqueuses stables à partir des milieux de fermentation basés sur des eaux usées d'amidon ou des boues secondaires. La composition des formulations stables est présentée au Tableau 8.1. Indépendamment du type de formulations de Bt, les formulations se détérioraient à pH 6 et 6,5 et à 40 et 50°C.

8. Les études technico-économiques ont mis en évidence que les formulations à base de boues hydrolysées offraient la meilleure rentabilité pour les investisseurs.

Les recherches effectuées dans le cadre de ce projet de doctorat a permis de créer une base solide de connaissances en vue de mettre sur pied un procédé industriel de production de biopesticides de Bt basés sur des matériaux économiques, procédé qui pourrait être adapté à la production d'autres produits comme des bio-fongicides à base de *Trichoderma* sp., des protéases, des biofertilisants à base de *Rhizobium*, des biosurfactants et bien d'autres. L'établissement de différentes techniques analytiques ajoutera une nouvelle dimension aux futures études pilotes pour la production de produits à valeur ajoutée en utilisant des eaux usées et des boues d'épuration comme substrat de fermentation.

8.2. Recommandations

Les prochaines recherches sur la bioconversion des eaux usées/boues d'épuration pourraient avantageusement tenir compte des recommandations suivantes :

1. Le Tween-80 est un modificateur des propriétés rhéologiques. L'addition de cet adjuvant aux eaux usées d'amidon pourrait permet d'augmenter la récupération des spores pendant la centrifugation. De plus, la fermentation en mode <<fed-batch>> et l'addition de chitine pourraient être testées pour évaluer leur contribution à l'augmentation de l'entomotoxicité. La récupération d'entomotoxicité par centrifugation dans les bouillons fermentés à base des eaux usées d'amidon peut être améliorée par l'utilisation d'aides à la floculation ou à la filtration qui peuvent faciliter l'adsorption de différents facteurs de virulence.

2. Des études doivent être effectuées pour vérifier la présence et comprendre l'action synergique d'autres facteurs de virulence, à savoir, les phospholipases et les protéines insecticides végétatives afin de maximiser l'entomotoxicité lors de la fermentation.
3. Le potentiel de suspension des formulations liquides doit être corrélé avec le potentiel zêta pour éviter d'utiliser la méthode de mesure du potentiel de suspension qui est laborieuse.
4. Les adjuvants alternatifs à identifier doivent être multifonctionnels pour réduire le coût final des formulations. Des formulations sèches comme les poudres et les granules humides, doivent être étudiées pour augmenter la gamme de produits issus de ce procédé de production de Bt. Des formulations avancées comme des microencapsulations doivent également être développées afin de pouvoir mettre en marché des produits hautement efficaces et durables.
5. Des études sur le terrain doivent être effectuées pour établir l'efficacité réelle des formulations stables de Bt sous différentes conditions environnementales de pluie, de rosée, de vent, de rayonnement UV et de feuillage.

ANNEXES

Annexe – I

Données

Value Addition of Wastewater Sludge- Future Course in Sludge Re-utilization

Figure 5. Biopesticide production potential of wastewater (sewage) sludge in comparison to semi synthetic conventional medium (soyameal) covering different aspects of production: a) Fermentation (reported as biopesticidal potential, entomotoxicity, Tx-spruce budworm units/L); b) Downstream processing-centrifugation (reported as time required for 70-80% Tx recovery, minutes); c) Formulation development (shelf-life storage, reported in years based on physical and biological stability) and; d) Prospective field application (laboratory UV studies, reported as half-life, days)

	Wastewater Sludge	**Commercial medium-Soyameal**
Fermentation (Tx, 10^9 SBU/L)	15	9
Centrifugation Time (minutes)	60	120
Shelf-Life (years)	1.7	1.3
UV Half-Life (days)	11	3

Annexe – II

Données

Comparative Rheology and Particle Size Analysis of various types of *Bacillus thuringiensis* Fermented Sludges

Figure 1. Rheological behaviour of different sludges, a) viscosity vs. time and, b) viscosity vs. shear rate

	SECONDARY		MIXED		PRIMARY	
Time(minutes)	Viscosity(cP)	S.E.	Viscosity(cP)	S.E.	Viscosity(cP)	S.E.
0	209	20.9	56	5.6	217	21.7
3	193	19.3	56	5.6	212	21.2
6	186	18.6	57	5.7	213	21.3
9	163	16.3	84	8.4	211	21.1
12	150	15	83	8.3	211	21.1
15	139	13.9	80	8	199	19.9
18	128	12.8	66	6.6	196	19.6
21	125	12.5	64	6.4	195	19.5
24	125	12.5	60	6	195	19.5
27	121	12.1	58	5.8	196	19.6
30	120	12	59	5.9	197	19.7
33	120	12	60	6	199	19.9
36	115	11.5	54	5.4	201	20.1
39	111	11.1	46	4.6	200	20
41	112	11.2	39	3.9	198	19.8
44	113	11.3	38	3.8	194	19.4
47	113	11.3	38	3.8	192	19.2
50	114	11.4	38	3.8	189	18.9
53	111	11.1	38	3.8	188	18.8
56	107	10.7	37	3.7	187	18.7
59	108	10.8	36	3.6	185	18.5
61	108	10.8	36	3.6	184	18.4
64	107	10.7	35	3.5	182	18.2
67	107	10.7	34	3.4	180	18
71	108	10.8	35	3.5	178	17.8
74	108	10.8	34	3.4	177	17.7
77	108	10.8	34	3.4	178	17.8
80	107	10.7	33	3.3	177	17.7
83	106	10.6	33	3.3	176	17.6
86	105	10.5	33	3.3	176	17.6
89	106	10.6	33	3.3	174	17.4
91	105	10.5	32	3.2	171	17.1
94	107	10.7	32	3.2	168	16.8
97	106	10.6	32	3.2	164	16.4
100	105	10.5	32	3.2	164	16.4

Figure 1b. Viscosity vs. shear rate behaviour of different wastewater sludges

	Primary		Mixed		Secondary	
Shear rate (s^{-1})	Viscosity(cP)	S.E.	Viscosity(cP)	S.E.	Viscosity(cP)	S.E.
0.36	2036	204	1022	102	842	84.2
0.73	1171	117	847	84.7	556	55.6
1.83	553	55.3	432	43.2	354	35.4
3.67	320	32	276	27.6	223	22.3
7.34	202	16.2	154	12.3	121	9.68
14.68	145	11.6	95	7.6	84	6.72
36.71	95	7.6	56	4.48	47	3.76
73.42	50	5	18	1.8	10	1

Figure 2. Rheological pattern of different sludges at different stages of process treatment - a)NH; b)HF; c)NHF

NH						
	Primary		Secondary		Mixed	
TS (g/l)	Visc.(cP)	S.E.	Visc.(cP)	S.E.	Visc.(cP)	S.E.
10	8.87	0.89	3.36	0.34	4.5	0.45
15	33	0.17	4.56	0.02	6.6	0.03
20	95	6.65	9.64	0.67	18	1.26
30	102	8.16	96	7.68	46	3.68
40	361	28.88	156	12.48	72	5.76
HF						
	Primary		Secondary		Mixed	
TS (g/l)	Visc.(cP)	S.E.	Visc.(cP)	S.E.	Visc.(cP)	S.E.
10	19	1.90	3.8	0.38	7	0.70
15	71	0.36	6.2	0.03	19	0.10
20	92	6.44	7.6	0.53	33	2.31
30	378	30.24	210	16.80	66	5.28
40	385	30.80	240	19.20	100	8.00
NHF						
	Primary		Secondary		Mixed	
TS (g/l)	Visc.(cP)	S.E.	Visc.(cP)	S.E.	Visc.(cP)	S.E.
10	21	2.10	5.2	0.52	13	1.30
15	37	0.19	5.4	0.03	19	0.10
20	83	5.81	5.9	0.41	40	2.80
30	295	23.60	17	1.36	42	3.36
40	572	45.76	36	2.88	83	6.64

Figure 3. Viscosity and particle size distribution profile for different sludges at various stages of process treatment

	PARTICLE SIZES (µm)														
	NH					HF					NHF				
	NH1	NH1.5	NH2	NH3	NH4	HF1	HF1.5	HF2	HF3	HF4	NHF1	NHF1.5	NHF2	NHF3	NHF4
PRIMARY	54.06	52.4	70.5	61.9	60.8	110.1	81.8	60.7	50.4	38.6	34.7	38.2	75.6	95.6	134.8
S.E.	5.406	5.24	7.05	6.19	4.86	11.01	5.73	6.07	4.03	3.86	3.12	3.82	6.05	6.69	9.44
SECONDARY	42.24	44.5	49	50.9	56.7	25.9	29.8	35.9	44.4	24.8	58.1	47.3	46.5	44.2	40.5
S.E.	4.224	4.45	4.9	5.09	4.54	2.59	2.09	3.59	3.55	2.48	5.23	4.73	3.72	3.09	2.84
MIXED	27.54	29.19	30.23	28.18	32.31	22	22.54	20.6	41.5	28.8	47.9	58.6	54.9	36.5	58.4
S.E.	2.754	2.919	3.023	2.818	2.58	2.2	1.58	2.06	3.32	2.88	4.31	5.86	4.39	2.56	4.09
	VISCOSITIES (cP)														
	NH						**HF**						**NHF**		
	NH1	NH1.5	NH2	NH3	NH4	HF1	HF1.5	HF2	HF3	HF4	NHF1	NHF1.5	NHF2	NHF3	NHF4
PRIMARY	8.87	33	95	102	361	19	71	92	378	385	21	37	83	295	572
S.E.	0.887	3.3	9.5	10.2	28.88	1.9	4.97	9.2	30.24	38.5	1.89	3.7	6.64	20.65	40.04
SECONDARY	3.36	4.56	9.64	96	156	3.8	6.2	7.6	210	240	5.2	5.4	5.9	17	36
S.E.	0.336	0.456	0.964	9.6	12.48	0.38	0.43	0.76	16.80	24	0.47	0.54	0.47	1.19	2.52
MIXED	4.5	6.6	18	46	72	7	19	33	66	100	13	19	40	42	83
S.E.	0.45	0.66	1.8	4.6	5.76	0.7	1.33	3.3	5.28	10	1.17	1.9	3.20	2.94	5.81

CODES	NH1	NH1.5	NH2	NH3	NH4	HF1	HF1.5	HF2	HF3	HF4	NHF1	NHF1.5	NHF2	NHF3	NHF4
Stands for	NH-10	NH-15	NH-20	NH-30	NH-40	HF-10	HF-15	HF-20	HF-30	HF-40	NHF-10	NHF-15	NHF-20	NHF-30	NHF-40

Annexe – III

Données

Sludge based *Bacillus thuringiensis* Biopesticides: Viscosity Impacts

Figure 2. Thixotropic behaviour of secondary sludge (TS = 20 g/L)

Time(minutes)	Viscosity(cP)	S.E.
0	209	20.9
3	193	19.3
6	186	18.6
9	163	16.3
12	150	15
15	139	13.9
18	128	12.8
21	125	12.5
24	125	12.5
27	121	12.1
30	120	12
33	120	12
36	115	11.5
39	111	11.1
41	112	11.2
44	113	11.3
47	113	11.3
50	114	11.4
53	111	11.1
56	107	10.7
59	108	10.8
61	108	10.8
64	107	10.7
67	107	10.7
71	108	10.8
74	108	10.8
77	108	10.8
80	107	10.7
83	106	10.6
86	105	10.5
89	106	10.6
91	105	10.5
94	107	10.7
97	106	10.6
100	105	10.5

Figure 3. Effect of process treatments and solids concentration

TS (g/L)	NH	S.E.	ST	S.E.	TAH	S.E.	HST	S.E.	HF	S.E.	NHF	S.E.
10	3.4	0.34	2.74	0.27	3.67	0.37	2.77	0.28	3.83	0.38	5.16	0.52
15	6.3	0.63	3.2	0.32	4.5	0.45	5.23	0.52	4.3	0.43	5.48	0.55
20	9.64	0.96	4.77	0.48	5.86	0.59	7.76	0.78	5.55	0.56	12.7	1.27
25	59	5.90	24	2.40	17.8	1.78	43	4.3	10.58	1.06	25.13	2.51
30	96	7.68	14	1.12	26	2.08	55	4.4	14.11	1.13	51.28	4.10
40	156	12.48	70	5.60	49	3.92	125	10	33.53	2.68	122.65	9.81

Figure 4. Evolution of Bt growth profile with viscosity and k_La in a bench-scale fermenter (TS = 25g/L)

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Viscosity (cP)	S.E.
0	1.3E+07	1.0E+06	4.3E+03	3.4E+02	51.4	57	4.56
3	3.8E+07	2.7E+06	7.0E+03	4.9E+02	122.55	50	3.50
6	1.7E+08	1.4E+07	7.0E+05	5.6E+04	23.25	39	3.12
9	2.3E+08	1.8E+07	2.5E+07	2.0E+06	35.42	33	2.64
12	3.2E+08	2.5E+07	6.0E+07	4.8E+06	33.53	37.7	3.02
15	6.0E+08	3.0E+07	1.1E+08	5.5E+06	49.90	24	1.20
18	5.4E+08	3.8E+07	2.5E+08	1.8E+07	46.02	20	1.40
21	6.6E+08	3.3E+07	2.7E+08	1.4E+07	44.9	21	1.05
24	6.0E+08	3.0E+07	3.2E+08	1.6E+07	27.8	20	1.00
30	5.2E+08	3.1E+07	3.3E+08	2.0E+07	37.8	18	1.08
36	5.4E+08	3.2E+07	3.4E+08	2.0E+07	35.2	13	0.78
48	5.0E+08	3.0E+07	3.0E+08	1.8E+07	27.3	24	1.44

Figure 5. Dissolved solids concentration profile at different TS for different treatments

TS (g/L)	NH	S.E.	ST	S.E.	TAH	S.E.	HST	S.E.	HF	S.E.	NHF	S.E.
10	0.445	0.04	2.06	0.21	5.355	0.54	4.973	0.50	3.124	0.31	0.918	0.09
20	0.839	0.08	3.839	0.38	8.942	0.89	9.154	0.92	2.649	0.26	1.413	0.14
30	1.203	0.12	5.116	0.51	11.66	1.17	12.48	1.25	3.717	0.37	2.17	0.22
40	1.611	0.16	6.693	0.67	16	1.60	16.21	1.62	5.26	0.53	2.666	0.27

Annexe – IV

Données

Particle Size Variations during Production of Wastewater Sludge based *Bacillus thuringiensis* Biopesticides

Figure 5. Sedimentation velocity and SVI profile of different process stages with varying particle size

			SVI (ml/g)									
TS (g/L)	**NH**	**S.E.**	**ST**	**S.E.**	**TAH**	**S.E.**	**HST**	**S.E.**	**HF**	**S.E.**	**NHF**	**S.E.**
10	100.4	10.04	26.75	2.68	111.4	11.14	30.4	3.04	88.4	8.84	156.3	15.63
20	51.8	4.14	22.7	1.82	52.7	4.22	37.9	3.03	71.2	5.70	84.6	6.77
30	34.73	2.71	18	1.40	34.5	2.69	32.3	2.52	50.9	3.97	50.2	3.92
40	26	2.37	23.7	2.16	25.6	2.33	24.7	2.25	35.3	3.21	45.1	4.10

		sedimentation velocity, v_{sed}, m/s				
TS (g/L)	**NH**	**ST**	**TAH**	**HST**	**HF**	**NHF**
10	9.36E-09	2E-08	2.93518E-09	0	8.91E-09	1.2E-08
20	8.49E-10	8E-09	3.87446E-09	0	3.86E-09	2E-09
30	2.44E-10	5E-09	1.14707E-09	0	2.38E-09	5.4E-10
40	3.93E-11	5E-10	1.04387E-09	0	2.72E-10	1.6E-10

Annexe – V

Données

Broth Rheology and Process Performance of *Bacillus thuringiensis* Fermented Primary and Mixed Sludge in Fermenter

Figure 1. Bt growth (0.015 m^3 fermenter) profile in soya medium; b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	91.12	2.46	300.71
3.05	62.30	2.46	297.50
6.01	49.92	3.45	356.46
9.07	54.00	2.96	356.46
12.02	66.70	2.96	345.37
15.08	77.47	2.97	246.50
18.03	76.70	2.95	247.08
21.09	77.70	2.95	252.62
24.04	78.42	2.97	254.67
27.10	83.25	2.96	248.79
30.05	86.92	2.96	254.92
33.01	89.80	2.96	249.67
36.06	91.57	2.96	250.25
39.02	92.70	2.96	248.79
45.03	92.35	2.94	251.42
47.65	91.75	2.94	250.00

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	1.83E+07	1.5E+06	1.76E+06	1.4E+05	149.35	2.42	0.19	54.9	4.39	0.99235	0.08
3	1.77E+07	1.2E+06	1.21E+07	8.4E+05	51.52	2.21	0.15	33.9	2.37	0.99766	0.07
6	1.71E+08	1.4E+07	2.33E+07	1.9E+06	114.00	3.58	0.29	18.82	1.51	1.01608	0.08
9	2.27E+08	1.8E+07	5.03E+07	4.0E+06	200.78	5.18	0.41	15.8	1.26	1.02327	0.08
12	2.47E+08	2.0E+07	5.11E+07	4.1E+06	283.91	5.34	0.43	14.91	1.19	1.02423	0.08
15	2.53E+08	1.3E+07	2.01E+08	1.0E+07	85.42	4.38	0.22	14.3	0.72	1.00434	0.05
18	2.53E+08	1.8E+07	2.27E+08	1.6E+07	77.54	4.72	0.33	13.5	0.95	1.01345	0.07
21	2.67E+08	1.3E+07	2.53E+08	1.3E+07	79.99	3.15	0.16	26.8	1.34	1.00776	0.05
24	2.87E+08	1.4E+07	2.53E+08	1.3E+07	36.52	2.18	0.11	32.9	1.65	0.98084	0.05
30	2.77E+08	1.7E+07	2.43E+08	1.5E+07	84.71	2.69	0.16	26.3	1.58	0.99624	0.06
36	2.60E+08	2.1E+07	2.40E+08	1.9E+07	64.55	2.36	0.19	27.03	2.16	0.99831	0.08
48	2.70E+08	2.2E+07	2.43E+08	1.9E+07	112	2.46	0.20	21.83	1.75	0.977695	0.08

Figure 3. Bt growth (0.015 m^3 fermenter) profile in raw primary sludge; a) process parameters, and b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	96.34	1.95	352
3.08	52.57	1.87	354
6.07	49.67	3.94	500
9.05	57.04	3.95	499
12.03	67.00	2.95	499
15.02	82.14	2.95	500
18.00	86.97	2.95	503
21.08	90.00	2.95	500
24.07	89.74	1.95	401
27.05	90.57	1.95	400
30.03	92.04	1.95	398
33.02	92.49	1.95	401
36.00	89.44	1.95	302
39.08	90.47	1.95	299
42.07	90.92	1.96	302
45.05	91.24	1.95	303
0	96.34	1.95	352

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	9.95E+04	8.0E+03	4.50E+03	3.6E+02	85.88	127	10.16	8.6	0.69	1.014	0.08
3	1.23E+05	8.6E+03	5.50E+03	3.9E+02	45.54	140	9.80	30.7	2.15	0.9988	0.07
6	2.20E+05	1.8E+04	3.50E+04	2.8E+03	66.17	184	14.72	25.9	2.07	0.9765	0.08
9	2.20E+06	1.8E+05	1.50E+05	1.2E+04	76.9	50	4.00	17.8	1.42	0.9818	0.08
12	2.40E+06	1.9E+05	2.50E+05	2.0E+04	78.45	101	8.08	13.3	1.06	0.9862	0.08
15	2.60E+06	1.3E+05	1.60E+06	8.0E+04	81.84	216	10.80	43	2.15	1.0015	0.05
18	2.95E+07	2.1E+06	4.35E+06	3.0E+05	91.5	112	7.84	51.4	3.60	1.0023	0.07
21	3.05E+07	1.5E+06	9.90E+06	5.0E+05	98.52	157	7.85	56.2	2.81	0.9745	0.05
24	3.55E+07	1.8E+06	1.27E+07	6.4E+05	67.92	109	5.45	15.3	0.77	0.9789	0.05
30	3.70E+07	2.2E+06	1.32E+07	7.9E+05	83.39	197	11.82	23.4	1.40	0.9984	0.06
36	3.70E+07	3.0E+06	2.90E+07	2.3E+06	74.22	194	15.52	76.8	6.14	0.9815	0.08
48	3.80E+07	3.0E+06	2.90E+07	2.3E+06	77.45	156	12.48	47.9	3.83	0.9783	0.08

Figure 4. Bt growth (0.015 m^3fermenter) profile in hydrolyzed primary sludge; a) process parameters, and b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	96.29	1.95	355
3.08	52.92	1.95	350
6.07	60.37	2.95	454
9.05	71.19	2.95	451
12.03	85.07	2.95	450
15.02	90.62	2.95	447
18.00	96.67	2.95	458
21.08	97.09	1.95	402
24.07	86.12	1.95	301
27.05	91.99	1.95	303
30.03	95.42	1.95	303
33.02	98.04	1.95	300
36.00	92.57	1.46	251
39.08	95.62	1.46	251
42.07	98.94	1.46	253
45.05	99.72	1.46	252
48.03	99.72	1.46	250

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	9.90E+03	7.9E+02	3.50E+03	2.8E+02	27.09	117	9.36	21.7	1.74	0.9989	0.08
3	3.40E+04	2.4E+03	4.50E+03	3.2E+02	30.38	110	7.70	51.7	3.62	0.9948	0.07
6	1.20E+05	9.6E+03	5.50E+03	4.4E+02	55.46	87	6.96	28.9	2.31	0.9734	0.08
9	1.35E+06	1.1E+05	2.50E+04	2.0E+03	54.50	70	5.60	45.3	3.62	0.9811	0.08
12	1.70E+06	1.4E+05	1.50E+05	1.2E+04	55.28	71	5.68	33.1	2.65	0.9702	0.08
15	2.10E+06	1.1E+05	1.55E+06	7.8E+04	59.59	140	7.00	21.8	1.09	1.0033	0.05
18	2.75E+07	1.9E+06	3.85E+06	2.7E+05	59.74	114	7.98	29.8	2.09	1.0043	0.07
21	3.15E+07	1.6E+06	9.95E+06	5.0E+05	45.56	140	7.00	21.4	1.07	0.9794	0.05
24	3.20E+07	1.6E+06	1.35E+07	6.7E+05	58.99	83	4.15	17	0.85	0.9747	0.05
30	3.90E+07	2.3E+06	2.52E+07	1.5E+06	52.31	152	9.12	46.3	2.78	1.0017	0.06
36	3.90E+07	3.1E+06	3.25E+07	2.6E+06	58.42	128	10.24	62.4	4.99	0.9726	0.08
48	4.00E+07	3.2E+06	3.35E+07	2.7E+06	82.96	62	4.96	27.8	2.22	0.988	0.08

Figure 5. Bt growth (0.015 m^3 fermenter) profile in raw mixed sludge; a) process parameters, and b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	67.44	1.95	250
3.08	54.79	0.23	403.4
6.07	23.94	0.23	400.2
9.06	38.78	0.6	404.0
12.05	43.89	0.31	399.9
15.04	51.94	2.44	398.1
18.02	56.49	1.3	402.2
21.01	61.00	2.44	398.4
24	63.35	2.455	401.6
27.09	60.88	2.455	400.5
30.07	60.25	2.455	403.4
33.06	60.16	2.455	399.9
36.05	61.02	2.44	300
39.04	60.08	2.44	300
42.03	60.25	2.44	300
45.01	60.13	2.44	250
48	59.90	2.44	250

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	7.5E+06	6.0E+05	4.00E+03	3.2E+02	65.42	33	2.64	30.6	2.45	1.0046	0.08
3	2.8E+07	2.0E+06	3.50E+03	2.5E+02	42.12	26	1.82	16.3	1.14	1	0.07
6	8.5E+07	6.8E+06	2.00E+05	1.6E+04	40.28	33	2.64	16.9	1.35	0.9985	0.08
9	2.0E+08	1.6E+07	6.00E+06	4.8E+05	57.58	30	2.40	10.3	0.82	1.0003	0.08
12	2.2E+08	1.7E+07	1.05E+07	8.4E+05	49.27	36	2.88	11.1	0.89	1.0024	0.08
15	3.3E+08	1.6E+07	7.00E+07	3.5E+06	57.73	33	1.65	18.1	0.91	1.001	0.05
18	3.8E+08	2.6E+07	2.95E+08	2.1E+07	49.75	38	2.66	3.2	0.22	1.0016	0.07
21	3.8E+08	1.9E+07	3.10E+08	1.6E+07	62.28	40	2.00	78.5	3.93	1.001	0.05
24	4.0E+08	2.0E+07	3.75E+08	1.9E+07	46.34	38	1.90	30.1	1.51	1.0009	0.05
30	3.9E+08	2.3E+07	3.70E+08	2.2E+07	61.80	37	2.22	66.3	3.98	1.0009	0.06
36	4.0E+08	3.2E+07	3.60E+08	2.9E+07	69.33	35	2.80	9.8	0.78	1.0002	0.08
48	3.9E+08	3.1E+07	3.70E+08	3.0E+07	54.75	37	2.96	42.2	3.38	0.9999	0.08

