**Institut National de la Recherche Scientifique**

# DÉVELOPPEMENT D'UN PROCESSUS D'OBTENTION D'AGENTS BIOLOGIQUES À BASE DE *TRICHODERMA* SPP. EN UTILISANT DES EAUX USÉES OU DES BOUES D'ÉPURATION COMME SUBSTRATS DE FERMENTATION

**(Process development of *Trichoderma* spp. based biocontrol agent from different wastewaters and wastewater sludges)**

MAUSAM VERMA

Thèse présentée

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

Jury d'évaluation

| | |
|---|---|
| Examinateurs externes | Prof. Gérald J. Zagury (École Polytechnique de Montréal) |
| | Dr. Marc Gagné (NUVAC Environnement) |
| Examinateur interne | Prof. Jean-François Blais (INRS-ETE) |
| Directeur de recherche | Prof. Rajeshwar Dayal Tyagi (INRS-ETE) |
| Co-directeur de recherche | Dr. José R. Valéro (INRS-ETE) |



**Mai 2007**

EAU, TERRE ET ENVIRONNEMENT INRS

*Dedicated*

*To*

*God,*

*My parents and my brother and sister who have always been my moral strength in this great venture*

# *ACKNOWLEDGEMENTS*

*I take immense pleasure, in expressing my wholesome gratitude to my Ph.D. supervisor* ***Prof. R. D. Tyagi*** *for his expert guidance and constant encouragement throughout the period of my project. I am equally grateful to my co supervisor* ***Dr. J. R. Valéro*** *for his motivation, patience and precious time that he devoted for my project work. I also feel great pleasure in thanking my mini-project supervisor* ***Dr. R. Y. Surampalli****, who was always ready to help in my Ph.D. activities.*

*I exclusively thank my colleague and friend* ***Satinder Kaur Brar*** *for her technical suggestions, inspiration, emotional support, and wholehearted help in all part of project work and life. I also express my thanks to* ***all lab members*** *for their valuable help. They assisted me in my experiments and willingness to help in any aspect, whenever I required.*

*I am highly indebted to* ***Prof. J. F. Blais, Simon*** *and* ***José Anné*** *for their timely translation of the synopsis in French.*

*For collaborative work, I would like to thank,* ***Dr. Danielle Prévost*** *for the execution of plant bioassay experiment.*

*I bestow my special thanks to* ***Michelle, Carole, Stéfane, Pauline, Sébastien, René, Lise*** *and others for their technical supports regarding general experiment execution and chemical analyses. I am also thankful to all faculty members of* ***INRS-ETE*** *for their inspiring persona.*

*Last but not the least, I express my heartfelt indebtness to my* ***all family members and friends*** *for their love, encouragement and constant support throughout my academics.*

***Mausam Verma***

# AVANT-PROPOS

## PUBLICATIONS DANS CETTE THÈSE

1. **Verma, M.**, Brar, S.K., Tyagi, R.D. Surampalli, R.Y. and Valéro, J.R. (2007). Industrial wastewaters and dewatered sludge: rich nutrient source for production and formulation of biocontrol agent, *Trichoderma viride*. *World Journal of Microbiology and Biotechnology*. DOI 10.1007/s11274-007-9417-4.

2. **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. *Bioresource Technology*, 98, 2154–2162. DOI:10.1016/j.biortech.2006.08.032.

3. **Verma, M.,** Brar, S.K., Tyagi, R.D. Surampalli, R.Y. and Valéro, J.R. (2006). Pre-treatment of wastewater sludge - biodegradability and rheology study. *Environmental Technology*, 28, xxx–xxx (in press).

4. **Verma, M.,** Brar, S.K., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2006). Bioremediation with fungi. In: Remediation Technologies for Soil and Groundwater. EWRI, ASCE, Virginia, USA. ISBN – 13: 978-0-7844-0894-0. pp 223–258.

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. *Journal of Industrial Microbiology & Biotechnology*, 33, 941–952. DOI 10.1007/s10295-006-0164-6.

6. **Verma, M.**, Brar, S.K., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2005). Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. *Water Research* 39 (15), 3587-3596. DOI:10.1016/j.watres.2005.07.001.

7. **Verma, M.**, Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. (2006). Antagonistic Fungi, *Trichoderma* spp.: Panoply of Biological Control. *Biochemical Engineering Journal* (under review).

8. **Verma, M.**, Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. (2006). Bench-scale fermentation of *Trichoderma viride* on wastewater sludge: rheology, lytic enzymes and biocontrol activity. *Enzyme and Microbial Technology* (submitted).

9. **Verma, M.**, Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. (2006). Fed batch fermentation strategy for antagonist *Trichoderma viride* production using unstructured mathematical modeling in starch industry wastewater. *Biochemical Engineering Journal* (submitted).

10. **Verma, M.**, Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. (2006). Techno-economic evaluation of a *Trichoderma viride* production plant based on waste as raw material: Process feasibility at different scenarios. *World Journal of Microbiology and Biotechnology* (to be submitted).

## BREVET HORS THÈSE

**US Patent**

1. Barnabé S., **Verma, M.,** Tyagi, R. D., Valéro, J. R. 2005. Culture media for increasing biopesticide producing microorganism's entomotoxicity. **PCT/CA2005/000235 (Under review).**

## PUBLICATIONS HORS THÈSE

1. **Verma, M.**, Brar, S.K., Blais, J.F., Tyagi, R.D. and Surampalli, R.Y. (2006). Aerobic biofiltration processes – advances in wastewater treatment. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management*, 10(4), 264–276.

2. **Verma, M.**, Sreekrishnan, T.R. and Tyagi, R.D. (2005). Cell agglomeration in acidogenic, mixed, and methanogenic cultures at different loading and mixing conditions. *Environmental Technology,* 26 (12): 745-756.

3. **Verma, M.**, Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and T.R. Sreekrishnan (2006). Dimensionless groups as scale up parameters for wastewater and wastewater sludge based biopesticides production in a stirred tank reactor. *Journal of Residual Science and Technology*, (accepted, December 2006).

4. Brar, S.K., **Verma, M.,** Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2006). Rheology of hydrolyzed sludge *Bacillus thuringiensis* fermentation and development of stable liquid formulations. *Chemosphere* (accepted, November 2006).

5. Brar, S.K., **Verma, M.,** Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2006). *Bacillus thuringiensis* proteases: production, sporulation and synergism. *Process Biochemistry* (accepted, under revision).

6. 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.

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. 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.

9. Brar, S.K., **Verma, M.**, Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2006). Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. *Process Biochemistry* 41, 323-342.

10. 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.

11. 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.

12. 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 (13): 3001-3011.

13. 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.

14. 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.

15. 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.

16. 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. *J. Environmental Engineering* (submitted, 29 March, 2006).

17. Brar, S.K., **Verma, M.**, Misra, K., Tyagi, R.D. (2006). Lindane-A Contaminant of Global Concern. *Pollution Research* (submitted 16 March, 2006).

18. 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*. (submitted, 25 August 2006).

19. Brar, S.K., **Verma, M.**, Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2004). Value addition of sewage sludge - a clean management strategy. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management* 54 pages. (Submitted, March, 2006).

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

21. 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* (submitted).

22. Pham, H.T.T., **Verma, M.**, Tyagi, R.D. Surampalli, R.Y. (2006). Sludge Pre-treatment Processes and Biodegradability: A Review. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management* (to be submitted).

23. 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* (submitted).

24. 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* (submitted).

# CONGRÈS ET CONFÉRENCES

1. **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 – Sustainable sludge management: state of the art, challenges and perspectives.** Moscow, May, 27-28th, 2006.

2. **Verma, M.**, Brar, S.K., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. Potential of fungi *Trichoderma* spp. grown on wastewater and wastewater sludge for simultaneous biocontrol and degradation of estrogenic compounds. **41st Central Canadian symposium on Water Quality Research (CAWQ),** National Water Research Institute, Burlington, Ontario, Febraury **13-14th**, 2006.

3. Pham, H. T. T., **Verma M.,** Tyagi R. D., Brar S.K., and Surampalli R.Y.(2006). Sludge Pre-treatment Processes and Biodegradability: A Review and Research Initiatives. **41st Central Canadian symposium on Water Quality Research (CAWQ),** National Water Research Institute, Burlington, Ontario, Febraury **13-14th**, 2006.

4. Adjalle, K.D., Brar, S.K., **Verma, M.,** Tyagi, R.D. Valéro, J.R. and Surampalli, R.Y. (2006). Procédé en aval des bouillons fermentés de *Bacillus thuringiensis* à partir des eaux usées et boues d'épuration – Approche d'ultrafiltration. 22nd Central conference of the CAWQ (Canadian Association on Water Quality), Concordia University, Montreal, Canada. November 3, 2006. (submitted).

5. Brar, S.K., **Verma, M.,** Tyagi, R.D. Valéro, J.R. and Surampalli, R.Y. Techno-economic prospects of production of biopesticides from wastewater and wastewater sludge. IWA Conference on Wastewater Biosolids Sustainability – Technical, Managerial and Public Synergy, Moncton, New Brunswick, Canada, June 24 - 27, 2007 (submitted).

6. Adjalle, K.D., Brar, S.K., **Verma, M.,** Tyagi, R.D. Valéro, J.R. and Surampalli, R.Y. Ultrafiltration Recovery of Entomotoxicity from *Bacillus thuringiensis* Fermented Wastewater/wastewater Sludge and Subsequent Formulation Study. IWA Conference on Wastewater Biosolids Sustainability – Technical, Managerial and Public Synergy, Moncton, New Brunswick, Canada, June 24 - 27, 2007 (submitted).

7. Pham, T.T.H., Barnabé, S., **Verma, M.**, Tyagi, R. D. Optimization of ultrasonication treatment process for improvement of wastewater sludge biodegradability using response surface methodology. IWA Conference on Wastewater Biosolids Sustainability – Technical, Managerial and Public Synergy, Moncton, New Brunswick, Canada, June 24 - 27, 2007 (submitted).

8. Brar, S. K., **Verma, M.**, Tyagi, R. D. and Valéro, J. R. Do wastewater and wastewater sludge possess natural features to sustain *Bacillus thuringiensis* biopesticides production? **41st Central Canadian symposium on Water Quality Research**

**(CAWQ),** National Water Research Institute, Burlington, Ontario, Febraury 13-14, 2006.

9. **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 Canadian symposium on Water Quality Research (CAWQ)**, Institut nationale de la recherche scientifique, Québec city, Québec, November **4th**, 2005.

10. 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. **21st Eastern Canadian symposium on Water Quality Research (CAWQ),** Institut nationale de la recherche scientifique, Québec city, Québec, November 4th, 2005.

11. **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, Febraury 14-15, 2005.

12. 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, February 14-15, 2005.

13. **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.

14. 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.

15. **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, 2004,** February 6-8, 2004, Gananoque, Ontario.

16. 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, 2004,** February 6-8, 2004, Gananoque, Ontario.

17. **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.

18. 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.

19. 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 Specialised Conference,** Singapore, March 28-29, 2003.

20. **Verma, M.**, Sreekrishnan, T.R. and Tyagi, R.D. Study on cell agglomeration in mixed acidogenic and mixed methanogenic cultures at different loading and mixing conditions. **38th Central Canadian symposium on Water Quality Research (CAWQ),** National Water Research Institute, Burlington, Ontario, February 10-11, 2003.

21. **Verma, M.,** Sreekrishnan, T.R. (2002). Study of toxicity of chromate ions on methanogenic culture'', International conference on "Water and Wastewater Perspectives of Developing Countries", IWA series, Dec. 11-13, New Delhi, India.

22. Singh, S.K., **Verma, M.,** Sreekrishnan, T.R. (2002). Paper on, "*Study of toxicity of chromate ions on methanogenic culture*'', International conference on "Water and Wastewater Perspectives of Developing Countries", IWA series, Dec. 11-13, New Delhi, India.

23. **Participated** in organizing 15-days workshop on Membrane Technology which also included international participants organized by the Department of Biochemical Engineering and Biotechnology, IIT Delhi, India in March-2001.

24. **Verma, M.,** Sreekrishnan, T.R. Poster presentation on, "*Comparison of Attached and Suspended Cell Methods for the Treatment of Wastewater*" in Biotechnology seminar "Biohorizon2001" organized by the Department of Biochemical Engineering and Biotechnology, IIT Delhi, India in Feb. 2001.

25. **Verma, M.,** Sreekrishnan, T.R. Poster presentation on, "*A Novel High Cell Density Process for the Treatment of Wastewater at High Rate*" in Biotechnology seminar "Biohorizon2000" organized by Department of Biochemical Engineering and Biotechnology, IIT Delhi, India in Feb. 2000.

# TABLE OF CONTENTS

# LIST OF TABLES

**4. Les eaux usées industrielles pour la production et la formulation de *T. viride***

***Part I. Industrial wastewaters and dewatered sludge: rich nutrient source for production and formulation of biocontrol agent, Trichoderma viride***



***Part II. Starch industry wastewater as a substrate for antagonist, Trichoderma viride production***



***Part III. Dissolved oxygen as principal parameter for conidia production of biocontrol fungi Trichoderma viride in non-Newtonian wastewater***



***Part IV. Fed batch fermentation strategy for antagonist Trichoderma viride prodcution using unstructured mathematical modeling in starch industry wastewater***



**5. Étude technico-économique de la production de *Trichoderma* spp.**

# LIST OF FIGURES

**5. Étude technico-économique de la production de *Trichoderma* spp.**

# RÉSUMÉ

Compte tenu des avancées scientifiques, les pesticides biologiques destinés au contrôle des ravageurs deviennent des substituts efficaces à l'utilisation des agents chimiques. À ce propos, des biopesticides à base de champignons microscopiques présentent beaucoup de potentiel autant pour la répression des phytopathogènes que pour l'accroissement de la croissance des plantes. C'est le cas de plusieurs *Trichoderma* qui ont une action antagoniste contre plusieurs phytopathogènes et ce, tout en produisant des métabolites essentiels tels que vitamines, phytohormones de croissance et stimulation du système de défense des plantes. *Trichoderma sp.* possède trois formes principales soit le mycélium, les conidies et les chlamydospores. Par contre, parmi ces trois formes et pour une utilisation efficace de ce champignon en champ, les suspensions de *Trichoderma* spp. les plus rentables et les plus acceptables sont à base de conidies.

Dans ce contexte, contrairement au procédé de production par fermentation liquide, la production en masse des conidies et des métabolites de *Trichoderma* spp. est une option très valable. À ce titre, il a été constaté que les préparations de *Trichoderma* spp. contenant plus de $10^6$ conidia par g ou par ml, sont efficaces sur le terrain et ce, à un taux recommandé de $10^{10}$-$10^{11}$ conidies par hectare. Actuellement, différents biopesticides à base de *Trichoderma* spp. sont disponibles sur le marché. Toutefois, l'utilisation de milieux conventionnels tels que glucose, liqueurs de mélasses de maïs, tartrate de sodium, cellulose, sucrose, etc. pour la production de masse de ces agents biologiques réduisent la rentabilité, par exemple une récolte d'un faible rapport conidies/spores et un coût très élevé des matières premières. Afin de pallier à ces problèmes, les possibilités d'utilisation de résidus comme matières premières de fermentation pour la production de spores et de métabolites et l'obtention de biopesticides a fait l'objet de ces études.

Des boues secondaires et déshydratées d'usines de traitement des eaux avec ou sans traitement préalables (hydrolyse alcaline ou hydrolyse thermo-alcaline) ont été employées pour obtenir des concentrations très élevées de spores de *Trichoderma spp.*. Il a été aussi constaté que des résidus d'industries de fromage, d'amidon ou de pâtes et papiers sont

aussi très valables comme milieux de croissance de *Trichoderma* spp.. Des concentrations de spores de l'ordre de $10^7$–$10^{11}$ UFC/ml ont été obtenues avec ces résidus et les bio-essais préliminaires de préparations à base de boues secondaires ayant subi une hydrolyse thermo-alcaline ont montré une activité entomotoxique significative et qu'elles présentent donc un potentiel élevé pour le contrôle biologique des insectes nuisibles. Initialement, ces études avaient pour but de déterminer la production de conidies en employant ces résidus comme substrats. Par ailleurs, une méthode de détermination du nombre de spores a été modifiée pour utilisation dans le cadre de ces recherches. Ce procédé peut être aisément appliqué pour d'autres champignons. Des travaux d'optimisation concernant les conditions de bioréaction ont aussi été réalisés afin de faciliter la mise à l'échelle du procédé et d'augmenter son rendement. De plus, les facteurs impliqués dans la sporulation ont aussi fait l'objet d'études pour accroître si possible la concentration de spores éventuellement obtenue. Des travaux de recherche ont été accomplis pour développer un procédé de production d'un agent de contrôle biologique à base de *T. viride* et ce, en employant divers résidus tels que des eaux usées, des boues d'épuration d'origine municipale ou industrielle. Un procédé de production de *T. viride* a été défini et a fait l'objet d'une analyse technico-économique pour valider les possibilités de production de masse. Les résultats obtenus démontrent le potentiel très élevé des boues d'épuration et de certains résidus industriels pour l'obtention de *T. viride*, ce qui ouvre la voie pour d'une part la commercialisation de ce biopesticide obtenu en utilisant les résidus mentionnés comme matières premières et, d'autre part, pour le recyclage de ces rejets en produits à haute valeur ajoutée.

# ABSTRACT

Biological control agents are becoming effective substitutes for harmful chemical pesticides with advances in scientific research. Moreover, some fungal based biological control agents have tremendous potential in terms of disease control and plant growth promotion. *Trichoderma* genus is one of these fungi, which are antagonistic against several commercially important phytopathogens, in addition to producing metabolites like, vitamins, plant growth hormones, and induction of plant defense system. These fungi exist in three main microbial propagule forms, namely, mycelia, conidia, and chlamydospores. However, for efficient field application, conidial formulations of *Trichoderma* spp. are preferred, as conidia are the most tolerant form among the three propagules. Nevertheless, simultaneous production of mycelial mass is necessary for the presence of metabolites.

Further, in contrast to solid state fermentation, liquid fermentation of *Trichoderma* spp. conidia and metabolites could be a suitable option for their mass production. *Trichoderma* spp. formulations containing > $10^6$ conidia/(g or, ml) have been found to be effective for field applications at the recommended basis of $10^{10}$-$10^{11}$ conidia/hectare. At present, several *Trichoderma* spp. based biological control agents are available in market. However, the use of conventional production media like, glucose, molasses-corn steep liquor, sodium tartrate, cellulose, sucrose etc. for mass production of these biological control agents suffer drawbacks like, low conidia/spore yield and high raw material cost. In order to overcome these shortcomings, waste based *Trichoderma* spp. spores and metabolites production for biological control agent use is investigated in this study.

Municipal sludges (secondary sludge and dewatered sludge) with and without pretreatments (alkaline and thermal alkaline hydrolyses) have been used to obtain high spore concentration. Also, industrial wastewaters (cheese, starch, and pulp and paper) have been found to be suitable as *Trichoderma* spp. growth media. Spore concentrations in the order of $10^7$–$10^{11}$ CFU/ml could be obtained for the wastes employed, and the preliminary bioassay of fermented secondary sludge (thermal alkaline hydrolyzed) showed significant entomotoxicity, thereby, showing high biological control activity. The initial studies

presented challenges like measurement of conidia in complex fermentation raw materials. Hence, a modified fungal spore measurement method was also developed as a useful tool for this research. Nevertheless, the modified spore measurement method can be easily employed in most of the other fungi.

Optimization studies regarding fermentation conditions have been also carried out for scale up and further enhance the process performance. In addition, sporulation governing factors were also studied for any possible augmentation in spore concentration. In total, this study has been focused on developing a *T. viride* based biocontrol agent production process using several types of wastewaters and wastewater sludges of municipal and agro-industrial origin.

The selected *T. viride* production processes have been subjected to techno-economic analysis to ascertain their practicability of mass scale production. Thus, higher productivity results show intense potential of wastewater sludges and certain industrial wastewaters, which paves way to a new value addition pathway for sustainable waste management via *Trichoderma* spp. based biological control agent production.

# ABBREVIATIONS

| | |
|---|---|
| AA | Amylase activity |
| AH | Alkaline hydrolyzed sludge |
| ALB | Agents de lutte biologique |
| AM | Arbuscular mycorrhizae |
| ANOVA | Analysis of variance |
| ATP | Adénosine triphosphate |
| BCA | Biocontrol agent |
| BPC | Biphényls polychlorés |
| Bt | *Bacillus thuringiensis* |
| CA | Cellulase activity |
| CCB | Champignons causant la carie blanche |
| CFU | Colony forming units |
| CMC | Carboxy methyl cellulose |
| DDT | Dichloro diphenyl trichloro ethane |
| DH | Déshydrogénase |
| DNS | Dinitro salicylic acid |
| DO | Dissolved oxygen |
| DS | Dissolved solids (g/L) |
| ECM | Ectomycorrhizae |
| EDTA | Ethylene diamine tetra acetate |
| FL | Fermentation liquide |
| FS | Fermentation solide |
| GUI | Graphical user interface |
| HAP | Hydrocarbures aromatiques polycycliques |
| *I* | Inhibition index |
| IU | International units |
| $k_La$ | Coefficient volumétrique de transfert de l'oxygène/volumetric oxygen transfer coefficient |
| LB | Lutte biologique |
| LF | Liquid fermentation |
| LiP | Lignin peroxidase |
| MCPA | 2-methyl-4,6-dicholorophenoxy acetic acid |
| M-CSL | Mélasses - liqueur de maïs |
| MEA | Extrait de malt avec agar |
| MIP | Manganese independent peroxidase |
| MME | Milieu minimal essentiel |
| MnP | Manganese dependent peroxidase |
| NH | Non-hydrolyzed sludge |
| NS | Nitrate de sucrose |
| OD | Oxygène dissout |
| OTR | Oxygen transfer rate (mmol/L.h) |
| OUR | Oxygen uptake rate (mmol/L.h) |
| PA | Protease activity (IU/ml) |
| PAH | Polycyclic aromatic hydrocarbons |
| PCB | Polychloro biphenyls |
| PDA | Agar de dextrose de pomme de terre |
| PDA | Potato dextrose agar |
| PID | Proportional integral differential control |

| | |
|---|---|
| RH | Relative humidity |
| RQ | Quotient respiratoire |
| SB | Spruce budworm |
| SBU | Spruce budworm units |
| SCOD | Soluble chemical oxygen demand (mg/L) |
| SIW | Starch industry wastewater |
| SS | Suspended solids (g/L) |
| SSF | Solid state fermentation |
| TAH | Thermal alkaline hydrolyzed sludge |
| TG | Tartrate de glucose |
| TH25 | Thermal alkaline hydrolyzed sludge at 25 g/L suspended solids concentration |
| TH30 | Thermal alkaline hydrolyzed sludge at 30 g/L suspended solids concentration |
| TH35 | Thermal alkaline hydrolyzed sludge at 35 g/L suspended solids concentration |
| TNT | 2,4,6-trinitro toluene |
| TPH | Total petroleum hydrocarbons |
| TRS | Total reducing sugar (mg/L) |
| TS | Total solids (g/L) |
| TSA | Agar trypic du soja |
| TSB | Bouillon tryptique du soya |
| TSB | Tryptic soya broth |
| Tx | Entomotoxicity (SBU/µL)/Entomotoxicité |
| UFC | Unité de formation des colonies |
| WRF | White rot fungi |

* Each article contains its own set of abbreviation

# CHAPITRE 1.

# SYNTHÈSE

## 1.1. Introduction

Les agents de lutte biologique (ALBs) basés sur *Trichoderma* spp. ont retenu l'attention des chercheurs au cours des dernières décennies. Ces champignons antagonistes sont connus pour être très efficaces contre plusieurs maladies phytopathogéniques reliées aux sols, ainsi que pour la dégradation de composés aromatiques (toxiques) présents dans les sols. ***Les multiples enzymes lytiques, les antibiotiques et les hormones pour la croissance des plantes produits par ces champignons leur confèrent la capacité de protéger les plantes contre les phytopathogènes, ainsi que d'accroître le contenu en nutriments des sols*** (Bae *et* Knudsen, 2007; Singh *et al.*, 2007; Prabavathy *et al.*, 2006; Harman *et al.*, 2004; Papagianni, 2004; Whipps *et* Lumsden, 2001; Lewis *et* Papavizas, 1983). Ainsi, les ALBs basés sur *Trichoderma* spp. ont un grand potentiel parmi les autres ALBs fongiques. Cependant, malgré qu'il soit très efficace en lutte biologique (Suarez-Estrella *et al.*, 2007; Rabeendran *et al.*, 2006; Saraswathy *et* Hallberg, 2002; Baarschers *et* Heitland, 1986), la littérature sur le procédé de production de *Trichoderma* spp. est limitée. De plus, la présence de ces ALBs dans le marché est aussi marquée par un manque de recherche sur leur production de masse et c'est pourquoi des études sont requises.