Figure 6. Bt growth (0.015 m^3 fermenter) profile in hydrolyzed mixed sludge; a) process parameters, and b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	90.67	2.95	248
3.08	80.67	2.95	346
6.07	53.59	2.95	352
9.06	59.64	2.95	401
12.05	65.54	2.95	400
15.04	66.84	2.95	397
18.02	66.47	2.94	401
21.01	66.92	2.94	401
24	66.14	2.94	398
27.09	65.87	2.95	401
30.07	66.02	2.96	399
33.06	64.82	2.96	399
36.05	64.12	2.95	401
39.04	63.69	2.95	396
42.03	63.69	2.94	402
45.01	63.59	2.94	400
48	57.47	2.95	398

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	8.50E+06	6.8E+05	5.00E+03	4.0E+02	86.00	12	0.96	16.9	1.35	0.9996	0.08
3	4.15E+07	2.9E+06	1.70E+04	1.2E+03	80.31	15	1.05	18.6	1.30	0.9995	0.07
6	2.70E+08	2.2E+07	3.00E+05	2.4E+04	72.24	9	0.72	7.4	0.59	0.9943	0.08
9	2.85E+08	2.3E+07	5.00E+06	4.0E+05	94.26	10	0.80	30.6	2.45	0.9989	0.08
12	3.40E+08	2.7E+07	1.05E+07	8.4E+05	70.05	17	1.36	13.1	1.05	1.0004	0.08
15	4.10E+08	2.1E+07	9.50E+07	4.8E+06	58.34	13	0.65	7.9	0.40	1.0027	0.05
18	3.85E+08	2.7E+07	3.20E+08	2.2E+07	69.06	11	0.77	7.8	0.55	1.0013	0.07
21	4.00E+08	2.0E+07	2.90E+08	1.5E+07	64.20	10	0.50	2.3	0.12	1.0016	0.05
24	3.90E+08	2.0E+07	3.60E+08	1.8E+07	56.97	13	0.65	3.6	0.18	1.0014	0.05
30	5.60E+08	3.4E+07	5.25E+08	3.2E+07	56.60	13	0.78	3.2	0.19	1.0015	0.06
36	5.65E+08	4.5E+07	5.10E+08	4.1E+07	56.57	11	0.88	29.1	2.33	0.9968	0.08
48	5.60E+08	4.5E+07	5.15E+08	4.1E+07	53.21	9	0.72	9.2	0.74	0.9959	0.08

Figure 7. OUR profiles of different fermented media

Time (h)	Soya	NHP	THP	NHM	THM
3	0.18	1.107	0.94	0.99	1.396
6	1.44	3.087	2.94	3.762	4.804
9	4.95	1.773	3.12	4.887	5.764
12	3.758	1.305	1.7	1.854	4.772
15	1.8	1.053	1.15	0.882	3.448
18	1.197	0.684	1.29	0.657	2.076
21	1.35	0.468	0.86	0.423	2.6
24	0.09	0.333	0.99	0.486	3.128
30	0.36	0.189	0.3	0.279	3.196
36	0.27	0.135	0.23	0.198	2.968
48	0.15	0.684	0.08	0.108	0.048

Figure 8a). Rheograms and shear rate profile of different fermented broths (at 48h)

	Shear Stress (dynes/cm^2)				
Shear Rate (s^{-1})	NHP	THP	NHM	THM	Soya
0.08	3.36	1.85	2.02	1.34	0.01
0.17	3.70	2.35	2.86	1.34	0.02
0.42	4.37	3.53	3.36	2.52	0.05
0.84	4.87	4.87	4.20	3.36	0.10
1.68	5.71	6.21	5.04	4.54	0.18
2.52	6.72	6.72	5.88	5.37	0.26
3.36	7.56	7.56	6.72	5.88	0.32
4.20	8.40	8.73	7.56	7.05	0.40
5.60	9.24	10.08	9.07	8.57	0.52
7.00	10.25	11.25	10.41	9.57	0.62
8.40	10.92	12.93	11.42	10.92	0.75
9.80	12.09	14.28	12.09	11.76	0.87
11.20	12.77	15.79	11.93	12.26	0.98
12.60	13.94	17.97	12.60	14.11	1.09
14.00	14.61	19.99	10.25	16.96	1.21
15.40	15.28	22.00	10.75	16.29	1.31
16.80	15.62	24.19	14.61	21.84	1.43
18.20	16.29	25.87	15.62	19.15	1.55
19.60	17.80	27.38	14.95	16.63	1.67
21.00	18.48	27.21	14.78	15.96	1.78
22.40	18.64	27.21	12.09	16.80	1.89
23.80	19.65	25.36	11.93	15.96	2.00
25.20	22.34	26.03	12.43	16.29	2.11
26.60	24.35	24.52	12.26	14.61	2.23
28.00	25.87	22.51	11.93	14.44	2.34
29.40	26.03	27.71	12.26	14.28	2.46
30.80	26.03	27.04	12.43	14.44	2.57
32.20	25.70	28.39	12.43	14.44	2.69
33.60	26.20	33.93	11.93	14.61	2.80

Shear Stress (dynes/cm^2)					
Shear Rate (s^{-1})	NHP	THP	NHM	THM	Soya
35.00	26.37	36.95	11.59	14.61	2.91
36.40	33.09	34.26	11.76	14.61	3.03
37.80	34.94	34.43	11.42	13.94	3.15
39.20	24.35	35.10	11.59	11.42	3.28
40.60	22.84	37.29	10.75	12.93	3.40
42.00	21.00	30.91	9.07	13.44	3.68
43.40	19.99	29.56	10.75	16.96	4.04
44.80	20.32	30.40	10.75	14.28	4.34
46.20	19.82	30.57	9.57	14.61	4.58

Figure 8b). Rheograms and shear rate profile of different fermented broths (at 48h)

Viscosity (mPa.s)					
Shear Rate (s^{-1})	NHP	THP	NHM	THM	Soya
0.08	3999.15	2199.53	2399.49	1599.66	199.96
0.17	2199.53	1399.70	1699.64	799.83	299.94
0.42	1039.78	839.82	799.83	599.87	279.94
0.84	579.88	579.88	499.89	399.91	259.94
1.68	339.93	369.92	299.94	269.94	239.95
2.52	266.61	266.61	233.28	213.29	233.28
3.36	224.95	224.95	199.96	174.96	214.95
4.20	199.96	207.96	179.96	167.96	215.95
5.60	164.96	179.96	161.97	152.97	212.95
7.00	146.37	160.77	148.77	136.77	203.96
8.40	129.97	153.97	135.97	129.97	203.96
9.80	123.40	145.68	123.40	119.97	203.96
11.20	113.98	140.97	106.48	109.48	200.96
12.60	110.64	142.64	99.98	111.98	198.62
14.00	104.38	142.77	73.18	121.17	197.96
15.40	99.25	142.88	69.80	105.80	195.23
16.80	92.98	143.97	86.98	129.97	194.96
18.20	89.52	142.12	85.83	105.21	194.73
19.60	90.84	139.68	76.27	84.84	194.53
21.00	87.98	129.57	70.38	75.98	194.36
22.40	83.23	121.47	53.99	74.98	192.71
23.80	82.57	106.57	50.11	67.04	191.96
25.20	88.65	103.31	49.32	64.65	191.96
26.60	91.56	92.19	46.10	54.94	191.96
28.00	92.38	80.38	42.59	51.59	191.36
29.40	88.55	94.27	41.71	48.56	191.39
30.80	84.53	87.80	40.36	46.90	190.87
32.20	79.81	88.16	38.60	44.86	191.44
33.60	77.98	100.98	35.49	43.49	190.96
35.00	75.34	105.58	33.11	41.75	190.52
36.40	90.90	94.13	32.30	40.15	190.57
37.80	92.42	91.09	30.22	36.88	191.07
39.20	62.13	89.55	29.57	29.14	191.53

	Viscosity (mPa.s)				
Shear Rate (s^{-1})	NHP	THP	NHM	THM	Soya
40.60	56.26	91.84	26.48	31.86	191.96
42.00	49.99	73.58	21.60	31.99	200.76
43.40	46.05	68.11	24.77	39.09	212.86
44.80	45.37	67.86	23.99	31.87	221.95
46.20	42.90	66.17	20.72	31.63	226.86

Figure 9. Consistency index (K) and flow behaviour index (n) profiles soya, secondary sludge (non-hydrolyzed, NHS); secondary sludge (hydrolyzed, THS) and starch industry wastewater fermentation

	Soya		SIW		NH		TH	
Time (h)	K ($mPa.s^n$)	n	K ($mPa.s^n$)	n	K ($mPa.s^n$)	n	K ($mPa.s^n$)	n
0	1.61	1.01	0.67	-	160.2	0.49	150.6	0.22
3	2.57	0.95	1.22	0.98	127.2	0.54	100	0.29
6	6.88	0.75	1.46	0.94	180.5	0.48	123.2	0.25
9	10.3	0.68	1.91	0.92	116.8	0.57	114.4	0.29
12	30.4	0.54	2.32	0.91	483.2	0.35	177.7	0.32
15	20.1	0.58	2.67	0.89	368.9	0.4	140.1	0.33
18	10.5	0.68	2.8	0.92	205.1	0.49	105.2	0.36
21	2.94	0.91	2.2	0.93	98	0.6	76.6	0.41
24	2.95	0.91	1.83	0.96	105.4	0.56	79.6	0.41
30	2.51	0.94	1.6	0.99	125.9	0.56	34.6	0.61
36	2.52	0.95	1.7	1.01	125.2	0.56	26	0.62
48	2.5	0.95	1.43	1.04	78.6	0.65	21.3	0.66

Annexe – VI

Données

Impact of Tween 80 during *Bacillus thuringiensis* Fermentation of Wastewater Sludges

Figure 1. Non-hydrolyzed sludges without Tween 80: (a) operational parameters; (b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	76.45	2.55	401
3.08	63.25	2.55	402
6.07	39.87	4.7	500
9.06	47.87	4.6	601
12.05	59.45	9.55	649
15.04	60.50	9.55	651
18.02	61.00	9.7	651
21.3	90.04	3.44	404
24.1	90.99	2.44	397
27.18	92.27	1.95	400
30.07	93.27	1.95	399
33.16	94.34	1.95	401
36.24	95.02	1.95	400
39.23	95.54	1.95	399
42.12	96.17	1.95	401
45.11	96.17	1.95	399
47.81	96.69	1.95	398

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	1.30E+07	1.0E+06	4.30E+03	3.4E+02	51.4	57	4.56	24.3	1.94	0.916	0.07
3	3.80E+07	2.7E+06	7.00E+03	4.9E+02	122.54	50	3.50	18.1	1.27	0.927	0.06
6	1.73E+08	1.4E+07	7.00E+05	5.6E+04	23.25	39	3.12	19	1.52	0.971	0.08
9	2.30E+08	1.8E+07	2.47E+07	2.0E+06	35.42	33	2.64	11.6	0.93	0.98	0.08
12	3.17E+08	2.5E+07	6.00E+07	4.8E+06	33.53	37.7	3.02	10.1	0.81	0.978	0.08
15	5.98E+08	3.0E+07	1.10E+08	5.5E+06	49.89	24	1.20	6.4	0.32	0.968	0.05
18	5.40E+08	3.8E+07	2.50E+08	1.8E+07	46.02	20	1.40	4.96	0.35	0.953	0.07
21	6.60E+08	3.3E+07	2.70E+08	1.4E+07	44.9	21	1.05	5.1	0.26	0.998	0.05
24	5.95E+08	3.0E+07	3.20E+08	1.6E+07	27.8	20	1.00	6.96	0.35	0.9786	0.05
30	5.20E+08	3.1E+07	3.30E+08	2.0E+07	37.8	18	1.08	6.99	0.42	0.9564	0.06
36	5.40E+08	4.3E+07	3.35E+08	2.7E+07	35.2	13	1.04	5.27	0.42	0.947	0.08
48	5.00E+08	4.0E+07	3.00E+08	2.4E+07	27.3	24	1.92	1.7	0.14	0.9507	0.08

Figure 2. Hydrolyzed sludges without Tween 80: (a) operational parameters; (b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	84.12	1.98	403
3.08	76.50	1.98	399
6.07	57.65	1.98	402
9.06	49.62	1.96	542
12.05	72.70	1.96	550
15.04	75.07	1.97	552
18.02	75.52	1.98	551
21.01	74.55	1.98	551
24	74.17	1.98	549
27.09	73.70	1.98	550
30.07	72.95	1.96	553
33.06	72.10	1.96	549
36.05	72.22	1.96	548
39.04	71.65	1.97	556
42.03	70.55	1.97	547
45.01	71.85	1.97	549
48	67.35	2.00	552

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	7.50E+06	6.0E+05	3.33E+03	2.7E+02	248.28	3.74	0.30	13.18	1.05	1.002	0.08
3	3.00E+07	2.1E+06	9.33E+03	6.5E+02	198.26	3.59	0.25	10.79	0.76	1.001	0.07
6	1.93E+08	1.5E+07	3.20E+06	2.6E+05	105.25	3.6	0.29	10.83	0.87	1.012	0.08
9	3.40E+08	2.7E+07	6.00E+07	4.8E+06	78.26	5.71	0.46	7.27	0.58	1.007	0.08
12	4.37E+08	3.5E+07	1.33E+08	1.1E+07	21.18	7.85	0.63	6.64	0.53	1.006	0.08
15	4.00E+08	2.0E+07	2.95E+08	1.5E+07	62.69	6.94	0.35	6.74	0.34	1.009	0.05
18	4.60E+08	3.2E+07	3.00E+08	2.1E+07	47.49	6.21	0.43	6.1	0.43	1.026	0.07
21	3.43E+08	1.7E+07	3.27E+08	1.6E+07	51.57	6.15	0.31	6.05	0.30	0.989	0.05
24	4.37E+08	2.2E+07	3.50E+08	1.8E+07	53.86	5.39	0.27	4.99	0.25	1.037	0.05
30	4.33E+08	2.6E+07	3.60E+08	2.2E+07	68.65	4.81	0.29	4.66	0.28	1.032	0.06
36	2.90E+08	2.3E+07	2.80E+08	2.2E+07	93.24	3.61	0.29	5.09	0.41	0.953	0.08
48	3.97E+08	3.2E+07	3.93E+08	3.1E+07	30.19	4.09	0.33	3.98	0.32	0.978	0.08

Figure 3. Non-hydrolyzed sludges with Tween 80: (a) operational parameters; (b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	98.54	2.52	452
3.08	90.74	0.19	454
6.07	50.09	3.45	401
9.06	56.09	3.45	401
12.05	76.09	3.45	401
15.04	82.54	3.44	399
18.02	86.29	3.44	401
21.01	89.84	3.44	400
24	90.99	2.44	398
27.09	92.12	1.95	402
30.07	93.27	1.95	399
33.06	94.34	1.95	398
36.05	95.02	1.95	401
39.04	95.37	1.95	397
42.03	96.17	1.95	401
45.01	96.17	1.95	401
48	96.69	1.95	402

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	6.15E+06	6.0E+05	1.20E+04	2.7E+02	88.1	12	0.30	257.3	1.05	0.998	0.08
3	5.65E+07	2.1E+06	1.05E+05	6.5E+02	117.3	5.7	0.25	24.2	0.76	0.99	0.07
6	3.55E+08	1.5E+07	2.70E+06	2.6E+05	87	6.1	0.29	54.3	0.87	0.98	0.08
9	3.90E+08	2.7E+07	6.95E+07	4.8E+06	84.7	10.33	0.46	17.9	0.58	0.997	0.08
12	4.15E+08	3.5E+07	7.90E+07	1.1E+07	63.8	9.29	0.63	16.6	0.53	1.003	0.08
15	4.15E+08	2.0E+07	3.80E+08	1.5E+07	92.9	8.18	0.35	17.3	0.34	1.01	0.05
18	4.10E+08	3.2E+07	3.90E+08	2.1E+07	72	6.46	0.43	15.9	0.43	1.032	0.07
21	3.90E+08	1.7E+07	3.75E+08	1.6E+07	65.9	6.48	0.31	11.6	0.30	0.998	0.05
24	9.95E+08	2.2E+07	9.45E+08	1.8E+07	79.2	6.23	0.27	18.3	0.25	1.007	0.05
30	1.13E+09	2.6E+07	1.07E+09	2.2E+07	72	5.96	0.29	10.6	0.28	1.009	0.06
36	1.14E+09	2.3E+07	1.08E+09	2.2E+07	80	5.4	0.29	9.4	0.41	1.005	0.08
48	1.15E+09	3.2E+07	1.15E+09	3.1E+07	72	5.79	0.33	10.8	0.32	1.003	0.08

Figure 4. Hydrolyzed sludges with Tween 80: (a) operational parameters; (b) performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	97.9	2.45	245
3.08	79.5	2.45	249
6.07	81.8	2.95	401
9.06	85.9	1.47	900
12.05	81.5	1.96	399
15.04	89.3	1.96	399
18.02	91.8	1.96	402
21.01	93.4	1.95	400
24	95.9	1.95	402
27.09	94.7	1.95	398
30.07	94.2	1.95	403
33.06	94.1	1.96	401
36.05	94.7	1.95	400
39.04	95.4	1.95	399
42.03	96.3	1.95	402
45.01	97.2	1.95	400
48	97.8	1.95	400

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	6.00E+06	1.0E+06	1.30E+04	3.4E+02	28.1	6.01	4.56	336.8	1.94	0.997	0.07
3	2.55E+07	2.7E+06	2.20E+04	4.9E+02	145.8	3.82	3.50	24.6	1.27	0.99	0.06
6	3.55E+08	1.4E+07	2.75E+06	5.6E+04	47.5	4.54	3.12	12.8	1.52	1	0.08
9	4.15E+08	1.8E+07	3.55E+07	2.0E+06	74.6	6.78	2.64	8	0.93	1.005	0.08
12	4.50E+08	2.5E+07	7.00E+07	4.8E+06	53.3	6.12	3.02	10.3	0.81	1.008	0.08
15	4.45E+08	3.0E+07	3.95E+08	5.5E+06	68.6	4.61	1.20	5.8	0.32	1.002	0.05
18	4.60E+08	3.8E+07	4.65E+08	1.8E+07	30.9	4.29	1.40	8.9	0.35	1.03	0.07
21	5.25E+08	3.3E+07	4.85E+08	1.4E+07	30.6	4.45	1.05	5.4	0.26	1.003	0.05
24	5.55E+08	3.0E+07	5.45E+08	1.6E+07	48.1	4.39	1.00	4.1	0.35	1.009	0.05
30	5.65E+08	3.1E+07	5.50E+08	2.0E+07	45	4.5	1.08	5	0.42	1.007	0.06
36	6.50E+08	4.3E+07	6.30E+08	2.7E+07	70.7	3.98	1.04	3.4	0.42	1.006	0.08
48	9.50E+08	4.0E+07	9.50E+08	2.4E+07	36.9	5	1.92	4.2	0.14	1.005	0.08

Figure 6. OUR profiles during fermentation of sludge for different treatment conditions

Time (h)	NH-25	TH-25	NHT-25	THT-25
0	0.25	0.28	0.08	0.63
3	0.62	1.13	2.36	3.94
6	0.39	0.27	2.36	3.94
9	1.60	2.81	4.73	3.15
12	1.80	1.35	2.36	3.15
15	1.25	0.94	1.58	3.15
18	1.01	0.90	0.95	1.58
21	1.35	1.41	0.47	0.79
24	1.13	1.35	0.32	0.47
30	0.39	0.27	0.16	0.24
36	0.42	0.18	0.16	0.16
48	0.16	0.24	0.12	0.16

Annexe – VII

Données

Ramification of Solids Concentration on Entomotoxicity and Enzyme Activity of *Bacillus thuringiensis* Fermented Wastewater Sludge

Figure 1. Bt fermentation profiles (15L fermenter) of non-hydrolyzed sludge (without Tween-80):a) Operational parameters and; b) Growth parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0.03	82.87	1.97	450
3.00	79.45	1.98	454
6.01	78.47	1.98	454
9.01	78.30	1.98	447
12.01	55.68	2.47	399
15.02	25.00	2.47	398
18.02	77.97	2.46	401
21.02	82.02	2.46	406
24.03	84.50	2.46	399
27.03	84.32	2.47	402
30.00	85.07	2.47	399
33.00	83.42	2.46	399
36.01	85.42	2.47	403
39.01	83.50	2.46	400
42.01	83.70	2.46	399
45.02	83.35	2.46	400
47.92	82.42	2.46	401

Time (h)	TC (CFU/ml)	VS (CFU/ml)	k_La (h^{-1})	Visc. (cP)
0	2.85E+05	5.00E+02	-	72
3	7.00E+05	4.50E+02	32.35	37
6	7.50E+06	1.05E+04	47.82	33
9	9.50E+06	5.50E+04	70.11	19
12	1.35E+07	8.00E+05	56.44	19.1
15	1.85E+08	1.15E+07	61.96	18.3
18	2.60E+08	1.90E+07	76.09	17.4
21	2.80E+08	1.32E+08	66.43	17.8
24	2.65E+08	1.42E+08	69.23	14.9
30	2.75E+08	1.42E+08	95.67	15.3
36	2.80E+08	1.45E+08	123.58	17.4
48	2.90E+08	1.55E+08	108.79	15.43

Figure 2. Bt fermentation profiles (15 L fermenter) of non-hydrolyzed sludge (with Tween-80): a) Operational parameters and; b) Growth parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0.03	99.82	2.00	455
3.00	81.75	2.00	249
6.01	63.60	2.00	251
9.01	33.45	2.00	298
12.01	47.70	2.50	408
15.02	57.40	2.50	405
18.02	63.70	2.50	405
21.02	65.70	2.50	397
24.03	76.92	2.50	401
27.03	79.10	2.50	402
30.00	82.60	2.50	398
33.00	82.52	2.50	401
36.01	80.85	2.50	403
39.01	83.40	2.50	398
42.01	84.80	2.50	401
45.02	86.27	2.50	401
47.92	87.42	2.50	403

Time (h)	TC (CFU/ml)	VS (CFU/ml)	k_La (h^{-1})	Visc. (cP)	D_{50} (µm)
0	3.00E+06	6.00E+02	-	28.2	3.55
3	7.60E+06	7.00E+03	42.74	26.48	7.4
6	1.25E+07	5.50E+05	17.87	27.18	4.74
9	1.55E+08	1.05E+06	35.05	25.85	4.1
12	1.85E+08	1.15E+07	31.94	28.45	3.3
15	3.60E+08	1.70E+07	13.98	44.75	4
18	4.35E+08	2.65E+07	35.81	34.35	3.3
21	4.50E+08	7.95E+07	29.94	22.23	3.2
24	4.60E+08	1.65E+08	37.72	17.43	3.9
30	4.75E+08	1.65E+08	38.26	15.85	3.44
36	4.90E+08	1.90E+08	35.49	18.65	2.63
48	4.70E+08	2.00E+08	100.30	13.73	2.38

Figure 3. OUR profile of non-hydrolyzed sludge (with and without Tween-80) at SS=30 g/L

Time (h)	NH-30	NHT-30
3	0.08	0.35
6	0.12	0.92
9	0.79	2.93
12	1.57	3.65
15	1.47	2.54
18	0.97	2.26
21	0.97	1.76
24	0.62	1.17
30	0.41	0.61
36	0.30	0.83
48	0.12	0.38

Figure 5. a) Chitinase and protease activity profiles of non-hydrolyzed Bt fermented broths (with and without Tween-80) and; b) correlation of protease activity with TC. "C" in parentheses represents chitinase activity

	Protease (IU/ml)				Chitinase (U/ml)			
Time (h)	NH-30	S.E.	NHT-30	S.E.	NH-30	S.E.	NHT-30	S.E.
0	0.035	0.003	0.207	0.017	10.79	0.863	9.81	0.785
3	0.037	0.003	0.290	0.020	24.45	1.712	14.44	1.011
6	0.030	0.002	0.411	0.033	47.33	3.786	32.42	2.594
9	0.077	0.006	0.382	0.031	50.87	4.069	30.55	2.444
12	0.141	0.011	0.406	0.032	60.28	4.822	35.80	2.864
15	0.355	0.018	0.635	0.032	75.87	3.794	54.03	2.701
18	0.601	0.042	0.490	0.034	55.85	3.910	28.78	2.014
21	0.511	0.026	0.656	0.033	51.96	2.598	31.87	1.594
24	0.697	0.035	0.706	0.035	54.70	2.735	30.43	1.522
30	1.203	0.072	0.881	0.053	51.31	3.079	27.60	1.656
36	0.659	0.053	0.686	0.055	53.23	4.258	44.44	3.556
48	0.547	0.044	0.382	0.031	49.46	3.957	28.58	2.286

	NH-30				NHT-30			
TC (CFU/ml)	S.E.	PA (IU/ml)	S.E.	TC (CFU/ml)	S.E.	PA (IU/ml)	S.E.
1.35E+07	1.08E+06	0.141	0.011	1.85E+08	1.48E+07	0.249	0.020
1.85E+08	1.30E+07	0.355	0.025	3.60E+08	2.52E+07	0.635	0.044
2.60E+08	2.08E+07	0.511	0.041	4.35E+08	3.48E+07	0.656	0.052
2.80E+08	2.24E+07	0.697	0.056	4.50E+08	3.60E+07	0.706	0.056
2.65E+08	2.12E+07	0.889	0.071	4.60E+08	3.68E+07	1.038	0.083

Figure 6. Entomotoxicity profiles of NH-30 and NHT-30 with fermentation time. The number after the hyphen denotes the suspended solids concentration

	NH-30		NHT-30	
Time (h)	Tx(SBU/µl)	S.E.	Tx(SBU/µl)	S.E.
9	5842	584.20	8802	880.20
12	6312	511.27	11501	931.58
15	7110	526.14	12100	895.40
21	7301	474.57	12423	807.50
24	8112	811.20	12798	1279.80
30	9834	983.40	14696	1469.60
36	10789	863.12	15598	1247.84
48	11632	767.71	16521	1189.51

Figure 7. a) Correlation profiles of spTx (Tx/1000 spores) with VS (inset "b" shows higher resolution of the dotted area). The data represented as NH<30 have been derived from Yezza et al. (2004, 2005 a,b) and comprises NH -15, NH-20, NH-25 and NH-25-150L data. The starch industry wastewater (SIW) was derived from Brar et al. (2005a,b)

NH-30				NHT-30				NH<30				SIW				Soya			
VS (CFU/ml)	S.E.	Tx/1000 spores	S.E.	VS (CFU/ml)	S.E.	Tx/1000 spores	S.E.	VS (CFU/ml)	S.E.	Tx/1000 spores	S.E.	VS (CFU/ml)	S.E.	Tx/1000 spores	S.E.	VS (CFU/ml)	S.E.	Tx/1000 spores	S.E.
1.05E+06	1.05E+05	5563.81	556.38	5.50E+05	5.50E+04	10116.02	1011.60	2.47E+07	2.47E+06	202.43	20.24	2.43E+07	2.43E+06	493.83	49.38	5.67E+07	5.67E+06	71.16	7.12
1.15E+07	9.32E+05	548.87	44.46	8.00E+05	6.48E+04	686.09	55.57	2.57E+08	2.08E+07	31.13	2.52	3.20E+08	2.59E+07	42.19	3.42	2.37E+08	1.92E+07	27.14	2.20
1.70E+07	1.26E+06	418.24	30.95	1.15E+07	8.51E+05	36.37	2.69	3.20E+08	2.37E+07	28.13	2.08	3.30E+08	2.44E+07	48.48	3.59	2.40E+08	1.78E+07	36.62	2.71
7.95E+07	5.17E+06	91.84	5.97	1.32E+08	8.58E+06	0.70	0.05	3.67E+08	2.39E+07	27.25	1.77	3.31E+08	2.15E+07	51.36	3.80	2.43E+08	1.58E+07	39.27	2.91
1.65E+08	1.65E+07	49.31	4.93	1.42E+08	1.42E+07	0.35	0.03	3.47E+07	3.47E+06	172.91	17.29								
1.65E+08	1.65E+07	59.60	5.96	1.42E+08	1.42E+07	0.42	0.04	3.83E+08	3.83E+07	23.50	2.35								
1.90E+08	1.52E+07	56.78	4.54	1.45E+08	1.16E+07	0.39	0.03	4.73E+08	3.78E+07	23.26	1.86								
2.00E+08	1.80E+07	58.16	5.23	1.55E+08	1.40E+07	0.38	0.03	4.83E+08	4.35E+07	24.84	2.24								
				4.80E+07	4.32E+06	125.00	12.50												
				5.40E+08	4.86E+07	16.67	1.67												
				5.17E+08	4.65E+07	21.28	2.13												

Figure 8. Correlation profiles of Tx with protease activity. The number after the hyphen denotes the suspended solids concentration

NH-30				NHT-30				NH<30				Soya			
Tx (SBU/µl)	S.E.	PA (IU/ml)	S.E.	Tx (SBU/µl)	S.E.	PA (IU/ml)	S.E.	Tx (SBU/µl)	S.E.	PA (IU/ml)	S.E.	Tx (SBU/µl)	S.E.	PA (IU/ml)	S.E.
6312	631.20	0.141	0.01	11501	1150.10	0.249	0.02	5000	500.00	1.46	0.15	4035	403.50	0.742	0.07
7110	575.91	0.355	0.03	12100	980.10	0.635	0.05	8000	648.00	1.51	0.12	5213	422.25	0.790	0.06
7301	540.27	0.511	0.04	12423	919.30	0.656	0.05	8234	609.32	1.63	0.12	6432	475.97	1.017	0.08
8112	527.28	0.697	0.05	12798	831.87	0.706	0.05	6000	390.00	1.58	0.10				
9834	983.40	0.889	0.09	14696	1469.60	1.038	0.10	9000	900.00	1.82	0.18				
								11000	1100.00	2.31	0.23				
								6000	480.00	1.28	0.10				

Annexe – VIII

Données

Efficient Centrifugal Recovery of *Bacillus thuringiensis* Biopesticides from Fermented Wastewater and Wastewater Sludge

Figure 1. VS and Tx in centrifugate at different solids concentration (''S'' represents VS); continuous lines represent Tx_c profiles and scatter points represent VS_c profile (regression equations represent relations between Tx_c and TS)

	NH				TH				SOYA				STARCH WW			
Tot solids (g/L)	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.
25	2.1E+08	1.7E+07	12.4	0.99	1.1E+09	8.8E+07	16.1	1.29	1.3E+08	1.1E+07	10.4	0.83	1.3E+08	1.0E+07	15.5	1.24
40	2.3E+08	1.6E+07	13.5	0.95	1.3E+09	9.1E+07	17.6	1.23	1.5E+08	1.0E+07	11.4	0.80	1.4E+08	1.0E+07	16.8	1.18
50	2.6E+08	2.1E+07	14.8	1.18	1.7E+09	1.4E+08	19.9	1.59	1.7E+08	1.3E+07	13	1.04	1.5E+08	1.2E+07	18.4	1.47
60	3.0E+08	2.4E+07	15.8	1.26	2.0E+09	1.6E+08	23.6	1.89	1.8E+08	1.4E+07	15	1.20	1.6E+08	1.3E+07	23.4	1.87
70	**3.1E+08**	2.5E+07	**17**	1.36	**2.2E+09**	1.8E+08	**25.5**	2.04	**1.9E+08**	1.5E+07	**17**	1.36	**1.9E+08**	1.5E+07	**24.7**	1.98
100	3.5E+08	1.7E+07	20.5	1.03	2.4E+09	1.2E+08	27	1.35	3.3E+08	1.7E+07	18	0.90	3.2E+08	1.6E+07	25.2	1.26
120	3.5E+08	2.5E+07	24.6	1.72	5.5E+09	3.8E+08	31	2.17	3.2E+08	2.3E+07	17.5	1.23	3.2E+08	2.2E+07	25.4	1.78
140	5.7E+08	2.8E+07	26.7	1.34	7.5E+09	3.8E+08	33	1.65	3.3E+08	1.6E+07	18.3	0.92	3.2E+08	1.6E+07	25.6	1.28
160	1.7E+09	8.4E+07	31	1.55	1.6E+10	7.9E+08	33	1.65	3.3E+08	1.7E+07	18.23	0.91	3.2E+08	1.6E+07	25.4	1.27

Figure2 – Tx and VS in centrifugate at different (a) pH and (b) temperatures (''S'' represents VS). The regression data belong to Tx_c (value on which biopesticidal potential is based)

	NH				TH				SIW				Soya			
pH	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.
4	1.98E+09	1.6E+08	18.69565217	1.50	2.20E+10	1.8E+09	25.3	2.02	2.30E+10	1.8E+09	24.30	1.94	1.87E+09	1.5E+08	13.60	1.09
5	1.96E+09	1.4E+08	16.43478261	1.15	2.10E+10	1.5E+09	24.1	1.69	1.84E+10	1.3E+09	23.10	1.62	1.77E+09	1.2E+08	12.50	0.88
7	1.71E+09	1.4E+08	13.04347826	1.04	1.85E+10	1.5E+09	20.3	1.62	7.76E+09	6.2E+08	21.00	1.68	1.63E+09	1.3E+08	11.40	0.91
8.5	1.80E+09	1.4E+08	10.86956522	0.87	1.62E+10	1.3E+09	19.2	1.54	6.66E+09	5.3E+08	18.30	1.46	1.56E+08	1.2E+07	10.50	0.84

	NH				TH				SIW				Soya			
Temp. (°C)	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS CFU/ml)	S.E.	Tx (BSBU/L)	S.E.	VS (CFU/ml)	S.E.	Tx (BSBU/L)	S.E.
4	1.68E+09	1.3E+08	17.57	1.41	1.58E+10	1.3E+09	21.2	1.70	6.87E+09	5.5E+08	20.50	1.64	1.67E+09	1.3E+08	12.50	1.00
10	1.74E+09	1.2E+08	18.09	1.27	1.69E+10	1.2E+09	23.5	1.65	1.90E+10	1.3E+09	22.60	1.58	1.71E+09	1.2E+08	13.60	0.95
20	2.00E+09	1.6E+08	19.60	1.57	2.20E+10	1.8E+09	25.4	2.03	2.50E+10	2.0E+09	24.70	1.98	1.90E+09	1.5E+08	14.70	1.18
30	1.94E+09	1.6E+08	13.70	1.10	2.00E+10	1.6E+09	20.6	1.65	5.93E+09	4.7E+08	18.50	1.48	1.71E+09	1.4E+08	11.80	0.94

Figure 3. ERE profiles for different fermented broths at different RCFs (regression equations represent relations between % ERE and RCF

	NH	TH	SIW	Soya	
RCF (g)	% ERE	% ERE	% ERE	% ERE	v_g (m/s)
4303	33.07	60.44	52.73	25.58	1.34E-06
7650	52.76	71.52	61.21	30.82	1.15E-06
9682	57.48	72.05	60.61	31.87	1.17E-06
14463	65.35	77.32	73.94	39.20	9.56E-07
23428	71.65	84.18	73.33	54.93	9.63E-07
30600	70.87	86.81	81.82	61.22	8.64E-07
38729	76.38	91.03	84.85	65.41	8.33E-07
47813	89.76	94.73	87.88	77.99	8.04E-07
50000	90.55	94.78	88.00	76.94	8.03E-07

Figure 4. ERE profiles for different fermented broths at (a) 9000g and (b) 48 000g (regression equations represent relations between % ERE and RCF)

	9000 g				
	NH	**TH**	**SIW**	**Soya**	
Time (min.)	**% ERE**	**% ERE**	**% ERE**	**% ERE**	**v_g (m/s)**
5	26.77	27.22	42.06	2.52	8.66E-09
10	36.22	47.94	48.24	8.81	4.92E-09
20	46.46	55.17	52.73	11.95	4.27E-09
30	57.48	72.05	60.61	29.77	3.27E-09
45	59.06	76.79	62.42	31.87	3.07E-09
60	66.14	75.74	72.73	31.87	3.11E-09
90	67.72	76.79	71.52	32.91	3.07E-09
120	70.87	81.54	80.61	42.35	2.89E-09

	48000 g				
	NH	**TH**	**SIW**	**Soya**	
Time (min.)	**% ERE**	**% ERE**	**% ERE**	**% ERE**	**v_g (m/s)**
5	71.20	84.22	66.41	30.21	5.26E-10
10	76.44	91.23	73.41	48.93	4.86E-10
20	89.53	94.74	78.92	63.70	4.68E-10
30	89.53	94.74	85.45	69.92	4.68E-10
45	92.15	94.74	85.53	80.30	4.68E-10
60	92.15	94.74	88.01	84.53	4.68E-10
90	92.15	96.49	89.78	89.65	4.59E-10
120	92.15	96.49	90.38	91.01	4.59E-10

Figure 5. Sigma profiles for different fermented broths: (a) sigma versus ERE and (b) power required versus sigma

% ERE (desired)	NH Σ (m^2)	TH Σ (m^2)	SIW Σ (m^2)	Soya Σ (m^2)
50	33736.34	18797.81	16835.38	354874.3
55	43379.28	23082.89	23363.72	481962.6
60	56243.32	28580.99	32693.78	660018.7
65	73432.09	35636.14	46069.63	910176.4
70	96444.69	44697.33	65304.3	1262619
75	127318.7	56350.01	93044.4	1760518
80	168826.5	71357.71	133159.8	2465717
85	224744.3	90716.77	191318.1	3466935

Power, Watts	Σ (m^2)	Rated Current, Amp
3177.70	0.00	5.53
3431.92	28.82	5.97
3686.14	115.28	6.41
3940.35	259.38	6.85
4086.53	689.08	7.11
4530.50	4896.33	7.88
4992.81	7831.90	8.68
6355.41	10416.67	11.05
7036.39	14962.52	12.24

Annexe – IX

Données

Presence and Characterization of Chitinases present in *Bacillus thuringiensis* Fermented Wastewater and Wastewater Sludge

Figure 2. Bt fermentation of wastewater and/or wastewater sludge: a) sporulation profile and, b) chitinase profile

	NH			TH			SIW		
Time (h)	Chitinase (U/ml)	S.E.	VS (CFU/ml)	Chitinase (U/ml)	S.E.	VS (CFU/ml)	Chitinase (U/ml)	S.E.	VS (CFU/ml)
0	-2.07	0.30	4.3E+03	0	0.00	3.3E+03	0	0.00	6.5E+04
3	4.14	0.80	7.0E+03	0	0.00	9.3E+03	0.50	0.01	1.3E+06
6	10.96	1.30	7.0E+05	13.15	1.30	3.2E+06	1	0.08	3.5E+07
9	21.19	2.40	2.5E+07	18.21	1.40	6.0E+07	0.18	0.07	1.2E+08
12	31.49	2.50	6.0E+07	24.65	1.00	1.3E+08	0.25	0.02	2.5E+08
15	36.92	3.30	1.1E+08	30.63	1.30	3.0E+08	2.50	0.30	5.2E+08
18	31.67	4.60	2.5E+08	26.34	2.20	3.0E+08	0.26	0.04	5.7E+08
21	35.23	5.30	2.7E+08	21.59	3.10	3.3E+08	0.22	0.05	8.2E+08
24	29.40	3.70	3.2E+08	16.68	1.70	3.5E+08	2.90	0.07	8.1E+08
30	64.21	4.60	3.3E+08	53.63	3.60	3.6E+08	0.54	0.01	8.2E+08
36	46.75	3.80	3.4E+08	44.12	2.80	2.8E+08	0.44	0.01	8.2E+08
48	43.88	4.40	3.0E+08	36.47	3.30	3.9E+08	0.36	0.01	8.0E+08

Figure 3. Stability of chitinases in various adjuvant amended Bt fermented broths (CA – Chitinase Activity)

	NH	TH	SIW
Time (min)	CA (U/ml)	CA (U/ml)	CA (U/ml)
0	45.14	35.12	2.02
20	45.16	35.13	1.98
40	44.95	35.11	1.97
60	45.11	35.1	1.98
80	45.02	34.97	1.98
100	45.04	34.95	2.01
120	44.95	35.08	2.03
140	45.01	35.05	1.98
160	45.09	35.07	1.99
2880	44.59	35.00	1.99
10080	44.10	34.93	1.98
20160	43.62	34.86	1.98

Figure 4. Characterization of wastewater sludge chitinases; a) NH, and; b) TH sludge

NH sludge														
	T = 10°C		T =20°C		T =30°C		T =40°C		T = 50°C		T = 60°C		T = 70°C	
pH	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.
3	58.67	4.69	62.78	5.02	66.95	5.36	75.31	6.02	77.71	6.22	72.48	5.80	68.76	5.50
4	66.37	4.65	64.18	4.49	57.98	4.06	72.09	5.05	73.99	5.18	70.49	4.93	70.92	4.96
5	66.21	5.30	43.00	3.44	49.30	3.94	55.30	4.42	53.43	4.27	51.04	4.08	55.57	4.45
6	32.90	2.63	26.09	2.09	26.09	2.09	22.18	1.77	34.42	2.75	21.48	1.72	17.78	1.42
7	34.68	2.77	39.51	3.16	42.05	3.36	30.17	2.41	33.97	2.72	38.74	3.10	22.64	1.81
8	51.54	2.58	48.25	2.41	56.97	2.85	42.97	2.15	32.36	1.62	57.06	2.85	28.71	1.44
9	35.06	2.45	26.22	1.84	19.96	1.40	34.19	2.39	29.77	2.08	12.57	0.88	22.95	1.61
10	25.56	1.28	46.35	2.32	53.01	2.65	40.46	2.02	44.37	2.22	24.58	1.23	34.25	1.71
11	50.55	2.53	42.54	2.13	39.29	1.96	35.41	1.77	44.08	2.20	41.39	2.07	50.45	2.52
12	29.57	1.77	41.39	2.48	40.96	2.46	42.56	2.55	41.10	2.47	40.14	2.41	41.24	2.47

TH sludge														
	T = 10°C		T =20°C		T =30°C		T =40°C		T = 50°C		T = 60°C		T = 70°C	
pH	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.	Chitinase (Units/ml)	S.E.
3	41.84	3.35	43.22	3.46	33.54	2.68	28.48	2.28	69.51	5.56	36.75	2.94	24.45	1.96
4	34.22	2.40	63.12	4.42	56.36	3.94	36.61	2.56	72.64	5.08	38.13	2.67	33.36	2.34
5	17.69	1.42	44.00	3.52	43.23	3.46	1.12	0.09	66.12	5.29	31.74	2.54	25.89	2.07
6	0.32	0.03	16.27	1.30	7.94	0.64	16.53	1.32	47.94	3.84	52.49	4.20	7.56	0.60
7	0.22	0.02	21.25	1.70	21.59	1.73	6.26	0.50	63.81	5.10	10.16	0.81	5.83	0.47
8	0.11	0.01	12.16	0.61	32.50	1.63	19.59	0.98	63.01	3.15	2.05	0.10	2.22	0.11
9	0.23	0.02	1.30	0.09	19.45	1.36	0.74	0.05	25.94	1.82	7.56	0.53	1.35	0.09
10	0.12	0.01	7.05	0.35	25.30	1.26	1.10	0.06	48.65	2.43	1.02	0.05	4.12	0.21
11	0.38	0.02	29.86	1.49	36.15	1.81	28.79	1.44	59.59	2.98	9.64	0.48	0.76	0.04
12	45.55	2.73	27.98	1.68	22.49	1.35	14.99	0.90	43.68	2.62	17.17	1.03	47.76	2.87

Annexe – X

Données

Recent Advances in Downstream Processing and Formulations of *Bacillus thuringiensis* based Biopesticides- Review

Figure 2. Half lives for different Bt formulations (without UV radiation screen) on UV radiation exposure

	Media	Half life (d)	S.E.
Non-Hydrolyzed	NH sludge	11.14	1.11
Hydrolyzed	H sludge	9.51	0.95
Starch industry	SIW	9.02	0.90
Synthetic	Soya	2.86	0.29

Annexe – XI

Données

Bacillus thuringiensis Proteases: Production, Sporulation and Synergism – Review

Figure 1. a) Total cells (TC), and b) PA profile in different Bt fermented raw materials; SIW: starch industry wastewater; SHWW: slaughterhouse wastewater; BLS: Black Lake sludge (sequencing batch reactor sludge); JQS: Jonquière sludge (sludge from activated sludge process); CUQS: Communauté Urbaine de Québec sludge (secondary sludge from biofiltration process); CUQHS: CUQ hydrolyzed secondary sludge; CUQHOS: CUQ hydrolyzed oxidized secondary sludge; CUQP: CUQ primary sludge (sludge from physical-chemical treatment) and, CUQM: CUQ mixed sludge (sludge from thickening tank)

Time (h)	Soya (10E+09 SBU/L)	SIW (17E+09 SBU/L)	SHWW (6E+09 SBU/L)	BLS (15E+09 SBU/L)	JQS (12E+09 SBU/L)	CUQS (12.5E+09 SBU/L)	CUQHS (18E+09 SBU/L)	CUQHOS (13E+09 SBU/L)	CUQP (5E+09 SBU/L)	CUQM (15E+09 SBU/L)
0	1.30E+07	1.70E+06	2.03E+06	1.47E+06	9.40E+05	2.97E+06	7.50E+06	2.22E+06	9.95E+04	7.50E+06
3	1.67E+07	8.00E+06	2.90E+06	2.80E+06	5.60E+06	1.07E+07	3.00E+07	4.17E+06	1.23E+05	2.80E+07
6	1.73E+08	1.70E+07	3.07E+06	1.41E+07	2.67E+07	1.60E+08	1.93E+08	2.19E+07	2.20E+05	8.50E+07
9	2.30E+08	7.33E+07	7.00E+06	3.31E+07	5.70E+07	3.50E+07	3.40E+08	7.12E+07	2.20E+06	1.95E+08
12	3.17E+08	3.00E+08	1.47E+07	6.37E+07	1.23E+08	3.90E+08	4.37E+08		2.40E+06	2.15E+08
15	3.30E+08	3.33E+08	2.63E+07	1.60E+08	1.83E+08	5.43E+08	4.00E+08		2.60E+06	3.25E+08
18	3.23E+08	3.57E+08	3.33E+07	2.63E+08	2.20E+08	5.13E+08	4.60E+08		2.95E+07	3.75E+08
21	3.57E+08	5.13E+08	6.00E+07	3.53E+08	3.63E+08	5.80E+08	3.43E+08		3.05E+07	3.75E+08
24	3.43E+08	8.13E+08	1.20E+08	4.83E+08	4.23E+08	7.27E+08	4.37E+08	9.89E+07	3.55E+07	4.00E+08
30	2.73E+08	1.00E+09	2.57E+08	6.83E+08	3.60E+08	6.13E+08	4.33E+08		3.70E+07	3.90E+08
36	3.87E+08	1.20E+09	2.87E+08	5.30E+08	3.70E+08	5.00E+08	2.90E+08	8.00E+07	3.70E+07	3.95E+08
48	4.90E+08	1.67E+09	2.93E+08	5.83E+08	5.70E+08	6.67E+08	3.97E+08	5.00E+07	3.80E+07	3.90E+08

Time (h)	PA (Soya)	PA (SIW)	PA (SHWW)	PA (BLS)	PA (JQS)	PA (CUQS)	PA (CUQHS)	PA (CUQHOS)	PA (CUQP)	PA (CUQM)
3	0.3	0.2	0.2	0.1	0.3	0.4	0.3		0.2	0.5
6	0.6	0.3	0.2	0.1	0.3	0.3	0.4	0.13	0.1	0.5
9	1	0.4	1.2	0.1	0.9	0.3	0.3		0.2	0.4
12	1.3	0.8	1	0.2	0.3	0.4	0.4	0.64	0.2	0.5
15	1.5	1	0.9	1	0.6	1.2	1.2		0.6	1.4
18	1.3	1.1	0.7	1.25	0.7	1.0	0.6		0.5	1.1
21	0.9	1.2	0.6	1.52	1.3	1.0	0.4		0.5	1.1
24	0.8	1.1	0.5	2.26	1.4	1.3	0.7	1.11	0.7	1.4
30	1	1.4	0.8	2.19	2.2	1.5	1.2	1.51	0.8	1.8
36	1.1	0.9	0.1	1.82	2.3	0.8	0.8	1.32	0.4	0.8
48	1.2	0.7	0.1	1.54	1.5	0.7	0.6	1.3	0.4	0.8

Figure 2. Protease activity profiles during Bt fermentation (15 L fermenter) of: a) soyameal (semi-synthetic commercial medium) with temperature optima of 40-50°C and pH 6-11; and b) wastewater sludge temperature optima of 50°C and pH 6-11

				Soya	PA (IU/ml)				
Time (h)	INRS4	INRS6	INRS8	INRS12	INRS14	INRS21	INRS24	Bta	Btk
0	0.17	0.13	0.53	0.26	0.05	0.17	0.52	0.07	0
3	0.32	0.59	2.62	0.29	0.36	2.66	0.87	0.15	0
6	0.63	2.15	4.61	1.18	0.54	4.5	0.93	0.41	0.14
9	2.41	3.49	3.57	2.64	0.61	3.47	1.89	1.91	0.89
12	2.92	0.45	3.18	2.56	4.02	3.96	3.25	2.95	2.39
15	2.67	0.33	3.51	2.7	4.23	3.93	1.81	2.83	1.37
18	2.26	0.33	3.34	1.79	3.32	3.66	1.32	0.94	1.19
21	2.23	0.47	3.52	1.28	3.3	3.66	1.29	1.25	1.21
24	2.22	0.32	3.18	0.87	1.35	3.59	2.1	1.02	1.93
27	2.62	0.48	2.76	0.76	2.4	3.24	2.07	1	1.89
30	2	0.38	2.73	0.7	0.3	2.9	2.1	1.27	1.76
33	2.13	0.3	2.51	0.75	0.18	3.25	2	1.25	2.04
36	1.67	0.47	2.11	0.83	0.21	2.92	1.95	1.13	2.33
48	1.92	0.47	1.96	0.89	0.93	2.75	2.29	1.24	2.56
				WWS	PA (IU/ml)				
Time (h)	INRS4	INRS6	INRS8	INRS12	INRS14	INRS21	INRS24	Bta	Btk
0	0.11	0.15	0.01	0.01	0.28	0.05	0	0.21	0
3	0.14	0.23	0.01	0.01	0.54	0.58	0.06	0.31	0
6	0.92	0.52	0.62	0.21	1.09	0.7	0.04	0.41	0.06
9	1.61	1.44	1.03	0.38	1.08	1.11	0.04	0.48	0.1
12	1.69	1.58	1.96	0.25	1.08	0.94	0.24	0.53	0.41
15	1.65	1.65	1.54	0.98	0.9	0.51	1.04	0.64	0.58
18	1.62	1.51	1.58	0.8	0.76	1.56	1.2	0.42	0.65
21	1.97	1.43	0.9	0.08	1	1.58	0.82	0.35	1.3
24	2.18	1.16	1.39	0.12	1.34	1.48	1.9	0.41	1.5
27	1.97	1.36	1.75	0.03	1.51	1.94	1.85	0.7	1.68
30	2.38	1.23	1.35	0.36	1.66	1.87	2.24	0.73	1.92
33	2.12	1.29	1.15	0.84	1.89	2.34	2.07	0.83	1.87
36	2.25	1.05	1.16	1	2.13	3.16	2.21	0.79	2.03
48	2.27	0.78	0.21	1.25	1.93	3.37	2.29	1.08	2.13