En général, les ALBs sont définis comme étant des formulations biologiques, capables d'améliorer le rendement des cultures à la fois comme pesticide et promoteur de la croissance végétale. Présentement, il existe une vaste gamme d'ALBs qui sont utilisés avec succès dans plusieurs applications sur le terrain. Ces ALBs peuvent être des plantes, des virus, des bactéries, des champignons, des protozoaires, des nématodes et presque n'importe quelle forme de vie (organisme tel quel et/ou le(s) métabolites produits). Cependant, après les bactéries, les ALBs fongiques sont de plus en plus acceptés, principalement en raison de leurs applications étendues en termes de ***contrôle de maladie et de rendement de production***.

Tel que mentionné précédemment, les espèces de *Trichoderma* spp. ont été étudiées par plusieurs chercheurs pour produire de nombreux produits fongiques détenant un fort potentiel comme ALBs. Selon Whipps *et* Lumsden (2001), les espèces de *Trichoderma* spp. partagent environ 50% du marché d'ALBs fongiques et cela fait d'eux des candidats intéressants pour la recherche. Les champignons *Trichoderma* sont bien connus pour leur action simultanée comme antagonistes et promoteurs de croissance végétale. Ils ont été

utilisés contre plusieurs phytopathogènes comme par exemple *Rhizoctonia* spp., *Pythium* spp., et *Fusarium* spp.. L'activité comme ALBs des champignons *Trichoderma* est due à différentes enzymes et composés antibiotiques produits ainsi qu'à la nature compétitive de leur croissance et reproduction (Leandro *et al.*, 2007; Prabavathy *et al.*, 2006; Benhamou *et* Picard, 1999; Esposito *et* da Silva, 1998; Ejechia, 1997).

Il a été reporté que les espèces de *Trichoderma* spp. peuvent être utilisées efficacement en terme de spores (spécialement les conidies) qui sont plus tolérants aux conditions environnementales défavorables lors de la formulation du produit et de l'application sur le terrain (Lewis *et* Papavizas, 1983). D'un autre côté, la présence de la biomasse mycélienne des champignons *Trichoderma* a été rapportée comme cruciale pour l'action antagoniste. Ainsi, un procédé de production d'ALBs basés sur des champignons *Trichoderma* devrait considérer les conidies et le mycélium. Les conidies et le mycélium peuvent être produits soit par fermentation solide ou liquide. Néanmoins, la plupart des chercheurs ont suggéré la fermentation liquide plutôt que la fermentation solide pour la production de masse en raison des problèmes associés à l'automatisation du procédé ainsi qu'à la manipulation de la matière première et du produit.

Malgré leur grand potentiel comme ALBs, l'exploitation efficace des *Trichoderma* spp. à leur plein potentiel n'est pas encore faite. De plus, la plupart des ALBs basés sur *Trichoderma* spp. ne sont pas homologués et sont simplement commercialisés comme des « améliorateurs de sols », probablement en raison des modes d'action plus ou moins bien définis de ces champignons. Des matières premières comme **le glucose, le sucrose, la liqueur de macération du maïs, le blé entier, la farine de soja, la farine de poisson, les déchets agricoles et quelques déchets industriels (ex. : pâtes et papiers, culture des canneberges)** ont déjà été utilisés pour leur production. Cependant, plusieurs facteurs tels que le coût élevé de production, la faible efficacité, le rendement faible en spores et les difficultés à quantifier leur activité comme ALBs sont des raisons évidentes de la commercialisation infructueuse de ces ALBs. D'un autre côté, il a été démontré que ***diverses eaux usées et boues d'épuration*** peuvent être une riche source de nutriments et qu'elles peuvent être employées pour la production de biopesticides basés sur *Bacillus thuringiensis*. En fait, ***aucune étude n'est disponible jusqu'à maintenant en ce qui concerne la production d'ALBs basés sur Trichoderma spp. en utilisant ces matières premières à coût moindre.*** Ainsi, le développement méticuleux d'un procédé pour obtenir

des ALBs basés sur *Trichoderma* spp. et ces matières premières apparaît comme une approche prometteuse pour un environnement sécuritaire et propre.

Le présent chapitre a été organisé de la manière suivante. La revue de littérature contient des généralités sur les champignons utilisés en bioremédiation et pour l'amélioration des sols avec des exemples spécifiques de *Trichoderma* spp. De plus, *Trichoderma* spp. fait l'objet d'une discussion sur son rôle comme ALBs. La biodégradation et la rhéologie des boues d'épuration sont aussi présente pour évaluer si les boues d'épuration sont adéquates comme matière première pour la fermentation de *Trichoderma* spp. Ceci est suivi par une discussion des problèmes dans ce secteur, des hypothèses et des objectives proposés pour les vérifier. Les faits saillants sur l'utilisation des boues d'épuration comme milieu de production en fioles et en fermenteur de laboratoire sont ensuite présentés. L'étude de différents rejets industriels pour la production d'ALBs basés sur *Trichoderma* spp. est également exposée. Plus particulièrement, la possibilité d'utiliser les eaux usées de l'industrie de l'amidon comme une matière première et les différentes stratégies de production pour accroître le potentiel de *Trichoderma* spp. comme ALBs sont discutées. Finalement, cette synthèse de termine par une étude technico-économique du procédé de production de *Trichoderma* spp. à partir de différentes matières premières.

## 1.2. Agents fongiques de lutte biologique

### 1.2.1. Rôle dans l'amélioration du contenu en nutriments des sols et en bioremédiation

Au cours des deux dernières décennies, la sensibilisation du public envers les pesticides chimiques toxiques a accru la demande pour les aliments biologiques et par conséquent, la demande pour les ALBs. Les champignons sont de forts agents de bioremédiation des sols qui peuvent transformer les contaminants comme les déchets de munition, les pesticides, les organochlorés, les biphényls polychlorés (BPCs), les hydrocarbures aromatiques polycycliques (HAP), les colorants synthétiques, les préservatifs du bois et les polymères synthétiques, lesquels peuvent être dégradés en des formes moins nuisibles (Pointing, 2001). Plus particulièrement, les ALBs fongiques comme *Trichoderma* spp. ont été utilisés pour la bioremédiation des sols et l'accroissement de leur contenu en nutriments. Présentement, le rôle des ALBs est devenu plus important en rivalisant et même en remplaçant leurs homologues chimiques, spécialement avec

l'avenue des champignons antagonistes (Whipps *et* Lumsden, 2001; Chet, 1993; Templeton *et* Heiny, 1989). Les ALBs basés sur des champignons fongiques suivent de près ceux basés sur des bactéries (principalement *Bacillus thuringiensis*) en raison de leur action combinée en termes de contrôle de plusieurs maladies et de stimulation de la croissance végétale via l'amélioration des nutriments dans les sols (Copping *et* Menn, 2000). Plusieurs chercheurs ont spécialement exploré l'application des champignons *Trichoderma* spp. pour améliorer leur contenu en nutriments des sols ou pour contrôler les maladies en raison de leur croissance agressive et leur nature antagoniste (Héraux *et al.*, 2005; Ortiz *et* Orduz, 2001; Yedidia *et al.*, 1999; Zheng *et* Shetty, 1998; Aziz *et al.*, 1997; Bonnarme *et al.*, 1997; Calistru *et al.*, 1997a, b; Ejechia, 1997; Benhamou *et* Chet, 1993; Ahmad *et* Baker, 1987).

### 1.2.2. *Trichoderma* spp. comme agents de lutte biologique

*Trichoderma* [*tricho* (ressemblant à des cheveux) + *derma* (peau)] est un genre de champignons imparfaits qui habitent les sols *et* qui fait partie de la classe des *Hyphomycètes* et de la famille des *Moniliaceae.* Quelques espèces produisent des trichothécènes (une toxine) et peuvent causer l'aleucie alimentaire toxique. Les *Trichoderma* spp. sont des champignons de l'ordre des *Hypocreaceae*, omniprésents et anamorphiques dans l'environnement (se reproduisent de façon asexuée), spécialement dans le sol, les plantes, la végétation en décomposition et le bois. Ces champignons sont communément reconnus pour leurs colonies à croissance rapide avec des filaments blanchâtres, jaunâtres ou verdâtres contenant souvent des spores. L'activité des *Trichoderma* spp. contre plusieurs champignons phytopathogéniques du sol a été reportée tardivement (Papavizas *et* Lewis, 1982), même s'ils étaient connus depuis 1865. Cependant, basés sur les études antérieures, la classification taxonomique suivante (figure 1.1) pourrait être attribuée à ces champignons :

1 Royaume: Fungi
2 Division: *Ascomycota*
*3* Classe: *Euascomycetes*
*4* Ordre: *Hypocreales*
5 Famille: *Hypocreaceae*
6 Genre: *Trichoderma*

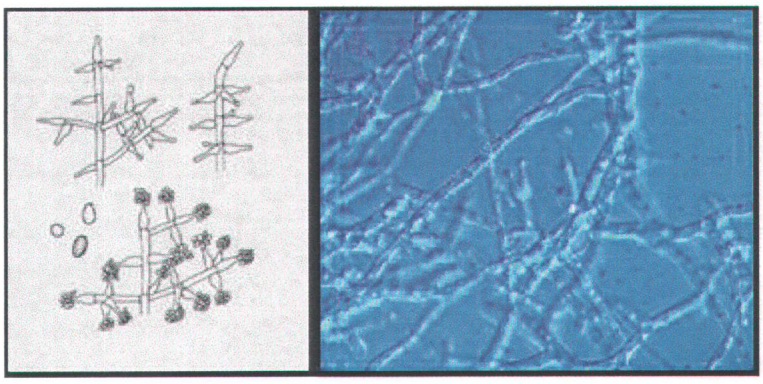


**Figure 1.1. Diagramme et image actuelle de *Trichoderma* spp.**

Un maximum de 33 taxons a été reporté jusqu'à maintenant pour ce genre (Samuels *et al*. 2004). Cependant, *Trichoderma viride, Trichoderma ressei, Trichoderma harzianum, Trichoderma virens* (autrefois connue comme *Gliocladium virens*), *Trichoderma koningii, Trichoderma longibrachiatum* et *Trichoderma pseudokoningii* sont les espèces les plus communes et sont très importantes pour leur application comme ALBs (Suarez-Estrella *et al*., 2007; Rabeendran *et al*., 2006; Ejechia, 1997; Papavizas, 1985). Quelques uns de ces champignons qui sont utilisés commercialement comme ALBs sont présentés au Tableau 1.1.

### 1.2.3 Innocuité environnementale de *Trichoderma* spp.

Comme il a été mentionné dans les sections précédentes, les *Trichoderma* spp. sont considérés comme des candidats potentiels pour des ALBs multi-fonctionnels et efficaces (Celar 2003; Calistru *et al*., 1997a,b; Lin *et al*., 1994). Bien que les *Trichoderma* spp. sont potentiellement des champignons non pathogènes et sont généralement considérés comme des microorganismes sécuritaires (Suarez-Estrella *et al*., 2007; Headon *et* Walsh, 1994), ils peuvent causer des infections dans des circonstances particulières comme par exemple chez les patients immunodéprimés (cas de neutropénie ou de transplantation), ayant des problèmes chroniques au niveau des reins et des poumons ou souffrant d'amyloïdose (Groll *et* Walsh, 2001). Cependant, cela peut s'appliquer pour pratiquement tous les microorganismes et les *Trichoderma* spp. ne devraient pas être considérés comme des organismes pathogènes ou écologiquement nuisibles. De plus, plusieurs études appuient la nature non pathogène des *Trichoderma* spp. (Prabavathy *et al*., 2006; Benhamou *et*

Brodeur, 2000; Benhamou *et al.*, 1999; Chet, 1993; Chet, 1987). Fekete *et al.* (1997) ont testé des *Trichoderma* spp. pour leur production de trichodiènes, soit des époxides sesquiterpenoïdes produites par quelques espèces de différents genres de champignons et connues pour causer des maladies sérieuses auprès des humains et des animaux (Marasas *et al.*, 1984). Cependant, les auteurs pouvaient seulement obtenir des signaux d'hybridisation cohérents, mais faibles, de la séquence codant pour la synthèse de la trichodiène durant le test de séquençage des *Trichoderma* spp. Cela suggère aussi que les *Trichoderma* spp. sont de faibles producteurs de trichodiène et qu'ils ne s'imposent pas comme un agent toxique dans l'écosystème alors que cette particularité contribue peut-être à améliorer la performance de ces champignons comme ALBs.

### 1.3. Caractéristiques essentielles des ALBs fongiques

Au cours des 25 dernières années, les *Trichoderma* spp. ont reçu beaucoup d'attention des chercheurs en raison de leurs caractéristiques adéquates pour leurs applications comme ALBs. Toutefois, pour connaître des succès commerciaux avec les *Trichoderma* spp., les éléments suivants sont requis :

- ❒ Production économique et abondante de propagules microbiens (spores)
- ❒ Tolérance envers plusieurs unités d'opération durant le traitement en aval
- ❒ Stabilité et durée de conservation adéquates de la formulation du produit
- ❒ Faible coût de préservation intensive et d'entreposage de la formulation de produit
- ❒ Commercialement faisable, efficace sur le terrain sous différentes conditions
- ❒ Interactions distinctes avec les microorganismes pathogéniques et bénéfiques
- ❒ Sécuritaire envers la santé humaine et environnementale
- ❒ Compatibilité avec les équipements existants pour l'application
- ❒ Activité résiduelle prolongée durant l'application sur le terrain

*Trichoderma* spp. remplissent la plupart des critères mentionnés précédemment en plus d'avoir certaines particularités qui placent ces champignons dans la catégorie des ALB à large spectre. Il est estimé qu'en améliorant efficacement les procédés de production, *Trichoderma* spp. pourraient s'avéré être d'excellents ALB, remplaçant

efficacement certains pesticides toxiques (Gerhardson, 2002; Harmann *et* Björjmann, 1998; Howell *et al.*, 1997), si le mécanisme impliqué dans l'effet antagoniste de ces champignons est bien compris. L'analyse des études mentionnées révèlent que malgré le fait qu'ils soient d'excellents ALB, les espèces de *Trichoderma* ne sont pas suffisamment exploitées, de plus, leur mode d'action n'est pas encore totalement élucidé. Les faits précédemment mentionnés sont peut-être la raison expliquant l'importante part du marché de *Trichoderma* spp. (50% des ALB fongiques) mais seulement une fraction de *Trichoderma* spp. sont homologués (20% de ALB sont basés sur *Trichoderma*) (Whipps *et* Lumsden 2001). Les connaissances fragmentées sur les produits à base de champignon *Trichoderma* apportent des conséquences durant l'application ou les effets secondaires (Chet, 1993). Par conséquent, la compréhension des nombreux facteurs concernant les interactions antagonistes de ces champignons serait d'un grand intérêt.

**Tableau 1.1.** Liste de quelques *Trichoderma* spp. et leur caractéristiques en tant que ALB (Agent de Lutte Biologique)

| **Agent actif** | **Antagoniste contre** | **Enzymes responsalbes/ Métabolites** | **Maladie/ contrôle épidémiologique ; Références** |
|---|---|---|---|
| *T. harzianum* 1051<br>*T. harzianum 39.1* | *Crinipellis perniciosa* (champignon) | Chitinase, N-acetylglucosaminidase, β-1,3-glucanase, cellulase totale, endoglucanase, aryl- β-glucosidase, β-glucosidase, protéase et amylase | "Balais de sorcière"(*Marasmius perniciosus* Stahel) de cacao (Lisboa de Marco *et al.*, 2003) |
| *T. lignorum* | *Rhizoctonia solani* (champignon) | Substances inhibitrices inconnues | "Damping-off"de haricot (Aziz *et al.*, 1997) |
| *T. viride*<br>*T. harzianum* | *Aspergillus flavus* et *Fusarium moniliforme* (champignon) | Enzymes lipolytiques, protéolytiques, pectinolytiques et cellulolytiques. Composé antibiotique inconnu (mycotoxines) (ex : peptides, polypeptides cycliques) | Fongique – associé à la semence (Calistru *et al.*, 1997a,b) |
| *T. harzianum* BAFC 742 | *Sclerotinia sclerotiorum* BAFC 2232 (champignon) | 1,3-β-glucanase et chitinase | Fongique – graine de soya (Menendez et Godeas, 1998) |
| *T. viride, T. koningii*<br>*T. longibrachiatum*<br>*T. hamatum, T. harzianum* | *Atta cephalotes* (fourmis champignonniste coupeuses de feuilles) | 1,3-β-glucanase, chitinases, protéases, et lipases. Composés antibiotiques inconnus | Dommage des feuilles par les fourmis (Lopez *et* Orduz, 2003; Ortiz *et* Orduz, 2000) |
| *T. harzianum* Rifai ITEM 908 et ITEM 910 | *Schizaphis graminum* (puceron) | Polysaccharide lyases, protéases, et lipases | Toxicité en cultures céréalières (Ganassi *et al.*, 2000) |
| *T. sp.* | *Sclerotium rolfsii* (champignon) | – – | Putréfaction de légumes communs (Mukherjee *et* Raghu, 1997) |
| *T. harzianum* 25<br>*T. viride* | *Serpula lacrymans* (champignon) | Antibiotique; anthraquinones | Dégradation fongique du bois (Score *et* Palfreyman, 1994) |
| *T. harzianum* | *Alternaria alternata* (champignon) | Endo-chitinase | Phytopathologies fongiques (Roco *et* Pérez, 2001) |

## 1.4. Mode d'action de *Trichoderma* spp.

Les connaissances sur le mode d'action de *Trichoderma* spp. – son application dans l'environnement, incluant pathogène, plante, et sol, de même qu'une classification en accord avec la littérature seront discutées dans cette section.

### 1.4.1. Activité indirecte induisant la croissance (lutte biologique direct)

#### 1.4.1.1. Antibiose:

Les métabolites antagonistes (antibiotiques) produits par *Trichoderma* spp. tuent les pathogènes. Il y a une énorme quantité d'antibiotiques (ex. peptides, polypeptides cycliques) qui sont produits par *Trichoderma* spp., comme la trichodermine isolé d'un bouillon de culture d'un souche de *Trichoderma viride* (Godtfredsen, 1965), et la gliotoxine isolée d'une souche de *Trichoderma viride* qui est efficace contre *Pythium* sp. (Whipps *et* Lumsden, 1991). Howell (1982) a mentionné la production d'un large spectre d'antibiotiques par *Trichoderma virens* tels, la gliovirine, la gliotoxine, le viridian, ainsi que le viridiol, un dihydrodérivé de la viridine qui inhibe la croissance de certaines plantes considérées comme des mauvaises herbes en agriculture. On note également la production d'un inhibiteur ribosomal fongicide, la tricholine, obtenu de *Trichoderma viride* qui agit sur *Rhizoctonia solani* grâce à un mécanisme « multi-coups » (Trillas *et al.*, 2006; Lin, 1994). De plus, l'acide heptelidique isolé de *Trichoderma viride* possède une activité antibiotique contre des bactéries anaérobies (Orgaz *et al.*, 2006; Stipanovic *et* Howell, 1983), *Pythium ultimum* et *Rhizoctonia solani* (Howell, 1982).

#### 1.4.1.2. Mycoparasitisme:

*Trichoderma* spp. s'enroulent autour du champignon pathogène, pénètre la paroi cellulaire, introduits ses toxines (tuant alors l'hôte), puis le consument. Ejechia (1997) a étudié la capacité de *Trichoderma viride* à retarder la pourriture du bois d'obeche (*Triplochiton sceleroxylon*) sur le terrain sur une période de onze mois couvrant les saisons sèches et humides du climat tropical. L'utilisation de *Trichoderma viride* a été très encourageante car cela exprimait une inhibition totale de la pourriture par la présence de mycoparasitisme. La même expérience a été effectuée avec *Penicillium sp.* démontrant que *Trichoderma* sp. était deux fois plus efficace que *Penicillium sp.* Le mycoparasitisme est associé et suivi de "l'antibiose". Cook *et* Rayner (1984) classifient les interactions

mycoparasitiques comme (1) un remplacement (antagonisme unilatéral), (2) une impasse (antagonisme mutuel), et (3) le mélange (pas d'antagonisme). Le processus du mycoparasitisme semble être assez bien expliqué. Cependant, le phénomène exact impliqué dans l'action antagoniste de *Trichoderma* spp. n'est pas encore bien compris, mais il semble que ces champignons antagonistes tuent l'organisme (généralement des champignons pathogènes) avec certaines toxines et les consument subséquemment en utilisant une combinaison de lysozymes (http://www.botany.utoronto.ca/ ResearchLabs/MallochLab/index.html).