Figure 5. Temporal profile of viable cells during shelf-storage of different Bt formulations

Time (d)	NH(CFU/ml)	TH(CFU/ml)	SIW(CFU/ml)	Soya(CFU/ml)
0	1.00E+07	2.70E+07	8.90E+07	3.40E+06
7	1.10E+07	2.56E+07	7.80E+07	3.00E+06
15	1.10E+06	2.60E+06	6.10E+06	2.56E+05
30	4.00E+05	1.80E+06	5.90E+06	1.40E+05
60	3.80E+04	8.00E+05	5.90E+05	1.10E+04
180	4.00E+04	1.60E+05	5.90E+04	9.00E+03
270	3.10E+03	3.00E+04	6.70E+03	5.90E+03
365	2.90E+02	1.70E+04	8.00E+02	4.50E+02
540	1.00E+01	1.10E+02	7.60E+01	5.40E+01

Figure 6. Protease activity (PA) and entomotoxicity (Tx) of different samples of: a) Non-hydrolyzed sludge and; b) Hydrolyzed sludge; FB- Fermented broth (48h), Pellet-Centrifuged fermented broth (9000 g for 30 min), SN-supernatant, W-Saline water (for dilution)

NH sludges				
Sample	PA(IU/ml)	S.E.	Tx (SBU/µL)	S.E.
FB	2.1	0.17	12000	960
Pellet	0.5	0.16	14000	970
SN	1.3	0.11	1100	550
1SN:1W	0.7	0.1	1350	357
2SN:1W	1.5	0.17	4200	378
3SN:1W	1.9	0.19	5800	476
TH sludges				
Sample	PA(IU/ml)	S.E.	Tx (SBU/µL)	S.E.
FB	1.6	0.19	17800	1012
Pellet	0.4	0.06	19520	1021
SN	1.1	0.2	1300	630
1SN:1W	0.5	0.03	1560	432
2SN:1W	1.01	0.18	3780	657
3SN:1W	1.32	0.11	4960	745

Annexe – XII

Données

Screening of different Adjuvants for Wastewater/Wastewater Sludge based *Bacillus thuringiensis* Formulations

Figure 2. Screening profile of different adjuvants for different media, namely, non-hydrolyzed sludge (NH); hydrolyzed sludge (H); starch industry wastewater (SIW) and soya: a) suspending agents; b) phagostimulants; c) stickers; d) and e) UV screens (UV1 to UV5). Error bars represent standard deviations. Control refers to sample without adjuvants. All symbols D_x, P_x, R_x, UV_x (x=1, 2, 3, 4, 5, 6) are defined in Table 2

Suspendibility (%)														
MEDIA	**D1**	**S.E.**	**D2**	**S.E.**	**D3**	**S.E.**	**D4**	**S.E.**	**D5**	**S.E.**	**D6**	**S.E.**	**Cont**	**S.E.**
NH	72	7.20	51	5.10	68	6.80	73	7.30	42	4.20	37	3.70	26	2.60
TH	92	7.36	60	4.80	86.5	6.92	81.4	6.51	64	5.12	53	4.24	41	3.28
SIW	70	5.46	47	3.67	71	5.54	72	5.62	37	2.89	25	1.95	31	2.42
SOYA	87	7.92	63	5.73	85.4	7.77	82.3	7.49	79	7.19	65	5.92	38	3.46

% Tx increase														
MEDIA	**P1**	**S.E.**	**P2**	**S.E.**	**P3**	**S.E.**	**P4**	**S.E.**	**P5**	**S.E.**	**P6**	**S.E.**	**Cont**	**S.E.**
NH	6.44	0.64	2.01	0.20	13.47	1.35	7.46	0.75	13.57	1.36	13.59	1.36	-0.43	0.04
TH	4.76	0.38	0.40	0.03	9.94	0.79	4.30	0.34	3.79	0.30	7.13	0.57	-1.00	0.08
SIW	4.25	0.33	0.49	0.04	7.65	0.60	11.17	0.87	13.82	1.08	4.40	0.34	-1.10	0.09
SOYA	0.16	0.01	2.58	0.23	6.97	0.63	14.48	1.32	13.94	1.27	0.31	0.03	-0.20	0.02

% sticking														
MEDIA	**R1**	**S.E.**	**R2**	**S.E.**	**R3**	**S.E.**	**R4**	**S.E.**	**R5**	**S.E.**	**R6**	**S.E.**	**Cont**	**S.E.**
NH	84.44	8.44	48.14	4.81	77.16	7.72	91.10	9.11	5.30	0.53	15.13	1.51	1.50	0.15
TH	85.42	6.83	29.85	2.39	27.42	2.19	71.24	5.70	20.49	1.64	8.23	0.66	1.50	0.12
SIW	95.34	7.44	19.28	1.50	56.24	4.39	28.38	2.21	32.76	2.56	0.33	0.03	0.00	0.00
SOYA	53.90	4.90	23.19	2.11	33.67	3.06	70.44	6.41	11.84	1.08	28.24	2.57	0.50	0.05

	UV screens				% spore survival									
MEDIA	**UV1**	**S.E.**	**UV2**	**S.E.**	**UV3**	**S.E.**	**UV4**	**S.E.**	**UV5**	**S.E.**	**UV6**	**S.E.**	**Cont**	**S.E.**
NH	93.64	9.36	98.75	9.88	99.16	9.92	9.13	0.91	98.35	9.84	6.50	0.65	68.50	6.85
TH	93.64	7.49	97.87	7.83	95.00	7.60	7.02	0.56	98.34	7.87	4.60	0.37	63.60	5.09
SIW	91.76	7.16	95.38	7.44	8.95	0.70	7.92	0.62	95.21	7.43	7.39	0.58	68.50	5.34
SOYA	94.81	8.63	96.25	8.76	91.77	8.35	54.78	4.98	76.69	6.98	9.19	0.84	75.30	6.85

	UV screens				% Tx losses									
MEDIA	**UV1**	**S.E.**	**UV2**	**S.E.**	**UV3**	**S.E.**	**UV4**	**S.E.**	**UV5**	**S.E.**	**UV6**	**S.E.**	**Cont**	**S.E.**
NH	6.59	0.66	1.58	0.16	2.28	0.23	13.97	1.40	0.72	0.07	22.11	2.21	14.40	1.44
TH	3.73	0.30	4.62	0.37	7.29	0.58	7.13	0.57	14.68	1.17	93.11	7.45	14.20	1.14
SIW	5.27	0.41	0.49	0.04	16.76	1.31	18.83	1.47	5.42	0.42	29.48	2.30	46.40	3.62
SOYA	3.76	0.34	4.12	0.37	6.85	0.62	7.94	0.72	18.26	1.66	94.60	8.61	52.30	4.76

Figure 3. Screening of different anti-microbial agents (AMAs) for non–hydrolyzed sludge (NH); hydrolyzed sludge (TH); starch industry wastewater (SIW) and soyameal formulations (period – two months) ; (Y&M – yeast and mold, Ent – *Enterococcus*, C – Coliforms, E.c – *Escherichia coli*, S- *Salmonella*, Sta.- *Staphylococcus,* TC–total cell count)

	NH				All TC (CFU/ml)							
Anti-microbial agents	Yeast and Moulds	S.E.	Enterococcus	S.E.	Coliforms (total and feacal)	S.E.	E.coli	S.E.	Salmonella	S.E.	Staphylococcus	S.E.
AM-1	26	2.60E+00	0	0.00E+00	4	4.00E-01	0	0.00E+00	0	0.00E+00	0	0.00E+00
AM-2	1.30E+03	1.04E+02	124	9.92E+00	15	1.20E+00	265	2.12E+01	89	7.12E+00	6	4.80E-01
AM-3	1.20E+03	9.36E+01	68	5.30E+00	49	3.82E+00	68	5.30E+00	91	7.10E+00	18	1.40E+00
AM-4	1.40E+05	1.27E+04	45	4.10E+00	79	7.19E+00	69	6.28E+00	186	1.69E+01	28	2.55E+00
AM-5	1.20E+04	1.20E+03	62	6.20E+00	81	8.10E+00	72	7.20E+00	0	0.00E+00	11	1.10E+00
AM-6	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00

	TH											
Anti-microbial agents	Yeast and Moulds	S.E.	Enterococcus	S.E.	Coliforms (total and feacal)	S.E.	E.coli	S.E.	Salmonella	S.E.	Staphylococcus	S.E.
AM-1	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00
AM-2	1.50E+04	1.20E+03	83	6.64E+00	234	1.87E+01	114	9.12E+00	119	9.52E+00	256	2.05E+01
AM-3	1.70E+05	1.33E+04	0	0.00E+00	28	2.18E+00	76	5.93E+00	143	1.12E+01	8	6.24E-01
AM-4	8.00E+03	7.28E+02	48	4.37E+00	63	5.73E+00	0	0.00E+00	154	1.40E+01	15	1.37E+00
AM-5	9.00E+03	9.00E+02	39	3.90E+00	58	5.80E+00	81	8.10E+00	128	1.28E+01	9	9.00E-01
AM-6	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00

Figure 3. Screening of different anti-microbial agents (AMAs) for non–hydrolyzed sludge (NH); hydrolyzed sludge (TH); starch industry wastewater (SIW) and soyameal formulations (period – two months) ; (Y&M – yeast and mold, Ent – *Enterococcus*, C – Coliforms, E.c – *Escherichia coli*, S- *Salmonella*, Sta.- *Staphylococcus*, TC–total cell count) contd…

SIW												
Anti-microbial agents	Yeast and Moulds	S.E.	Entero coccus	S.E.	Coliforms (total and feacal)	S.E.	E.coli	S.E.	Salmonella	S.E.	Staphylococcus	S.E.
AM-1	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	11	1.10E+00	0	0.00E+00
AM-2	3.20E+05	2.56E+04	183	1.46E+01	126	1.01E+01	324	2.59E+01	119	9.52E+00	148	1.18E+01
AM-3	1.78E+06	1.39E+05	259	2.02E+01	168	1.31E+01	186	1.45E+01	143	1.12E+01	625	4.88E+01
AM-4	2.90E+06	2.64E+05	96	8.74E+00	458	4.17E+01	245	2.23E+01	154	1.40E+01	546	4.97E+01
AM-5	1.80E+05	1.80E+04	81	8.10E+00	226	2.26E+01	214	2.14E+01	98	9.80E+00	340	3.40E+01
AM-6	34	2.72E+00	5	4.00E-01	0	0.00E+00	0	0.00E+00	5	4.00E-01	0	0.00E+00

Soya												
Anti-microbial agents	Yeast and Moulds	S.E.	Enterococcus	S.E.	Coliforms (total and feacal)	S.E.	E.coli	S.E.	Salmonella	S.E.	Staphylococcus	S.E.
AM-1	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00
AM-2	3.70E+01	2.96E+00	3	2.40E-01	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00
AM-3	1.70E+05	1.33E+04	259	2.02E+01	168	1.31E+01	186	1.45E+01	143	1.12E+01	625	4.88E+01
AM-4	2.90E+06	2.64E+05	96	8.74E+00	458	4.17E+01	245	2.23E+01	154	1.40E+01	546	4.97E+01
AM-5	3.20E+06	3.20E+05	84	8.40E+00	351	3.51E+01	186	1.86E+01	154	1.54E+01	621	6.21E+01
AM-6	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00	0	0.00E+00

Figure 5. Half-lives of entomotoxicity potential (Tx) for different fermented media (control) and formulations when exposed to UV radiation; based on pooled data. Error bars represent standard deviations

	Formulations		Fermented media		Formulations with UV Screen	
Media	**Half-life (d)**	**S.E.**	**Half-life (d)**	**S.E.**	**Half-life (d)**	**S.E.**
NH sludge	11.14	1.11	3.99	0.40	10.72	1.07
H sludge	9.51	0.95	6.20	0.62	9.54	0.95
SIW	9.02	0.90	2.64	0.26	10.9	1.09
Soya	2.86	0.29	1.19	0.12	6.43	0.64
Commercial formulation					7.23	0.72

Annexe – XIII

Données

Starch industry Wastewater based Stable *Bacillus thuringiensis* Liquid Formulations

Figure 1. Bt growth profile in SIW. (A) Operational parameters. (B) Process performance parameters

Time (h)	D.O. (%)	Air flow rate (LPM)	Agit. (rpm)
0	99.72	1.96	298
3.08	77.00	1.96	302
6.07	46.65	1.96	398
9.06	58.52	2.47	300
12.05	73.02	2.45	302
15.04	76.72	2.45	301
18.02	82.62	2.45	301
21.01	86.92	2.45	270
24.00	80.62	2.45	270
27.09	83.32	2.45	267
30.07	84.27	2.45	270
33.06	86.15	2.45	273
36.05	87.10	2.45	268
39.04	87.80	2.45	270
42.03	88.70	2.45	273
45.01	88.52	2.95	271
48.00	88.77	2.95	270

Time (h)	TC (CFU/ml)	S.E.	VS (CFU/ml)	S.E.	k_La (h^{-1})	Visc. (cP)	S.E.	D_{50} (µm)	S.E.	Density (g/ml)	S.E.
0	1.07E+06	8.5E+04	6.50E+04	5.2E+03	160	2.7	0.22	149.5	11.96	0.99823	0.08
3	1.10E+07	7.7E+05	1.25E+06	8.8E+04	88.52	2.5	0.18	106.4	7.45	0.9865	0.07
6	1.15E+08	9.2E+06	3.50E+07	2.8E+06	121.00	2.7	0.22	106.4	8.51	0.9786	0.08
9	4.17E+08	3.3E+07	1.25E+08	1.0E+07	169.78	2.1	0.17	102.9	8.23	0.9976	0.08
12	6.20E+08	5.0E+07	2.46E+08	2.0E+07	235.91	3.4	0.27	39.3	3.14	0.9999	0.08
15	7.85E+08	3.9E+07	5.15E+08	2.6E+07	77.42	4.5	0.23	24.2	1.21	1.021	0.05
18	9.45E+08	6.6E+07	5.70E+08	4.0E+07	87.54	6.7	0.47	19.1	1.34	1.022	0.07
21	1.10E+09	5.5E+07	8.20E+08	4.1E+07	52.52	3.03	0.15	9.8	0.49	1.023	0.05
24	1.35E+09	6.8E+07	8.05E+08	4.0E+07	74.71	2.97	0.15	7.6	0.38	1.024	0.05
30	1.55E+09	9.3E+07	8.15E+08	4.9E+07	65.55	2.54	0.15	5.2	0.31	1.04	0.06
36	1.65E+09	1.3E+08	8.15E+08	6.5E+07	66.45	2.34	0.19	2.02	0.16	0.9974	0.08
48	1.67E+09	1.3E+08	8.00E+08	6.4E+07	71.44	2.36	0.19	2.53	0.20	1.031	0.08

Figure 2. Optimized formulation of soya (Sy-3). (A) Rheological proÞle at different pH. (B) Rheological profile at different temperatures. (C) Biological efficacy (P, particle size); bars shown with different letters are significantly different at $P \leq 0.05$

Sy3	Viscosity, cP						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.6	18.4	18.5	18.4	18.3	18	17.4	17	16.8	16.5	15.8	15.4
4.5	18.4	18.5	18.4	18.1	18	17.6	17.5	17	16.7	16.5	16.1	15.6
5	18.5	18.4	18.3	18.1	17.8	17.5	17.1	17	16.8	16.5	16.2	15.7
6	18.4	18.4	18.2	18	17.6	17.4	17	16.7	16.5	16.1	15.7	14.6
6.5	18.5	18.4	18.2	18	17.4	17.3	17	16.8	16.4	16	15.6	14.3

Sy3	Particle-size, D 50			pH stability studies								
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3	3	2.7	2.5	2.4	2.2	2.1	2	1.8	1.5	1.4	1.2
4.5	3.2	3	2.7	2.6	2.4	2.1	2	1.8	1.5	1.3	1.1	1
5	3.2	3	2.5	2.4	2.3	2	1.9	1.7	1.4	1.3	1	0.8
6	3.1	3	2.4	2.2	2	1.7	1.5	1.4	1.1	1	1	0.7
6.5	3.1	2.5	2.2	2	1.8	1.3	1	0.8	0.4	0.2	ND	ND

Sy3	Viscosity, cP			Temperature stability studies								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.6	18.2	18.4	18.2	18	17.7	17.2	17	16.6	16.3	16.1	15.7
10	18.4	18.3	18.3	18	17.6	17.3	16.8	16.3	15.7	15.3	15	14.7
30	18.5	18.2	18.2	17.8	17.4	17.3	16.7	16.4	15.6	15.3	14.6	13.6
40	18.4	18.3	18.2	17.6	17.3	17	15.8	15.3	15.4	13.6	13	12.6
50	18.5	18.2	18.1	16.7	15.5	14.6	14.3	13.8	12.6	11.9	11.2	10.6

Figure 2. Optimized formulation of soya (Sy-3). (A) Rheological proÞle at different pH. (B) Rheological profile at different temperatures. (C) Biological efficacy (P, particle size); bars shown with different letters are significantly different at $P \leq 0.05$ contd...

Sy3	Particle-size, D_{50}	Temperature stability studies										
Tem\Day	0	3	7	10	15	30	60	90	120	150	180	365
4	3	3	2.7	2.5	2.4	2.3	2.1	2	1.7	1.5	1.4	1.3
10	3.2	3.1	2.8	2.3	2.2	2.1	2	1.8	1.6	1.5	1.4	1.2
30	3.2	3.1	2.7	2.3	2.2	2.1	2	1.9	1.7	1.7	1.6	1.5
40	3.1	3	2.6	2.4	2.1	2	1.8	1.6	1.4	1.2	1	0.9
50	3.1	2.5	2.2	2	1.7	1.4	1.3	1.1	1	0.8	0.5	0.2

Sy3	Entomotoxicity, SBU/µL	pH stability studies										
pH\Days	0	3	7	10	15	30	60	90	120	150	180	365
4	15698	15645	15456	15345	15268	15164	15116	15111	15102	15101	15000	14887
5	15879	15465	15354	15264	15164	15089	15065	15006	15000	14886	14687	14564
6.5	15879	15725	15645	15468	15328	15245	15062	15000	14877	14555	14366	14234

Sy3	Entomotoxicity, SBU/µL	Temperature stability studies										
Tem\Day	0	3	7	10	15	30	60	90	120	150	180	365
4	15698	15459	15364	15224	15000	14786	14648	14452	14325	14320	14225	14000
30	15879	15669	15423	15108	15000	14805	14762	14326	14200	14100	13954	13875
50	15879	15800	15600	15126	15000	14523	14209	14100	13876	13614	13452	13008

Figure 3. Stability profile of soya formulations at different pH and temperatures (time = 365 d). (A) Percentage of spore survival. (B) Percentage of suspendibility (T, temperature). "T" indicates the effect of temperature on the two variables, whereas the bars indicate effects of pH

pH	S-1	S.E.	S-2	S.E.	S-3	S.E.	S-4	S.E.	S-5	S.E.
4	83.00	2.47	97.00	0.97	99.00	4.14	93.00	3.96	97.00	3.73
4.5	79.00	1.71	92.00	2.41	94.00	3.42	83.00	3.62	90.00	3.27
5	78.00	2.32	86.00	2.41	90.00	1.78	91.00	4.82	93.00	2.32
6	70.00	1.59	82.00	1.26	86.00	4.50	90.00	5.22	87.00	3.71
6.5	74.00	1.34	80.00	2.66	86.00	3.23	88.00	3.19	84.00	1.90
Temp (°C)	**S-1**	**S.E.**	**S-2**	**S.E.**	**S-3**	**S.E.**	**S-4**	**S.E.**	**S-5**	**S.E.**
4	85.00	0.87	93.00	2.22	98.00	3.07	91.90	2.01	92.00	1.14
10	86.40	0.22	87.60	1.50	94.70	3.83	87.10	1.71	85.70	3.21
30	86.20	1.83	85.70	2.31	89.80	3.12	87.40	3.62	85.50	2.21
50	74.30	1.39	82.40	2.52	87.40	3.57	84.10	1.83	78.60	1.68

pH	S-1	S.E.	S-2	S.E.	S-3	S.E.	S-4	S.E.	S-5	S.E.
4	36.00	0.97	37.00	1.40	65.00	1.76	51.00	0.69	48.00	1.61
4.5	41.00	1.55	38.00	1.71	72.00	1.28	64.00	2.21	63.00	0.93
5	42.00	1.36	45.00	1.92	77.00	2.46	66.00	1.52	65.00	1.84
6	41.00	0.95	47.00	1.42	78.00	3.84	68.00	0.49	67.00	1.18
6.5	37.00	1.02	36.00	1.23	75.00	1.74	56.00	1.50	52.00	1.54

Temp (°C)	S-1	S.E.	S-2	S.E.	S-3	S.E.	S-4	S.E.	S-5	S.E.
4	37.00	0.82	38.00	0.89	66.00	1.46	58.00	0.98	58.00	2.54
10	38.00	1.91	41.00	1.10	74.00	2.19	64.00	0.93	64.00	2.60
30	37.00	0.49	40.00	0.81	76.00	1.65	63.00	2.69	61.00	1.36
50	45.00	0.26	48.00	0.42	83.00	1.01	71.00	1.33	74.00	1.28

Annexe – XIV

Données

Bacillus thuringiensis Fermentation of Hydrolyzed Sludge - Rheology and Formulation Studies

Figure 1. Rheograms of hydrolyzed sludge during fermentation and formulation: a) shear stress profile and; b) viscosity profile

	Time = 0h	Time = 3h	Time = 6h	Time = 9h	Time = 12h	Time = 15h	Time = 18h	Time = 21h	Time = 24h	Time = 30h	Time = 36h	Time = 48h
Shear Rate (s^{-1})	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress	Sh Stress
0.37	1.27	0.99	1.08	1.48	1.04	0.98	0.98	0.64	0.71	0.22	0.15	0.10
0.73	1.86	0.88	1.51	1.61	1.59	1.12	1.12	0.80	0.81	0.29	0.23	0.19
1.83	2.85	1.59	2.03	1.92	1.62	1.20	1.20	0.95	0.95	0.48	0.43	0.35
3.67	3.84	2.00	2.58	2.42	1.90	1.45	1.45	1.24	1.20	0.68	0.59	0.54
7.34	2.97	2.35	2.46	3.26	2.55	1.91	1.91	1.60	1.58	1.03	0.86	0.80
11.01	1.82	2.01	1.99	3.92	2.93	2.23	2.23	1.86	1.91	1.33	1.08	1.03
14.68	2.37	2.40	1.87	4.22	3.22	2.47	2.47	2.10	2.17	1.83	1.27	1.22
18.35	1.89	1.46	1.62	4.53	3.47	2.67	2.67	2.26	2.38	2.10	1.46	1.39
24.46	1.70	1.72	1.84	5.00	3.85	3.05	3.05	2.58	2.72	2.47	1.75	1.67
30.58	1.87	1.86	1.79	5.38	4.19	3.32	3.32	2.83	3.05	2.80	2.02	1.91
36.69	2.14	2.12	1.89	5.69	4.50	3.57	3.57	3.06	3.29	3.10	2.29	2.17
42.81	2.23	2.30	4.04	5.91	4.80	3.77	3.77	3.27	3.57	3.38	2.56	2.43
48.92	2.37	2.52	2.23	6.22	5.03	4.00	4.00	3.52	3.76	3.67	2.82	2.67
55.04	2.58	2.28	2.44	6.46	5.28	4.23	4.23	3.73	4.00	3.93	3.04	2.90
61.15	2.80	2.53	2.67	6.76	5.48	4.43	4.43	3.93	4.22	4.20	3.27	3.13
67.27	2.95	2.66	2.78	7.01	5.73	4.61	4.61	4.12	4.43	4.47	3.49	3.32
73.38	3.15	2.85	2.99	7.25	5.96	4.83	4.83	4.28	4.66	4.74	3.71	3.54
79.50	3.34	3.03	3.21		6.15	5.03	5.03	4.49	4.84	4.99	3.93	3.75
85.61	3.52	3.19	3.32		6.35	5.23	5.23	4.70	5.05	5.25	4.15	3.95
91.73	3.69	3.35	3.56		6.60	5.44	5.44	4.88	5.25	5.50	4.37	4.16
97.84	3.79	3.54	3.70		6.80	5.60	5.60	5.06	5.42	5.72	4.57	4.36
103.96	3.99	3.73	3.84		7.05	5.80	5.80	5.25	5.62	5.96	4.78	4.56
110.07	4.12	3.87	3.93		7.20	5.99	5.99	5.44	5.81	6.16	4.97	4.75
116.19	4.37	4.08	4.11			6.16	6.16	5.62	6.00	6.38	5.16	4.94
122.30	4.57	4.26	4.34			6.36	6.36	5.79	6.21	6.60	5.36	5.14
128.42	4.77	4.45	4.48			6.54	6.54	5.96	6.40	6.81	5.56	5.33
134.53	4.91	4.61	4.69			6.71	6.71	6.15	6.58	7.04	5.77	5.53
140.65	5.15	4.73	4.83			6.90	6.90	6.31	6.76	7.23	5.95	5.72