1.4.1.3. Compétition:

Contrairement à la plupart des pathogènes, étant à croissance rapide, *Trichoderma* spp. limitent la croissance des pathogènes en exerçant une forte compétition pour les nutriments limités. Les nutriments essentiels sont souvent présents à l'échelle du microgramme dans les habitats naturels de tous les microorganismes, spécialement ceux présents dans le sol et les plantes. Ceci résulte en une compétition subséquente pour les nutriments parmi les microorganismes (Leandro *et al.*, 2007; Singh *et al.*, 2007; Trillas *et al.*, 2006). Récemment, des chercheurs ont également conclu qu'une telle compétition pour les nutriments occasionnait un stress et menait à augmenter la formation de métabolites avec des effets antagonistes. Cette compétition affecte donc grandement le caractère antagoniste *Trichoderma* spp. De plus, ces champignons antagonistes peuvent aussi compétitionner et séquestrer les ions métalliques (essentiels pour le phytopathogène *Serpula lacrymans* pour la formation d'un complexe non-enzymatique) en relâchent des composés connus sous le nom de sidérophores (Srinivasan *et al.*, 1992). La faisabilité *in situ* de l'exemple précédent n'a pas été vérifiée. Cependant, la possibilité d'une action multi-antagoniste sur une grande quantité de substrats par *Trichoderma* spp. ne peut être écartée.

1.4.2. Activité inductrice de croissance directe (activité de lutte biologique indirecte)

1.4.2.1. Phytohormone de croissance, vitamines et induction de la défense:

Par le fait qu'il soit un champignon, la production de métabolites comme des hormones et des vitamines est très commune dans *Trichoderma* spp. Plusieurs chercheurs ont suggéré que la production d'hormones de croissance et l'augmentation du transfert des

minéraux à la rhizosphère agissait comme facteur primordiaux permettant la performance spectaculaire des ALB composés de champignons *Trichoderma* (Prabavathy *et al.*, 2006; Benhamou *et* Picard, 1999).

En plus de la nature mycoparasitaire des champignons *Trichoderma*, l'induction de la résistance contre les pathogènes des plantes a été reportée par Prabavathy *et al.* (2006), Yedidia *et al.* (2000), Benhamou *et* Picard (1999) et Zeilinger *et al.* (1999). Yedidia *et al.* (1999) a suggéré que l'association de *Trichoderma* avec la rhizosphère réduirait les maladies racinaires par l'activation des mécanismes de défense de la plante plutôt que seulement l'action antagoniste du champignon. L'induction des protéines reliées à la pathogénicité (PR: *pathogenesis related*) est le principal facteur contrôlant l'action apparente de *Trichoderma* spp. L'auteur à également mentionné la possibilité de la présence de différents mécanismes d'induction pour chaque type d'enzyme chitinolytique et la nécessité de caractériser ces enzymes et leur importance dans les mécanismes de lutte biologique.

1.4.2.2. Production enzymatique – rôle dans l'activité des ALB:

Les composés simples (à l'état assimilable par la plante) sont fournis par les enzymes lytiques produits.

Outre les composés antibiotiques, *Trichoderma* spp. sont aussi connus pour être des synthétiseurs prolifiques de polysaccharides lyases, de protéases et de lipases, tous ces enzymes pouvant jouer un rôle crucial dans la dégradation des cellules de l'hôte. (Benhamou *et* Chet, 1993). De plus, des souches de *Trichoderma viride* ont été testées avec succès pour la présence de chitinase *in vitro* (Domsch *et al.*, 1980) alors que *Trichoderma harzianum* est connu pour produire des enzymes cellulotytiques (Liu *et* Xia, 2006; Donzelli *et al.*, 2003; Petenbauer *et* Heidenreich, 1992; Harman *et al.*, 1993; Ahmad *et* Baker, 1987). L'importance de ces enzymes pour une activité d'ALB a été répertoriée au Tableau 1.1.

1.4.2.3. Enrichissement de la rhizosphère:

La présence physique de masse mycélienne dans la rhizosphère sert d'engrais pour la rhizosphère normale des plantes.

Lindsey *et* Baker (1967) ont démontré que la fortification de la rhizosphère causée par *Trichoderma viride,* en aidant la croissance de tomates gnotobiotiques (soit, bien définie, ou aucune microflore), démontre alors l'activité inductrice de croissance par ce champignon.

### 1.5. Eaux usées, boues d'épuration et *Trichoderma* spp.

*Trichoderma* spp. n'ont pas été beaucoup exploités, premièrement dû à l'absence de procédé commercialement viable. En d'autre mots, la plupart des procédés actuels utilisent des matières brutes dispendieuses et obtiennent un faible rendement en spores, un facteur important pour leur succès comme ALB. Par ailleurs, les eaux usées et les boues d'épuration constituent d'importants problèmes de pollution environnementale et représentent un défi qu'en à leur destruction. Par conséquent, l'utilisation des eaux usées municipales et de certaines eaux usées industrielles et leur boue respective pour la production de ALB à base de champignons *Trichoderma* semble très prometteuse. Il a été reporté que plusieurs boues et eaux usées sont riches en composés biodégradables. Ceux-ci pourraient donc être utilisées comme substrat à coût « négatif » pour la production de *Trichoderma* spp. Cependant, ***jusqu'à ce jour, aucune étude n'a été réalisée sur l'utilisation des eaux usées municipales ou industrielles, ou de leur boues respectives pour la production de ALB à base de Trichoderma.*** Cependant, il existe quelques études rapportant la production efficace à partir de substrats renouvelables (notamment avec des eaux usées et des boues d'épuration) de biopesticides à base *Bacillus thuringiensis* (Tirado-Montiel *et al.*, 2003, 2001; Vidyarthi *et al.*, 2002, 2001; Lachhab *et al.*, 2001; Sachdeva *et al.*, 2000).

Tel que mentionné plus tôt, les informations sur l'utilisation des eaux usées et des boues d'épuration comme matière brutes pour la production de champignon *Trichoderma* demeurent insuffisantes, mais il existe cependant quelques références à ce sujet qui seront discutées ultérieurement (ex : Karim *et* Kamil, 1989). Toutefois, ***aucune de ces études ne portent sur la production de ALB à partir de ces substrats renouvelables.*** Récemment, Molla *et al.* (2004), Alam *et* Fakhru'l-Razi (2003) et Molla *et al.* (2002) mentionnaient l'utilisation de *Trichoderma* spp. pour le traitement des eaux usées et des boues d'épuration pour dégrader la matière organique et les composés organiques toxiques. Cependant, ces auteurs ne pouvaient envisager l'idée d'utiliser ces déchets comme matière

première pour la production de produits à valeur ajoutée comme des ALB à base de *Trichoderma* spp. (métabolites antagonistes et spores).

## 1.6. Production de *Trichoderma* spp.

Les propagules microbiennes de *Trichoderma* spp. peuvent être produites sous trois formes, à savoir, le mycélium, les conidies et les chlamydospores (Lewis *et* Papavizas, 1983). Cependant, les trois propagules possèdent des caractéristiques distinctes en terme de productivité, de stabilité et d'activité comme ALB. Il est alors impératif de choisir la forme idéale de *Trichoderma* spp. pour leur utilisation en ALB de façon à activer efficacement les modes d'action des champignons.

En fait, le mycélium peut avoir une très bonne activité comme ALB, mais celui-ci ne survit pas lors des procédés en aval tel que le séchage, donc demeure inutilisable comme ALB (Amsellem *et al.*, 1999). Par ailleurs, les chlamydospores requièrent une croissance de 2 à 3 semaines et ne peuvent non plus survivre au séchage (Lewis *et* Papavizas, 1983); cependant, ils demeurent préférables au mycélium. Les conidies, par contre, possèdent une très bonne activité de lutte biologique, sont moins susceptibles aux conditions environnementales et peuvent être produits rapidement. La production de *Trichoderma* spp. sous forme de conidies s'avère donc la meilleure option en regard à son utilisation comme ALB. Cependant, la présence de mycélium parmi les conidies ne peut être éliminée. De plus, la présence simultanée de mycélium et de conidies peut assurer la présence de nombreux métabolites essentiels (ex. d'antibiotiques) utiles pour l'activité de ALB. Pour cette raison, ***la production de Trichoderma spp. contenant des conidies comme propagules principales ainsi que du mycélium semble être la meilleure stratégie.*** Malheureusement des informations cruciales sur les facteurs contrôlant la sporulation chez les champignons n'ont pas été étudiés en détail, c'est pourquoi ***la production de spores demeure un défi de taille.***

Les procédés de production de *Trichoderma* spp. consistent en des conditions environnementales et de cultures appropriées et un milieu de culture optimisé. Généralement, ce procédé peut être classé en deux catégories ; a) la fermentation liquide (FL) et b) la fermentation sous forme solide (FS). Ceux deux techniques (FL et FS possèdent certains avantages et désavantages qui sont résumés au Tableau 1.2. De ce tableau, on retire que la fermentation liquide est plus avantageuse que la fermentation avec

solides. Cependant, la sporulation doit être améliorée pour la FL afin de réaliser le plein potentiel de cette technique.

**Tableau 1.2.** Avantages et inconvénients des procédés de FL et de FS

| *FL* | *FS* |
|---|---|
| ➢ Moins exigeant | 👎 Demande beaucoup de travail |
| ➢ S'adapte à la production de masse | 👎 Difficile à faire la mise à échelle |
| ➢ Paramètres opérationnels faciles à contrôler | 👎 Paramètres opérationnels difficiles à contrôler |
| 👎 Coût d'opération élevé– forte agitation requise et bonne aération | ➢ Faible coût d'opération – besoin minimal d'agitation et d'aération |
| ➢ Meilleure productivité (rendement/temps) | 👎 Faible productivité (rendement/temps) |
| ➢ Facile à manipuler (pompage, lignes pressurisé) | 👎 Matériel difficile à manipuler |
| ➢ Compatible avec les bioréacteurs existants | ➢ Plus adaptée à la production de petits volumes |
| 👎 Sporulation plus faible | ➢ Bonne sporulation |
| ➢ Meilleur pour la production de certains métabolites | ➢ Efficace pour certains métabolites |

1.6.1. Fermentation liquide

Lewis *et* Papavizas (1982) ont examiné la faisabilité de certains milieux liquides, par exemple mélasses - liqueur de maïs (M-CSL), le nitrate de sucrose (NS), et le tartrate de glucose (TG) comme milieu de culture (fermentation statique et submergée). Ces auteurs ont observé que le milieu M-CSL, et le TG étaient supérieurs au milieu NS en FL. Une concentration maximale de chlamydospores de l'ordre de $10^9$ spores $g^{-1}$ poids sec de mycélium a été atteinte, ce qui ne représente pas une valeur très élevée si l'on transforme la base de poids sec de mycélium en unité de volume de milieu (par exemple., le nombre de spores par ml de bouillon fermenté) car le calcul mathématique « du poids sec de mycélium » était très inférieur en quantité si on le compare au « volume du bouillon fermenté », le dénominateur dans cette expression mathématique pour la « concentration de spores » devient inférieure, donnant alors une valeur apparemment élevée de

l'expression de la concentration de spores. Une différence de 550 fois a été estimée entre ces deux unités conventionnelles. Ce problème requière une attention particulière. Par conséquent, une attention appropriée devrait être portée à ce fait, sans quoi il faut estimer le potentiel des ALB d'un produit à base de spores de *Trichoderma* spp. Par ailleurs, Lewis *et* Papavizas (1982) ont démontré avec succès que la fermentation liquide produit également un nombre comparable de conidies ($\approx 10^8$-$10^9$ spores/g mycélium sec) et davantage de chlamydospores sont formés comparativement au milieu solide. Un inconvénient du procédé demeure le temps d'incubation très long (3 semaines) et le coût des matières premières, ce qui rend difficile son application à l'échelle industrielle. Par dessus tout, la concentration actuelle de spores par ce procédé reste faible, ce qui s'avère un désavantage majeur pour ce procédé.

Pour la production de spores de *Trichoderma* spp., l'utilisation de milieu à base de D-glucose, de cellulose, ou d'amidon soluble a également été rapportée par quelques auteurs (Gupta *et al.*, 1997; Saxena *et al.*, 1993). Ceux-ci ont obtenu une sporulation maximale de l'ordre de $10^8$ spores par ml de bouillon fermenté. Cependant, la production de spores de *Trichoderma* spp. pour leur usage en ALB, en utilisant ces substrats, semble ne pas être rentable, dû au coût élevé des matières premières. Karim *et* Kamil (1989) ont suggéré l'utilisation des rejets liquides de l'industrie de l'huile comme substrat pour la production de *Trichoderma* spp. de manière a obtenir des protéines brutes, des enzymes et une masse de mycélium. Cependant, le temps de fermentation était plus long ($\geq$ 2 semaines) et le potentiel des produits fermentés pour être utilisés comme ALB n'a pas été mesuré par ces auteurs. Felse *et* Panda (2000, 1999) ont reporté l'utilisation de carapaces de crabes non traitées. Olsson *et al.* (2003) ont utilisé la cellulose, de même que la chair de betterave à sucre crue ou traitée sans l'ajout de nutriments, mais ceux-ci ont utilisé des composés du milieu de Mendels pour le développement de leur inoculation. Dans l'exemple précédent, les auteurs n'ont pas mesuré les spores mais ont noté une bonne production d'enzymes cellulolytique et hémicellulolytique. Malgré tout, la seule présence d'enzyme lytique dans les ALB à base de *Trichoderma* spp n'est pas suffisante pour assurer la viabilité lors de son utilisation. En effet, ***une concentration en spores plus élevée est également importante, car pendant l'application au sol, un nombre plus élevé de spores pourra assurer une meilleure prolifération de biomasse de mycélium de Trichoderma spp, ce qui aura une activité antagoniste contre les pathogènes.***

### 1.6.2. Fermentation solide

Hutchinson (1999) a évalué la possibilité d'utiliser un compost de fumier de poulet inoculé avec *Trichoderma virens* comme ALB contre les mauvaises herbes. Évidemment, ce procédé apparaît plus abordable que l'utilisation de grains de riz humidifié (Howell *et* Stipanovic, 1984), ou la mousse de tourbe enrichie. Néanmoins, il est moins efficace (exige plus de temps ≈8 semaines) donc demande plus d'études avant son application commerciale. Aussi, l'utilisation d'un rejet animal spécifique n'est pas nécessairement une solution durable (limitation possible sur la disponibilité en regard au moment et à l'endroit) pour la production de ALB à base de *Trichoderma* spp. De plus, la sporulation n'a pas été considérée comme un facteur important, pour cette raison, ce procédé pourrait ne pas être efficace à l'échelle de la production. De façon similaire, Trillas *et al.* (2006) ont évalué le potentiel de *T. viride* pour le compost agricole de *T. viride*, cependant, aucun procédé de production n'a été discuté.

Zheng *et* Shetty (1998) ont examiné les résidus des usines de transformation alimentaire (canneberge pommasse) pour la production de ALB orienté sur un inoculum de *Trichoderma* spp. Ces procédés se sont avérés efficaces avec une durée de fermentation d'environ quatre jours avec une croissance de mycélium suffisante pour avoir une activité de ALB (en utilisant l'inoculum fongique pour la dégradation de pesticides organochlorés dans le sol). Malheureusement, les auteurs n'ont pas mis beaucoup d'attention sur le niveau de sporulation et le choix de la matière première pourrait limiter géographiquement l'installation d'usines de production.

Score *et* Palfreyman (1994) ont observé l'effet des ALB sur la cellulose et un milieu de base contenant du glucose. Cependant, leur milieu ne peut être commercialement viable dû au coût élevé des matières premières. Certaines matières brutes comme la chitine de langoustines (Donzelli *et al.*, 2003), le vieux papier (van Wyk *et* Mohulatsi, 2003), la paille de blé oxydée et humide (Thygesen *et al.*, 2003) et les résidus d'épis de maïs (Xia *et* Shen, 2004) ont été évalués pour leur potentiel comme substrat pour la production d'enzymes par *Trichoderma* spp. Malheureusement, aucun auteur n'a essayé ces procédés pour la production de ALB à base de *Trichoderma* spp. Néanmoins, ces matières brutes pourraient aussi être examinées pour leur potentiel comme milieu de croissance pour la production de ALB par *Trichoderma* spp.

Il existe plusieurs autres exemples sur l'utilisation de milieux semi-synthétiques basée sur des milieux minimaux essentiels pour la production *Trichoderma* spp. (ex. PDA, Mandels' pectin agar) (Roco *et* Pérez, 2001). Ces milieux pourraient être seulement utilisés pour la préservation des souches et des recherches avancées sur ces champignons, et non pour la production à grande échelle dû simplement à leur coût élevé.

Par conséquent, la validation de la viabilité de ces procédés ne peut être faite. Reczey *et al.* (1996) et Vlaev *et al.* (1997) ont proposé l'utilisation du bois de saule traité à la vapeur (une matière lignocellulosique) comme une source de remplacement du glucose et de la liqueur de maïs en milieu de Tanaka. Kansoh *et al.* (2002) et Petre *et al.* (1999) sont quelques exemples supportant l'utilisation de matières brutes économiques pour des procédés de production de *Trichoderma* spp. Tel que mentionné précédemment, la plupart de ces études se sont ***concentrées sur la production de métabolites (enzymes et/ou antibiotiques) et non sur la sporulation***, qui est un facteur important pour une application efficace.

En général, il est rationnel de déduire que les procédés précédemment mentionné ne peuvent être exploités simplement à cause du coût trop élevé des matières brutes, du manque d'information sur le potentiel en ALB et la faible productivité de ces milieux en terme de sporulation et de durée de fermentation.

## 1.7. Comptage des spores

Une observation commune sur un problème dans le comptage des spores a été notée dans toutes les études discutées jusqu'à maintenant. Dans la plupart des cas, la microscopie à fond clair utilisant un hémacytomètre était utilisée pour le dénombrement des spores, mais cela ne représentait pas véritablement les spores viables (Stentelaire *et al.*, 2001). Ainsi, le potentiel comme ALB de ces procédés ne pouvait pas être estimé convenablement. De plus, quelques auteurs ont utilisé la technique d'étalement pour le comptage des unités formatrices de colonies, mais cette méthode est très difficile à appliquer avec les champignons en raison de la filtration qu'elle requière et du risque élevé de faire des erreurs. D'autres auteurs n'ont pas utilisé la technique d'étalement et ont plutôt opté pour les techniques alternatives suivantes : (1) mesure de l'ATP- (Gikas *et* Livingston, 1993) – dans cette technique, la biomasse cellulaire (mycélium et/ou spores) pouvait être corrélée avec la concentration totale et dissoute de l'ATP, mais la quantification précise des spores

est difficile ou impossible; (2) mesure de l'activité de la déshydrogénase (DH) – un sel de tétrazolium est réduit en une molécule colorée de formazan par les DH cellulaires spécifiquement produites par des cellules viables (Stentelaire *et al.*, 2001). Cependant, l'estimation du nombre de spores des ALBs à base de *Trichoderma* spp. peut seulement être effectuée avec la technique d'étalement pour le comptage des unités formatrices de colonies. ***Ainsi, il faut accorder une grande importance à ce problème pour faciliter l'évaluation de la production de conidies par Trichoderma spp.***

## 1.8. Facteurs régissant la sporulation et l'activité comme ALB

### 1.8.1. Inoculum

La morphologie des champignons et le déroulement de la fermentation sont principalement affectés par le volume, le type (spores ou cellules végétatives) et l'âge de l'inoculum. Dans le passé, plusieurs auteurs ont tenté de standardiser un inoculum du champignon *Aspergillus niger* pour la production d'acide citrique en fermentation sous culture submergée (Domingues *et al.*, 2000; van Suijdam *et al.*, 1980; Clark, 1961; Steel *et al.*, 1954; Martin *et* Waters, 1952). Leurs résultats suggéraient que de fortes concentrations en spores ($\geq 10^5$-$10^8$ spores/ml) n'étaient pas bonnes pour la formation d'agrégats *et* la sporulation. Ainsi, ces études suggèrent que les inoculums (sous la forme de spores) ne jouent pas un rôle direct dans la sporulation. Néanmoins, ils pourraient être importants pour la production de métabolites et de masse mycélienne qui sont responsables pour l'activité comme lutte biologique (LB). ***Éventuellement, le type, la quantité et l'âge de l'inoculum doivent être explorés pour améliorer la sporulation.***

### 1.8.2. Composition du milieu

#### 1.8.2.1. Ratio C:N

Une variation du ratio carbone : azote (C:N) dans le milieu peut contribuer à la sporulation (production de conidies) de *Trichoderma* spp.. Olsson *et al.* (2003) ont étudié l'initiation de la phase de sporulation de *Trichoderma reesei* Rut-30 et ont remarqué que la diminution du ratio C:N ratio avait un effet défavorable sur la sporulation. Cependant, les auteurs n'avaient pas quantifié la sporulation. Une étude réalisée par Maheshwari (1999) sur *Neurospora crassa* a donné des résultats en accord avec ceux de Olsson *et al.* (2003). Bosch *et* Yantorno (1999) ont observé des résultats différents avec *Beauveria* sp. Les

auteurs ont rapporté qu'une diminution du ratio C:N pouvait être favorable pour la sporulation lorsque la concentration en carbone est faible, alors que le contraire survenait à une forte concentration en carbone. ***Ainsi, la modification du ratio C:N reste une option viable pour des études ultérieures sur processus de sporulation de Trichoderma spp. dans le but d'augmenter la concentration en spores.***

Score *et* Palfreyman (1994) ont utilisé un milieu complexe standard (extrait de malt avec agar, MEA) et un milieu minimal essentiel (MME) conçus pour simuler le ratio C:N du bois infecté par des pathogènes. Dans le MME, la composition de l'azote (L-asparagine) et du fer ($FeCl_3$) variait respectivement de 0.4 mM à 7.9 mM et de 0.01 mM à 0.1 mM alors que, dans le MEA, les concentrations étaient de 40 mM et de 0.34 mM pour l'azote et le fer respectivement. Encore une fois, ces auteurs n'ont pas mesuré la concentration en spores. Cependant, leur étude supporte le fait que les composés chimiques du milieu peuvent avoir un effet significatif sur la nature de l'interaction comme ALB. Leurs résultats ont aussi révélé le fait que *Trichoderma* spp. peut être significativement antagoniste même à un faible contenu en azote. Ainsi, toute augmentation du ratio C:N qui diminue le contenu en azote n'affecterait pas l'activité comme ALB de ces champignons. En d'autres mots, ***les substrats avec des ratios C:N élevés (>8) pourraient être bénéfiques pour la sporulation et l'activité comme ALB***. Il est intéressant de constater que, tel que mentionné dans les sections précédentes, ***diverses eaux usées et boues d'épuration dont le ratio C:N se situe dans cette intervalle (>8–12) de valeur pourraient être des substrats de choix***. En conclusion, le ratio C:N affecte le processus de sporulation (concentration finale en spores) et doit être étudié dans le cas de matières premières économiques comme les eaux usées et les boues d'épuration.

1.8.2.2. Nature des sources de carbone et d'azote

La sporulation de *Trichoderma* spp. est grandement affectée par la nature des sources de carbone et d'azote utilisées comme substrat. Olsson *et al.* (2003) ont observé que la sporulation était retardée en augmentant la disponibilité des sources de carbone (par des prétraitements). Malheureusement, les auteurs n'ont pas mesuré la quantité de spores et des conclusions exactes n'ont pu être émises. Cependant, Maheshwari (1999) et Bosch *et* Yantorno (1999) ont mentionné que la complexité de la source de carbone induit la sporulation de divers champignons. D'un autre côté, Pascual (1997) a observé que la

peptone, les acides aminés libres (ex. : arginine), le mannose, le xylose et le fructose induisent des niveaux élevés de sporulation. Basés sur ces faits, il est possible que ***l'exploration de diverses sources de carbone et d'azote et leur pré-traitement soient une façon d'induire la sporulation***. Ainsi, l'addition de quelques sources de carbone complexes ou simples et/ou des sources d'azote aux eaux usées ou aux boues d'épuration pourrait être favorable au processus de sporulation.

1.8.2.3. Addition de composés induisant la sporulation

L'introduction de quelques agents déclencheurs peut être aussi favorable au processus de sporulation. Ces agents peuvent être des ions métalliques comme le manganèse (Papagianni, 2004) ou des composés organiques complexes. Roncal *et al.* (2002) ont identifié le conidiogénol, la coniodiogénone et les diterpènes tétracycliques qui ont des activités fortes et sélectives sur la conidiogénèse chez *Penicillium cyclopium.* Ces découvertes sont encore à une étape initiale et elles pourraient être utiles dans les applications à une étape plus avancée. Néanmoins, selon ces études, il apparaît que ***l'introduction de quelques ions métalliques et/ou des composés complexes dans le milieu de culture pourrait induire la sporulation.*** Heureusement, les eaux usées et les boues d'épuration sont des sources riches de différents métaux et de composés complexes, ce qui pourrait alors être utile pour atteindre une forte concentration en spores.

1.8.3. Paramètres environnementaux

Des paramètres environnementaux, soit la température, le pH, l'oxygène dissout, le gaz carbonique dissout, l'humidité et la rhéologie, sont cruciaux pour la croissance de tous les champignons et peuvent aussi jouer un rôle important dans la sporulation. D'après la littérature, la température est un paramètre peu important pour la sporulation (Carlsen, 1995) alors que le pH du milieu joue un rôle important. ***Bien que les champignons formant des conidies peuvent croître à une vaste gamme de pH, ils croissent et sporulent de façon maximale à une valeur près du pH neutre*** (Papagianni, 2004). ***L'oxygène dissout et le carbone organique dissout peuvent jouer un rôle dans l'induction de la sporulation, probablement en imposant des conditions de stress à la culture.*** La sporulation chez les champignons est aussi un important mode de reproduction et c'est pourquoi il est attendu que la sporulation de *Trichoderma* spp. pourrait être corrélée à leur

quotient respiratoire (RQ). ***Ainsi, une étude en fermenteur devrait être réalisée afin de vérifier ces faits***. Felse *et* Panda (2000) ont étudié l'effet des conditions d'agitation sur *Trichoderma* sp. et les auteurs ont déduit que des conditions d'agitation extrêmes n'étaient pas bonnes pour la croissance et la sporulation. De plus, ces paramètres environnementaux ont été optimisés par plusieurs chercheurs pour différents milieux conventionnels. ***Ces paramètres pourraient être optimisés pour atteindre la sporulation dans le cas des eaux usées et des boues d'épuration comme matière première, principalement à cause des caractéristiques non Newtoniennes de ces milieux.***

1.8.4. Technique de fermentation

Afin d'effectuer les manipulations proposées en considérant les paramètres régissant la sporulation et l'activité ALB (tels que mentionnés précédemment), des techniques de fermentation spécifiques sont requises. Des études concernant les modes de culture ***comme les cultures en continu ou en "fed-batch" permettraient de mieux contrôler la concentration de substrat (concentration en solides, ratio C:N), de la biomasse (mycélium et/ou concentration en spores), d'addition simple ou multiple de nutriments (stratégie d'alimentation pour des substrats complexes ou simples).*** Cela permettrait alors d'élaborer une stratégie pour induire la sporulation.

## 1.9. Besoins pour l'application et solutions conventionnelles ou futures

Pour *Trichoderma* spp., les besoins d'application peuvent varier de $10^{10}$ à $10^{11}$ spores viables par hectare de terrain (http://www.shaktibiotech.com; http://www.nutri-tech.com). De plus, pour quelques champignons (*Beauveria* sp., *Paecilomyces* sp., *Metarhizium* sp.), Vega *et al.* (2003) ont suggéré que la concentration maximale à appliquer sur le terrain soit de $10^{13}$ spores viables par hectare. Ainsi, ***une base standard de $10^{10}$- $10^{11}$ spores par hectare comme standard pour les besoins de l'application sur le terrain de Trichoderma spp.*** serait efficace et économique.

Il existe sur le marché quelques formulations de $10^{7}$-$10^{8}$ spores par gramme de produit. Cependant, les substrats coûteux à rendement faible ou modéré (en termes de spores) comme le glucose, la liqueur de macération du maïs et l'amidon soluble de ces formulations ne leur permettent pas d'être des ALBs économiquement viables. D'un autre côté, les produits basés sur des substrats conventionnels (glucose, amidon soluble et

liqueur de maïs) ou bons marchés contiennent normalement de $10^6$-$10^7$ spores par gramme de produit (Cho *et* Lee, 1999), ce qui demande de grandes quantités de produits pour obtenir une action antagoniste efficace. Afin de surmonter la limite liée au nombre de spores, Amsellem *et al.* (1999) ont proposé des méthodes de préservation de la masse mycélienne de quelques champignons antagonistes. Cependant, il y avait des inconvénients (forte perte de viabilité des mycéliums) et les méthodes ne pouvaient pas être directement appliquées à d'autres champignons comme *Trichoderma* spp. dans le cas présent. Quelques chercheurs ont même proposé des techniques avancées comme la manipulation génétique pour accroître la production de spores et/ou de métabolites impliqués dans la lutte biologique. Puyesky *et al.* (1999) ont exploré une protéine de surface d'une conidiospore de *Trichoderma* sp. afin de comprendre et d'améliorer le processus de sporulation. Cependant, leurs travaux étaient encore à l'étape préliminaire et requièrent des efforts additionnels avant toute application. D'autres auteurs ont aussi proposé d'utiliser la manipulation génétique pour accroître la production d'enzymes impliquées dans l'action antagoniste (Mandels *et* Andreotti, 1978; Nevalainen *et al.*, 1980; Durand *et al.*, 1988; Szengyel *et al.*, 2000; Zaldívar *et al.*, 2001) (Tableau 1.2). De plus, Hanson *et* Howell (2002) ont utilisé la manipulation génétique pour combiner les meilleures caractéristiques de deux différentes souches de champignons *Trichoderma* par la fusion de protoplastes. Ils ont combiné une souche fortement antagoniste et une souche hautement tolérante de *Trichoderma* sp. pour obtenir une seule souche avec les deux propriétés. Il est possible que des manipulations génétiques méticuleuses puissent fournir des solutions durables pour des ALBs fortement efficaces (forte production de spores et forte viabilité) puisque des auteurs ont rapporté des souches stables de *Trichoderma* spp. génétiquement modifiées (Prabavathy *et al.*, 2006; Harmann *et* Björjmann, 1998). Malheureusement, l'utilisation d'organismes génétiquement modifiés et de produits font face à des restrictions dans plusieurs pays, ce qui décourage l'utilisation de cette option potentiellement viable jusqu'à un certain point. Finalement, des composés induisant la sporulation ont aussi été proposés, mais ces options sont encore trop élémentaires pour leur utilisation à l'échelle réelle.

Il est possible d'affirmer que, pour une production économique et efficace d'ALBs à base de *Trichoderma* spp., une nouvelle gamme de substrats doit être explorée pour surmonter les faibles rendements en spores et les coûts élevés de la matière première. La réalisation d'expériences avec différentes eaux usées et boues d'épuration pourrait fournir

une solution durable à ce type de production comme c'est le cas pour *Bacillus thuringiensis*. De plus, certaines eaux usées municipales et industrielles et boues d'épuration peuvent être des sources très riches de carbone, d'azote, de phosphore et d'autres nutriments essentiels pour plusieurs procédés microbiens. Ces rejets contiennent souvent des polluants organiques comme des résidus de pesticides (ex. : pesticides organochlorés) et les souches de *Trichoderma* spp. sont très efficaces pour dégrader ces polluants (Zheng *et* Shetty, 1998). Ces rejets peuvent aussi contenir des métaux toxiques et les souches de *Trichoderma* spp. peuvent tolérer et même normaliser les fortes concentrations en métaux (par chélation des ions métalliques; Morales-Barrera *et al.*, 2006; Srinivasan *et al.*, 1992). Le fait soulevé précédemment renforce et encourage l'idée d'utiliser ces rejets comme substrats pour les ALBs basés sur *Trichoderma* spp.. Cette gamme de substrats alternatifs contient divers composés complexes avec les composantes essentielles. Ainsi, il est possible que l'un d'eux détienne des composés susceptibles d'induire la sporulation et peut-être même la croissance végétative du champignon.

Enfin, la demande importante pour des ALBs basés sur *Trichoderma* spp. et la demande subséquente pour de grandes quantités de déchets serviront à deux fins : (1) production durable d'ALBs basés sur *Trichoderma* spp.; et (2) gestion des déchets par une pratique novatrice et compatible avec l'environnement (réduction des émissions de gaz à effet de serre générés par l'incinération et des risques d'épandage des déchets dans les champs). Ces boues pourraient être utilisés dès maintenant pour la production d'ALBs basés sur *Trichoderma* spp. en autant qu'ils respectent les normes réglementaires pour le contenu en métaux.

## 1.10. Conclusions

Ainsi, l'interprétation faite de la littérature indique que le succès des ALBs basés sur *Trichoderma* spp. est possible si les facteurs pertinents dans le procédé de production sont considérés dans l'ensemble, c'est-à-dire, l'approvisionnement en matière première, du procédé de fermentation, la commercialisation, l'utilisation sur le terrain et les impacts environnementaux. De plus, toutes les études sur *Trichoderma* spp. répertoriées précédemment étaient marquées par des inconvénients au niveau de l'efficacité du produit et de l'aspect économique du procédé. D'un autre côté, plusieurs eaux usées et boues d'épuration possèdent tous les nutriments essentiels pour la fermentation microbienne et

leur potentiel comme matières premières alternatives peut être exploré. Ainsi, selon la revue de la littérature, les problèmes majeurs qui ont été examinés dans cette étude sont présentés comme suit :

1.10.1. Production économique de conidies et de mycélium de *Trichoderma* spp.

L'utilisation de matières premières semi-synthétiques comme le milieu de Mendel, M-CSL (mélasses - liqueur de maïs) et tartrate de sodium (ST) permet d'obtenir un rendement en spores ($10^6$–$10^7$ spores $ml^{-1}$/ spores $g^{-1}$) qui est satisfaisant jusqu'à un certain point pour l'application sur le terrain. Cependant, les coûts élevés pour ces matières premières nuisent à leur commercialisation. De plus, les besoins standards pour l'application des ALBs basés sur *Trichoderma* spp. ($10^{10}$–$10^{11}$ spores/ hectare) pourraient ne pas être accomplis avec ces substrats semi-synthétiques.

Afin de contrer les coûts élevés, les milieux semi-synthétiques ont été substitués par des déchets agricoles et industriels comme les résidus de la transformation du blé ou du riz ou de la culture de canneberges. Malgré leur coût moindre, de faibles rendements en spores et en activité comme ALB limitent la commercialisation des procédés basés sur ces matières premières alternatives. De plus, la nature non ubiquiste de ces déchets s'avère contraignante pour la production d'ALBs basés sur *Trichoderma* spp. tout au long de l'année.

Des modes d'action bien définis pour les ALBs jouent un rôle crucial dans le succès commercial. À titre d'exemple, depuis les quatre dernières décennies, les biopesticides basés sur *Bacillus thuringiensis* ont dominé le marché des biopesticides et en raison des modes d'action et des techniques de bioessai ou de quantification de la croissance qui sont bien établis.

1.10.2. Gestion des eaux usées et des boues d'épuration

Une station typique de traitement des eaux usées génère des quantités considérables de déchets solides désignés comme des « boues ». La gestion des boues est depuis longtemps considérée comme un enjeu principal dans le traitement des eaux usées. Il existe différentes méthodes pour la disposition et la réutilisation des boues comme l'incinération, l'enfouissement et l'épandage agricole. Les avenues de disposition sont souvent des options inévitables et englobent plusieurs problèmes secondaires en réintroduisant des

polluants. Cependant, l'emphase est maintenant mise sur les avenues durables de la gestion des boues. Cela est défini comme "la valorisation" des boues où elles sont recyclées et retournées dans les cycles biogéochimiques à travers des options compatibles avec l'environnement. Divers produits à valeur ajoutée ont été développés ou sont en voie de l'être comme des adsorbants, des matériaux de construction, des huiles, des biopesticides, des enzymes, des biofloculants, des amendements pour les sols, etc.

Quelques auteurs ont reporté l'utilisation de *Trichoderma* spp. pour le traitement des eaux usées et des boues d'épuration, mais pas pour la production d'ALBs. Ainsi, la production d'ALBs basés sur *Trichoderma* spp. à partir de ces rejets reste un sujet d'intérêt à explorer.

## 1.11. Hypothèses et objectifs

Le développement d'un procédé de production pour des ALBs à base de *Trichoderma* spp. commercialement viables requiert de faire divers essais pour : (i) sélectionner les matières premières compatibles avec les champignons *Trichoderma*; (ii) développer de méthodes rapides et fiables pour la mesure de croissance du champignon; (iii) optimiser les paramètres de fermentation; (iv) mettre à l'échelle le procédé optimisé; (v) formuler le produit antagoniste; (vi) les bioessais du produit antagoniste formulé (*in vitro* et *in situ*); et (vii) évaluer le produit final pour son enregistrement comme ALBs. Dans ce contexte, l'objectif global de cette étude est divisé comme suit :

### 1.11.1. Hypothèses

Les hypothèses suivantes ont été formulées en se basant sur les connaissances actuelles de la production de *Trichoderma* spp. et de leur utilisation comme ALBs:

1. Un procédé de production d'ALBs basés sur *Trichoderma* spp. peut être commercialement viable si des eaux usées et des boues d'épuration sont utilisées comme matière première lors de la fermentation liquide.
2. Il y a une grande possibilité que la présence de substrats complexes dans les eaux usées et les boues d'épuration contribue à atteindre une plus forte sporulation et activité comme ALBs lors de la fermentation par *Trichoderma* spp.

3. La production de diverses enzymes lytiques par *Trichoderma* spp. dans les boues pourrait à la fois servir à la lutte biologique et à la dégradation de composés organiques toxiques présents dans le sol.
4. Un grand marché d'ALBs à large spectre (biopesticide et promoteur de croissance végétale) en agriculture utiliserait une quantité substantielle d'eaux usées et de boues d'épuration et aurait par conséquent un impact significatif sur leur gestion.

### 1.11.2. Objectifs majeurs en ordre chronologique

1. Développement d'une technique modifiée d'étalage sur plaque pour le compte des unités formatrices de colonies conidiales de champignon (*Trichoderma* spp.).
2. Étude des boues d'épuration des eaux usées comme milieu pour la croissance de *Trichoderma* sp. et des prétraitements de boues pour accroître la production de spores et l'activité d'ALB. Des études d'optimisation pour le pH, la température, le volume de l'inoculum, le type d'inoculum (spores et/ou mycélium), et l'âge de l'inoculum dans le cas de l'utilisation des boues d'épuration comme milieu.
3. Étude des eaux usées industrielles et des boues d'épuration comme milieux pour la production de spores de *Trichoderma* sp. et l'obtention de l'activité comme ALB.
4. Estimation de la sporulation de *Trichoderma* sp. par une technique alternative (mesure de l'ATP).
5. Réalisation de bioessais avec les cultures sur Pétri et des formulations de boues déshydratées de *Trichoderma* sp. pour évaluer la stimulation de la croissance végétale et le potentiel antagoniste envers des phytopathogènes importants.
6. Les études préliminaires de production industrielle en fermenteur avec des eaux usées et des boues d'épuration.
7. Étude technico-économique du procédé développé pour diverses eaux usées et boues d'épuration.

## 1.12. Originalité

Selon la revue de littérature, ***la production de propagules de Trichoderma (spores et/ou mycélium) à partir d'eaux usées industrielles et de boues d'épuration pour leur utilisation comme ALBs n'a pas encore été explorée***. Ainsi, le développement de procédé pour la production de propagules de *Trichoderma* sera unique. De plus, ***les bioessais proposés pour les formulations de Trichoderma basées sur ces matières premières fourniront des résultats exclusifs sur leur potentiel comme ALBs.***

## 1.13. Sommaire du contenu de la thèse

### 1.13.1. Chapitre 2: Les champignons pour la bioremédiation et la lutte biologique

Ce chapitre comprend deux parties. La première partie informe de façon exhaustive sur le rôle des champignons en bioremédiation de sol. Il a été conclu que les champignons sont d'importants agents de bioremédiation. Actuellement, divers champignons sont utilisés avec succès au niveau du laboratoire et aux échelles pilotes et commerciales. De plus, leur grand potentiel comme bioremédiateurs a été classé sur la base de leur capacité de bioremédiation comme les saprophytes et les champignons mycorhiziens. Les champignons *Trichoderma* spp. ont été rapportés par plusieurs auteurs pour leur habilité à améliorer les sols en raison de leur capacité à dégrader plusieurs pesticides chimiques toxiques dans le sol. Par conséquent, l'application de *Trichoderma* spp. dans les sols pourraient servir à la fois pour dégrader les toxines et pour la lutte biologique. Cependant, malgré les utilisations commerciales, seulement très peu d'information est disponible dans la littérature en ce qui concerne leur production comme antagoniste. Ainsi, la deuxième partie du chapitre 2 présente le potentiel de lutte biologique des champignons et discute en détails des avantages additionnels quant à l'utilisation *Trichoderma* spp. antagonistes comme ALBs.

### 1.13.2. Chapitre 3: Biodégradation et rhéologie des boues d'épuration et production d'ALBs à base de *T. viride*

Dans le présent travail de recherche, *T. viride* a été considéré comme une souche antagoniste commerciale de champignon *Trichoderma*. Dans le chapitre 3, la production de *T. viride* à partir des boues d'épuration a été discutée en trois étapes. Dans la première partie, la biodégradation des boues d'épuration a été étudiée de façon détaillée en utilisant

un consortium de microorganismes aérobies et en comparant avec *T. viride*. Les résultats de cette étude suggéraient que les boues d'épuration pouvaient être directement biodégradées par *Trichoderma* spp. Cependant, les boues requéraient une étape additionnelle de prétraitement en raison de leur rhéologie complexe. Dans la seconde partie, la concentration en solides des boues et différentes étapes de prétraitement ont été étudiées en détails. De plus, afin d'établir le potentiel comme ALB, un bioessai avec les boues fermentées par *T. viride* contre des insectes est présenté. Finalement, dans la troisième partie, une étude en fermenteur de *T. viride* sur boues d'épuration est présentée afin de valider le potentiel de ce champignon comme ALB avec des résultats de bioessais sur des plantes, des insectes et des phytopathogènes fongiques.

1.13.3. Chapitre 4: Eaux usées industrielles et boues déshydratées comme matière première potentielle pour la production de *T. viride* et boues déshydratées comme agent épaississant pour la formulation.

Ce chapitre est divisé en quatre parties. La première partie aborde une étude détaillée sur différentes matières premières pour atteindre une plus forte activité comme ALBs en réalisant une culture axénique de champignon *T. viride* en fioles. Particulièrement, un milieu synthétique sur de l'amidon soluble, des boues d'épuration déshydratées, des boues d'épuration de fromagerie, des eaux usées prétraitées et traitées de papetière et des eaux usées d'abattoir ont été testés pour la production de conidies et de protéases de *T. viride*. Cette étude indiquait que les eaux usées d'abattoir avaient l'activité antifongique la plus élevée avec une production modérée de conidies. Les résultats ont montré que la formulation sèche de conidies de *T. viride* (jusqu'à 19,2 kg de boues déshydratées par litre de bouillon fermenté) était viable en termes d'unités formatrices de colonies résiduelles (environ 70% de viabilité après un mois à 25±1°C et environ 90% après six mois à 4±1°C). La deuxième partie du chapitre 4 aborde une étude intensive sur les eaux usées de l'industrie de l'amidon qui ont permis d'atteindre la plus forte production de conidies. De plus, les eaux usées de l'industrie de l'amidon ont aussi été testées dans des fermenteurs de laboratoire pour déterminer la faisabilité d'une production de conidies de *T. viride* à plus grande échelle. Une étude sur l'aération et l'agitation a aussi été réalisée avec les eaux usées de l'industrie de l'amidon pour minimiser la consommation d'énergie et elle fait l'objet de la troisième partie. Dans la quatrième partie, l'étude de la

fermentation en mode "fed-batch" avec les eaux usées de l'industrie de l'amidon est présentée et les bioessais sur les plantes avec les eaux usées de l'industrie de l'amidon fermentées sont aussi discutés.

1.13.4. Chapitre 5: Analyse technico-économique du procédé de production d'ALBs basés sur *T. viride*

Ce chapitre comprend une analyse de la faisabilité pratique des ALBs basés sur *T. viride*. L'analyse faite avec différentes matières premières alternatives suggère que les boues d'épuration prétraitées et les eaux usées de l'industrie de l'amidon sont des options faisables pour la production commerciale de *T. viride*.

## References


Ahmad, J.S. and Baker, R. 1987. Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutants of *Trichoderma harzianum*. *Phytopathology,* **77:** 358-362.

Amsellem, Z., Zidack, N.K., Quimby Jr., P.C., Gressel, J. 1999. Long-term dry preservation of viable mycelia of two mycoherbicidal organisms. *Crop Proctection,* **18:** 643-649.

Aziz, N.H., El-Fouly, M.Z., El-Essawy A.A., Khalaf, M.A. 1997. Influence of bean seedling root exudates on the rhizosphere colonization by *Trichoderma lignorum* for the control of *Rhizoctonia solani*. *Bot. Bull. Acad. Sin.,* **38:** 33-39.