	Time = 0h	Time = 3h	Time = 6h	Time = 9h	Time = 12h	Time = 15h	Time = 18h	Time = 21h	Time = 24h	Time = 30h	Time = 36h	Time = 48h
Shear Rate (s^{-1})	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**	**Sh Stress**
146.76	5.31	4.95	5.01			7.07	7.07	6.47	6.95		6.15	5.91
152.88	5.46	5.12	5.15			7.27	7.27	6.63	7.14		6.34	6.10
158.99	5.67	5.28	5.33					6.81	7.31		6.53	6.29
165.11	5.84	5.47	5.52					6.98			6.72	6.49
171.22	6.02	5.63	5.72					7.15			6.93	6.68
177.34	6.21	5.81	5.92					7.32				6.85
183.45	6.41	5.91	6.02									7.04
189.57	6.62	6.09	6.19									7.23

Figure 1. Rheograms of hydrolyzed sludge during fermentation and formulation: a) shear stress profile and; b) viscosity profile contd…

	0h	3h	6h	9h	12h	15h	21h	24h	30h	36h	48h
Shear Rate	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)	Viscosity (mPa.s)
0.37	345.93	269.94	293.94	449.90	403.91	283.94	173.96	193.96	59.99	39.99	27.99
0.73	253.95	119.97	205.96	167.96	219.95	216.95	108.98	110.98	39.99	31.99	25.99
1.83	155.57	86.78	110.78	95.98	104.78	88.38	51.99	51.99	26.39	23.20	19.20
3.67	104.58	54.39	70.19	52.19	65.99	51.79	33.79	32.59	18.60	16.20	14.80
7.34	40.49	32.09	33.49	24.79	44.39	34.69	21.80	21.60	14.00	11.70	10.90
11.01	16.53	18.26	18.06	15.06	35.59	26.59	16.93	17.40	12.06	9.80	9.33
14.68	16.15	16.35	12.75	11.85	28.74	21.95	14.30	14.80	12.50	8.65	8.30
18.35	10.28	7.96	8.84	9.44	24.67	18.92	12.32	13.00	11.44	7.96	7.56
24.46	6.96	7.05	7.53	7.95	20.46	15.75	10.53	11.13	10.08	7.14	6.81
30.58	6.12	6.09	5.85	6.93	17.61	13.70	9.26	9.98	9.17	6.62	6.26
36.69	5.84	5.78	5.14	6.30	15.52	12.26	8.34	8.98	8.44	6.24	5.92
42.81	5.21	5.36	9.43	5.91	13.80	11.21	7.64	8.35	7.90	5.98	5.67
48.92	4.84	5.14	4.56	5.43	12.72	10.29	7.20	7.69	7.50	5.76	5.46
55.04	4.69	4.15	4.44	5.17	11.73	9.60	6.77	7.27	7.15	5.53	5.27
61.15	4.57	4.14	4.37	4.92	11.05	8.96	6.43	6.90	6.86	5.35	5.11
67.27	4.38	3.95	4.13	4.71	10.42	8.52	6.12	6.59	6.64	5.19	4.94
73.38	4.30	3.88	4.07	4.53	9.88	8.12	5.84	6.35	6.46	5.06	4.82
79.50	4.20	3.81	4.04	4.37		7.73	5.65	6.09	6.28	4.95	4.72
85.61	4.11	3.73	3.87	4.28		7.42	5.48	5.90	6.14	4.85	4.62
91.73	4.02	3.65	3.88	4.18		7.19	5.32	5.72	6.00	4.77	4.54
97.84	3.88	3.61	3.79	4.08		6.95	5.17	5.54	5.84	4.67	4.45
103.96	3.84	3.59	3.69	4.04		6.78	5.05	5.41	5.73	4.59	4.38
110.07	3.75	3.51	3.57	4.02		6.55	4.95	5.28	5.60	4.51	4.31
116.19	3.76	3.51	3.54	3.98			4.84	5.17	5.49	4.45	4.26
122.30	3.74	3.49	3.55	3.93			4.73	5.07	5.40	4.39	4.21
128.42	3.71	3.47	3.49	3.88			4.64	4.98	5.30	4.33	4.15
134.53	3.65	3.42	3.48	3.86			4.57	4.89	5.23	4.29	4.11

	0h	3h	6h	9h	12h	15h	21h	24h	30h	36h	48h
Shear Rate	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**	**Viscosity (mPa.s)**
140.65	3.66	3.36	3.44	3.84			4.49	4.81	5.14	4.23	4.07
146.76	3.62	3.37	3.41	3.78			4.41	4.73		4.19	4.03
152.88	3.57	3.35	3.37	3.75			4.34	4.67		4.15	3.99
158.99	3.57	3.32	3.35	3.71			4.28	4.60		4.11	3.96
165.11	3.54	3.31	3.34	3.68			4.23			4.07	3.93
171.22	3.51	3.29	3.34	3.64			4.18			4.05	3.90
177.34	3.50	3.28	3.34	3.63			4.13			4.01	3.86
183.45	3.50	3.22	3.28	3.60						3.98	3.84
189.57	3.49	3.21	3.27	3.56							3.81
195.68	3.46	3.20	3.23	3.55							
201.80	3.43	3.18	3.23	3.54							

Figure 2. Power law behaviour of hydrolyzed sludge and its correlation with Bt growth (15 L fermenter)

Time, h	D.O. (%)
0.03	97.95
3.00	81.00
6.01	48.08
9.01	67.97
12.01	65.42
15.02	76.12
18.02	81.55
21.02	87.12
24.03	78.90
27.03	87.75
30.00	89.92
33.00	92.77
36.01	94.15
39.01	94.35
42.01	95.02
45.02	96.90
47.89	96.70

	TC		VS			
Time (h)	(CFU/ml)	S.E.	(CFU/ml)	S.E.	CI (mPa.s^n)	FI
0	2.43E+06	2.43E+05	9.00E+02	9.00E+01	150.6	0.22
3	3.50E+06	2.84E+05	1.87E+03	1.51E+02	100	0.29
6	7.27E+06	5.38E+05	3.67E+04	2.71E+03	123.2	0.25
9	1.43E+07	9.32E+05	8.00E+04	5.20E+03	114.4	0.29
12	2.60E+07	2.60E+06	1.00E+06	1.00E+05	177.7	0.32
15	5.53E+07	5.53E+06	1.50E+06	1.50E+05	140.1	0.33
18	1.30E+08	1.04E+07	1.20E+07	9.60E+05	105.2	0.36
21	7.17E+08	6.45E+07	4.60E+07	4.14E+06	76.6	0.41
24	8.90E+08	8.01E+07	5.17E+07	4.65E+06	79.6	0.41
30	1.11E+09	1.00E+08	5.83E+08	5.25E+07	34.6	0.61
36	1.00E+09	9.03E+07	7.83E+08	7.05E+07	26	0.62
48	1.00E+09	9.03E+07	7.63E+08	6.87E+07	21.3	0.66

Figure 3. Stability profiles of TH-30 formulation (FH-4); (a) Rheological profile at different pH; (b) rheological profile at different temperatures; and (c) Biological efficacy (P, particle size)

F4	Viscosity, cP			pH stability studies										
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**	**545**	**730**
4	47	48	49	47	46	47	48	49	47	46	44	43	42	43
4.5	46	46	48	45	48	47	47	48	47	46	47	42	44	43
5	47	46	47	46	47	47	46	47	47	46	47	43	41	40
6	48	47	46	47	44	46	45	31	30	28	25	26	25	25
6.5	47	48	47	47	45	47	48	32	25	24	30	28	27	27

F4	Particle-size, D_{50}			pH stability studies										
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**	**545**	**730**
4	34.6	36.8	37.4	36.8	36.4	37.2	37.4	36.9	36.4	37.1	36.9	32	32	32
4.5	35.1	36.2	34.8	34.6	34.8	35.2	36.4	36.2	34.8	35.7	36.2	35	37	36
5	36.2	36.6	34.8	36.4	36.1	35.6	36.2	35.9	34.9	36.3	34.8	33	32	32
6	37.4	35.9	35.2	36.2	36.1	36.1	35.4	34.8	26.2	24.8	25.8	32	30	30
6.5	36.8	37.1	34.3	35.6	35.4	35.5	36.1	33.9	26.3	26.1	25.7	35	33	32

F4	Viscosity, cP			Temperature stability studies										
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**	**545**	**730**
4	44	43	42	40	40	40	38	40	41	42	42	41	42	43
10	45	43	43	44	45	46	46	45	46	45	45	42	44	43
30	46	43	43	45	46	45	45	43	44	48	45	43	41	41
40	47	46	47	48	46	39	36	34	31	30	27	26	25	25
50	44	45	43	42	42	40	39	38	37	35	30	27	26	26

Figure 3. Stability profiles of TH-30 formulation (FH-4); (a) Rheological profile at different pH; (b) rheological profile at different temperatures; and (c) Biological efficacy (P, particle size) contd...

F4	Particle-size, D_{50}			Temperature stability studies										
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**	**545**	**730**
4	31.7	30.6	30.2	30.4	30.1	31.3	31.5	30.7	30.4	30.6	30.8	30	32	31
10	30.7	30.2	30.1	30.4	31.2	31.4	31.8	30.8	30.2	30.6	31.4	28.5	31	30
30	30.8	30.4	31.2	31.2	31.7	31.4	31.6	30.9	30.8	30.7	29.5	27.5	29	28
40	31.6	32.4	31.7	31.5	31.4	31.2	30.6	30.4	30.7	30.2	28.9	26.6	25	26
50	31.4	31.5	31.5	31.6	30.9	30.7	30.5	30	29.7	28.2	26.5	24.8	24	23

F4	Entomotoxicity, SBU/µL	pH stability studies												
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**	**545**	**730**
4	19096	19062	18098	18076	18066	18072	18066	18076	18064	18056	18058	17898	18000	17900
5	20002	19062	19026	19042	18072	18062	18092	18096	17534	17082	17092	16556	17345	16788
7	19086	19048	19018	19036	18068	18064	18036	18082	17212	17068	17089	16989	16455	16234

F4	Entomotoxicity, SBU/µL	Temperature stability studies												
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**	**545**	**730**
4	20000	20300	19600	19800	18076	18022	18100	18400	18036	18072	18042	17899	17456	17000
30	20300	20100	19400	19300	19400	19200	18600	18700	18600	18546	18846	18344	18210	18000
50	20260	20200	20100	19400	19500	19600	19800	19356	18756	18248	18064	17348	17301	17220

Annexe - XV

Optimization of Different Liquid Formulations of Various Fermented Broths

Table 1. Stability studies of various NH formulations at different pH and temperatures

a) **Viscosity**

F1	Viscosity, cP					pH stability studies						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	60	60	60	60	61	61	58	60	60	60	61	45
4.5	61	59	59	59	56	55	59	60	59	58	59	34
5	56	54	53	57	54	52	54	57	57	56	57	22
6	58	57	58	56	55	54	57	58	58	57	55	29
6.5	59	56	59	58	57	58	59	59	59	59	56	30
F2	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	108	109	108	107	107	108	105	106	107	106	104	132
4.5	107	108	110	108	108	109	108	107	108	105	104	100
5	106	109	106	109	109	107	109	106	107	104	106	94
6	109	96	98	97	99	96	97	98	95	94	92	86
6.5	101	99	95	96	96	96	96	96	95	94	90	77
F3	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	100	99	97	98	99	97	95	94	93	92	94	86
4.5	95	98	97	94	94	92	96	97	98	94	91	76
5	96	99	98	95	95	94	93	99	96	95	97	68
6	97	97	96	97	97	97	95	100	87	84	80	59
6.5	98	96	95	94	98	98	94	98	80	82	75	44
F4	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	86	85	87	88	88	86	87	85	85	83	85	79
4.5	85	81	88	86	88	87	86	88	89	88	89	80
5	82	88	87	87	85	87	88	89	85	87	88	77
6	84	89	86	88	88	86	87	74	76	77	76	72
6.5	83	85	85	89	89	84	80	77	76	75	71	65
F5	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	81	82	81	80	80	82	80	80	80	77	80	74
4.5	80	83	83	82	85	83	81	82	81	79	79	72
5	83	83	86	83	84	82	81	81	80	81	81	63
6	82	85	84	85	82	81	81	80	80	82	82	61
6.5	81	84	85	84	81	85	74	81	80	81	81	66

Table 1. Stability studies of various NH formulations at different pH and temperatures contd...

39) **Viscosity**

F1		Viscosity, cP		Temperature stability studies								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	60	60	60	60	58	57	56	57	58	57	58	44
10	61	60	58	58	57	56	57	56	56	54	52	35
30	59	59	59	59	56	54	52	54	52	51	52	33
40	58	58	57	56	44	42	40	38	36	34	32	28
50	58	57	56	54	44	42	41	39	37	36	33	28
F2		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	107	106	107	106	104	103	102	101	100	99	98	80
10	106	105	106	107	103	101	101	100	101	96	97	87
30	104	104	105	104	103	100	100	100	101	101	100	90
40	105	103	104	102	101	100	100	100	101	101	100	92
50	101	104	101	100	99	98	84	82	80	76	70	49
F3		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	106	107	106	104	103	99	98	99	100	99	100	93
10	104	104	104	101	100	99	98	98	99	100	99	84
30	105	104	102	100	96	97	98	99	100	101	100	82
40	107	106	101	100	99	98	81	76	75	70	65	58
50	105	105	103	102	100	100	80	74	71	69	62	53
F4		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	104	103	102	101	100	99	98	99	98	97	96	83
10	105	103	101	100	102	100	99	98	97	95	94	92
30	106	102	100	101	101	100	98	96	97	96	95	90
40	107	106	105	104	100	99	81	75	71	75	66	55
50	108	107	106	103	101	98	82	74	72	73	69	52
F5		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	107	108	109	107	106	107	109	108	104	103	101	92
10	106	106	107	106	105	107	106	103	102	101	100	92
30	107	104	102	100	98	105	102	101	100	101	100	90
40	108	107	107	85	80	82	84	70	74	71	70	55
50	106	105	105	106	86	84	81	85	82	76	70	49

b) Particle size

F1	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	52	50	50.6	46.9	48.7	48.6	50.6	50.1	53.6	51.6	51.3	43.2
4.5	52	51	50.4	50.6	49.6	49.1	49.7	50.6	52.1	50.4	50.6	36.7
5	48.6	52	53	53.4	52.4	52.6	50.6	52.1	51.6	51.8	50.6	37.6
6	47.6	45.4	48.4	49	48.1	49.2	49.6	49.6	49.1	49.2	49.1	35.4
6.5	48.6	50.4	50.3	50.2	50.1	50.6	51.2	50.6	50.1	50.1	50.1	33.2
F2	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	47.6	46.3	46.4	47.3	46.9	46.9	47.1	48.1	47.4	48.1	46.8	40.4
4.5	48.1	47.1	45.6	46.3	45.8	46.4	49.1	49.2	48.1	50.6	47.6	42.2
5	46.9	46.3	47.8	48.3	48.1	47.6	48.6	49.3	49.1	51.2	48.4	40.8
6	45.3	45.2	45.4	46.1	44.1	45.6	48.6	49.1	50.1	53.6	47.4	42.4
6.5	45.3	45.3	46.3	47.4	46.1	45.4	49.6	54.6	56.1	54.5	44.5	40.4
F3	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	45.4	46.7	48.1	47.6	47.4	48.1	44.1	48.1	44.5	45.1	45.3	41.4
4.5	44.1	46.4	46.1	45.2	46.4	48.1	47.5	46.8	48.2	48.1	44.6	40.8
5	45.6	46.7	47.4	46.1	46.5	47.6	46.9	46.7	47.6	46.8	47.6	40.4
6	45.3	46.9	46.9	45.9	47.6	48.2	46.8	55.6	57	58.3	56.4	40.5
6.5	46.2	47.8	47.3	46.7	48.1	48.3	45.8	56.8	54.8	56.8	57.1	40.3
F4	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	47.1	46.9	47.4	48.1	47.6	46.9	47.8	48.2	48.1	49.1	46.4	39.9
4.5	48.2	46.6	46.5	47.8	46.8	47.2	48.3	47.8	46.1	47.4	48.1	37.9
5	48.2	47.1	46.3	46.8	47.1	48.1	47.9	48.1	45.9	48.1	47.6	39.2
6	47.5	46.1	47.2	46.4	46.1	48.2	46.9	47.6	50	52	56	38.9
6.5	48.3	48	46.2	45.9	47.2	48.3	48.3	48.1	55	54	58.1	39.4
F5	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	46	44	47	46	46	48	46.9	47.4	48	47.6	46.8	42.9
4.5	45	48	45	45	44	47.4	44.5	47.6	48.2	44.6	45.4	40.6
5	46.4	46.3	48.2	41.3	45	46.1	47.6	46.9	48.1	45.6	46.1	43.3
6	46.6	47.1	46.9	40.9	44	46.9	44.5	44.9	48	48.1	47.2	44.3
6.5	47.5	44.7	47.3	46.9	48.1	45.6	46.4	48.1	47.6	48.9	43.5	39.8

c) Particle size contd...

F1	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	51	56.2	56.2	56.1	54.2	55.7	55.6	52.3	53.6	57.4	50.7	45.7
10	54	56.8	57.2	56.1	53.6	50.7	50.6	50.7	51.4	50.7	49.8	45.5
30	55.6	54.5	57.6	57.2	54.2	50.3	50.4	51.4	51.6	50.8	50.6	45.3
40	54.8	56.2	54.8	56.3	55.6	51.4	49.2	48.1	46.4	42.4	40.9	38.9
50	56.1	55.7	56.8	53.7	54.8	57.2	49.5	49.6	47.4	44.6	41.6	36.7
F2	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	46.4	44.5	45.6	46.3	46.3	47.2	48.3	47.6	47.6	47.1	47.2	46.8
10	45.2	45.7	45.2	45.4	46.3	47.6	46.3	46.8	46.4	46.3	46.4	42.4
30	44.9	46.2	44.1	45.2	44.8	45.6	45.2	46.1	45.2	43.2	44.8	39.5
40	45.6	47.2	46.1	44.7	45.6	46.2	46.7	45.8	47.8	37.8	36.7	33.6
50	46.8	47.3	46.2	45.7	46.2	47.3	44.1	40.4	35.1	30.2	28.9	27.4
F3	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	47.8	47.1	46.2	46.1	46.2	47.6	47.4	47.2	47.4	47.3	46.2	45.7
10	46.2	45.8	44.7	43.6	44.7	46.9	47.4	46.1	40.1	46.1	46.9	44.3
30	45.4	44.7	45.1	45.3	47.8	47.4	46.9	47.3	45.4	47.6	45.1	42.6
40	45.3	45.1	44.6	43.1	48.2	47.6	42.3	42.4	37.6	37.2	34.1	34.5
50	44.9	45.2	44.7	43.2	45.7	42.1	38.4	36.3	34.6	29.1	27.8	29.5
F4	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	48.1	47.6	47.4	48.2	47.6	47.2	46.4	45.4	45.8	46.2	46.3	44.6
10	46.4	48.1	48.2	47.1	47.4	48.2	47.4	48.1	47.2	47.1	46.2	46.5
30	47.2	46.4	47.2	47.6	46.4	46.8	47.4	48.4	47.2	46.9	47.2	45.7
40	48.2	46.7	46.9	46.8	48.1	39	37.4	36.2	34.3	30.1	29.8	27.8
50	47.4	47.4	48.2	49.1	49.2	36	35	32.6	30.4	30.2	36.4	39.3
F5	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	46.2	46.3	47.1	48.2	48.1	46.1	45.8	45.1	44.6	44.9	43.7	45.6
10	45.8	48.1	48.1	47.6	47.4	46.4	46.1	46.2	46.4	46.1	44.8	41.4
30	47.2	47.6	46.9	47.3	47.4	47.2	45.8	47.3	46.3	45.8	45.3	42.6
40	46.9	46.2	47.2	45.6	45.8	45.6	42.1	36.9	32.6	33.7	34.6	43.5
50	44.1	46.3	47.3	46.2	45.9	45.7	42.8	35.8	33.7	34.8	35.2	46.7

d) VS concentration

F1	Spores, x 10E+07 CFU/ml						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	10	6	6.2	8.2	8.4	8.2	7.8	7.6	7.5	7.2	7.1	5
4.5	10	6.8	6.6	8.3	8.2	8.1	7.6	7.4	7.2	7.6	8	4.5
5	11	9.7	6.8	8.6	8.2	7.8	8.6	7.2	7.06	7.9	7.5	4.7
6	10	9.8	7.2	7.1	7	6.8	7	6	6.9	6.5	6.3	3.6
6.5	10	8.9	7.6	7.4	6.8	6.4	7.1	7	6.2	6.2	6	3.3
F2	**Spores, x 10E+07 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	10	6	6.2	8.2	8.4	8.2	7.8	7.6	7.5	7.2	7.1	5
4.5	10	6.8	6.6	8.3	8.2	8.1	7.6	7.4	7.2	7.6	8	4.5
5	11	9.7	6.8	8.6	8.2	7.8	8.6	7.2	7.06	7.9	7.5	4.7
6	10	9.8	7.2	7.1	7	6.8	7	6	6.9	6.5	6.3	3.6
6.5	10	8.9	7.6	7.4	6.8	6.4	7.1	7	6.2	6.2	6	3.3
F3	**Spores, x 10E+07 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19.8	19.4	19.1	19	19	18.6	18.9	18.7	18.8	18.8	18.4	18
4.5	19.9	19.6	19.6	19.4	19.4	19.5	19.1	18.8	17.5	17.6	17.6	17.2
5	20	20	19.4	19.8	19.7	19.2	18.8	18.6	17.6	18	18	17.4
6	21.5	20	19.6	17.7	17.7	19.4	18.5	18.4	18.6	17.6	18	17.3
6.5	20	21	20	19.6	19.8	19.4	18.1	17.9	18.3	17.7	17.4	15.7
F4	**Spores, x 10E+07 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	22	19.8	19.6	19.7	19.4	19.3	19.1	19	18.7	18.4	18.2	17.5
4.5	21	20	19.8	19.5	19.1	18.9	18.7	18.6	18.9	18.7	18.8	17.6
5	23	20	19.7	19.6	19.7	19.8	19.4	18.7	18.9	18.7	18.5	14.7
6	20	20	19.4	19.2	19.8	19.6	19.8	19.2	18.7	18.8	18.6	13.7
6.5	21	21	20	19.8	19.5	19.4	19.7	19.2	19.1	18.5	18	13.5
F5	**Spores, x 10E+07 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	22	20	19.8	19.7	19.6	19.6	19.4	19.2	19.2	19.1	19	18.4
4.5	24	21	19.6	19.6	19.6	19.5	19.4	19.4	19.2	19.1	19	18.3
5	21	21	19.7	20	19.8	19.5	19.6	19.4	19	18.8	18.9	18
6	20	20	19.9	20	19.9	19.7	19.6	19.2	19.1	18.7	18.6	18
6.5	24	22	19.8	20	19.9	19.8	19.8	19.6	19	18.7	18.4	18

c) VS concentration contd...