Baarschers, W.H., Heitland, H.S. 1986. Biodegradation of Fenitrothion and Fenitrooxon by the fungus *Trichoderma viridae*. *J. Agric. Food. Chem.* 34, 707–709.

Bae,Y.S., Knudsen, G.R. (2007). Effect of sclerotial distribution pattern of *Sclerotinia sclerotiorum* on biocontrol efficacy of *Trichoderma harzianum*. *Applied Soil Ecology,* **35:** 21–24. doi:10.1016/j.apsoil.2006.05.014

Benhamou, N. and Brodeur, J. 2000. Evidence for Antibiosis and Induced Host Defense Reactions in the Interaction Between *Verticillium lecanii* and *Penicillium digitatum,* the Casual Agent for Green Mold. *Biochemistry and Cell Biology,* **90(9):** 932-943.

Benhamou, N. and Picard, K. 1999. La résistance induite : une nouvelle stratégie de défense des plantes contre les agents pathogènes. *Phytoprotection,* **80(3):** 137-168.

Benhamou, N., Chet, I. 1993. Hyphal interaction between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold chemistry of the mycoparasitic process. *Phytopathology,* **83:** 1062-1071.

Benhamou, N., Rey, P., Picard, K., Tirilly, Y. 1999. Ultrastructural and cytochemical aspects of the interaction between the mycoparasite, *Pythium oligandrum*, and soilborne pathogens. *Phytopathology,* **89:** 506–517.

Bonnarme, P., Djian, A., Latrasse, A., Féron, G., Giniès, C., Durand, A., Le Quéré, J.-L. 1997. Production of 6-pentyl-α-pyrone by *Trichoderma* sp. from vegetable oils. *Journal of Biotechnology,* **56:** 143-150.

Bosch, A., Yantorno, O. 1999. Microcycle conidiation in the entomopathogenic fungus *Beauveria bassiana* bals. (vuill.). *Process Biochemistry,* **34:** 707-716.

Calistru, C., McLean, M., Berjak, P. 1997a. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. *A study of the production of extracellular metabolites by Trichoderma species. Mycopathologia,* **137:** 115-124.

Calistru, C., McLean, M., Berjak, P. 1997b. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species *1. Macroscopical and microscopical observations of fungal interactions. Mycopathologia,* **139:** 115-121.

Carlsen, M., Spohr, A.B., Nielsen, J, Villadsen, J. 1995. Morphology and physiology of an α-amylase producing strain of *Aspergillus oryzae* during batch cultivations. *Biotechnol. Bioeng.,* **49:** 266-76.

Celar, F. 2003. Competition for ammonium and nitrate forms of nitrogen between some phytopathogenic and antagonistic soil fungi. *Biological Control,* **28:** 19-24.

Chet, I. 1987. *Trichoderma*-Applications, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. Pages 137-160 in: *Innovative Approaches to Plant Disease Control. I.* Chet, ed. John Wiley & Sons, New York.

Chet, I. 1993. *Biotechnology in Plant Disease Control.* John Wiley & Sons, New York.

Cho, C-F., Lee, W-C. 1999. Formulation of a biocontrol agent by entrapping biomass of *Trichoderma viride* in gluten matrix. *Journal of Bioscience and Bioengineering,* **87(6):** 822-824.

Clark, D.S. 1961. Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of Aspergillus niger. *Can J Microbiol.,* **8:**133-136.

Cook, R.C., Rayner, A.D.M. 1984. *Ecology of saprophytic fungi.* Longman, London.

Copping LG, Menn JJ. Biopesticides: their action, applications and efficacy – a review. Pest Management Science 2000; 56(8): 651–676.

Domingues, F.C., Queiroz, J.A., Cabral, J.M.S., Fonseca, L.P. 2000. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. *Enzyme and Microbial Technology,* **26:** 394-401.

Domsch, K.H., Gams, W., Anderson, T-W., 1980. In: *Trichoderma. Compendium of Soil fungi.* Academic Press, **1:** 795-809.

Donzelli, B.G.G., Ostroff, G., Harman, G.E. 2003. Enhanced enzymatic hydrolysis of langostino shell chitin with mixtures of enzymes from bacterial and fungal sources *Carbohydrate Research,* **338:** 1823-1833.

Durand, H., Clanet, M., Tiraby, G. 1988. Genetic improvement of *Trichoderma reesei* for large scale cellulase production. *Enzyme and Microbial Technology,* **10:** 341-346.

Ejechia, B.O. 1997. Biological control of wood decay in an open tropical environment with *Penicillium* spp. and *Trichoderma viride. International Biodeterioration and Biodegradation,* **39:** 295-299.

Esposito, E., da Silva, M. 1998. Systematics and Environmental Application of the Genus *Trichoderma, Critical Reviews in Microbiology,* **24(2):** 89-98.

Fekete, C., Logrieco, A., Giczey, G., Hornok, L. 1997. Screening of fungi for the presence of the trichodiene synthase encoding sequence by hybridization to the *Tri5* gene cloned from *Fusarium poae. Mycopathologia,* **138:** 91-97.

Felse, P.A., Panda, T. 1999. Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. *Process Biochemistry,* **34:** 563-566.

Felse, P.A., Panda, T. 2000. Submerged culture production of chitinase by *Trichoderma harzianum* in stirred tank bioreactors – the influence of agitator speed. *Biochemical Engineering Journal,* **4:** 115-120.

Ganassi, S., Moretti, A., Stornelli, C., Fratello, B., Bonvicini Pagliai, A.M., Logrieco, A., Sabatini, M.A. 2000. Effect of *Fusarium*, *Paecilomyces* and *Trichoderma* formulations against aphid *Schizaphis graminum. Mycopathologia,* **151:** 131-138.

Gerhardson, B. 2002. Biological substitutes for pesticides. *Trends in Biotechnology* **20(8):** 338-343.

Gikas, P., Livingston, A.G. 1993. Use of ATP to characterize biomass viability in freely suspended and immobilized cell bioreactors. *Biotech. Bioeng.,* **42:** 1337-1351.

Godtfredsen, W.O., Vangedal, S. 1965. Trichodermin, a new sesquiterpene antibiotic. *Acta Chemica Scandinavia,* **19(5):** 1089-1102.

Groll, A.H., Walsh, T.J. 2001. Uncommon opportunistic fungi: new nosocomial threats. *Clin Microbiol Infect,* **7:** 8-24.

Hanson, L.E., Howell, C.R. 2002. Biocontrol efficacy and other characteristics of protoplast fusants between *Trichoderma koningii* and *T. virens*. *Mycol. Res.,* **106(3):** 321-328.

Harman, G.E., Haynes, C.K., Lorito, M., Broadway, R.M., di Pietro, A., Peterbauer, C., Tronsmo, A. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Phytopathology,* **83:** 313-318.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M. 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2: 43–56.

Harmann, G.E. and Björjmann, T. 1998. Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In *Trichoderma and Gliocladium Vol. 2.* (Harmann, G.E. and Kubicek, C.K., eds), pp 229–265, Taylor and Francis Ltd., London.

Headon, D.R., Walsh, G. 1994. The industrial production of enzymes. *Biotechnology Advances,* **12:** 635-646.

Héraux FMG, Hallett, SG, Weller, SC. Combining *Trichoderma virens*-inoculated compost and a rye cover crop for weed control in transplanted vegetables. Biological Control 2005; 34: 21–26.

Howell, C.R. 1982. Effect of *Gliocladium virens* on *Pythium ultimum*, *Rhizoctonia solani*, and damping-off of cotton seeds. *Phytopathology,* **72:** 496-498.

Howell, C.R., Devay, J.E., Garber, R.H. Batson, W.E. 1997. Field control of cotton seedling diseases with *Trichoderma virens* in combination with fungicide seed treatments. *Journal of Cotton Science,* **1:** 15-20.

Howell, C.R., Stipanovic, R.D. 1984. Phytotoxicity to crop plants and herbicidal effects on weeds of viridiol produced by *Gliocladium virens. Phytopathology* **74:** 1346-1349.f

http://www.shaktibiotech.com/shakti-trichoderma-viride.html, cited on Dec. 12, 2003.

Hutchinson, C.M. 1999. *Trichoderma virens*-Inoculated Composted Chicken Manure for Biological Weed Control. *Biological Control,* **16:** 217-222.

Kansoh, A.L., Essam, S.A., Zeinat, A.N. 1999. Biodegradation and utilization of bagasse with *Trichoderma reesie. Polymer Degradation and Stability,* **63:** 273-278.

Karim, M.I.A. and Kamil, A.Q.A. 1989. Biological treatment of palm oil mill effluent using *Trichoderma viride. Biological Wastes,* **27:** 143-152.

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.

Leandro, L.F.S., Guzman, T., Ferguson, L.M., Fernandez, G.E., Louws, F.J. (2007). Population dynamics of *Trichoderma* in fumigated and compost-amended soil and on strawberry roots. *Applied Soil Ecology,* **35:** 237–246. doi:10.1016/j.apsoil.2006.04.008

Lewis, J.A., Papavizas, G.C. 1983. Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. *Soil Biol. Biochem.* **15(3):** 351-357.

Lin, A., Lee, T.M. and Rern, J.C. 1994. Tricholin, a new antifungal agent from *Trichoderma viride*, and its action in biological control of Rhizoctonia solani. *Journal of Antibiotics,* **47:** 799-805.

Lindsey, D. L., and R. Baker. 1967. Effect of certain fungi on dwarf tomatoes grown under gnotobiotic conditions. Phytopathology **57:**1262–1263.

Lisboa De Marco, J., Valadares-Inglis, M.C., Felix, C.R. 2003. Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity against *Crinipellis perniciosa*, the causal agent of witches' broom of cocoa. *Brazilian Journal of Microbiology,* **34:** 33-38.

Liu, J., Xia, W. (2006). Purification and characterization of a bifunctional enzyme with chitosanase and cellulase activity from commercial cellulase. *Biochemical Engineering Journal,* **30:** 82–87. doi:10.1016/j.bej.2006.02.005

Lopez, E. and Orduz, S. 2003. *Metarhizium anisopliae* and *Trichoderma viride* for control of nests of the fungus-growing ant, *Atta cephalotes*. *Biological Control,* **27:** 194-200.

Maheshwari, R. 1999. Microconidia of *Neurospora crassa. Fungal Genetics and Biology,* 26(1): 1-18.

Mandels, M. and Andreotti, R.E. 1978. Problems and challenges in the cellulose to cellulase fermentation. *Process Biochemistry,* **13:** 6-13.

Marasas, W.F.O., Nelson, P.E., Toussoun, T.A. 1984. Toxigenic *Fusarium* species. Identity and Mycotoxicology. University Park/London: *The Pennsylvania State University Press.*

Martin, S.M., Waters, W.R. 1952. Production of citric acid by submerged fermentation. *Ind. Eng. Chem.,* **44:** 2229–2233.

Menendez, A. B., Godeas, A. 1998. Biological control of *Sclerotinia sclerotiorum* attacking soybean plants. Degradation of the cell walls of this pathogen by *Trichoderma harzianum* (BAFC 742). *Mycopathologia,* **142:** 153-160.

Morales-Barrera, L., Cristiani-Urbina, E. (2006). Removal of hexavalent chromium by *Trichoderma viride* in an airlift bioreactor. *Enzyme and Microbial Technology,* **40:** 107–113. doi:10.1016/j.enzmictec.2005.10.044

Mukherjee, P.K., Raghu, K. 1997. *Trichoderma* sp. as a microbial suppressive agent of *Sclerotium rolfsii* on vegetables. *World Journal of Microbiology and Biotechnology,* **13:** 497-499.

Nevalainen, K.M.H. Palva, E.T., Bailey, M.J. 1980. A high cellulase producing mutant strain of *Trichoderma reesei. Enzyme and Microbial Technology,* **2:** 59-60.

Olsson, L., Christensen, T.M.I.E., Hansen, K.P., Palmqvist, E.A. 2003. Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C-30. *Enzyme and Microbial Technology,* **33:** 612-619.

Orgaz, B., Kives, J., Pedregosa, A.M., Monistrol, I.F., Laborda, F., Carmen, S. (2006). Bacterial biofilm removal using fungal enzymes. *Enzyme and Microbial Technology,* **40:** 51–56. doi:10.1016/j.enzmictec.2005.10.037

Ortiz, A., Orduz, S. 2001. *In vitro* evaluation of *Trichoderma* and *Gliocladium* antagonism against the symbiotic fungus of the leaf-cutting ant *Atta cephalotes. Mycopathologia,* **150:** 53-60.

Papagianni, M. 2004. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances,* **22:** 189-259.

Papagianni, M. 2004. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances,* **22:** 189-259.

Papavizas, G.C. 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. *Annual Review of Phytopathology,* **23:** 23-54.

Papavizas, G.C., Lewis, J.A., Abd-El Moity, T.H. 1982. Evaluation of new biotypes of *Trichoderma harzianum* for tolerance to benomyl and enhanced biocontrol capabilities. *Phytopathology,* **72:** 126-132.

Pascual, S., Rico, J.R., De Cal, A., Melgarejo, P. 1997. Ecophysiological factors affecting growth, sporulation and survival of the biocontrol agent *Penicillium oxalicum. Mycopathologia,* **139:** 43-50.

Petenbauer, C.K. and Heidenreich, E. 1992. Effect of benomyl and benomyl resistance on cellulase formation by *Trichoderma reesei* and *Trichoderma harzianum. Canadian Journal of Microbiology,* **38:** 1292-1297.

Pointing, S.B. 2001. Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology.* 57, 20–33.

Prabavathy, V.R., Mathivanan, N., Sagadevan, E., Murugesan, K., Lalithakumari, D. (2006). Self-fusion of protoplasts enhances chitinase production and biocontrol activity in *Trichoderma harzianum. Bioresource Technology* **97:** 2330–2334. doi:10.1016/j.bior tech.2005.10.031

Puyesky, M., Benhamou, N., Noyola, P.P., Bauw, G., Ziv, T., Montagu, M.V., Herrera-Estrella, A., Horwitz, B.A. 1999. Developmental Regulation of cmp1, a Gene Encoding a Multidomain Conidiospore Surface Protein of *Trichoderma*. *Fungal Genetics and Biology,* **27:** 88-99.

Rabeendran, N., Jones, E.E., Moot, D.J., Stewart, A. (2006). Biocontrol of *Sclerotinia* lettuce drop by *Coniothyrium minitans* and *Trichoderma hamatum. Biological Control,* **39:** 352–362. doi:10.1016/j.biocontrol.2006.06.004

Reczey, K., Szengyel, Zs., Eklund, R., Zacchi, G. 1996. Cellulase production by *T. reesei. Bioresource Technology,* **57:** 25-30.

Roco, A., Pérez, L.M. 2001. *In vitro* biocontrol activity of *Trichoderma harzianum* on *Alternaria alternata* in the presence of growth regulators. *Electronic Journal of Biotechnology,* **4(2):** 68-73.

Roncal, T., Cordobés, S., Ugalde, U., He, Y., Sterner, O. 2002. Novel diterpenes with potent conidiation inducing activity. *Tetrahedron Letters,* **43:** 6799-6802.

Sachdeva, V., Tyagi, R.D., Valéro, J.R. 2000. Production of biopesticides as a novel method of wastewater sludge utilization/disposal. *Water science and technology,* **42:** 211-216.

Samuels, G.J., Chaverri, P., Farr, D.F., McCray, E.B. *Trichoderma* Online, Systematic Botany & Mycology Laboratory, ARS, USDA. Retrieved June 15, 2004, from http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm.

Saraswathy, A., Hallberg, R. 2002. Degradation of pyrene by indigenous fungi from a former gasworks site. *FEMS Microbiology Letters.* 210, 227–232.

Score, A.J., Palfreyman, J.W. 1994. Biological control of the dry rot fungus *Serpula lacrymans* by *Trichiderma* secies: The effects of complex and synthetic media on interaction and hyphal extension rates. *International Biodeterioration and Biodegradation,* **33:** 115-128.

Sharma SK, Kalra KL, Grewal HS 2002. Fermentation of enzymatically saccharified sunflower stalks for ethanol production and its scale up. *Bioresource Technology,* **85:** 31-33.

Singh, A., Srivastava, S., Singh, H.B. (2007). Effect of substrates on growth and shelf life of *Trichoderma harzianum* and its use in biocontrol of diseases. *Bioresource Technology*, **98:** 470–473. doi:10.1016/j.biortech.2006.01.002

Srinivasan, U., Staines, H.J., Bruce, A. 1992. Influence of media type on antagonistic modes of *Trichoderma* spp. against wood decay basidiomycetes. *Material and Organismen,* **27(4):** 301-321.

Steel, R., Lentz, C.P., Martin, S.M. 1954. A standard inoculum for citric acid production in submerged culture. *Can J Microbiol,* **1:** 150-157.

Stentelaire, C., Antoine, N., Cabrol, C., Feron, G., Durand, A. 2001. Development of rapid and highly sensitive biochemical method for the measurement of fungal spore viability. An alternative to the CFU method. *Enzyme and Microbial Technology,* **29:** 560-566.

Stipanovic RD and Howell CR. The x-ray crystal structure determination, and biosynthetic studies of the antibiotic, heptelidic acid. Tetrahedron 1983; 39: 1103-1107.

Suarez-Estrella, F., Vargas-Garcia, C., Lopez, M.J., Capel, C., Moreno, J. (2007). Antagonistic activity of bacteria and fungi from horticultural compost against *Fusarium oxysporum* f. sp. *melonis. Crop Protection*, **26:** 46–53. doi:10.1016/j.cropro.2006.04.003

Szengyel, Z., Zacchi, G., Varga, A., Reczey, K. 2000. Cellulase production of *Trichoderma reesei* RUT C30 using steam- pretreated spruce. Hydrolytic potential of cellulases on differnt substrate. *Applied Biochemistry and Biotechnology Spring,* **84-86:** 679-691.

Templeton GE, Heiny DK. 1989. Improvement of fungi to enhance mycoherbicide potential. In: *Biotechnology of fungi for improving plant growth* (Whipps, J. M.; Lumsden, R. D. eds.). Cambridge University Press, UK. Chapter 6 pages 127–151.

Thygesen, A., Thomsena, A.B., Schmidt, A.S., Jørgensen, H., Ahring, B.K., Olsson, L. (2003). Production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidised wheat straw. *Enzyme and Microbial Technology,* **32:** 606–615.

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:** 3807-3816.

Tirado-Montiel, ML, Tyagi, RD, Valéro, JR, Surampalli, RY. 2003. Production biopesticides using wastewater sludge as a raw material: Effect of process parameters. Water Science and technology. **48:** 239-246.

Trillas, M.I., Casanova, E., Cotxarrera, L., Ordovás, J., Borrero, C., Avilés, M. (2006). Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. *Biological Control,* **39:** 32–38. doi:10.1016/j.biocontrol.2006.05.007

van Suijdam, J.C., Kossen, N.W.F., Paul, P.G. 1980. An inoculum technique for the production of fungal pellets. *Eur J Appl Microbiol Biotechnol,* **10:** 211-221.

van Wyk, J.P.H., Mohulatsi, M. (2003). Biodegradation of wastepaper by cellulase from *Trichoderma viride. Bioresource Technology,* **86:** 21–23.

Vega, F.E., Jackson, M.A., Mercadier, G., Poprawski, T.J. 2003. The impact of nutrition on spore yields for various fungal entomopathogens in liquid culture. *World Journal of Microbiology & Biotechnology,* **19:** 363-368.

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:** 253-260.

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 Research,* **36:** 4850-4860.

Vlaev, S.D., Djejeva, G., Raykovska, V., Schugerl, K. 1997. Cellulase production by *Trichoderma* sp. Grown on corn fibre substrate. *Process Biochemistry,* **32:** 561-565.

Whipps, J.M. and Lumsden, R.D. 2001. Commercial use of fungi as plant disease biological control agents: status and prospects. In: *Fungal Biocontrol Agents: Progress;, Problems and Potential,* (eds T. Butt, C. Jackson & N. Magan), Wallingford: CABI Publishing, pp 9-22.

Xia L., Shen X. (2004). High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. *Bioresource Technology,* **91:** 259–262.

Yedidia, I., Benhamou, N., Chet, I. 1999. Induction of defense responses in cucumber by the biocontrol agent *Trichoderma harzianum. Appl. Environ. Microbiol.,* **65:** 1061-1070.

Yedidia, I., Benhamou, N., Kapulnik, Y., Chet, I. 2000. Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. *Plant Physiol. Biochem.*, **38:** 863-873.

Zaldívar, M., Velásquez, J.C., Contreras, I., Pérez, L.M. 2001. *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. *Electronic Journal of Biotechnology,* **4(3):** 160-168. (This article is available online at, http://www.ejb.org/content/vol4/issue3/full/7)

Zeilinger, S., Galhaup, C., Payer K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M., Kubicek, C.P. 1999. Chitinase Gene Expression during Mycoparasitic Interaction of *Trichoderma harzianum* with Its Host. *Fungal Genetics and Biology,* **26:** 131-140.

Zheng, Z. and Shetty, K. 1998. Cranberry processing waste for solid state fungal inoculant production. *Process Biochemistry,* **33(3):** 323-329.

# CHAPITRE 2.
# BIORÉMÉDIATION ET LUTTE BIOLOGIQUE PAR LES CHAMPIGNONS

# Part I

# Bioremediation with Fungi

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

[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

**American Society of Chemical Engineers Book Series: Chapter 9**
**(ISBN – 13: 978-0-7844-0894-0. pp 223-258)**

## Biorémédiation par les champignons

### Résumé

Les champignons sont des agents de biorémédiation importants. Ils ont été utilisés avec succès à différents niveaux (laboratoire, pilote, commercial) avec de fréquents ajustements. Leur grand potentiel comme biorémédiateurs a incité la communauté scientifique à les classer dans diverses catégories basées sur leurs capacités en biorémédiation, par exemple les saprophytes (champignons causant la carie blanche ou brune) et les champignons mycorhiziens. Les contaminants potentiels comme les déchets de munition, les pesticides, les organochlorés, les biphényls polychlorés (BPCs), les hydrocarbures aromatiques polycycliques (HAP), les colorants synthétiques, les préservatifs du bois et les polymères synthétiques peuvent être dégradés en des formes moins nuisibles suite à un traitement par les champignons. Cependant, plusieurs champignons lignolytiques sont éprouvés pour décomposer une grande variété de composés récalcitrants grâce à leur système enzymatique non spécifique. Les champignons lignolytiques apparaissent comme étant tout aussi efficaces pour traiter les contaminants dans les sols et les eaux. Les symbioses mycorhiziennes (champignons ectomycorhizes, à arbuscules, éricoïdes et orchides) contribuent à la dégradation de plusieurs composés xénobiotiques par un consortium de champignons qui résistent différemment à l'attaque par un ou quelques organismes. En opposition avec l'application de champignons causant la carie blanche (CCB), la présence naturelle des champignons mycorhiziens dans la rhizosphère serait un important avantage. Cependant, leur croissance lente constitue l'une des contraintes à l'utilisation des champignons mycorhiziens en biorémédiation.