F1	Spores, x 10E+07 CFU/ml					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	11	9.96	9.96	9.84	9.76	9.82	9.84	9.87	9.89	9.96	9.94	8.7
10	10	9.84	10.07	10.06	10	10	9.6	9.9	9.9	9.86	9.92	8.2
30	11	9.76	11	11	9.87	9.86	8.6	8.2	8	7.6	7.4	5.6
50	12	9.84	11	10	9.92	9.89	8.4	7.5	7.2	7	6.94	6.3
F2	**Spores, x 10E+07 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	23	20	21	20	20	19	19	19.9	19.6	18.6	18.9	16.9
10	21	20	24	23	22	18	18.2	18.1	18.3	19.2	19.3	17.3
30	20	20	23	22	21	17	18.4	18.1	18.3	18.4	18.6	17.4
50	24	23	25	24	23	19	17.6	17	17.2	16.8	17.6	17.2
F3	**Spores, x 10E+07 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	24	23	20	21	19.8	19.9	19.5	19.6	19.7	19.8	19.7	18.4
10	25	21	21	20	19.6	19.7	19.8	19.7	19.6	19.4	19.8	18.8
30	23	22	20	20	19.8	19.8	19.7	19.2	19	18.9	18.1	17.6
50	22	21	21	20	19.9	19.8	19.7	19	18.6	18.2	18	17.5
F4	**Spores, x 10E+07 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	23	22	21	19.6	19.7	19.8	19.9	19.8	19.7	19.8	19.9	18.5
10	24	23	22	19.7	19.8	19.7	19.6	19.5	19.4	19.3	18.7	18.1
30	23	22	21	19.6	19.6	19.9	18.2	17.6	17.4	17.3	16.9	15.6
50	22	21	20	19.8	19.7	19.7	18.6	18.2	17.9	17.6	17	16.4
F5	**Spores, x 10E+07 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	24	23	19.8	19.9	19.6	19.8	19.7	19.8	19.9	19.8	19.9	18.8
10	22	21	19.6	20	19.8	19.9	19.6	19.4	19.5	19.3	19.2	18.4
30	22	23	19.6	19.2	19.3	18.8	18.2	17.9	17.6	17.1	16.6	16.4
50	21	20	19.5	19.4	19.2	18.9	18.6	17.8	17.1	16.9	16.2	15.9

d) Entomotoxicity

F1	Entomotoxicity, SBU/µL						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	10000	10000	9000	9000	9000	9000	9000	9000	9000	9000	9080	6658
5	10012	9069	9065	9001	9023	9041	9021	8143	8123	8096	9021	5687
6.5	10001	9049	9052	9056	10003	9012	9004	9001	8069	8099	8045	4786
F2	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	14000	14030	14021	14001	13049	13061	13042	13061	13091	13049	13061	12678
5	13069	13076	13091	13069	13089	13069	13062	13069	13065	13092	13096	12873
6.5	13049	13051	13061	13046	13091	13061	13049	13081	13086	13090	13094	12000
F3	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15000	14070	14060	13090	13070	13069	13089	13061	13012	13069	13071	12987
5	14090	14080	14070	14080	13080	14010	14001	13071	13069	14002	13049	12076
6.5	14060	14060	14050	13070	14032	14024	13086	13069	14003	13089	14001	12064
F4	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	14012	14031	13086	12092	13016	13012	13001	13000	13092	13016	13004	12983
5	14032	14010	13062	13059	13091	13092	13014	13001	13002	13000	12096	12045
6.5	13096	13089	13016	14000	13016	13021	13061	13024	13023	13014	12094	11879
F5	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	14036	14016	14037	14001	13091	12061	12041	12089	12062	12076	12082	12045
5	14001	14001	14041	14006	13089	13061	14021	13082	13061	13001	13021	12798
6.5	13091	13097	14052	14023	13067	13022	14032	13047	13041	14023	14071	13267

d) Entomotoxicity contd...

F1		Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day		**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
	4	10001	9982	9894	9890	9794	9746	9792	9891	9912	9806	9807	7896
	30	10003	9891	9806	9816	9804	9816	9800	9706	8482	8721	8623	6323
	50	9896	9891	9807	8923	8921	8947	8946	8841	8838	8804	8416	4423
F2		**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day		**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
	4	14000	13098	13076	13082	13074	13082	13092	13091	13099	13096	13094	11564
	30	13099	14001	14000	14002	13080	13030	13050	13000	13040	12090	12070	11465
	50	14004	14001	14000	14003	13090	13040	12080	12040	11080	11060	11010	10789
F3		**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day		**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
	4	13080	14001	14060	14050	14020	14010	13090	13080	13070	13040	13011	11398
	30	14000	13090	13090	14000	14001	13090	13040	12080	12040	11080	11060	10257
	50	14060	13090	13090	14010	14022	13080	13060	12090	12060	11060	11000	10045
F4		**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day		**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
	4	14030	14020	14000	13090	13080	13089	13096	13097	13096	13097	13098	12054
	30	14060	14050	13070	13060	13050	13060	12010	11080	11070	11040	11036	11211
	50	14050	14040	13060	13050	13082	13076	13072	12050	12010	11090	11070	10897
F5		**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day		**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
	4	14100	14000	13096	13098	13097	13096	13094	13024	13048	13052	13049	12087
	30	14040	13098	13097	13096	13081	13090	12090	12080	12060	11080	11040	11012
	50	14020	13099	13096	13097	13080	13020	12060	12040	12070	11080	11020	10876

Table 2. Stability studies of various TH formulations at different pH and temperatures

a) Viscosity

F1	Viscosity, cP					pH stability studies						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18	19	18	18	17	19	18	16	18	19	18	14.3
4.5	16	17	14	15	16	17	18	17	18	17	16	14.5
5	17	16	19	16	17	19	19	18	19	18	18	15
6	10	19	14	17	18	18	16	17	20	18	17	14
6.5	13	18	16	17	16	17	18	19	17	16	17	12.9
F2	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	47	46	48	49	47	46	47	46	48	46	49	46
4.5	48	49	47	48	47	48	46	48	49	48	49	43
5	47	48	47	48	47	50	49	47	47	48	50	43
6	46	46	47	45	48	47	49	48	48	47	50	45
6.5	47	48	43	48	49	48	49	49	48	48	49	46
F3	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	46	46	48	47	46	47	48	47	46	47	46	44
4.5	47	48	47	48	49	47	47	48	46	48	47	42
5	44	47	45	44	47	46	46	47	44	47	48	43
6	46	46	47	45	48	47	49	48	36	34	35	31
6.5	48	47	48	47	47	48	48	49	38	34	35	30
F4	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	47	48	49	47	46	47	48	49	47	46	44	43
4.5	46	46	48	45	48	47	47	48	47	46	47	42
5	47	46	47	46	47	47	46	47	47	46	47	43
6	48	47	46	47	44	46	45	31	30	28	25	26
6.5	47	48	47	47	45	47	48	60	25	24	30	28
F5	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	48	47	46	47	48	47	46	47	48	47	46	44
4.5	46	46	47	49	46	48	47	46	46	47	48	45
5	45	48	45	48	49	49	49	48	47	46	49	45
6	47	47	46	47	47	47	48	47	47	48	47	46
6.5	48	44	45	46	47	48	48	47	46	45	44	43

a) Viscosity contd...

F1		Viscosity, cP		Temperature stability studies								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18	17	18	16	17	16.9	16.8	16.6	16.4	16.4	16.1	14.3
10	17	16	18	16	16.1	16.3	16.1	15.4	15.3	15.4	15.2	12.4
30	16	18	18	16.4	17.8	17	17	16.4	16	15	15	13
40	17	16	18	16	16	16	16	14	13	13	11	10
50	18	15	17	16	16	15	15	13	13	13	10.5	10
F2		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	47	48	46	46	46	46	48	46	46	46	45.6	43
10	46	47	46	47	47	46	46	45	45	45	45	42
30	47	48	46	48	47	47	45	48	47	46	45	37
40	48	47	46	47	47	46	45	35	30	29	28	21
50	47	46	46	47	47	47	45	35	31	32	29	24
F3		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	46	46	47	46	46	47	47	48	47	46	46	42
10	47	47	48	46	47	46	46	47	46	47	47	43
30	48	47	47	46	46	47	47	48	48	48	47	44
40	46	46	47	46	47	35	36	37	34	31	29	26
50	47	47	46	48	46	34	32	30	29	28	26	23
F4		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	44	43	42	40	40	40	38	40	41	42	42	41
10	45	43	43	44	45	46	46	45	46	45	45	42
30	46	43	43	45	46	45	45	43	44	48	45	43
40	47	46	47	48	46	39	36	34	31	30	27	26
50	44	45	43	42	42	40	39	38	37	35	30	27
F5		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	41	42	40	43	45	44	45	46	41	40	42	40
10	40	40	41	42	40	41	40	41	39	37	34	33
30	40	39	40	42	41	40	41	39	36	37	32	30
40	40	37	38	37	34	32	29	26	22	21	20	18
50	41	38	34	30	28	27	24	21	19	17	14	10

b) Particle size

F1	Particle-size, D_{50} (µm)					pH stability studies						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	36.1	36.4	36.1	37.4	36.4	35.4	35.8	35.4	35.1	35.6	36.1	32
4.5	35.8	36.1	35.9	36.9	37.1	36.4	34.8	36.1	36.1	36.1	36.3	33.5
5	36.9	36.2	36.1	36.4	36.9	35.6	35.6	35.6	34.8	35.1	34.6	33.2
6	36.4	36.4	36.5	36.2	37.6	36.4	35.4	29.6	30.1	30.1	28.9	27.5
6.5	35.8	37.4	36.4	37.3	36.3	36.3	37.4	36.1	28.4	30.2	30.5	27.4
F2	**Particle-size, D_{50} (µm)**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34.6	36.8	36.1	36.4	38.1	37.6	38.1	36.1	34.5	36.1	37.5	35.6
4.5	38.1	37.6	36.3	36.1	36.9	36.9	37.4	36.8	37.3	36.5	36.7	34.9
5	37.6	36.2	36.2	37.1	37.4	37.4	36.9	35.1	36.2	36.8	34.6	33.4
6	36.1	36.3	34.6	37.4	36.9	36.4	36.4	36.4	34.9	34.6	36.7	35.4
6.5	34.1	34.6	35.4	36.2	37.4	36.4	37.5	35.2	36	36.7	36.9	33.7
F3	**Particle-size, D_{50} (µm)**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34.5	36.1	36.9	36.8	36.4	37.1	36.9	36.4	36.4	37.2	36.4	34.6
4.5	36.2	36.9	37.1	38.1	37.3	36.4	37.4	36.8	36.2	36.9	36.1	33.1
5	37.4	37.1	36.1	37.4	38	38.1	38.4	36.4	37.5	35.6	38.1	32
6	38.4	37.1	35.4	35.1	34.9	36.1	37.1	36.9	37.2	36.9	38.4	31
6.5	36.9	37.1	35.4	35.1	34.9	36.1	37.4	36.9	37.2	36.9	34.8	30.4
F4	**Particle-size, D_{50} (µm)**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34.6	36.8	37.4	36.8	36.4	37.2	37.4	36.9	36.4	37.1	36.9	32
4.5	35.1	36.2	34.8	34.6	34.8	35.2	36.4	36.2	34.8	35.7	36.2	35
5	36.2	36.6	34.8	36.4	36.1	35.6	36.2	35.9	34.9	36.3	34.8	33
6	37.4	35.9	35.2	36.2	36.1	36.1	35.4	34.8	26.2	24.8	25.8	32
6.5	36.8	37.1	34.3	35.6	35.4	35.5	36.1	33.9	26.3	26.1	25.7	35
F5	**Particle-size, D_{50} (µm)**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	35.3	36.2	34.6	35.4	35.8	36.1	36.2	35.8	35.6	36.2	36.2	37
4.5	35.4	36.1	36.2	36.1	35.1	34.6	35.8	34.6	35.1	35.3	36.1	34.5
5	36.1	35.6	34.8	36.2	34.8	36.1	36.1	34.9	35.4	32.1	37.1	36.4
6	36.1	35.2	35.4	34.8	35.3	37.1	35.1	34.7	36.8	34.1	35.2	34
6.5	36.2	34.6	36.1	36.2	36.2	38.2	34.8	36.2	34.9	35.3	33.9	32.4

b) Particle size contd...

F1	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	36.1	34.2	34.5	34.8	36.1	36.3	36.5	36.1	35.8	34.9	34.7	36.1
10	34.1	34.1	36.3	37.8	38.1	26.7	34.2	36.3	36.7	39.2	38.4	34.1
30	35.6	36.1	36.4	37.3	37.2	36.4	37.3	38.1	38.2	39.1	38.4	35.6
40	36.2	36.3	34.1	28.6	24.1	24.1	24.1	24.3	24.6	24.4	24.5	36.2
50	37.2	36.4	30.8	30.4	31	31.2	30.6	30.2	26.4	22.3	20.2	37.2
F2	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	31.6	30.4	28.6	26.2	26.3	24.6	24.3	24.1	24	21.7	20.8	19.6
10	31.7	31.4	31.7	30.6	30.8	30.7	30.6	30.4	31.2	31.4	31.6	30.4
30	31.3	31.2	30.7	31.2	26.2	28.2	28.4	27.7	27.3	30.4	31.2	33.3
40	31.3	31.2	30.7	31.2	26.2	24.7	21.2	21	21	20.6	20.4	19.4
50	31.7	28.9	29.1	30.4	31.2	29.8	29.7	29.3	29.1	22.3	21.6	17.5
F3	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	31.2	30.7	30.6	30.7	30.8	30.9	30.4	31.2	31.7	30.6	30.9	28.9
10	31.2	31.6	31.6	31.4	31.7	31.5	31.3	31.5	31.7	31.9	30.6	28.9
30	30.7	30.8	30.3	31.7	30.8	30.7	30.7	30.6	30.8	32.3	30.5	27.5
40	31.4	31.6	31.1	31.7	31.5	31.1	31.4	30.7	30.6	30.6	27.4	26.5
50	30.1	30.2	30.7	28.4	29.1	27.1	26.4	26	25.9	25.6	23.7	20.7
F4	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	31.7	30.6	30.2	30.4	30.1	31.3	31.5	30.7	30.4	30.6	30.8	30
10	30.7	30.2	30.1	30.4	31.2	31.4	31.8	30.8	30.2	30.6	31.4	28.5
30	30.8	30.4	31.2	31.2	31.7	31.4	31.6	30.9	30.8	30.7	29.5	27.5
40	31.6	32.4	31.7	31.5	31.4	31.2	30.6	30.4	30.7	30.2	28.9	26.6
50	31.4	31.5	31.5	31.6	30.9	30.7	30.5	30	29.7	28.2	26.5	24.8
F5	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	30.6	31.4	36.2	31.4	31.2	30.7	30.6	30.8	30.4	30.3	30.4	29.7
10	30.1	31.2	31.6	31.7	31.3	30.6	31.2	31.3	31.7	31.6	31.8	28.6
30	30.3	30.2	30.8	30.2	31.4	30.6	31.2	30.7	30.6	31.4	31.3	29.6
40	30.6	30.3	30.4	30.5	30.7	30.4	30.3	30.7	29.6	29.7	28.6	28.5
50	30.2	31.2	31	31.3	31.0	30.7	30.8	30.9	29.6	28.4	27.2	25.7

c) VS concentration

F1	Spores, x 10E+08 CFU/ml						pH stability studies					
pH\Days	0	3	7	10	15	30	60	90	120	150	180	365
4	11	10	10	10	9.9	9.6	9.7	9.7	9.4	9	9	8.7
4.5	12	10	10	9.9	9.8	9.4	9.5	9.3	9.2	9	9.1	8.5
5	10	10	11	9.9	9.7	9.6	9.5	9.4	9.3	9.1	9	8.4
6	10	10	10	9.9	9.6	9.4	9.4	9.3	9.2	8.1	8.4	7.5
6.5	10	10	9.9	9.9	9.5	9.3	9.2	9.1	8.1	7.6	7.2	6.6
F2	Spores, x 10E+08 CFU/ml						pH stability studies					
pH\Days	0	3	7	10	15	30	60	90	120	150	180	365
4	24	21	19.6	19.8	19.9	19.6	19.9	19.4	19.6	19.8	19.4	19
4.5	23	20	19.7	19.8	19.4	19.5	19.7	19.8	19.6	19.5	19.8	17.8
5	21	20	19.8	19.6	19.4	19.8	19.4	19.2	19.1	19	18.6	17.7
6	22	20	19.9	19.4	19.5	19.7	19.6	19	18.2	18.7	18.6	17.8
6.5	24	21	19.6	19.8	19.9	19.6	19.9	19.4	19.6	19.8	19.4	19
F3	Spores, x 10E+08 CFU/ml						pH stability studies					
pH\Days	0	3	7	10	15	30	60	90	120	150	180	365
4	23	20	19.9	19.8	19.6	19.7	19.6	19.7	19.8	19.4	19.6	19.3
4.5	24	21	19.8	19.7	19.6	19.7	19.7	19.8	19.7	19.6	19.4	19.3
5	21	20	19.9	19.5	19.8	19.9	20	19.8	19.6	19.6	19.2	19
6	22	21	19.8	19.7	19.6	19.9	21	20	19.6	19.6	19.4	19.2
6.5	21	20	19.9	19.8	19.7	19.8	20	20	19.9	19.8	19.2	17.3
F4	Spores, x 10E+08 CFU/ml						pH stability studies					
pH\Days	0	3	7	10	15	30	60	90	120	150	180	365
4	23	22	22	21	19.8	19.6	19.4	19.3	19.2	19	19	18.7
4.5	24	23	23	22	19.6	19.4	19.2	19.1	19	19	19	18.5
5	22	21	21	21	21	19.7	19.6	19.7	19.6	19.4	19.5	18.4
6	21	20	20	20	19.8	19.9	19.8	19.7	19.2	19.2	19	18.3
6.5	20	20	21	21	19.9	19.8	19.8	19.7	19.5	19	19.8	17.6
F5	Spores, x 10E+08 CFU/ml						pH stability studies					
pH\Days	0	3	7	10	15	30	60	90	120	150	180	365
4	24	23	19.6	19.7	19.4	19.2	19	19	19	18.9	18.6	18.5
4.5	23	22	19.7	19.6	19.4	19.5	19.4	19.3	19.1	18.9	18.6	18.5
5	21	21	19.8	19.7	19.5	19.4	19.5	19.4	19	18.9	18.6	18
6	20	20	19.8	19.8	19.6	19.5	19.6	19.1	18.9	18.6	18.1	17.7
6.5	20	20	19.9	19.6	19.4	19.3	19.3	19.2	19	18.9	18.6	17.3

c) VS concentration contd...

F1	Spores, x 10E+08 CFU/ml					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	10	10	9.9	9.9	9.9	9.9	9.8	9.9	9.9	9.9	9.9	9
10	9.8	10	9.9	9.97	9.93	9.9	9.8	9.9	9.9	9.8	9.9	8.7
30	9.7	10	9.8	9.7	9.7	9.8	9.8	9.8	9.7	9.8	9.7	8.5
50	9.9	9.9	9.8	9.8	9.8	9.8	9.8	9.7	9.6	9.8	9.4	7.6
F2	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	23	22	19.9	19.8	19.7	19.6	19.8	19.7	19.6	19.5	19.3	18.6
10	20	21	19.9	19.8	19.6	19.2	19.3	19.4	19.6	19.4	19.2	18.8
30	20	20	19.8	19.1	19.2	19.3	19.5	19.6	19.7	19.8	19.6	18.5
50	21	24	19.7	19.4	19.5	19.7	19.6	19.3	19.2	19.4	19.5	18.4
F3	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	23	19.7	19.4	19.3	19.4	19.5	19.7	19.4	19.3	19.6	19.7	18.8
10	22	19.6	19.3	19.2	19.1	19	19	19	19.1	19.3	19.2	18.4
30	21	19.8	19.6	19.8	19.6	19.7	19.4	19.5	19.7	19.6	19.8	18.5
50	22	19.2	18.7	18.5	18.4	18.3	18.2	18.1	18	18	17.6	16.2
F4	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	23	20	20	20	19.8	19.7	19.4	19.3	19.2	19.7	19.6	18.8
10	22	19.7	19.5	19.7	19.8	19.5	19.6	19.4	19.3	19.2	19.4	18.7
30	24	19.6	19.7	19.5	19.3	19.4	19.2	19.5	19.3	19.1	19	18.7
50	23	19.8	19.7	19.9	19.6	19.5	19.7	19.4	19.4	19.6	19.5	19.2
F5	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	23	21	21	20	19.9	19.8	19.7	19.8	19.8	19.9	19.7	18.4
10	24	21	20	20	19.9	20	19.7	19.6	19.5	19.8	19.7	18.2
30	23	22	21	20	19.8	19.7	19.6	19.4	19.3	19.5	19.3	17.8
50	25	22	21	20	20	18.4	18.2	18.1	17.9	17.6	17.8	15.3

d) Entomotoxicity

F1	Entomotoxicity, SBU/µL						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	16000	16043	16041	16001	15001	15023	14099	14069	14089	14071	14042	13434
5	16012	16029	16028	15069	15002	15016	15014	14016	15001	14062	14032	13678
6.5	16004	16032	16001	15004	15042	15000	14032	15022	14066	14024	13098	12778
F2	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19082	20042	20032	19074	19072	19082	19094	19076	19028	19044	19040	18097
5	20001	20000	20032	19098	19048	19062	19044	19052	18042	18022	19052	18000
6.5	20032	20000	20004	19046	19028	19098	19048	19062	17012	17066	17042	16789
F3	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19062	19460	19412	19042	19028	19012	19042	19080	19076	19096	18076	17589
5	19022	19512	19624	19016	19046	18026	18046	18510	18210	18236	18612	17546
6.5	19480	19368	19364	19012	18476	18046	18624	18412	18314	18456	18818	17487
F4	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19096	19062	18098	18076	18066	18072	18066	18076	18064	18056	18058	17898
5	20002	19062	19026	19042	18072	18062	18092	18096	17534	17082	17092	16556
6.5	19086	19048	19018	19036	18068	18064	18036	18082	17212	17068	17089	16989
F5	**Entomotoxicity, SBU/µL**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19076	20046	20038	20042	20016	20000	20000	19012	18016	18042	18036	17574
5	19026	19048	18064	18082	18069	18042	18069	18072	19004	18024	18092	17888
6.5	19004	19028	18026	18048	18096	18064	18072	18068	19008	19026	19018	18023

d) Entomotoxicity contd...

F1	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	14500	14600	14600	14600	14200	13600	13700	13200	13100	12800	12220	11666
30	15100	14300	14300	14000	13700	13500	13100	13000	12900	12700	12600	12347
50	15200	13400	13200	13100	12800	12600	12400	12200	11400	11300	11101	11000
F2	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18600	18410	18340	18230	18100	18600	18300	18200	18300	18400	18500	17955
30	18960	18710	18100	17600	17400	17300	17400	17600	17700	17800	17900	17656
50	18820	18300	18100	16500	16200	16000	16000	16350	15780	15810	15560	14983
F3	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	20100	19400	19300	19400	19100	19600	19200	19100	19060	19000	18700	17885
30	19200	19500	19400	19500	19300	19100	19100	19006	18078	18084	18092	17988
50	19600	19700	19800	19200	19300	19400	19500	19700	18900	18880	18776	18233
F4	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	20000	20300	19600	19800	18076	18022	18100	18400	18036	18072	18042	17899
30	20300	20100	19400	19300	19400	19200	18600	18700	18600	18546	18846	18344
50	20260	20200	20100	19400	19500	19600	19800	19356	18756	18248	18064	17348
F5	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	20000	19600	19800	19700	19666	19486	19526	19836	19788	19468	19382	19000
30	20200	20500	19600	19826	19996	19868	19442	19300	19600	19200	19300	18859
50	19990	19100	19000	19500	19500	19600	19300	19200	19500	19700	19586	18438

Table 3. Stability studies of various SIW formulations at different pH and temperatures

a) Viscosity

S1	Viscosity, cP					pH stability studies						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.3	15.4	14.9	15.4	15.6	15.8	15.7	16.3	15.3	15.1	15	15
4.5	15.2	15.3	15.1	15	15	15.4	15.2	15.3	14.8	15	14.9	14
5	15	15.4	15.3	15	15	15.4	15.1	15.6	14.9	15.2	14.8	13.9
6	15	15	14.8	14.6	15	15.2	15.1	15.3	14.6	15	13	12
6.5	14.9	14.8	15	15	15	15	15.3	15	14.9	15.3	12	10
S2	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.4	16.1	15.4	15	15.2	15.1	15.2	15.3	15.1	15.3	15.1	15
4.5	15	15.6	15.7	14	15.3	15.2	15.3	15.3	15.2	15.2	15.2	14.8
5	15.1	15.3	15.6	15	15.2	15.4	15.3	15.2	15.3	15.3	15.4	15.2
6	15.4	15.4	15.3	15	15.3	15.3	15.3	15.1	15.4	15.1	15.6	12
6.5	15.3	15.3	15.4	14	15.3	15.3	15.3	15.3	15.3	14.9	16.1	9.5
S3	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.3	15.6	15.4	15.5	15.6	15.7	15.4	15.4	15.3	15.4	15.3	15
4.5	15.3	15.3	15.5	15.6	15.4	15.4	15.4	15.4	15.4	15.4	14.9	15.3
5	15.4	15.2	15.6	15.4	15.6	15.3	15.3	15.3	15.3	15.3	14.7	15.4
6	15.2	15.1	15.4	15.7	15.3	15.5	15.4	15.4	15.2	15.1	14.9	15.2
6.5	15.2	15.3	15.5	15.6	15.4	15.3	15.5	15.3	15.3	15	15.2	15.3
S4	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.2	15.3	15.3	15.1	15.3	15.4	15.2	15.3	15.3	15.6	15.3	15
4.5	15.1	15.2	15.3	15.3	15.4	15.3	15.2	15.3	15.2	15.3	15.2	14.9
5	15.2	15.2	15.4	15.4	15.3	15.3	15.3	15.4	15	15.1	15.3	14.7
6	15.2	15.2	15.4	15.3	15.4	15.3	15.4	15.5	14	13.7	12	11
6.5	15.2	15.3	15.2	15.3	15.3	15.2	15.3	15.2	14.2	13.4	12.2	8.6
S5	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.3	15.4	15.3	15.4	15.3	15.4	15.4	15.4	15.3	15.1	15.3	15.2
4.5	15.2	15.3	15.4	15.3	15.4	15.5	15.4	15.4	15.1	15.3	15.2	15.2
5	15.1	15.3	15.3	15.4	15.4	15.4	15.4	15.6	15.4	15.4	15.4	15.2
6	15.3	15.4	15.4	15.4	15.6	15.5	15.6	15.4	15.3	14.8	14.6	13
6.5	15	15.6	15.3	15.4	15.5	15.6	15.6	15.3	15.2	14.6	14.4	12

a) Viscosity contd...

S1		Viscosity, cP		Temperature stability studies								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.3	15.2	15.3	15.2	15.3	15.1	14.6	15.1	15.2	15.3	15.2	15.1
10	15.3	15.4	15.3	15.3	15.3	14.9	15.2	15	15.1	15	15.3	15
30	15.4	15.2	15.2	15.3	15.1	15.2	15.3	15.2	15	14.9	15.4	14.8
40	15.3	15.3	14.9	15.2	14.9	13	12.8	11.8	11.4	11.1	10.8	10.1
50	15.3	15.3	14.8	14.4	14.2	13.4	12.6	11.4	11	10.8	10.4	9.8
S2		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.2	15.2	15.4	15.2	15.1	15.3	15.1	15	15.2	15	15.2	15.1
10	15.4	15.3	15.2	15.6	15.3	15	14.9	15	15.2	15.1	15	15
30	15.4	15.4	15.3	15.4	14.9	15	15.3	15.4	14.9	14.7	14.7	14.8
40	15.3	15.3	15.1	14.8	14.2	14	13.7	13.4	13.1	12.4	12	11
50	15.2	15.2	15	14.4	13.8	13.7	13.3	13	12.8	12.2	11.6	10.5
S3		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15.1	15	15.3	15.2	15	15	15.2	15.3	15.2	15.3	15.2	15
10	15.2	15	15.2	15.1	15	15.1	15.1	15	15.1	15.2	15.3	15.2
30	15.2	15	15.1	15	14.8	15.2	15	15.2	15	15	15.3	15.2
40	15.3	14.9	14.7	14.6	14.5	14.2	14.3	14.2	14.3	14.4	14.5	14.1
50	15.1	14.8	14.7	14.3	14.3	14.1	14	14.2	14.2	14.1	13.9	13.6
S4		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15	15.2	15	15.3	15.2	15.3	15.2	15	15	14.8	15.1	15
10	15.2	15.2	15.2	15	15	15.2	15	15	15.2	14.9	15.1	15
30	15.3	15	15.2	15.2	15	15	15	14.9	14.8	14.7	15	14.8
40	15.1	15	15.1	15.1	14.7	14.5	14	13.4	13	12.5	12.1	11.6
50	15	15	15	14.8	14.6	14.4	13.8	13	12.7	12.5	11.8	11.2
S5		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15	15	15.2	15	15.2	15.2	15	14.9	14.8	14.4	14.8	15.1
10	15.3	15.1	15.3	15	15.4	15.3	15.1	14.8	15	14.9	14.8	15
30	15.2	14.9	15.4	15	15.5	15	14.9	14.7	15	15.1	15.4	15.2
40	15.1	15.3	15.4	15	14.8	14.7	14.4	14	13.6	13	12.3	10.8
50	15	15.3	15.2	15	14.8	14.5	14.3	13.8	13.2	12.5	11.6	8.9

b) Particle size

S1	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	5.7	5.7	5.8	5.7	5.7	5.7	5.7	5.6	5.7	6	5.6	5.6
4.5	5.8	5.7	5.7	5.7	5.8	5.8	5.6	5.5	5.7	5.8	5.7	5.4
5	6	5.6	5.6	5.8	5.6	5.6	5.5	5.6	5.6	5.8	5.6	5.6
6	7	5.8	5.5	5.9	5.8	5.5	6	5.4	5.8	4.6	4	4.2
6.5	7.1	5.6	5.8	5.6	5.7	5.7	5.6	5.8	5.6	4.3	4.2	4.3
S2	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	5.6	5.6	6	5.8	5.6	5.6	5.6	5.7	5.6	5.7	5.6	5.4
4.5	5.7	5.5	5.6	5.4	5.4	5.7	5.7	5.6	5.7	5.6	5.4	5.6
5	5.6	5.4	5.5	5.5	5.5	5.8	5.6	5.5	5.4	5.4	5.5	5.6
6	5.8	5.3	5.3	5.6	5.6	5.6	5.7	5.6	5.4	5.2	5.3	4.6
6.5	5.7	5.2	5	5.2	5.7	5.7	5.6	5.5	5.4	4.9	4.2	4
S3	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	5.7	5.8	5.7	5.6	5.7	5.6	5.7	5.7	5.6	5.7	5.8	5.6
4.5	6	5.6	5.4	5.3	5.4	5.4	5.6	5.6	5.5	5.4	5.3	5.4
5	6.3	6	5.8	5.6	5.7	5.8	6	5.4	5.6	5.5	5.4	5.3
6	6.4	6.2	6.1	5.8	5.9	6.1	6	5.5	5.4	5.3	5.3	5
6.5	6.2	6	5.9	5.6	5.7	5.8	5.6	5.7	5.7	5.6	5.4	5.2
S4	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	6.3	6.3	6.2	5.9	5.8	6	5.7	5.8	5.6	5.4	5.4	5.5
4.5	6.1	6.4	6.3	5.9	5.7	5.9	5.6	5.9	5.7	5.6	5.5	5.4
5	6.3	6.2	6.4	5.8	6	6.1	6	5.9	5.6	5.5	5.5	5.4
6	6.4	6.3	6.3	6.1	5.9	6.2	5.8	5.7	5.4	5.2	5.1	4.4
6.5	6	6	6.1	6	5.8	6	5.9	5.8	4.8	4.5	4.2	3.8
S5	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	6.5	6.2	6	6.3	6.3	6.4	6.3	6.3	6.4	6.3	6.4	6.6
4.5	6.4	6.3	6.3	6.4	6.4	6.5	6.4	6.5	6.5	6.4	6.3	6.5
5	6.7	6.2	6.2	6.3	6.3	6.2	6.3	6.7	6.7	6.2	6.5	6.4
6	6.5	6.4	6.2	6.5	6.2	6.4	6.3	6.4	6.2	6	6.1	5.2
6.5	6.6	6.3	6.4	6.4	6.4	6.3	6.2	6.1	5.8	5.6	5.3	4.6

b) Particle size contd...

S1	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	6.2	6.1	6	5.7	5.8	5.8	5.8	5.8	5.7	5.8	5.7	5.6
10	6.1	6	5.9	5.6	5.7	5.8	5.6	5.6	5.6	5.7	5.6	6.8
30	5.7	5.7	5.7	5.7	5.6	5.7	5.6	5.6	5.7	5.5	5.4	5.7
40	5.6	5.5	5.6	5.6	5.5	5.5	5.5	5.5	5.6	5.4	5.2	5
50	5.6	5.7	5.4	5.4	5.3	5.6	5.6	4.6	4.2	4.3	4.1	3.6
S2	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	5.7	5.6	5.6	5.6	5.6	5.7	5.6	5.7	5.4	5.3	5.4	5.5
10	5.8	5.6	5.7	5.8	5.7	5.6	5.7	5.4	5.4	5.4	5.2	5.4
30	5.6	5.7	5.6	5.6	5.8	5.7	5.7	5.3	5.5	5.4	5.3	5.3
40	5.6	5.4	5.8	5.7	5.6	5.6	4.9	5.4	4.8	4.8	4.5	4.2
50	5.8	5.7	5.7	5.8	5.7	5.7	4.5	4.4	4.2	4	3.8	3.6
S3	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	5.6	5.6	5.4	5.6	5.7	5.8	5.7	5.6	5.6	5.5	5.7	5.6
10	6.1	5.8	5.7	5.6	5.7	5.6	5.4	5.7	5.5	5.4	5.7	5.6
30	5.6	5.6	5.5	5.5	5.4	5.3	5.2	5.4	5.4	5.7	5.6	5.4
40	6.2	6	5.8	5.7	5.6	5.4	5.3	5.3	5.6	5.7	5.6	5.3
50	5.7	5.6	5.5	5.4	5.1	5	4.7	4.9	4.8	4.9	4.8	4.7
S4	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	6.3	6.2	5.9	5.8	5.8	5.6	5.7	5.6	5.7	5.8	5.6	5.5
10	6.4	6.1	5.8	5.7	5.6	5.7	5.6	5.7	5.7	5.7	5.6	5.4
30	6.3	6.2	6.1	6	5.7	5.6	5.4	5.6	5.4	5.5	5.4	5.3
40	6.2	6	5.8	5.7	5.6	5.4	5.7	5.3	5.1	5	4.7	4.5
50	6.1	6	5.7	5.8	5.7	4.8	4.6	4.3	4.2	4	3.6	3.4
S5	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	6.5	6.4	6.6	6.5	6.6	6.5	6.5	6.7	6.6	6.5	6.5	6.4
10	6.7	6.5	6.3	6.7	6.5	6.7	6.6	6.5	6.4	6.7	6.5	6.2
30	6.6	6.5	6.4	6.5	6.5	6.8	6.7	6.6	6.4	6.5	6.6	5.9
40	6.5	6.4	6.3	6.4	6.4	6.5	6.5	6.5	6.3	6.7	6.5	6.4
50	6.7	6.7	6.4	6.3	5.7	5.4	5	4.8	4.5	4	3.7	3.3

c) VS concentration

S1	Spores, x 10E+08 CFU/ml						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	20	23	22	20	21	20	20	23	24	20	19.9	19
4.5	22	24	21	20	22	19	19	18	23	20	19.7	19
5	21	25	21	20	23	21	21	20	22	20	19.8	19.5
6	22	26	24	22	22	21	23	23	19.7	18.7	18.2	18.1
6.5	23	24	22	21	20	19.8	21	24	19.6	18.5	17.8	17.8
S2	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	20	20	19.8	19.7	19.6	19.5	19.7	19.7	19.2	19.6	19.6	19.4
4.5	21	21	19.9	19.7	19.8	19.7	19.6	19.6	19.2	19.5	19.5	19.3
5	22	22	21	20	20	19.7	19.6	19.7	19.3	19.1	19	19
6	21	23	20.9	20.4	20.1	19.8	19.6	19.5	19.4	19.3	19.3	18.8
6.5	23	22	20	19.7	19.7	19.6	19.5	19.4	18.8	18.8	18.4	17.7
S3	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	21	20	23	22	23	20	19.8	19.7	19.8	19.7	19.8	19.7
4.5	20	21	22	21	23	19.8	19.9	19.7	19.8	19.6	19.8	19.8
5	23	22	21	20	24	19.7	19.9	19.8	19.7	19.7	19.7	19.7
6	24	23	23	22	21	19.8	19.8	19.7	19.9	19.5	19.6	19.7
6.5	22	23	24	23	23	19.8	19.7	19.8	19.7	19.6	19.7	19.7
S4	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	21	21	21	21	21	21	21	20	19.9	19.7	19.5	19.6
4.5	22	23	22	22	22	22	22	21.3	19.7	19.6	19.4	19.2
5	23	21	22	23	23	23	20	20.6	19.6	19.4	19.4	19.1
6	24	21	21	21	21	21	20.7	19.8	19.5	19.4	19.3	19
6.5	23	21	21	21	21	21	19.8	19.5	19.7	19.2	19.4	18.4
S5	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	21	20	20.3	20.4	20.6	20.4	20	20.1	19.7	19.4	19.4	19.3
4.5	22	21	20.8	20.4	20.4	20.3	19.8	19.8	19.7	19.5	19.3	19
5	23	20	19.8	19.7	19.8	19.7	19.5	19.4	19.5	19.3	19	18.5
6	22	21	20.6	20.4	20.3	20.4	19.8	19.6	19.3	19	18.8	18.3
6.5	21	21	20.8	20.5	20	19.6	19.3	19.1	19.4	18.6	18.3	17.6

c) VS concentration contd...

S1	Spores, x 10E+08 CFU/ml			Temperature stability studies								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	20	22	21	20.6	20	19.7	19.8	19.7	19.7	19.4	19	18.7
10	22	22	20.7	20.7	21.4	20	20	20	19.8	19.6	19.3	19
30	21	21	20.7	19.8	19.8	19.6	19.7	19.7	19	18.7	18.4	18.1
50	22	23	22.5	21	19.6	18.7	18.8	18.4	18	17.5	17.6	17.1
F2	**Spores, x 10E+08 CFU/ml**			**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	20	20	19.8	19.7	19.7	19.6	19.5	19.2	19	19	18.9	18.6
10	21	21	19.9	19.8	19.6	19.5	19.4	18.9	18.8	18.7	18.6	18.4
30	22	21	20.8	20.4	20.1	20	20	19.7	19.3	19	18.5	18
50	21	21	20.4	20.2	19.6	19.5	19.4	18.7	18.6	18.5	17.8	17.3
S3	**Spores, x 10E+08 CFU/ml**			**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	21	20	22	20.7	20.6	20.4	20.4	20.2	20.2	20	19.8	19.6
10	20	20.7	20.8	20.7	20.5	20.3	20.1	20	20	20	19.7	19.6
30	23	21.6	20	20	19.8	19.7	19.7	19.8	19.7	19.7	19.5	19.4
50	24	22.3	21.2	21	20.6	20.2	19.8	19.7	19.7	19.5	19.6	19.5
S4	**Spores, x 10E+08 CFU/ml**			**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	21	21	20.7	20.7	19.8	19.4	19.7	19.6	19.5	19.6	19.3	19.3
10	22	21	21	20.8	19.8	19.5	19.6	19.4	19.3	19.2	19.1	18.3
30	23	20.7	20.7	20.2	19.5	19.4	19.3	19.1	19	18.7	18.6	18.1
50	24	20.7	20.8	20.3	19.5	19.4	19.3	18.7	18.5	18.3	18.4	17.4
S5	**Spores, x 10E+08 CFU/ml**			**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	21	20	20.3	20.4	20	20	19.6	19.5	19.6	19.4	19	18.4
10	22	21	20.8	20.2	19.7	19.4	19.2	19.3	19	18.4	18.1	18
30	23	20	19.7	19.8	19.5	19.4	19	18.7	18.3	18	17.5	17.1
50	22	21	20.6	20	19.3	19	18.5	18.2	18	17.6	17	16.5

Entomotoxicity

S1	Entomotoxicity, SBU/µL						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19046	19066	19000	19034	19031	19003	19002	19034	19027	19032	19000
5	19034	19034	19037	19002	19032	19024	19000	19001	19031	19034	19000	18987
6.5	19047	19034	19043	19002	19021	19035	18978	18889	19002	18002	17689	17487
S2	Entomotoxicity, SBU/µL						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19032	19002	19000	19005	19000	19012	19000	18978	19003	18867	18156
5	19000	19032	19033	19031	19014	19007	19004	18967	19003	18034	18034	18002
6.5	19021	18999	18789	18893	18678	18700	19003	19002	18678	18023	18038	18015
S3	Entomotoxicity, SBU/µL						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19032	19022	19023	19000	19000	19022	19022	19000	18984	18978	18972	18927
5	19043	19036	19032	19016	19017	19032	19023	19000	19002	19000	19001	18875
6.5	19021	19018	19004	19000	19043	19011	19022	19000	18867	18745	18697	18670
S4	Entomotoxicity, SBU/µL						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19000	19000	19000	19000	19033	19033	19031	19000	18679	18457	18460
5	19031	19034	19022	19016	19000	19028	19018	19003	18867	18823	18345	18423
6.5	19004	19022	19008	19002	19000	19026	19000	18675	18324	18000	17453	17207
S5	Entomotoxicity, SBU/µL						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19000	19022	19017	19018	19016	19004	19003	18956	18768	18678	18349
5	19003	19000	19011	19005	19001	19004	19007	19004	18457	18455	18256	18105
6.5	19043	19043	19034	19027	19017	19006	19025	19002	18505	18487	18211	17693

d) Entomotoxicity contd…

S1	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19000	19022	19012	19010	19000	19011	19002	19000	19000	19002	19000
30	19023	19022	19002	19000	19000	18978	18789	18656	18442	18367	18112	18016
50	19011	19027	19000	18975	18789	18456	18233	18215	18017	17982	17678	17437
S2	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19000	19000	19000	19000	19000	19000	18798	18800	18798	18789	18567
30	19000	19002	19002	19001	19000	18896	18796	18634	18436	18342	18100	18076
50	19025	19032	19026	19016	18976	18645	18452	18367	18145	18245	17890	17867
S3	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19000	19000	19000	18956	18997	18907	18904	18996	18999	19000	18895
30	19024	19020	19018	19004	19000	18967	18906	18902	19000	18897	19002	18768
50	19036	19038	19025	19034	18978	18780	18678	18458	18500	18423	18289	18198
S4	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19006	19006	19004	19000	19001	19000	18896	18890	18709	18800	18657
30	19043	19007	19004	19002	19000	18798	18569	18500	18490	18234	18200	18108
50	19032	19005	19003	19000	18967	19478	18435	18347	18207	18004	17798	17658
S5	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19000	19000	19025	19027	19010	19000	19017	19000	19011	19000	18876	18834
30	19023	19018	19009	19015	19000	18879	18769	18679	18459	18329	18127	18025
50	19033	19023	19028	19017	18765	18568	18546	18543	18326	18211	18016	17689

Table 4. Stability studies of various Soya formulations at different pH and temperatures

a) Viscosity

Sy-1	Viscosity, cP					pH stability studies						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18	18	18.2	17.5	17.4	17.2	17.3	17.3	17.4	16.8	16.6	16.3
4.5	18.2	18.2	18.1	17.5	17.3	17.2	17.2	17.3	17.2	16.7	16.7	16.1
5	18.1	18.1	18.1	17.4	17.4	17.3	17.4	17.2	17.2	16.8	16.8	16.4
6	18.1	18.1	18	17.5	17.4	17.3	17.3	17.2	17.1	16.9	16.7	15.7
6.5	18.2	18.2	18	17.9	17.8	17.5	17.3	17.2	16.4	16.4	16.8	15.6
Sy-2	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19.3	19.1	19	18.8	18.5	18.2	17.9	17.6	17.5	16.9	16.7	16.5
4.5	19.2	19	18.7	18.6	18.4	18	17.8	17.6	17.4	16.8	16.7	16.6
5	19.1	18.9	18.6	18.5	18.2	17.8	17.7	17.5	17.3	16.6	16.5	16.4
6	18.9	18.7	18.5	18.4	18.2	17.7	17.5	17.3	17.1	16.5	16.4	16.1
6.5	18.8	18.6	18.4	18.3	18.1	17.8	17.5	17.3	17.1	16.7	16.5	15.8
Sy-3	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.6	18.4	18.5	18.4	18.3	18	17.4	17	16.8	16.5	15.8	15.4
4.5	18.4	18.5	18.4	18.1	18	17.6	17.5	17	16.7	16.5	16.1	15.6
5	18.5	18.4	18.3	18.1	17.8	17.5	17.1	17	16.8	16.5	16.2	15.7
6	18.4	18.4	18.2	18	17.6	17.4	17	16.7	16.5	16.1	15.7	14.6
6.5	18.5	18.4	18.2	18	17.4	17.3	17	16.8	16.4	16	15.6	14.3
Sy-4	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.3	18.2	18.1	17.8	17.4	17.2	16.8	16.5	16.2	16.1	15.8	15.4
4.5	18.4	18.3	18.1	17.7	17.5	17.3	17	16.7	16.4	16	15.6	15.1
5	18.4	18.3	18.1	17.6	17.4	17.2	16.9	16.6	16.3	16.1	15.7	15.5
6	18.5	18.3	18.1	17.5	17.4	17.3	16.7	16.3	16	15.8	15.2	15
6.5	18.4	18.2	18	17.6	17.4	17	16.5	16.3	15.7	15.4	14.7	14.3
Sy-5	**Viscosity, cP**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.3	18.4	18.4	18.2	18	17.5	17.3	17.1	16.7	16.3	16	15.4
4.5	18.4	18.4	18.4	18	17.8	17.3	17	16.8	16.4	16.1	16	15.3
5	18.3	18.3	18.5	18.3	17.9	17.4	17.1	16.7	16.3	16	15.4	15.4
6	18.4	18.3	18.2	18	17.7	17.5	17.1	16.6	16.3	15.8	15	14.6
6.5	18.3	18.4	18.2	17.8	17.5	17.2	16.8	16.6	16	15.6	14.7	14.2

a) Viscosity contd...

Sy-1		Viscosity, cP		Temperature stability studies								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18	18	18.2	17.5	17.2	17.1	17	16.5	16.1	15.8	15.7	15.2
10	18.2	18	17.8	17.6	17.5	17.2	17.1	16.4	16.1	15.7	15.6	15.4
30	18.1	18	17.8	17.6	17.4	17.1	16.7	16.3	16	15.5	15.4	15.1
40	18.1	18	17.8	17.3	17	16.5	15.2	14.3	14	13.4	13	12.5
50	18.2	18	17.5	16.5	16.3	16	15.8	14.5	13.1	12.8	12.3	11.3
Sy-2		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	19.3	19	19	18.4	18.2	17.6	17.3	17	16.6	16.3	16	15.4
10	19.2	19	18.5	18.3	18.1	17.4	17.2	16.7	16.2	15.6	15.2	14.7
30	19.1	18.7	18.4	18.4	18	17.5	17.4	16.7	16.1	15.6	15	14.8
40	18.9	18.7	18.3	18	17.6	17.2	16.4	15.7	15.2	14.7	14.2	13.6
50	18.8	18.5	18	17.4	17	16.4	15.7	15	14.6	13.7	13.3	12.7
Sy-3		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.6	18.2	18.4	18.2	18	17.7	17.2	17	16.6	16.3	16.1	15.7
10	18.4	18.3	18.3	18	17.6	17.3	16.8	16.3	15.7	15.3	15	14.7
30	18.5	18.2	18.2	17.8	17.4	17.3	16.7	16.4	15.6	15.3	14.6	13.6
40	18.4	18.3	18.2	17.6	17.3	17	15.8	15.3	15.4	13.6	13	12.6
50	18.5	18.2	18.1	16.7	15.5	14.6	14.3	13.8	12.6	11.9	11.2	10.6
Sy-4		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.3	18.2	18	17.8	17.3	17.1	17.2	16.7	16.1	16	15.6	15.2
10	18.4	18	17.8	17.5	17.2	17.2	17	16.6	16.2	15.7	15.3	15
30	18.4	18.1	17.6	17.6	17.3	17	16.5	16	15.6	15.4	14.6	14.3
40	18.5	18.2	17.4	17.1	16.5	15.8	15.6	15.3	14.5	14.2	13.8	13.1
50	18.4	18	17.2	16.7	15.6	14.8	14.5	14	13.4	12.5	12.2	11.6
Sy-5		**Viscosity, cP**		**Temperature stability studies**								
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	18.3	18.2	18.3	18	17.7	17.4	17	16.6	16.5	16.2	15.6	15.1
10	18.4	18	18	17.5	17	16.7	16.1	15.7	15.4	15.3	14.7	14.2
30	18.3	18.2	18.1	17.3	16.6	16.2	15.8	14.6	14.1	13.7	12.7	12.2
40	18.4	18	17.8	17	16.4	16	15.6	14.7	13.4	12.8	11.6	11.3
50	18.3	18	17.7	17.2	16.4	16.1	15.4	14.8	13.1	12.4	11.2	10.3

b) Particle size

Sy-1	Particle-size, D_{50} (µm)						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3	3.2	3.1	3.1	3.2	3.1	3.2	3	3.2	3	2.8	2.7
4.5	3	2.5	3.2	2.4	3.1	3.2	3.2	2.6	3	2.7	2.6	2.5
5	3.4	2.7	3.3	3.2	3.2	3	3.3	2.7	2.5	2.4	2.3	2.2
6	3.5	2.3	3.1	2.7	3.1	3	2.5	2.5	2.3	2.2	2.1	2
6.5	3.2	2.8	2.6	2.3	2.4	2.5	2.6	2.4	2.2	2.1	2	1.7
Sy-2	**Particle-size, D_{50} (µm)**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3.1	3	2.8	2.7	2.4	2.5	2.4	2.3	2.3	2.4	2.2	2
4.5	3.1	3	2.7	2.6	2.4	2.6	2.4	2.4	2.4	2.2	2.1	1.6
5	3.2	3	2.8	2.5	2.4	2.5	2.4	2.4	2.2	2.1	2	1.8
6	3.1	3.1	2.7	2.5	2.4	2.4	2.3	2.2	2	1.5	1.3	1.1
6.5	3.3	2.9	2.8	2.5	2.3	2.3	2.3	2.2	1.8	1.4	1.2	1
Sy-3	**Particle-size, D_{50} (µm)**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3	3	2.7	2.5	2.4	2.2	2.1	2	1.8	1.5	1.4	1.2
4.5	3.2	3	2.7	2.6	2.4	2.1	2	1.8	1.5	1.3	1.1	1
5	3.2	3	2.5	2.4	2.3	2	1.9	1.7	1.4	1.3	1	0.8
6	3.1	3	2.4	2.2	2	1.7	1.5	1.4	1.1	1	1	0.7
6.5	3.1	2.5	2.2	2	1.8	1.3	1	0.8	0.4	0.2	ND	ND
Sy-4	**Particle-size, D_{50} (µm)**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3.2	3.1	3	2.9	2.8	2.7	2.8	2.5	2.4	2.3	2.2	2
4.5	3.1	3	2.8	2.6	2.5	2.5	2.7	2.6	2.4	2.2	2	1.8
5	3.2	3.1	3	2.7	2.5	2.4	2.6	2.5	2.3	2	1.7	1.5
6	3.3	3.1	3	2.8	2.7	2.3	2.2	2	1.8	1.4	1.1	1
6.5	3.3	3.2	3	2.7	2.4	2.1	2	1.9	1.7	1.4	1	0.6
Sy-5	**Particle-size, D_{50} (µm)**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3.2	3.2	3.1	3	2.8	2.4	2.3	2.1	2	1.8	1.7	1.6
4.5	3.3	3.3	3.1	3	2.7	2.5	2.4	2.3	2.1	1.5	1.3	1.2
5	3.2	3.1	3	2.8	2.7	2.4	2.1	2	1.8	1.4	1.1	1
6	3.4	3.3	3	2.8	2.6	2.4	2.2	2	1.7	1.5	1.4	1
6.5	3.2	3.1	3	2.9	2.4	2.2	2	1.7	1.4	1.1	1	0.8

b) Particle size contd...