Les champignons peuvent également être exploités afin d'extraire (mise en solution des métaux par des procédés de biolixiviation) ou d'immobiliser des métaux (récupération des métaux par adsorption par des procédés de biosorption).

Il est proposé que la présence de conditions environnementales adéquates pour la flore microbienne indigène stimule le processus de biorémédiation et, par conséquent, la croissance de plantes. Il existe différentes formulations commerciales en biorémédiation conçues pour divers besoins et, de façon intéressante, une majorité d'entre-elles contiennent des inoculums de champignons, ou encore, des stimulateurs de croissance pour les champignons biorémédiateurs.

**Mots clés:** Biorémédiation, champignon, lignolytique, mycorhizes, saprophytes, champignon de la carie blanche.

**Abstract**

Fungi are important bioremediation agents. They have been successfully used at laboratory, pilot and commercial levels with frequent modifications. Their immense potential as bioremediators has stimulated the scientific community to further categorize fungi on the basis of their remediation capabilities, e.g. saprotrophs (white rot and brown rot fungi) and mycorrhizal fungi. Potential contaminants like munition wastes, pesticides, organochlorines, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, wood preservatives and synthetic polymers can be degraded or converted into less nuisance forms via fungal treatments. Several ligninolytic fungi, however, have been shown to decompose a large variety of recalcitrant compounds due to their non-specific enzyme systems. Ligninolytic fungi appear to be equally effective in treating contaminants associated with soil or water. Mycorrhizal symbioses (ectomycorrhizal, arbuscular, ericoid and orchid fungi) of fungi help in degradation of many xenobiotics by a consortium of large group of fungi, which are otherwise resistant to attack by a single or, a few organisms. In contrast to application of white rot fungi (WRF), the natural presence of mycorrhizal fungi in rhizosphere would be a definite advantage. However, slower growth, dependency on plant/tree root systems, and fungal biomass are some of the constraints of mycorrhizal fungi in bioremediation.

Benevolent speciation of metals in environment by fungi is another widely studied and implemented mode of bioremediation. In general, metal speciation ability of microorganisms is due to their mobilization or immobilization processes that control transportation of metal species between soluble and insoluble phases.

It is proposed that the presence of adequate environmental conditions for native microbial flora would stimulate the remediation process and consequently plant growth promotion. There are several of commercial/proprietary formulations for soil remediation designed for different requirements, interestingly, a majority of them either contain inocula of fungi or, growth enhancers for bioremediator fungi.

**Keywords:** Bioremediation, fungi, ligninolytic, mycorrhizal, saprotrophs, white-rot.

# 1 Introduction

Fungi are important bioremediation agents. They have been successfully used at laboratory, pilot and commercial levels with frequent modifications. Their immense potential as bioremediators has stimulated the scientific community to further categorize fungi on the basis of their remediation capabilities, e.g. saprotrophs (white rot and brown rot fungi) and mycorrhizal fungi (Meharg and Cairney, 2000). Potential contaminants like munition wastes, pesticides, organochlorines, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, wood preservatives and synthetic polymers can be degraded or converted into less nuisance forms via fungal treatments (Pointing, 2001). Even though bacteria, algae, and plants are able to perform decontamination, their applications are limited by their low treatment efficacies. In most cases, xenobiotic chemicals are extremely resistant to biodegradation by native flora and fauna (Fernando and Aust, 1994). Several ligninolytic fungi, however, have been shown to decompose a large variety of recalcitrant compounds due to their non-specific enzyme systems (Novotny et al., 2004). Ligninolytic fungi appear to be equally effective in treating contaminants associated with soil or water. However, their proliferation in contaminated soil is highly susceptible to competition from native microbes, thereby warrants, bulking agents (as supplementary nutrient) like peat moss, bran flakes, and pine wood shavings (Meysami and Baheri, 2003).

Mycorrhizal symbioses (ectomycorrhizal, arbuscular, ericoid and orchid fungi) of fungi help in degradation of many xenobiotics by a consortium of large group of fungi, which are otherwise resistant to attack by a single or, a few organisms (Meharg and Cairney, 2000; Allen et al., 1995; Clapp et al., 1995; Perotto et al., 1996; Gardes and Bruns, 1996; Dahlberg et al., 1997; Liu et al., 1998). The need of mycorrhizal symbioses arose from some of the drawbacks of phytoremediation technology which has serious environmental implications, e.g., bioaccumulation of contaminants into plants and their subsequent entry into wild-life food chains (Anderson et al., 1993). In contrast to application of white rot fungi (WRF), the natural presence of mycorrhizal fungi in rhizosphere would be a definite advantage. However, slower growth, dependency on plant/tree root systems, and fungal biomass are some of the constraints of mycorrhizal fungi in bioremediation (Lappin et al., 1985; Donnelly and Fletcher, 1994; Meharg and Cairney, 2000).

Benevolent speciation of metals in environment by fungi is another widely studied and implemented mode of bioremediation (Lovley and Coates, 1997; Eccles, 1999). Furthermore, in addition to metals, speciation and mobility of other elements, including carbon, nitrogen, sulfur and phosphorus are fundamental to biogeochemical cycles implications for plant productivity and human health (Gadd, 1999, 2002, 2004; Verrecchia and Dumont, 1996). In general, metal speciation ability of microorganisms is due to their mobilization or immobilization processes that control transportation of metal species between soluble and insoluble phases (White et al., 1997, 1998; Sreekrishnan and Tyagi, 1994; Vachon et al., 1994).

Currently, there are some commercialized/proprietary processes, claiming simultaneous execution of bioremediation and growth factor enhancement of soil for agricultural purposes (Walker, 2003). These processes normally comprise of application of fertilizer and pesticide in combination with specific carbohydrate-based surfactant. It is proposed that the presence of adequate environmental conditions for native microbial flora would stimulate the remediation process and consequently plant growth promotion. There are several other types of commercial/proprietary formulations for soil remediation designed for different requirements, interestingly, a majority of them either contain inocula of fungi or, growth enhancers for bioremediator fungi (Gill, 1996, 1997; Bennett et al., 2001; Walker, 2003).

This chapter will broadly circumscribe important developments in saprotroph and mycorrhizal fungi associated soil bioremediation processes aimed at application and their known modes of action. Few case studies are presented for well accounted processes.

## 2 Saprotrophic Fungal Processes

White-rot fungi (WRF) are the most studied fungi among saprotrophs (WRF, brown-rot fungi, litter-decaying fungi) for soil bioremediation (Figure 1). It is a physiological classification rather than taxonomic, comprising those fungi that have a panoply of lignin degrading enzymes (Pointing, 2001). The name white-rot signifies the appearance of wood attacked by these fungi, in which lignin removal results in a white appearance of the substrate. The lignin degrading characteristic of WRF facilitate in soil bioremediation in two ways – (1) capability to degrade a wide range of highly recalcitrant organopollutants with structural similarities to lignin (Figure 2, Pointing, 2001); (2) WRF

as well as other microbial flora nourish from bioavailable substrate obtained from lignin degradation. Mostly basidiomycetes, and a few ascomycete genera (Figure 1) are capable of white-rot decay (Eaton and Hale, 1993). Table 1 lists some WRF-mediated soil bioremediation processes, grouped on the basis of pollutants treated.

Various recalcitrant pollutants described in the following sections, have been categorized on the basis of their end-use and similarity in chemical class.

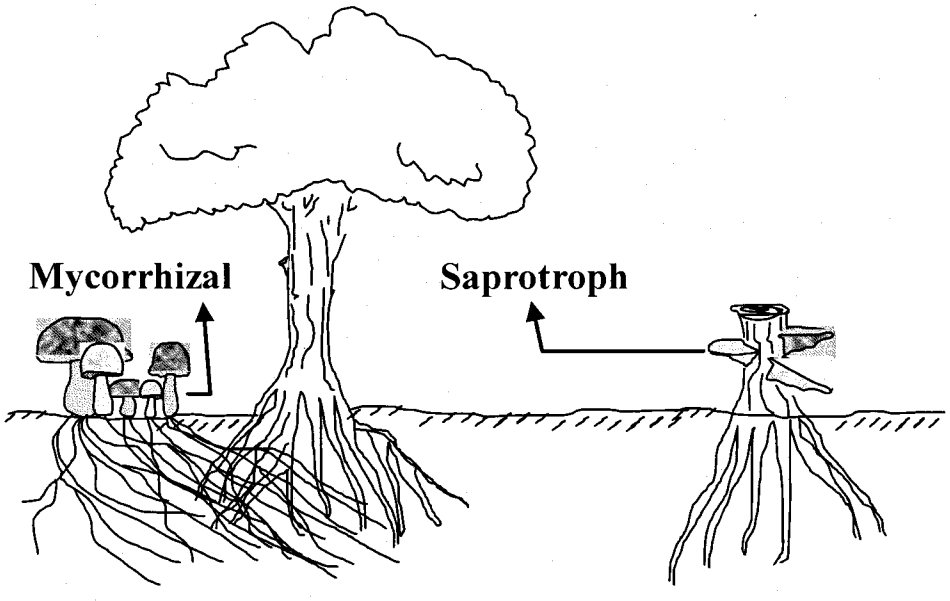



**Figure 1.** Schematic sketch of Saprotrophs, and mycorrhizal fungi.

### *2.1 Synthetic Dyes, Pesticides and Polychlorinated Biphenyls*

These pollutants are introduced into environment by agricultural sanitization, textile dyeing, paints, refineries and electrical industries (Novotny et al., 2001). These pollutants are of great environmental concern because of their abundance and high toxic, carcinogenic and reproductive effects on animal and humans. About 632 million tons of PCBs have been produced for transformer oil (1929–1970, Rojas-Avelizapa et al., 1999; Duke et al., 1970; INE., 1995; Hutzinger et al., 1974). Inadequate use of these oils, lack of proper storage and disposal of spent oils, and accidental spillage has caused serious

contamination problems (INE, 1995). Similarly, there are several other major sources of these pollutants (Meharg and Cairney, 2000).

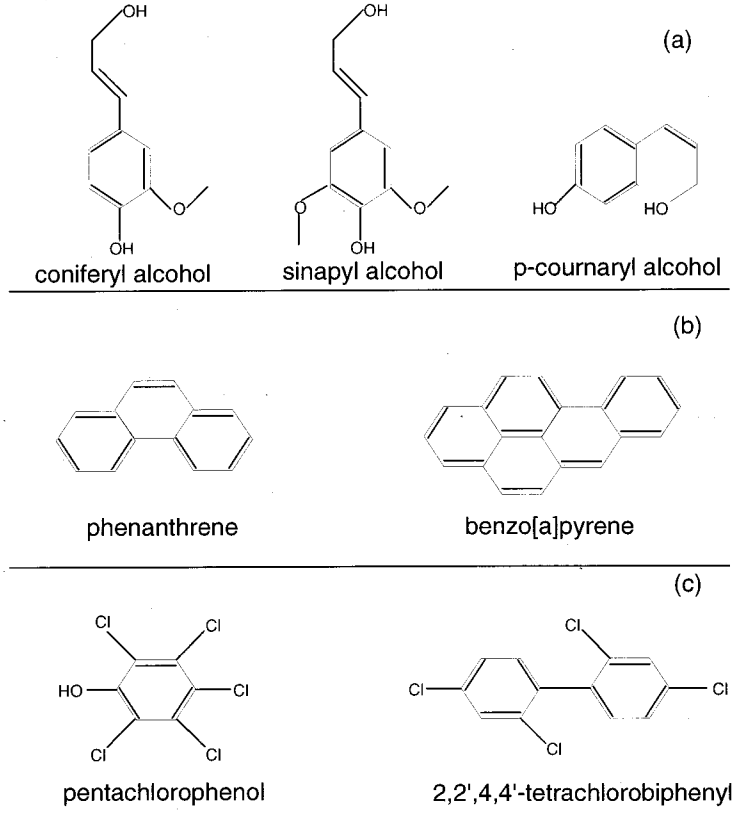



**Figure 2.** (a) Lignin monomers, (b) polycyclic aromatic hydrocarbons (PAH), and (c) halogenated compounds mineralized by the ligninolytic enzyme system of white-rot fungi (Pointing, 2001).

In particular, pesticides are persistent in the environment in the form of organochlorines, organophosphate and are linked to toxic effects and population declines at higher trophic levels (Alloway and Ayres 1993). Fortunately, bacteria and several genera of soil fungi (e.g. *Fusarium*, *Penicillium*) obtained from pesticide contaminated soils are now known to degrade pesticides with great efficacy (Twigg and Socha, 2001; Wong et al., 1992).

### 2.1.1. Mechanisms of Bioremediation

The oxidation of lignin takes place in the secondary metabolic process of WRF (Figure 3), in order to access wood polysaccharides bound in lignin-carbohydrate complexes (Jeffries, 1990). On the other hand, organophosphate insecticides are not generally persistent, and certain WRF e.g., *Phanerochaete chrysosporium* have been demonstrated to mineralize chloropyrifos, fonofos, and terbufos (Bumpus et al. 1993). The action of lignin-modifying enzymes was unclear, however, hydrolytic cleavage of the organophosphates, fenitrothion and fenitrooxon by non-ligninolytic fungus *Trichoderma viridae* (Baarschers and Heitland 1986) have been well known. Thus, it could be inferred that WRF have been able to degrade a wide range of pesticides simply due to their diversity in enzymes.

**Table 1.** White rot fungi based bioremediation of different pollutants.

| Saprotroph Fungi (White Rot and Brown Rot) | Bulking Agent(s)/ Synthetic Media | Pollutant | Pollutant Concentration | Reference |
|---|---|---|---|---|
| *Aspergillus fumigatus, A. sydowii, A. terreus, A. clavatus, A. niger, Fusarium oxysporum, F. decemcellulare, Penicillium oxalicum, P. restrictum, P. simplicissimum, P. verruculosum, P. montanense, P. pinophilum, P. janthinellum, Amorphoteca resinae, Neosartorya fischeri, Paecilomyces variotii, Talaromyces flavus, Graphium putredinis, Cunninghamella echinulata, Eupenicillium ochrosalmoneum* | KCl, 250 mg $l^{-1}$; $NaH_2PO_4$, 1 g $l^{-1}$; $MgSO_4$, 0.5 g $l^{-1}$; $NH_4NO_3$, 1 g $l^{-1}$, Chloramphenicol, 100 mg $l^{-1}$ | Arabian light crude oil (BAL 150) | 1 g $l^{-1}$ | Chaillan et al., (2004) |
| *Coriolopsis gallica, Bjerkandera adusta, Pleurotus pilmanarius, P. Ostreatus, Phanerochaete chrysos, Trametes versicolor* | Pine wood shavings, peat moss and bran flakes | Wheathered crude oil | 4000-14000 ppm | Meysami and Baheri, (2003) |
| *Phanerochaete chrysosporium* | 1% (w/v) malt extract; 1% (w/v) glucose; 0.2% (w/v) peptone; 0.2% (w/v) yeast extract; 1.5% (w/v) agar | TNT (2,4,6-trinitrotoluene) | 2-10 µg $l^{-1}$ | Stahl and Aust, (1993a, 1993b); Zheng and Obbard, (2002) |
| *Pleurotus osteratus* | Spent mushroom compost wetted with fish oil as surfactant | Creosote (containing 16 different PAHs) | 1900 mg PAH/kg creosote | Eggen, T. (1999) |

| **Saprotroph Fungi (White Rot and Brown Rot)** | **Bulking Agent(s)/ Synthetic Media** | **Pollutant** | **Pollutant Concentration** | **Reference** |
|---|---|---|---|---|
| *Lentinula edodes* | Spent mushroom compost | PAHs | -- | Buswell, (1994) |
| *Phytophthora* sp., *Mucor* sp., *Cladosporium* sp., *Coniothyrium* sp., *Doratomyces* sp., *Fusarium* sp., *Phialophora* sp., *Scedosporium* sp., *Sphaeropsis* sp., *Stachybotrys* sp., *Trichoderma* sp. | BSM–glycerol medium: Glycerol, 10 g $l^{-1}$; $KH_2PO_4$, 2 g $l^{-1}$; $CaCl_2.2H_2O$, 0.14 g $l^{-1}$; $MgSO_4.7H_2O$, 0.07 g $l^{-1}$; thiamine hypochloride, 2.5 mg $l^{-1}$; Tween 80, 7.5 ml $l^{-1}$; D- diammonium tartrate, 1.84 g $l^{-1}$; yeast extract, 1 g $l^{-1}$; $FeSO_4.7H_2O$, 70 mg $l^{-1}$; $ZnSO_4.7H_2O$, 46.2 mg $l^{-1}$; $MnSO_4.H_2O$, 35 mg $l^{-1}$; $CuSO_4.5H_2O$, 7 mg $l^{-1}$ | PAHs | 800–1100 mg PAHs /kg soil | Potin et al., (2004a, 2004b) |
| *Absidia cylindrospora, A. spinosa, Acremonium murorum, Alternaria alternata, Aspergillus flavipes, A. fumigatus, A. niger, A. oryzae, A. parasiticus, A. terreus, A. ustus, Botryotrichum piluliferum, Cladosporium herbarum, Cunninghamella bainieri, Cylindrocarpon lucidum, Doratomyces asperulus, D. stemonitis, Fusarium culmorum, F. lateritium, F. oxysporum, F. solani, Gliocladium roseum, G. virens, Humicola grisea, Minimedusa polyspora, Mucor hiemalis, Neosartorya fischeri, Paecilomyces lilacinus, Penicillium canescens, P. janczewskii, P. montanense, P. restrictum, P. simplicissimum, Phoma eupyrena, P. exigua, Pseudallescheria boydii, Seimatosporium sp., Stachybotrys bisbyi, S. chartarum, Talaromyces flavus, Trichoderma hamatum, T. harzianum, T. koningii. Trichurus spiralis, Verticillium tenerum, Westerdykella dispersa, Zygorhynchus heterogamus* | GS* liquid medium supplemented with 5 g $l^{-1}$ glucose | Fluorene | 100 µg /g soil | Garon et al. (2004) |

| **Saprotroph Fungi (White Rot and Brown Rot)** | **Bulking Agent(s)/ Synthetic Media** | **Pollutant** | **Pollutant Concentration** | **Reference** |
|---|---|---|---|---|
| *Kuehneromyces mutabilis and Agrocybe aegerita* | Mycelial mat grown over malt agar | Pyrene | 80 µg / g soil | Sack and Fritsche (1997) |
| *Pleurotus ostreatus, Phanerochaete chrysosporium, Hypholoma fasciculare* | Freshly ground, dried birch sticks – steel net-sieved (7 mm) – use as a fungal substrate | PAH (gasworks plant, Ystad, Sweden) | 5–30 µg / g soil | Andersson et al. (2000) |
| *Actinomyces* sp. and *Paecilomyces* sp. | $KNO_3$, 1 g $l^{-1}$; FeCl3, 0.02 g $l^{-1}$; MgSO4, 0.2 g $l^{-1}$; NaCl, 0.1 g $l^{-1}$; CaCl2, 0.1 g $l^{-1}$; K2HPO4, 1 g $l^{-1}$; yeast extract, 0.05 g $l^{-1}$, transformer oil, 1% v/v (after sterilization) | Transformer oil (PCB content, 88% w/v) | 7000 mg / kg soil | Rojas-Avelizapa et al. (1999) |
| *Pleurotus osteratus* | -- | PCB commercial mixture Delor 103 (1.02 mg/ml in acetone) | 10.21 g / kg soil | Kubatova et al. (2001) |
| *Irpex lacteus* and *Pleurotus ostreatus* | Growth medium – malt extract/glucose medium, malt extract broth medium, and low nitrogen mineral medium; Support medium – polyurethane foam/pinewood chips/coarsed-milled straw | Remazol Brilliant Blue R (Synthetic dye) | 150 µg / g soil | Novotny et al. (2001) |
| *Phanerochaete chrysosporium, Trametes versicolor* and *Pleurotus ostreatus* | Growth medium – malt extract/glucose medium, yeast extract/peptone/glucose medium, and phenazine induction medium; Support medium – polyurethane foam/pinewood chips/coarsed-milled straw | PCB mixture (Delor 106) and a number of synthetic dyes | 150 µg / g soil | Novotny et al. (2004) |

| Saprotroph Fungi (White Rot and Brown Rot) | Bulking Agent(s)/ Synthetic Media | Pollutant | Pollutant Concentration | Reference |
|---|---|---|---|---|
| *Aspergillus flavus, A. fumigatus, Fusarium avenaceum, F. compactum, F. equiseti, F. oxysporum, F. proliferatum, F. semitectum, F. solani, Penicillium spinulosum* | -- | Commercial pesticide – Sodium monofluoroacetate (1080) | 20 mM | Twigg and Socha (2001) |

* Galzy and Slonimski, (1957)

*In vitro* role of extracellular peroxidases and laccases in oxidizing recalcitrant compounds have been well documented (Figure 3) but the functional significance of individual enzyme levels *in vivo* is poorly understood (Novotny et al., 2001). In a majority of cases, the complexity of biodegradation processes where various interactions may determine the rate-limiting step renders enzymatic actions unclear. However, this has not been an obstacle in developing WRF based soil bioremediation of PCBs (Pointing, 2001; Van Acken et al., 1999). Unfortunately, there are many other pesticides which are known to contaminate soils, have not been studied well and need extensive research to develop effective bioremediation approaches.