Sy-1	Particle-size, D_{50} (µm)					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3	3.2	3.1	3	2.9	2.8	2.7	2.6	2.4	2.2	2	1.8
10	3	2.5	2.5	2.4	2.3	2.1	2	1.8	1.7	1.4	1.3	1.1
30	3.4	2.7	2.6	2.5	2.2	2.1	2	1.7	1.5	1.3	1.1	1
40	3.5	2.3	2.5	2.7	2.5	2.2	2	1.5	1.1	1	0.7	0.4
50	3.2	2.8	2.6	2.3	2	1.7	1.4	1.2	1	0.7	0.3	ND
Sy-2	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3.1	3	2.8	2.7	2.5	2.4	2.3	2.2	2	1.6	1.4	1.1
10	3.1	3	2.8	2.6	2.5	2.3	2.2	2.1	2	1.4	1.3	1
30	3.2	3	2.7	2.3	2.2	2	1.7	1.6	1.4	1.3	1.2	1
40	3.1	3	2.7	2.2	2.1	2	1.8	1.5	1.5	1.3	1.2	1
50	3.3	2.9	2.8	2.3	2	1.7	1.4	1	0.7	0.4	ND	ND
Sy-3	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3	3	2.7	2.5	2.4	2.3	2.1	2	1.7	1.5	1.4	1.3
10	3.2	3.1	2.8	2.3	2.2	2.1	2	1.8	1.6	1.5	1.4	1.2
30	3.2	3.1	2.7	2.3	2.2	2.1	2	1.9	1.7	1.7	1.6	1.5
40	3.1	3	2.6	2.4	2.1	2	1.8	1.6	1.4	1.2	1	0.9
50	3.1	2.5	2.2	2	1.7	1.4	1.3	1.1	1	0.8	0.5	0.2
Sy-4	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3.2	3.1	3	2.9	2.4	2.1	2	1.8	1.7	1.6	1.5	1.3
10	3.1	3	2.8	2.5	2.4	2.2	2.2	1.8	1.7	1.5	1.2	1.1
30	3.2	3	3	2.7	2.5	2.4	2.3	1.9	1.6	1.5	1.3	1.2
40	3.3	3.2	3.1	2.6	2.4	2.3	2.1	2	1.7	1.5	1	0.9
50	3.3	3.1	2.7	2.3	2.3	2.3	2.3	2.2	1.5	1	0.8	0.4
Sy-5	**Particle-size, D_{50} (µm)**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	3.2	3.1	3.1	2.8	2.5	2.4	2.3	2.4	2.3	2	1.7	1.5
10	3.3	3.2	3	2.8	2.4	2.4	2.4	2.3	2.2	2	1.8	1.4
30	3.2	3	3	2.7	2.5	2.3	2.2	2.1	2	1.8	1.7	1.5
40	3.4	3.2	3	2.8	2.3	2.2	2.1	2	1.8	1.5	1.2	1
50	3.2	3.1	3	2.5	2.1	1.6	1.7	1.5	1.1	0.8	0.7	0.2

c) VS concentration

Sy-1	Spores, x 10E+08 CFU/ml						pH stability studies					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	35	34	32	33	32	31	30	29	27	25	24
4.5	35	34	33	33	32	31	30	28	27	26	25	23
5	34	33	32	31	30	28	27	26	25	24	22	24
6	35	34	31	30	28	27	26	25	26	24	22	23
6.5	35	32	30	31	28	25	24	23	24	22	20	21
Sy-2	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	32	31	28	27	24	28	27	25	24	23	24
4.5	33	32	31	28	28	25	24	24	23	24	24	22
5	35	33	31	29	29	26	25	24	24	23	22	21
6	34	31	30	27	26	27	26	24	23	21	20	19
6.5	34	31	30	26	25	26	24	23	22	20	20	18
Sy-3	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	33	31	30	28	27	26	25	23	22	21	20
4.5	32	34	30	29	28	25	24	21	24	24	23	21
5	34	33	32	28	27	26	25	24	23	24	21	20
6	35	33	32	28	25	24	21	20	21	20	17	17
6.5	34	33	30	27	24	21	19	19	20	18	15	14.8
Sy-4	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	34	33	31	30	28	27	25	24	23	21	20
4.5	35	35	34	30	29	27	25	25	24	23	20	20
5	35	34	33	32	27	25	23	21	20.4	18.4	17.5	16.8
6	34	33	32	33	29	26	24	22	21.2	17.9	17.2	16.4
6.5	33	32	31	30	26	24	21	20	18.6	15.4	15.1	14.4
Sy-5	**Spores, x 10E+08 CFU/ml**						**pH stability studies**					
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	33	31	29	28	27	26	25	24	23	21	20
4.5	33	32	31	29	28	26	24	23	22	21	20	19.5
5	32	32	30	28	27	25	23	22	21.9	22	21	20
6	34	33	30	28	27	25	23	21	20.4	19.4	17.8	17.1
6.5	34	33	30	27	25	21	20	18.9	17.8	16.7	15.8	15.3

c) VS concentration contd...

Sy-1	Spores, x 10E+08 CFU/ml					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	34	32	31	30	28	27	26	25	24.8	23.4	23.2
10	35	33	31	32	30	29.1	27.5	25.4	24.6	24.5	22.9	22
30	34	32	31	30	27.8	27.5	27.3	25.6	24.2	24	23.1	22.6
50	35	31	30	27	24.5	24.3	23.8	22.5	21.7	20.4	18.5	16.5
Sy-2	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	33	31	28	25	24	23.4	22.8	22.4	21.4	21.2	21.2
10	33	31	31	27.8	26.1	26.1	24.4	23.2	22.4	21.5	21.4	21.4
30	35	32	31	28	26.1	25.4	25.1	23	22.7	21.5	21.4	21.3
50	34	31	29.7	27	25.4	23.4	23.1	23	20.9	19.4	19	15.8
Sy-3	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	31	31	30	29.7	29.4	28.9	28.7	28.6	28.4	27.8	27.2
10	32	33	30	29	28.9	28.4	28.3	28	27.8	26.4	25.9	24.5
30	34	32	31	28	27.8	27.2	26.8	25.9	25.4	24.6	23.2	22.1
50	35	33	30	27.8	26.4	26.1	25.8	25.3	23.7	22.3	21.2	19.4
Sy-4	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	33	32	31	29.8	29.4	28.7	28.2	28	27.7	26.2	26
10	35	32	31	30	29.5	29.5	28.6	28.4	27.7	26.8	26	25.1
30	35	33	32	30	29.6	29	28.4	28.3	27.3	26.4	25.8	25
50	34	31	31	30	29.3	28.9	27.3	27.1	26.4	23.4	21.5	20
Sy-5	**Spores, x 10E+08 CFU/ml**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	34	32	30.6	28.9	28.2	28.1	27.5	27.3	26.4	26.3	25.8	25.4
10	33	31	30.5	28.8	28.4	28.1	27.3	27	26.4	26.1	25.4	25.3
30	32	33	30	27.9	27.4	27.3	27	26.4	25.7	25.4	25	24.8
50	34	32	30	27.5	27.1	27.4	25.2	22.4	21.1	19.8	18.7	15.4

d) Entomotoxicity

Sy-1	Entomotoxicity, SBU/µL					pH stability studies						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15346	15671	15367	15346	15234	15489	15321	15221	15201	15000	14888	14565
5	14897	14872	14779	15346	15347	15126	15065	15033	15220	15000	14908	14689
6.5	15487	15487	14899	15623	15624	15233	15006	14889	14664	14444	14100	13455
Sy-2	**Entomotoxicity, SBU/µL**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15656	15662	15334	15216	15105	15009	14798	14662	14567	14432	14522	14569
5	15459	15358	15226	15118	15102	14896	14766	14598	14466	14255	14265	14113
6.5	15449	15235	15108	15088	15022	14890	14635	14456	14126	14236	14100	13887
Sy-3	**Entomotoxicity, SBU/µL**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15698	15645	15456	15345	15268	15164	15116	15111	15102	15101	15000	14887
5	15879	15465	15354	15264	15164	15089	15065	15006	15000	14886	14687	14564
6.5	15879	15725	15645	15468	15328	15245	15062	15000	14877	14555	14366	14234
Sy-4	**Entomotoxicity, SBU/µL**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15647	15545	15426	15324	15168	15148	15120	15000	15000	14856	14877	14776
5	15868	15456	15364	15235	15165	15468	15263	15000	15000	14652	14358	14200
6.5	15877	15466	15265	15128	15100	15200	15100	15000	15000	14426	14238	14000
Sy-5	**Entomotoxicity, SBU/µL**					**pH stability studies**						
pH\Days	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	16166	16098	16090	16100	16096	15768	15668	15623	15478	15593	15371	15261
5	16177	16096	16087	16122	16064	15796	15689	15533	15475	15688	15587	15261
6.5	16155	16150	15770	15871	15700	15645	15475	15476	15261	15055	14754	14677

d) Entomotoxicity contd...

Sy-1	Entomotoxicity, SBU/µL					Temperature stability studies						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15346	15269	15126	15000	14879	14800	14789	14583	14546	14319	14135	14000
30	14897	14800	14789	14689	14654	14532	14458	14235	14165	14008	14000	13495
50	15487	15482	14300	14235	14208	14100	14000	13568	13241	13100	12487	12200
Sy-2	**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15656	15621	15465	15109	15000	14879	14856	14568	14546	14410	14200	13789
30	15459	15247	15205	15132	15000	14856	14562	14463	14422	14000	13897	13678
50	15449	15226	15000	14875	14678	14459	14231	14000	13258	13100	12879	12645
Sy-3	**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15698	15459	15364	15224	15000	14786	14648	14452	14325	14320	14225	14000
30	15879	15669	15423	15108	15000	14805	14762	14326	14200	14100	13954	13875
50	15879	15800	15600	15126	15000	14523	14209	14100	13876	13614	13452	13008
Sy-4	**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15647	15487	15397	15215	15100	15000	14897	14736	14648	14410	14105	14000
30	15868	15689	15287	15162	15102	15000	14689	14547	14498	14365	14205	14000
50	15877	15687	15345	15000	14397	14008	13468	13368	13268	13100	12897	12108
Sy-5	**Entomotoxicity, SBU/µL**					**Temperature stability studies**						
Tem\Day	**0**	**3**	**7**	**10**	**15**	**30**	**60**	**90**	**120**	**150**	**180**	**365**
4	15890	15800	15720	15687	15446	15416	15129	15000	14865	14759	14520	14234
30	15900	15678	15526	15432	15169	15000	14897	14263	14000	13874	13587	13416
50	15879	15664	15304	14659	14428	14368	14000	13650	13426	13230	13000	12289

Annexe - XVI

Rheology Studies of Soya and Starch industry Wastewater Fermentation

Table 1. Different rheological model fits of Bt fermented starch industry wastewater

Time (h)	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**	**36**	**48**
Bingham Law →	$\tau = \tau_0 + \eta D$; τ = shear stress (dynes/cm^2); D = shear rate (s^{-1})											
Plastic Viscosity (η, mPa.s)	-	-	-	-	-	-	-	-	-	-	-	-
Yield Stress (τ_0, dynes/cm^2)	-	-	-	-	-	-	-	-	-	-	-	-
Confidence of fit (%)	-	-	-	-	-	-	-	-	-	-	-	-
Casson law	$\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{\eta D}$											
Plastic Viscosity (η, mPa.s)	1.84	1.86	1.95	1.92	1.87	1.8	1.8	1.91	1.82	1.87	1.94	1.86
Yield Stress (τ_0, dynes/cm^2)	0	0	0	0	0	0	0	0	0	0	0	0
Confidence of fit (%)	52.2	34.3	21.5	37.6	88.5	87.1	26.5	93.7	92.7	94.3	89.6	71.8
NCA/CMA Casson	$(1+a)\sqrt{\tau} = 2\sqrt{\tau_0} + (1+a)\sqrt{\eta D}$; a= spindle radius/inner cup radius											
Plastic Viscosity (η, mPa.s)	1.84	1.86	1.95	1.92	1.87	1.8	1.8	1.91	1.82	1.87	1.94	1.86
Yield Stress (τ_0, dynes/cm^2)	0	0	0	0	0	0	0	0	0	0	0	0
Confidence of fit (%)	52.2	34.3	21.5	37.6	88.5	87.1	26.5	93.7	92.7	94.3	89.6	71.8
Power law	$\tau = K D^n$											
Consistency index (K, mPa.s^n)	2.06	1.66	1.13	1.19	2.82	2.91	1.52	2	2.57	1.94	1.67	1.46
Flow behaviour index (n)	0.93	0.98	1.07	1.07	0.91	0.89	0.99	0.98	0.93	0.99	1.03	1.04
Confidence of fit (%)	82.8	85	90.6	90.8	87.8	84.9	84.8	96.6	92.7	96.1	92.6	91.6
IPC Paste	$\eta = K R^n$; K = consistency multiplier; R = rotational speed, rpm											
Shear sensitivity factor (n)	1.75	1.57	1.36	1.41	2.24	2.2	1.48	1.92	2.14	1.88	1.79	1.6
10 rpm viscosity (η)	0.07	0.02	0.07	0.07	0.09	0.11	0.01	0.02	0.07	0.01	0.03	0.04
Confidence of fit (%)	82.8	85	90.6	90.8	87.8	84.9	84.8	96.6	92.7	96.1	92.6	91.6

Shaded cells represent lower confidence of fits

Notes:

In general, Bt fermented starch industry wastewater (SIW) medium presented a pseudpoplastic and non-Newtonian behavior. The Bingham plastic law was not followed by SIW during the course of fermentation (48 h). In fact, Casson law and NCA-CMA Casson were also not obeyed during the first 9 h of fermentation as predicted by the lower confidence of fits (20 to 53 %, Table 1). However, the yield stress during the course of fermentation also remained zero suggesting the easy flowability of the medium which is a necessary pre-requisite for further downstream processing, namely, harvesting and formulation development steps. Plastic viscosity did not show much variation except for a small increase at 36 h which cannot be generalized as a trend. On the contrary, power law was obeyed with higher confidence of fits (82 to 96 %) which is a principal characteristic of any fermentation medium. Moreover, the consistency index decreased as the fermentation progressed towards 48 h and flow behavior index increased to 1 suggesting fairly Newtonian characteristics which is an excellent input for designing of pipes and pumps during downstream processing as well as enhanced feasibility of formulation amendment. IPC paste model also showed decrease in the shear sensitivity factor so that the fermented broth was not highly sensitive to shear changes as well as the decrease in 10 rpm viscosity was in concordance with the consistency index decrease discussed earlier.

Thus, SIW can rheologically serve as excellent substrate for Bt fermentation with possibility of higher oxygen transfer during fermentation, less messy handling during downstream processing and ease of amendment (low sensitivity to shear rate). Nevertheless, the lower shear sensitivity could also be a drawback as the viscosity would remain practically constant so that during pulverization, the spray has to be carried out using same pressure.

Table 2. Different rheological model fits of Bt fermented soya medium

Time (h) ⟶	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**30**	**36**	**48**
Bingham Law	$\tau = \tau_0 + \eta D$; τ = shear stress (dynes/cm^2); D = shear rate (s^{-1})											
Plastic Viscosity (η, mPa.s)	-	-	2.03	2.01	2.7	2.31	2.01	1.95	1.95	-	-	-
Yield Stress (τ_0, dynes/cm^2)	-	-	0.01	0.02	0.07	0.05	0.03	0	0	-	-	-
Confidence of fit (%)	-	-	88.3	84.1	82.2	82.3	76.7	88.4	85.9	-	-	-
Casson law	$\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{\eta D}$											
Plastic Viscosity (η, mPa.s)	1.99	2.01	1.75	1.63	1.88	1.69	1.62	1.8	1.8	1.86	1.91	1.93
Yield Stress (τ_0, dynes/cm^2)	0	0	0	0.01	0.03	0.02	0.01	0	0	0	0	0
Confidence of fit (%)	43.7	94.7	95.6	95.6	93.9	94.6	93.2	91.7	90.4	93	94.3	94.2
NCA/CMA Casson	$(1+a)\sqrt{\tau} = 2\sqrt{\tau_0} + (1+a)\sqrt{\eta D}$; a= spindle radius/inner cup radius											
Plastic Viscosity (η, mPa.s)	1.99	2.01	1.75	1.63	1.88	1.69	1.62	1.8	1.8	1.86	1.91	1.93
Yield Stress (τ_0, dynes/cm^2)	0	0	0	0.01	0.03	0.02	0.01	0	0	0	0	0
Confidence of fit (%)	43.7	94.7	95.6	95.6	93.9	94.6	93.2	91.7	90.4	93	94.3	94.2
Power law	$\tau = K D^n$											
Consistency index (K, mPa.s^n)	1.61	2.57	6.88	10.3	30.4	20.1	10.5	2.94	2.95	2.51	2.52	2.5
Flow behaviour index (n)	1.01	0.95	0.75	0.68	0.54	0.58	0.68	0.91	0.91	0.94	0.95	0.95
Confidence of fit (%)	90.4	95.1	91.9	91.8	92.4	92.6	94.2	92.4	92.1	93.5	94.8	95
IPC Paste	$\eta = K R^n$; K = consistency multiplier; R = rotational speed, rpm											
Shear sensitivity factor (n)	1.65	2.29	3.67	4.56	9.59	7.08	4.71	2.36	2.36	2.27	2.22	2.21
10 rpm viscosity (η)	0.01	0.05	0.25	0.32	0.46	0.42	0.32	0.09	0.09	0.08	0.05	0.05
Confidence of fit (%)	90.4	95.1	91.9	91.8	92.4	92.6	94.2	92.4	92.1	94.5	94.8	95

Shaded cells represent lower confidence of fits.

Notes:

Bt fermented soyameal medium showed a pseudoplastic profile during fermentation again suggesting non-Newtonian behavior similar to SIW medium. Interestingly, Bingham model was followed from 6 to 24 h (Table 2) which could be due to the increase in cell density during the fermentation which would have caused thickening of the medium. Casson and NCA-CMA Casson law showed no marked variation in plastic viscosity during the entire course of fermentation. However, a small change in yield stress was observed during active exponential phase from 9 to 18 h which could be due to the active growth of Bt cells causing resistance to flow.

Power law gave excellent confidence of fits. The consistency index increased from the lag phase to active exponential phase (0 to 15 h) and then started decreasing, former increase could be due to active Bt metabolism and latter decrease could be due to cell lysis and sporulation. The flow behavior index decreased proportionately with consistency index and reached a value closer to 1 (0.95) suggesting Newtonian behavior. Likewise, IPC paste model showed an increase in shear sensitivity factor during active exponential phase (0 to 15 h) and later decrease in the same in tandem with the 10 rpm viscosity increase (0.01 to 0.46) followed by a decrease towards the end of fermentation (48 h). Thus, the shear sensitivity of the soyameal medium was similar to SIW medium and will show the same characteristics during downstream processing and formulation development.

In totality, SIW is a good replacement candidate for soyameal medium as the proponent Bt fermentation substrate as well as formulations (discussed in the main text).

Annexe – XVII

Ultrafiltration of Bt Fermented Media

Table Screening of different membranes for ultrafiltration of Bt supernatant derived from different fermented broths

	Tx_R (x 10^9SBU/l)			
MWCO (kDa)	**100**	**30**	**10**	**5**
NH	5	9	11.5	13.5
TH	4	7.7	8.9	9.7
SIW	7	10.8	15.8	15
Soya	4.5	9.5	10.8	12
	Tx_P (x 10^9SBU/l)			
NH	4.2	3.5	2.5	n.d.
TH	3	2.6	1.7	n.d.
SIW	4.2	3.4	2.2	n.d.
Soya	3.8	2.5	1.5	n.d.
	VS_R (x 10^6CFU/ml)			
NH	1.5	2	5	20
TH	0.5	0.8	4.3	10
SIW	0.8	1.1	6.4	11
Soya	4.2	5	7.2	10
	VS_P (x 10^7CFU/ml)			
NH	11	1.5	0.5	n.d.
TH	11	0.5	0.1	n.d.
SIW	9	0.8	0.1	n.d.
Soya	7.3	4.2	0.35	n.d.

n.d.: not detectable, assumed to be negligible loss

Tx_R: Entomotoxicity in retentate
Tx_P: Entomotoxicity in permeate
VS_R: Viable spores in retentate
VS_P: Viable spores in permeate
MWCO: Molecular weight cut-off
kDa : kilo Dalton

Screening of different UF membranes

Before proceeding to the optimization of different process parameters, screening of different membranes was carried out to select the best membrane. Thus, the supernatant of different fermented broths, namely, NH, TH sludge, SIW and soya medium were passed at a fixed flux rate (900 L/m^2/h) through the different membranes of MWCO of 100, 30, 10 and 5 kDa. Biological parameters, namely, Tx and VS were used as the control parameters to select the best performance membrane. It was observed that 5 KDa gave the best performance in terms of concentration of Tx and VS in the retentate and negligible losses in the permeate. Hence, 5KDa will be used as the MWCO for future experiments on UF of Bt fermented broths of NH, TH, SIW and soya.

Annexe – XVIII

Bioassays - Method and Experimental Controls

The entomotoxicity (Tx) was evaluated by bioassays using eastern spruce budworm larvae (SB) (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) in second instar, provided by Natural Resources Canada (Sault Ste-Marie, Ontario) by using diet incorporation method. The larvae were raised on an artificial diet (diet compostion given in Table XVII.1) for 7 days to obtain the third and fourth instar (L3-L4) larvae. The bioassays were conducted using the diet incorporation method (Dulmage et al., 1971; Beegle, 1990). In this technique, 1.5 ml Btk preparations obtained from the sludge (or sludge grown Btk) and formulated sludges/wastewater/soya were incorporated into 30 mL molten agar based diet (at 60 ± 1°C). Afterwards, the mixture was distributed in aliquots of 1 mL in twenty 15 x 45 mm glass vials (VWR Canlab, Canada) with a perforated plastic cap. Sixty vials containing 1 mL artificial diet (C1) were used as control, another control with sterilized sludge (C2) was also used. Once the vials with diet were ready, one L3-L4 larva was introduced into each vial and left for feeding *adlibitum* for 7 days at 25 ± 1°C. Mortality was monitored after 7 days. If mortality in control vials was higher than 10%, the experiment was repeated.

Table XVII.1 Composition of artificial diet (1L) required for the growth of spruce budworm larvae (McMorran, 1965)

Ingredient (s)	**Quantity**	**Vitamin solution**[1]	**Quantity**
Distilled water	840 ml	Distilled water	100 mL
Casein (vitamin free)	35 g	Niacin	100 mg
Potassium hydroxide 4M	5 ml	Calcium pantothenate	10 mg
Alphacel	5 g	Riboflavin	50 mg
Wesson Salt mixture	10 g	Thiamine hydrochloride	25 mg
Wheat germ	45.7 g	Pyridoxine hydrochloride	25 mg
Vitamin solution[1]	10 g	Folic acid	25 mg
Ascorbic acid	4 g	Vitamin B12	0.2 mg
Formaldehyde 40%	0.4 mL	Biotin	2 mg
Sucrose	35 g		
Agar	16.7 g		
Aureomycine powder	5.6 g		

Tx of sample preparations was obtained by comparing the final mortality (percentages) of eastern spruce budworm larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) with that of standard commercial product (Foray 76B, Abbott Laboratories, Chicago, IL) and expressed as relative spruce budworm units/µl (10^{-3} SBU/m^3). Foray 76B contained spores and crystals of Bt var. *kurstaki* at a potency of 20.1 x 10^{12} IU/m^3 (International Unit) measured against cabbage looper (*Trichoplusia ni*). On comparison of Tx of Bt fermented sludge and soyameal samples, it was found

that specific SBU in this study was 20-25 % higher than IU. The standard deviation for Tx measurement was 8–10 %.

Different controls used for each of the bioassays were as follows:

Controls	**Tx (x 10^9 SBU/L)**
Diet (-ve)	0.2
Sterilized wastewater/wastewater sludge (-ve)†	0.3 to 0.5
Additives/adjuvants of formulations (-ve) ††	n.d.
Tween -80 (-ve)	0.1 to 0.2
Fermented soya broth (+ve)	9.5

n.d. = not detectable

†- Included primary/secondary/mixed/hydrolyzed sludges and starch industry wastewater

†† - Included different additives, namely, suspending agents, phagostimulants, rainfasteners, anti-microbial agents and UV screens added during formulation development.

Reference:

McMorran, A. (1965). A synthetic diet for the spruce budworm, *Choritoneura fumiferana* (Clem.) (Lepidoptera : Tortricidae). *Can. Entomol.*, 97 : 58-62.