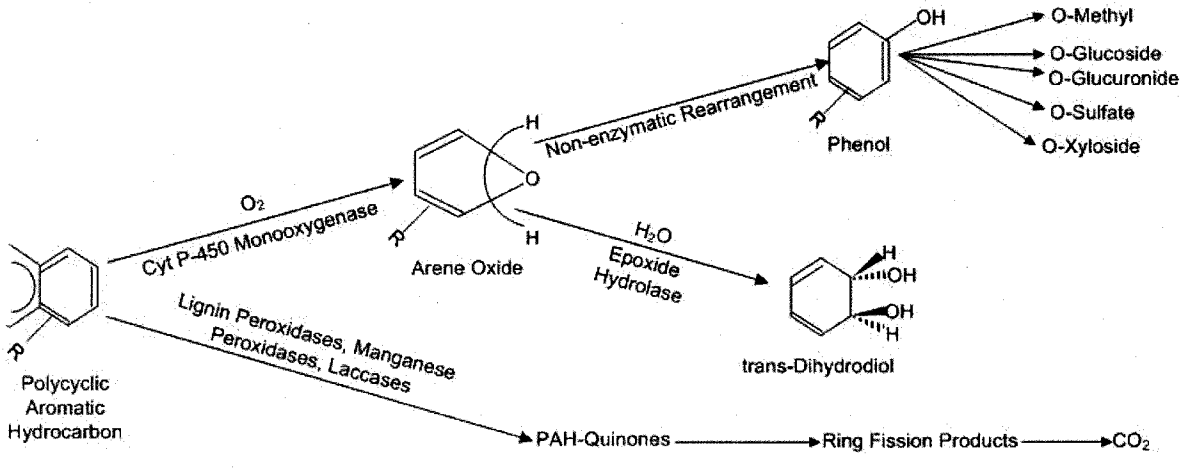



**Figure 3.** Fungal metabolism mechanisms of polycyclic aromatic hydrocarbons (Cerniglia, 1997).

### 2.1.2 Case Studies

While some soil microorganisms are ubiquitous and commonly occur in a variety of moist soils (Kelly, 1965; Bong et al., 1979), it is worthwhile to emphasize that fungal species have been more efficient in pesticide degradation even in arid and semi-arid soil conditions. Some case studies for fungal bioremediation of synthetic dyes, pesticides and polychlorinated biphenyls are presented below:

Many WRF have been efficient in mineralizing organochlorine pesticides, such as DDT, 2,4,5-trichlorophenoxyacetic (2,4,5-T) and 2,4-dichlorophenoxyacetic (2,4-D). Under ligninolytic growth conditions, many WRF strains, e.g., *Phanerochaete Chrysosporium*, *Pleurotus ostreatus*, *Phellinus weirii*, and *Polyporus versicolor* are able to mineralize 5.3–13.5% of added $^{14}$C-radiolabeled DDT, dicofol, and methoxychlor over 30 days (Bumpus and Aust 1987).

Highly recalcitrant pesticides like the chlorinated triazine herbicide 2-chloro-4-ethylamine-6-isopropylamino-1,3,4-triazine (atrazine) have been transformed by white-rot fungi *P. chrysosporium* (Mougin et al. 1994) and *Pleurotus pulmonarius* (Masaphy et al. 1993), yielding hydroxylated and *N*-dealkylated metabolites. The presence of healthy consortia of microorganisms in soil assures that certain pesticides can be used safely in environments, albeit under recommended application dosage.

In a safety study regarding pest control programs in Australia and New Zealand carried out by Twigg and Socha, (2001), role of fungal species of *Fusarium, Penicillium* and *Aspergillus* was assessed.. These researchers performed bioremediation of sodium monofluoroacetate (a commercial invertebrate pesticide) contaminated soil at pH 5.6 and 6.8 to distinguish the role of fungi in pesticide degradation in comparison to bacterial consortia and found that *Fusarium oxysporum* had greatest pesticide degradation ability (approximately 45% degradation of pesticide within 12 d). Degradation of the pesticides appeared to cease in a 28 d time course trial.

Novotny et al., (2001) successfully demonstrated that *in vivo* degradation of a broad selection of recalcitrant compounds (dyes, polyaromatic hydrocarbons–PAHs, PCBs) under a variety of conditions is possible, except that the enzyme levels are sufficiently high. These researchers used growth media like malt extract/glucose medium, yeast extract/peptone/glucose medium, and phenazine induction medium to produce mycelial mass and subsequently immobilized them to a support media like polyurethane

foam, pinewood chips or coarse-milled straw before application on contaminated soil (Novotny et al., 2004).

Previously, specially designed tube reactors were used to evaluate biodegradation experiments (Novotny et al., 1999). Each reactor was made of a tube (dia. 3.5 cm, length 24 cm) divided by a fine nylon net into two compartments. In one compartment, straw was inoculated with the WRF and fungal mycelium grew through the net to other compartment containing sterilized soil (10 g) that was contaminated with 100 µl of a Delor 103 solution in acetone. Subsequently, the system was aerated and moistened throughout the remediation period (2 month). Kubatova et al., (2001) could obtain a maximum removal efficiency of 40% of Delor 103 in two months using such a remediation system. However, such configurations are only applicable for micro-scale experiments and can not be feasible for field application.

Rojas-Avelizapa et al., (1999) used a mixed culture including fungi to degrade transformer oil. In liquid media amended with emulsifier Triton X-100 and supplemented with mineral salts, yeast extract (50 mg $l^{-1}$) and the transformer oil as sole carbon source, about 75% degradation occurred within 10 days. However, such studies could only provide partial solution of isolating or screening potential microbial strains for PCBs pollutants removal. Therefore, future studies should be more oriented towards field application of these potential fungi and their feasibility in soil bioremediation of PCBs.

## *2.2 Crude Oils and Polycyclic Aromatic Hydrocarbons*

Crude oil and its derivatives like polyaromatic hydrocarbons (PAHs) are highly toxic environmental contaminants introduced into the environment in huge quantities by several point sources (Meharg et al., 1998). The incidences of soil-contamination by PAH have been mostly noted for accidental spillage of crude oil besides, their perennial sources of emissions like, coal gasification processes, refineries and polymer industries (Leyval and Binet, 1998; Nicolotti and Egli, 1998). Different strains of bacteria have the ability to degrade simple PAHs like naphthalene (Lisowska and Dlugonski, 1999; Milstein et al., 1988; Katayama and Matsumura, 1991; Hofrichter et al., 1993; Lamar et al., 1993; Sack and Giinther, 1993).

### 2.2.1 Mechanisms of Bioremediation

In addition to the complexity of higher PAHs, temporal bioavailability of PAHs renders them more illusive towards bioremediation and requires concerted efforts of microbial consortia, which could simultaneously make PAHs bioavailable as well as degrade them, a phenomenon which is much easier for fungal species owing to their lytic enzyme systems (Bollag et al., 1992; McFarland et al., 1992; Eggen, 1999).

WRF produce an array of enzymes depending on their genetic constitution and environmental conditions. Some key degradation enzymes like lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), manganese-independent peroxidase (MIP), and laccase are well documented (Lamar, 1992; Vyas et al., 1994; Bogan and Lamar, 1996; Kotterman et al., 1996; Eggen, 1999). Extensive research has been focused on the role of these enzymes in bioremediation. Despite these serious efforts, the exact metabolic processes of biodegradation of PAHs are unavailable. For example, the PAHs degrading ability of *Pleurotus osteratus*, in the absence of LiP (Hatakka, 1990; Waldner et al., 1988) has been loosely correlated with laccase activity (Kerem et al., 1992; Thurston, 1994). In whole cultures (*Pleurotus ostreatus*, *Bjerkandera adusta*), attempts to link laccase activity with oxidation of phenanthrene and pyrene (Bezalel et al. 1996a) and the production of oxidative enzymes with PAH metabolism have been unsuccessful (Schutzendubel et al., 1999). It is likely that cytochrome P-450 monooxygenase produced by *Pleurotus osteratus* is responsible for the initial attack on PAHs, followed by subsequent degradation by laccase in similar manner to non-ligninolytic fungi (Bezalel et al., 1996b).

### 2.2.2 Case Studies

There are numerous examples where WRF have been incorporated in soil with or, without bulking/synthetic support media to treat PAHs like pyrene and fluorine (Sack and Fritsche, 1997; Garon et al., 2004). The use of PAH amended fungi (fungus pre-acclimatized to PAH or pollutant) culture may be more effective in bioremediation compared to non-acclimatized cultures (Garon et al., 2004). Certain fungi (*Gliocladium roseum, G. virens, Penicillium janczewskii, Stachybotrys chartarum, Trichoderma koningii* and *Zygorhynchus heterogamous*) show significant adaptability towards biodegradation of pollutants, while others (*Mucor hiemalis, Aspergillus terreus* and *A. ustus*) are not affected by acclimatization. Some case studies for fungal bioremediation of crude oils and polycyclic aromatic hydrocarbons are presented below:

Buswell, (1994) suggested use of spent mushroom culture (i.e. *Pleurotus ostreatus* – oyster mushroom, *Lentinula edodes* – shiitake mushroom; a by-product from commercial mushroom growers) as fungal inoculum for WRF in soil bioremediation. Further, Eggen, (1999) compared spent mushroom culture to colonized mushroom substrate (obtained from two commercial mushroom growers) before mushroom (fruiting body) production as an alternative for fungal inoculum. Eggen, (1999) investigated the modes of inoculum application, e.g., layering the fungi with soil and mixing fungi and soil. The PAH degradation potential of two commercial sources of fungi was dependent on the number of aromatic rings in the compounds. Spent mushroom compost was more effective than fungal substrate generated before mushroom production. Moreover, mixing of fungal inoculum and soil favored degradation of 4- and 5-ring compounds rather than layered incubation. After 7 weeks incubation at room temperature: 86% of total 16 PAHs, 89% of 3-ring PAHs, 87% of 4-ring PAHs and 48% of 5-ring PAHs were obtained.

A deuteromycete fungus, *Cladosporium sphaerospermum*, isolated from soil of an aged gas manufacturing plant was investigated by Potin et al., (2004a, b), to degrade PAHs. The average PAH (including high molecular weight PAHs) degradation capacity of this strain was 23%, after 4 weeks of incubation in aged PAH-contaminated soil. However, in liquid culture, it rapidly degraded benzo(α)pyrene during its early exponential phase of growth (18% after 4 days of incubation). In addition, only laccase activity was detected in liquid culture in the absence or in presence of benzo(α)pyrene. Therefore, bioremediation by *C. sphaerospermum* might be a potential and effective treatment approach for aged PAH-contaminated soils.

Laboratory scale experiments on axenic fungal biodegradation of aliphatic and aromatic hydrocarbons using à soil contaminated with 10% crude oil showed 65–74% reduction in 90 days (Colombo et al., 1996). Normal alkanes were almost completely degraded in the first 15 days, whereas slow dissociation of aromatic compounds (phenanthrene and methylphenanthrenes) was a rate–limiting step. *Aspergillus terreus* and *Fusarium solani*, isolated from oil-polluted areas, were more efficient in contaminant removal. Simultaneous multivariate analysis of parameters established a molecular weight dependant reactivity trend of oil components during biodegradation.

Meysami and Baheri, (2003) studied methods to support fungal growth and proliferation in soil contaminated with a weathered crude oil. They determined the ligninolytic enzyme activity and toxicity threshold of several white-rot fungi and attributed them to their hydrocarbon degradation ability. Approximately 100 g of artificially contaminated soil was inoculated with 7 days old mycelia of *Bjerkandera adusta* (approximately 60 ml buffer was added to 15 ml of mycelium) in jars at room temperature with loose caps until fungi mycelia had grown over the bulking agent surface completely. This was followed by complete mixing of contents and subsequent incubation for another 2 weeks. Pine wood chips, peat moss and Kellogg's bran flakes were examined for their properties as bulking agents and solid amendments. All strains developed severe toxicity at concentrations higher than 10000 ppm, a limitation in remediation process. The highest ligninolytic enzyme activities were shown by two strains of *Bjerkandera adusta* UAMH 7308 and 8258. A mixture of peat moss with bran flakes resulted in the best bulking agent composition for white-rot fungi, whereas, in the absence of any bulking agent, soil bioremediation was nominal. A maximum of 50% total petroleum hydrocarbon (TPH) reduction could be achieved by most of the fungal cultures within 4-5 weeks.

### *2.3 Munitions Waste*

*In-situ* (Lamar et al., 1993) and *in-vitro* (Barr and Aust, 1994b; Field et al., 1993) use of WRF in biodegradation of persistent organic chemicals containing aryl rings (e.g., explosives) has been extensively investigated. The role of lignolytic enzymes induced by white rots under stress conditions of carbon and nitrogen limitations (Barr and Aust, 1994b; Field et al., 1993) has been quoted as a principal one in bioremediation. It has been demonstrated by several researchers that WRFs have the ability to degrade explosive compounds like, 2,4,6- trinitrotoluene (TNT). For instance, TNT contamination of old munitions manufacturing and storage sites have been subjected to bioremediation utilising the white rot fungus *Phanerochaete chrysosporium* (Meharg et al., 1997a). White rots are promising solutions for the *in-situ* degradation of TNT in contaminated soils (Fernando et al., 1990; Michels and Gottshalk 1994; Stahl and Aust, 1993a, 1993b; Spiker et al., 1992).

### 2.3.1 Mechanisms of Bioremediation

The initial degradation mechanisms of TNT are common in all microorganisms (Meharg et al., 1997a). According to this mechanism, one of the nitro groups on aryl ring is reduced to form hydroxylamino-dinitrotoluenes and subsequent reduction results in the formation of amino-dinitrotoluenes (Michels and Gottshalk 1994; Goruntzy et al., 1994; Higson, 1992). The initial degradation of TNT by *P. chrysosporium* is subtly distinct from its degradation of many other organic chemicals, with respect to the role of lignolytic enzymes (Field et al., 1993). Also, the initial attack of *P. chrysosporium* on TNT is via reduction of nitro groups on the aryl ring takes place (Michels and Gottshalk 1994; Stahl and Aust, 1993a). However, once initial reduction occurs, lignolytic enzymes of *P. chrysosporium* can further degrade amino-dinitrotoluenes through oxidative attack, rather than subsequent reduction (Michels and Gottshalk 1994; Stahl and Aust, 1993a). Hydroxylamino-dinitrotoluene, an early intermediate of TNT reduction, inhibits *P. chryosporium* lignases, preventing its further degradation (Michels and Gottshalk 1994), thereby, limiting the potential of the fungi as bioremediators.

Stahl and Aust (1993b) concluded that reduction of TNT by *P. chryosporium* occurs via a redox potential driven reduction at plasmalemma. They conducted extensive assays of various cytological components to verify possible reduction of TNT and concluded that only intact *P. chryosporium* mycelium could reduce TNT. Therefore, it was established that TNT reduction occurs via plasmalemma redox potential.

### 2.3.2 Field Application

In spite of extensive research on understanding biochemical mechanisms of explosives degradation using WRF, present feasibility studies are not in unison with their future application. Some case studies for fungal bioremediation of munitions waste are presented below:

Craig et al., (1995) reported that WRF based processes were the least efficient among; (1) composting, (2) anaerobic bioslurry and (3) aerobic bioslurry for TNT degradation (Figure 4). In addition, selection criterian for explosive-contaminated soil bioremediation is also very narrow for WRF based process (Craig et al., 1995).

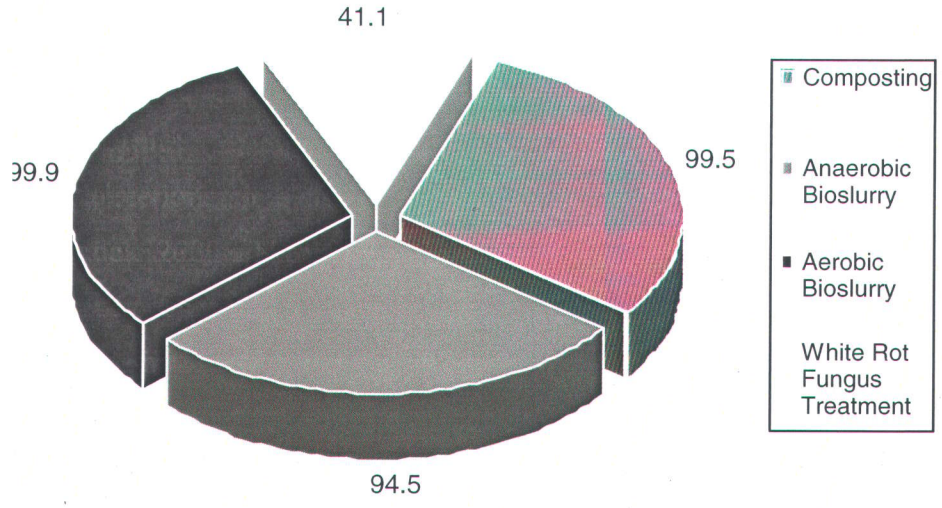



**Figure 4** Comparison chart for bench scale treatability as % destruction and removal efficiency of various processes for TNT contaminated soil bioremediation (Craig et al., 1995).

### *2.4 Metals*

Several methods for metal bioremediation have been investigated by many researchers.

#### 2.4.1 Mechanisms of Bioremediation

Treatment of metal-contaminated soil could take place via processes like heterotrophic (chemoorganotrophic) leaching – a strain of *Penicillium simplicissimum* has been employed to leach Zn from insoluble ZnO laden industrial filter dust by induced production of citric acid (>100 mM) (Schinner and Burgstaller, 1989; Franz et al., 1991, 1993); siderophore-mediated metal solubilization, where, metal is adsorbed to the biomass and or precipitated, with biomass separated from a soil slurry by flocculation (Gadd, 2004), resulting in a complete decrease in bioavailability of Cd, Zn and Pb (Diels et al., 1999); biomethylation of toxic metal complexes – several fungal species can methylate arsenic compounds such as arsenate [As(V), $AsO_4^{3-}$], arsenite [As(III), $AsO_2^-$] and methylarsonic acid ($CH_3H_2$ $AsO_3$) to volatile dimethyl-[$(CH_3)_2HAs$] or trimethylarsine [$(CH_3)_3As$] (Tamaki and Frankenberger, 1992), thereby, rendering them less bioavailable

to soil flora and fauna; redox transformations – most fungi can mobilize metals, metalloids and organometallic compounds by oxidation/reduction processes (Gadd, 1993; Gharieb et al., 1999; Lovley, 2000). Reduction of Hg(II) to Hg(0) by fungi results in diffusion of elemental Hg out of cells (Silver, 1996, 1998; Hobman et al., 2000) which can be utilized to mobilize Hg from contaminated soils.

Besides, biotransformation of metal and its complexes, fungi can also perform metal and metal-complex sorption to cellular surfaces, and even cationic species can be accumulated within cells via membrane transport systems of varying affinity and specificity. Inside cells, metal species may be incorporated within intracellular structures depending on the species concerned and the fungi (Gadd, 1996; White et al., 1997; Gadd and Sayer, 2000). In addition, there are numerous studies performed on free-living, pathogenic and plant symbiotic fungi associated with formation of calcium oxalate crystals from solubilised calcium (Gadd, 1999; Gharieb et al., 1998). This is important for biogeochemical processes in soils, acting as a buffering factor for calcium and phosphate availability. Other than calcium, fungi can also produce other metal oxalates and metal-bearing minerals, e.g., Cd, Co, Cu, Mn, Sr and Zn (White et al., 1997; Gadd, 1999; Sayer et al., 1999).

### 2.4.2 Case Studies

Metals bioremediation by fungi has been researched to a great extent by various researchers with very few field applications. Some case studies for fungal bioremediation of metals are presented below:

Barclay et al., (1998) demonstrated biodegradation of metallocyanide complexes by mixed fungi cultures by exploring the potential of different consortia like *Fusarium solani*, *Trichoderma polysporum*, *F. oxysporum, Scytalidium thermophilum,* and *Penicillium miczynski*. Under acidic conditions (pH 4), the metallocyanide complex degradation was slower (28 d for 90–95% cyanide removal), whereas, at pH 7 similar degradation was obtained within 5 days.

From the preceding examples, it becomes apparent that processes based on these fungal strains have the potential to provide efficient management and or degradation of toxic pollutants and or its derivatives. However, in order to fabricate a practical and

sustainable approach, proper measures should be taken before adapting WRF based bioremediation process in place of efficient physical and chemical methods or others.

## 3 Mycorrhizal Fungal Processes

The evolution of different guilds (fungal consortia) of mycorrhizal fungi (Figure 1) appears to have been driven by prevailing environmental conditions (Read, 1991, 1992). This has helped in the development and exploration of mycorrhizal processes, benevolent for soil remediation (Table 2). Although the naturally occurring symbiosis between higher plants and mycorrhizal fungi have been very well documented in many studies, the significance of functional diversity in mycorrhizal fungi (ectomycorrhizal, arbuscular, ericoid and orchid fungi) is still unclear (Cairney and Meharg, 1999; Cairney and Burke, 1994). It is believed that functional diversity of fungi is important for its symbiotic relationship with the host plant and to ecosystem level, which has the potential to offer an array of benefits to their plant hosts (Allen et al., 1995). Notably, in most of cases, the mycobionts present in rhizosphere are able to detoxify xenobiotics, thereby enhancing the quality of soil. Furthermore, close association of fungi to rhizosphere also includes nutritional needs of the fungi, thereby, resulting in a sustainable "eco-system", which could be of great interest in soil bioremediation. Mycorrhizal fungi dominate microbial ecology of heathlands, boreal and temperate forest biomes (Smith and Read, 1990), this fact is one of the many reasons that makes them suitable candidates in soil bioremediation. In the following sections, several pollutants have been discussed in relation to mycorrhizal fungi bioremediation.

### *3.1 Synthetic Dyes, Pesticides and Polychlorinated Biphenyls*

Mycorrhizal fungi, in particular, ectomycorrhizal (ECM), and ericoid collected from rural locations have been efficient remediators of a wide range of PCBs (Green et al., 1999; Meharg et al., 1997b; Donnelly et al., 1993). They have an innate ability to catabolize PCBs and pesticides like atrazine, 2,4-dichlorophenoxyacetic acid and chlorophenols (Green et al., 1999).

#### 3.1.1 Mechanisms of Bioremediation

Though mycorrhizal fungi may not degrade PCBs to yield energy, they may co-metabolize them as a consequence of consuming cyclic compounds, exuded by plants. For

example, plant phenolics, such as catechin and coumarin co-metabolize degradation of PCBs by bacteria (Salt et al., 1998).

### 3.1.2 Case Studies

The recommended dose of pesticide in field might be deleterious to a newly reclaimed calcareous soil with low populations of ECM (Abd-Alla et al., 2000). Therefore, information about pesticide contamination could be very important in assessing the soil bioremediation potential of ECM. Some case studies for fungal bioremediation of synthetic dyes, pesticides and polychlorinated biphenyls are presented below.

**Table 2.** Mycorrhizal soil fungi and respective pollutants.

| Mycorrhizal Fungi | Host Plant and Medium | Pollutant | Concentration | Reference |
|---|---|---|---|---|
| *Amanita pantherina, Amphinema bissoides, Armillaria mellea, Cenococcum geophilum, Hebeloma crustuliniforme, Laccaria amethystea, L. bicolor, L. laccata, Pisolithus tinctorius, Tricholoma vaccinum* | Black poplar (*Populus nigra*), Norway spruce (*Picea abies*) | Crude oil | 0.1–50 g $kg^{-1}$ | Nicolotti and Egli (1998) |
| *Trichoderma harzianum, Penicillium simplicissimum, P. janthinellum, P. funiculosum and P. terrestre* | Basal salts medium | Pyrene | 100 mg $l^{-1}$ | Saraswathy and Hallberg, (2002) |
| *Aspergillus niger* | -- | 2,4-D and MCPA | -- | Faulkner and Woodcock, (1964) |
| Arbuscular mycorrhizal fungi | -- | Pesticides – Afugan, Brominal, Gramoxone, Selecron and Sumi Oil | 0.13–3 mg/kg soil | Abd-Alla et al. (2000) |
| Arbuscular mycorrhizal fungi | -- | PAHs | 8–10 g $l^{-1}$ | Cabello (1997); Leyval and Binet (1998) |

| Mycorrhizal Fungi | Host Plant and Medium | Pollutant | Concentration | Reference |
|---|---|---|---|---|
| *Trichoderma* sp. | -- | Penta-chlorophenol, endosulfan and DDT | -- | Katayama and Matsumura (1991) |
| *Amanita muscaria, A. rubescens, A. Spissa, Bysporia terrestris, Gautieria crispa, G. othii, Hebeloma crustuliniforme, H. hiemale, H. sinapizans, Lactarius deliciosus, L. deterrimus, L. torminosus, Morchella conica, M. elata, M. esculenta, Paxillus involutus, Piloderma croceum, Radiigera atrogleba, Suillus granulatus, S. variegatus, Tricholoma lascivum, T. terreum* | -- | Phenanthrene, anthracene, fluronthene, pyrene, perylene, 4-fluorobiphenyl, TNT, 2,4-dichlorophenol, chlorpropham, | -- | Gramss et al. (1999); Donnelly and Fletcher (1995); Green et al. (1999); Meharg et al. (1997a,b) |
| *Trichoderma koningii* and *Fusarium culmorum* | Rye plant | $CdCl_2$ | -- | Kurek and Majewska (2004) |
| *Suillus bovines* | -- | Cd and Zn | 30–200 fungal/soil concentration ratio | Colpaert and Van Assche (1992) |

Biodegradation of organochlorine herbicides 2,4,5-T and 2,4-D have been also found to be mediated by mycorrhizal fungi association. However, the role of ligninolytic enzymes of *Phanerochaete chrysosporium* in this process was not confirmed (Ryan and Bumpus 1989; Yadav and Reddy 1993). Meanwhile, catabolic degradation of 2,4-D and 2-methyl-4,6-dichlorophenoxyacetic acid (MCPA) by *Aspergillus niger* (not a white-rot fungus) has been already established in previous studies on these herbicides (Faulkner and Woodcock 1964). Katayama and Matsumura, (1991) had shown degradation potential of rhizosphere-competent fungus *Trichoderma* sp. against several synthetic dyes, pentachlorophenol, endosulfan, and DDT.

### *3.2 Crude Oils and Polycyclic Aromatic Hydrocarbons*

Fungi in general have been successful in degradation of Polycyclic Aromatic Hydrocarbons (PAHs), and crude oil. There are increasing number of examples, where

ectomycorrhizal (ECM) fungi have been shown to degrade major environmentally deleterious PAHs. The ratio of ECM fungi screened for being a potential PAHs remediator is high (e.g., in a study, 33 ECM species out of 42 screened were efficient remediators, Meharg and Cairney, 2000).

### 3.2.1 Mechanisms of Bioremediation

Lower (2–3) chlorinated PCBs were readily degraded by majority of ECM species screened, and a limited number of species were able to degrade 4–5 chlorinated biphenyls (Donnelly and Fletcher, 1995). Gramss et al., (1999) have extensively studied selective PAH degradation by many ECM species and found that 4–5 ring PAHs were preferentially degraded by some species. Also, pollutants like chlorpropham, dichlorophenol, trinitrotoluene and monofluorobiphenyl have also been shown to be degraded by many ECM fungi (Meharg and Cairney, 2000; Green et al., 1999). Nevertheless, their degradation rate for the pollutants in comparison to WRF were somewhat low (Gramms et al., 1999), owing to inactive growth phase of the mycelia (Meharg and Cairney, 2000).

The PAH degradation efficiencies for ECM fungi have been high, for example, 90% trinitrotoluene (Meharg et al., 1997a), 95% monofluorobiphenyl (Green et al., 1999) and 50% benzo-(a)-pyrene from solution culture (Braun-Lullemann et al., 1999). It would be imperative to suggest that, isolation of ECM fungi from unpolluted soils may express PAH-degrading activities in their natural habitats.

In addition to saprotrophs, there is evidence of moderate to high tolerant arbuscular mycorrhizal (AM) fungi towards soil contaminated with PAHs as high as 8–10 $gl^{-1}$ (Cabello 1997; Leyval and Binet, 1998). Ganesan et al., (1991) found decreased diversity of AM fungal propagules in undisturbed soils in comparison to coal wastes, lignite spoils and calcite mine spoils, indicating negative effect of PAH on fungal population, whilst it indicated natural selection of fungal strains tolerant to PAH toxicity.

### 3.2.2 Case Studies

In a sagebrush-grassland ecosystem, alterations in AM fungal populations associated with application of waste water from oil shale processing units was observed by Stahl and Williams (1986). They observed good response from AM fungal taxa with respect to sensitivity towards crude oil pollutants – showing both decrease as well as

increase in density of spores in the treated soils. Nevertheless, decreased competition with other AM fungal taxa might be accepted as one of the valid reasons behind this phenomenon.

In order to assess the damage caused to ecosystems in Trecate's affected area Nicolotti and Egli (1998) studied the effect of artificial crude oil (88% hydrocarbons, 8% PAHs) contamination on ECM infection on the plant *Populus nigra* and *Picea abies*. The ECM infection was reduced in case of *Populus nigra* seedlings, due to oil contamination, however, for soils exposed to oil contamination prior to planting seedlings, ECM infection was stimulated, instead. Oil generally had no significant effect on percentage infection of *Picea abies*, but there was a decrease in infection in the soils exposed to oil for longer period of time (> 5 weeks) before planting. In addition, for diverse ECM fungi species exposed to same crude oil in axenic culture, some exhibited dose-dependent toxicity to crude oil, while others showed no response or stimulated growth, suggesting differential sensitivity to crude oil by ECM fungi (Nicolotti and Egli, 1998).

Another interesting finding of Nicolotti and Egli, (1998) was that after contamination, seedling growth of two plants reduced with time. Under normal conditions, soil would have recovered with time due to decrease in phytotoxic compounds. They hypothesized that seedling growth should be affected by mycorrhizal colonization. As a matter of fact, after contamination, the ectomycorrhizal infection potential on spruce and poplar decreased over time, contributing to seedlings better growth during contamination period. This incidence suggested that a crude oil spill in mixed agriculture or, forest area does not cause long-term environmental damage in contrast to coastal ecosystems. Many of the mycorrhizal fungi are able to survive in contaminated soil (Barr and Aust, 1994a). They use crude oil as their principal nutrient (Nicolotti and Egli, 1998), thereby, contributing to soil bioremediation.

There are ample evidences of various other soil fungi contributing to PAHs degradation e.g., *Trichoderma* sp., *Penicillium* spp., *Gliocladium* sp., whilst affecting mycorrhizal fungi both positively and/or, negatively (Azcbn-Aguilar and Barea, 1997). Saraswathy and Hallberg, (2002) reported a maximum of 75% removal for pyrene (4-ring PAH) at 50 mg $l^{-1}$ for axenic cultures of *Trichoderma* sp. and *Penicillium* spp. They also claimed that these soil fungi used pyrene as sole carbon source.

Therefore, it could be inferred that PAHs/crude oil contamination could be detoxified to a greater extent by naturally occurring mycobionts of mycorrhizal fungi and other soil fungi.

### *3.3 Munitions Waste*

In contrast to WRF, ectomycorrhizal (ECM) basidiomycetes also have a considerable potential to facilitate degradation of explosive chemical compounds (Donnelly and Fletcher, 1995; Donnelly et al., 1993, 1994). Meharg et al., (1997a) demonstrated that ECM fungi have the capacity to biotransform TNT.

#### 3.3.1 Mechanisms of Bioremediation

The munitions bioremediation potential of ECM was exhibited by both intact mycelial mass and extra-cellular enzymes under symbiotic conditions with the host plant. Meharg et al., (1997a) found that the biotransformation rate decreased with nitrogen limitation, contrary to basidiomycetes, whilst no decrease was observed under short term carbon starvation. This is crucial in the context to soil bioremediation since under symbiotic conditions, ECM fungi would have carbon and nitrogen sufficient environment.

Similar to *P. chryosporium* (Dass et al., 1995), ECM basidiomycetes may also release considerable amounts of non-specific extracellular proteases in liquid culture (Griffiths and Caldwell, 1992). Dass et al., (1995) concluded that *P. chryosporium* produces a number of proteases under different nutritional regimes, each having different abilities to degrade extracellular enzymes. Therefore, for *P. chryosporium* culture, in absence of protease inhibitors, there is greater possibility that other enzymes present might be degraded. Thus, TNT biodegradation capacity of culture filtrate must be viewed as a minimum as some residual proteolytic activity could have been present in filtrates. Further, the fact that extracellular enzymes are capable of degrading TNT could be regarded as additive phenomenon to plasmalemma redox mediated reduction or cytosolic biotransformation. In fact, intact cells were much more efficient at biotransformation than a cocktail of cell components. Furthermore, the apparent role of ECM basidiomycetes to biotransform TNT via extracellular enzymes has considerable significance in the potential use of ECM as bioremediators. It is expected that the efficiency of ECM fungi in biodegradation would augment in the presence of extracellular enzymes. Finally,

extracellular enzymes may modify the soil environment which are rather inaccessible to fungal hyphae, hence, contributing to efficacy in soil bioremediation.

### *3.4 Metals*

In soil, bioavailability of metals is dependant upon its various forms such as free metal ions, soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides, or they may form part of the structure of silicate minerals (Leyval et al., 1997). Furthermore, toxicity of metals in soil depends on their bioavailability, defined as their ability to be transferred from soil compartment to a living organism (Juste 1988).

Under axenic conditions, metal uptake and accumulation in the mycelium of ectomycorrhizal fungi have also been very efficient. For example, fungal/soil concentration ratios around 200 and 80 for Cd, and 40 and 30 for Zn of non-tolerant and metal-tolerant isolates of *Suillus bovinus,* respectively were reported by Colpaert and Van Assche (1992). ECM fungi have also been known to increase availability of metals in the rhizosphere by solubilizing minerals, including metal-containing rock phosphates, by production of organic acids or proton extrusion (Leyval et al. 1997).

Bradley et al. (1981, 1982) showed that ericoid mycorrhizal colonization led to a significant decrease in metal content of the shoot and an increase in the plant roots grown in sand amended with Cu or Zn. This explains mycorrhizal protection against excess heavy metal uptake by plants, in an ecosystem, where heavy metal availability may be higher due to soil acidity (Leyval et al. 1997). Consequently, excess translocation of metals within mycorrhizal fungi would exert more toxicity on fungal biomass, thereby, decline of fungal occurrence (propagule density) and infectivity in metal-polluted soils are possible. In fact, this has been used as bioindicator of soil contamination (Grodzinskaya et al. 1995). On the other hand, mycorrhizal colonization of plant roots after soil remediation can be an indication of metal detoxification/non-bioavailability.

These finding collectively suggest that mycorrhizal fungi based soil bioremediation of PCBs or metals is possible, however, due to their higher susceptibility and slower metabolism, mycorrhizal fungi would be inefficient in contrast to white-rot fungi.

## 4 Conclusions

Fungi demonstrate an excellent ability to carry out soil bioremediation. White rot fungi need supplementary-nutrient source other than pollutants. Hence, once the growth supporting bulking agent(s)/substrate(s) (wood chips, peat moss, cereal flakes) deplete(s), the soil must be reinoculated with fresh white rot fungal inoculum, a major drawback for its large-scale sustainable use. A self-sustaining establishment of fungal-consortia in soil is possible with mycorrhizal fungi-plant symbiosis. In fact, all tree species are usually infected by mycorrhizal symbionts and ectomycorrhizal mycelia are extensive in forest soils. In some instances, almost half the soil biomass could be constituent of mycorrhizal symbionts. Both WRF and mycorrhizal fungi have their own advantages and disadvantages. For example, although WRF inoculum density can be regulated, their success is greatly dependent on treatment location, e.g., cellulose-rich residues would support WRF growth. Similarly, mycorrhizal fungi are comparatively slower and their growth mainly relies on rhizosphere of plant and other soil microbial consortia. Whilst, WRF are more extensively studied and applied in soil bioremediation, there are only a few mycorrhizal fungi, screened against a limited number of pollutants. Nevertheless, WRF showed greater efficiency in soil bioremediation in contrast to mycorrhizal fungi, limited bioavailability of pollutants would be better dealt with sustainable ecological niche of mycorrhizal fungi in soil. In other words, WRF are at their best but potential of mycorrhizal fungi should also be intelligently realized to facilitate sustainable remediation of soil.

## 5 References

Abd-Alla, M.H., Omar, S.A., Karanxha, S. 2000. The impact of pesticides on arbuscular mycorrhizal and nitrogen-fixing symbioses in legumes. *Applied Soil Ecology.* 14, 191–200.

Allen, E.B., Allen, M.F., Helm, D.J., Trappe, J.M., Molina, R., Rincon, E. 1995. Patterns and regulation of mycorrhizal plant and fungal communities. *Plant and Soil.* 170, 47–62.

Alloway, J.B., Ayres, D.C. 1993. Chemical principles of environmental pollution, Chapman and Hall, London.

Anderson, T.A., Guthrie, E.A., Walton, B.T. 1993. Bioremediation in the rhizosphere. *Environmental Science and Technology.* 27, 2630–2636.

Andersson, B.E., Welinder, L., Olsson, P.A., Olsson, S., Henrysson, T. 2000. Growth of inoculated white-rot fungi and their interactions with the bacterial community in soil contaminated with polycyclic aromatic hydrocarbons, as measured by phospholipid fatty acids. *Bioresource Technology.* 73, 29–36.

Azcbn-Aguilar, C., Barea, J.M. 1997. Applying mycorrhiza biotechnology to horticulture: significance and potentials. *Scientia Horticulturae.* 68, 1–24.

Barclay, M., Hart, A., Knowles, C.J., Meeussen, J.C.L., Tett, V.A. 1998. Biodegradation of metal cyanides mixed and pure cultures of fungi. *Enzyme and Microbial Technology.* 22, 223–231.

Barr, P.D., Aust, S.D., 1994a. Mechanisms white rot fungi use to degrade pollutants. *Environmental Science and Technology.* 28, 78–87.

Barr, P.D., Aust, S.D. 1994b. Pollutant degradation by white rot fungi. Rev. Env. *Contam. Toxicol.* 138, 49–72.

Baarschers, W.H., Heitland, H.S. 1986. Biodegradation of Fenitrothion and Fenitrooxon by the fungus *Trichoderma viridae. J. Agric. Food. Chem.* 34, 707–709.

Bogan, B.W., Lamar, R.T. 1996. Polycyclic Aromatic Hydrocarbon degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. *Applied Environmental Microbiololgy.* 62, 1597–1603.

Bollag, J.-M., Myers, C.J., Minard, R.D. 1992. Biological and chemical interactions of pesticides with soil organic matter. *The Science of the Total Environment.* 123/124, 205–217.

Bezalel, L., Hadar, Y., Cerniglia, C.E. 1996a. Mineralization of Polycyclic Aromatic Hydrocarbons by the white-rot fungus *Pleurotus ostreatus. Applied Environmental Microbiololgy.* 62, 292–295.

Bezalel, L., Hadar, Y., Fu, P.P., Freeman, J.P., Cerniglia, C.E. 1996b. Initial oxidation products in the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by the white rot fungus *Pleurotus ostreatus. Applied Environmental Microbiololgy.* 62, 2554–2559.

Bennett, J.W., Childress, A.M., Wunch, K.G., Connick, Jr. W.J. 2001. Fungal compositions for bioremediation. United States Patent 6,204,049.

Bradley, R., Burt, A.J., Read, D.J. 1981. Mycorrhizal infection and resistance to heavy metal toxicity in *Calluna vulgaris. Nature.* 292, 335–337.

Bradley, R., Burt, A.J., Read, D.J. 1982. The biology of mycorrhiza in the Ericaceae. VIII. The role of mycorrhizal infection in heavy metal resistance. *New. Phytol.* 91, 197–209.

Braun-Lullemann, A., Huttermann, A., Majcherczyk, A. 1999. Screening of ectomycorrhizal fungi for degradation of polycyclic aromatic hydrocarbons. *Applied Microbiology and Biotechnology.* 53, 127–132.

Bumpus, J.A., Aust, S.D. 1987. Biodegradation of DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] by the white-rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 53, 2001–2008.

Bumpus, J.A., Kakar, S.N., Coleman, R.D. 1993. Fungal degradation of organophosphorous insecticides. *Appl. Biochem. Biotechnol.* 39, 715–726.

Chaillan, F., Flèche, A.L., Bury, E., Phantavong, Y., Grimont, P., Saliot, A., Oudot, J. 2004. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Research in Microbiology.* 155, 587–595.

Cabello, M.N. 1997. Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). *FEMS Microbiology Ecology.* 22, 233–236.

Cairney, J.W.G. and Burke, R.M. 1994. Fungal enzymes degrading plant cell walls: their possible significance in ectomycorrhizal symbiosis. *Mycol. Res.* 98, 1345–1356.

Cerniglia, C.E. 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *Journal of Industrial Microbiology & Biotech.* 19, 324–333.

Clapp, J.P., Young, J.P.W., Merryweather, J.W., Fitter, A.H. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytologist.* 130, 259–265.

Colombo, J., Cabello, M., Arambarri, A.M. 1996. Biodegradation of aliphatic and aromatic Hydrocarbons by natural soil microflora and Pure cultures of imperfect and lignolitic fungi. *Environmental Pollution.* 94, 355–362.

Colpaert, J.V., Van Assche, J.A. 1992. The effects of cadmium and the cadmium-zinc interaction on the axenic growth of ectomycorrhizal fungi. *Plant Soil.* 145, 237–243.

Craig, H.D., Sisk, W.E., Nelson, M.D., Dana, W.H. Bioremediation of Explosives- Contaminated Soils: A Status Review. Presented in: *Proceedings of the 10th Annual Conference on Hazardous Waste Research.* pp. 164–179. Kansas State University, Manhattan, Kansas, May 23-24, 1995.

Dahlberg, A., Jonsson, L., Nylund, J.-E. 1997. Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. *Canadian Journal of Botany.* 75, 1323–1335.

Dass, S.B. Dosoretz, C.G., Reddy, C.A., Grethlein, H.E. 1995. Extracellular proteases produced by the wood-degrading fungus *Phanerochaeta chrysosporium* under lignolytic and non-lignolytic conditions. *Arch. Microbiol.* 163, 254–258.

Diels, L., DeSmet, M., Hooyberghs, L., Corbisier, P. 1999. Heavy metals bioremediation of soil. *Mol. Biotechnol.* 12, 149–158.

Dittmann, J., Heyser, W., Bucking, H. 2002. Biodegradation of aromatic compounds by white rot and ectomycorrhizal fungal species and the accumulation of chlorinated benzoic acid in ectomycorrhizal pine seedlings. *Chemosphere.* 49, 297–306.

Donnelly, P.K., Fletcher, J.S. Potential use of mycorrhizal fungi as bioremediation agents. In: *Bioremediation Through Rhizosphere Technology* (Edited by T. A Anderson & J. IL Coats), pp. 93-98. American Chemical Society, Washington DC (1994).

Donnelly, P.K., Fletcher, J.S. 1995. PCB metabolism by ectomycorrhizal fungi. Bull. *Environ. Contain. Toxicol.*, 54, 507–513.

Donnelly, P.K., Entry, J.A., Crawford, D.L. 1993. Degradation of atrazine and 2,4-dichlorophenoxyacetic acid by mycorrhizal fungi at three nitrogen concentrations *in vitro. Appl. Environ. Microbiol.*, 59, 2642–2647.

Duke, T.W., Lowe, J.I., Wilson, A.J. 1970. A polychlorinated biphenyl (Arochlor 1254) in the water, sediment and biota of Escambia Bay, Florida. *Bull. Environ. Contam. Toxicol.* 5, 3171–80.

Eccles, H. 1999. Treatment of metal-contaminated wastes: why select a biological process? *Trends Biotechnol.* 17, 462–465.

Eggen, T. 1999. Application of fungal substrate from commercial mushroom production – *Pleuorotus ostreatus* – for bioremediation of creosote contaminated soil. *International Biodeterioration & Biodegradation.* 44, 117–126.

Faulkner, J.K., Woodcock, D. 1964. Metabolism of 2,4-dichlorophenoxyacetic acid by *Aspergillus niger* Van Tiegh. Nature. 203, 865–866.

Fernando, T., Bumpus, J.A., Aust, S.D. 1990. Biodegradatiun of TNT (2,4,6-trinitrotoluene) by *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. 56, 1666–1671.

Field, J.A., Jong, E., Feijoo-Costa, Bont, G.J.A.M. 1993. Screening for lignolytic fungi applicable to the biodegradation of xenobiotics. *Trend. Biotech.*, 11, 44–49.

Franz, A., Burgstaller, W., Schinner, F. 1991. Leaching with *Penicillium simplicissimum*: influence of metals and buffers on proton extrusion and citric acid production. *Appl. Environ. Microbiol.* 57, 769–774.

Franz, A., Burgstaller, W., Muller, B., Schinner, F. 1993. Influence of medium components and metabolic inhibitors on citric acid production by *Penicillium simplicissimum. J. Gen. Microbiol.* 139, 2101–2107.

Gadd, G.M. 1993. Microbial formation and transformation of organometallic and organometalloid compounds. *FEMS Microbiol. Rev.* 11, 297–316.

Gadd, G.M. 1996. Influence of microorganisms on the environmental fate of radionuclides. *Endeavour.* 20, 150–156.

Gadd, G.M., Sayer, J.A. 2000. Fungal transformations of metals and metalloids. In: Lovley, D.R. (Ed.), *Environmental Microbe –Metal Interactions.* Am. Soc. Microbiol, Washington, pp. 237– 256.

Gadd, G.M. 1999. Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. *Adv. Microb. Physiol.* 41, 47–92.

Gadd, G. M. 2004. Microbial influence on metal mobility and application for bioremediation. *Geoderma.* 122, 109– 119.

Galzy, P., Slonimski, S. 1957. Variations physiologiques de la levure au cours de la croissance sur l'acide lactique comme seule source de carbone. *Cri. Rev. Acad. Sci. Paris.* 245 sér. D, 2423–2426.

Ganesan, V., Ragupathy, S., Parthipan, B., Rajini Rani, D.B., Mahadevan, A. 1991. Distribution of vesicular-arbuscular mycorrhizal fungi in coal, lignite and calcite mine spoils of India. *Biology and Fertility of Soils.* 12, 131–136.

Gardes, M., Bruns, T. 1996. Community structure of ectomycorrhizal fungi in a Pinus muricata forest: above- and below-ground views. *Canadian Journal of Botany.* 74, 1572–1583.

Garon, D., Sage, L., Wouessidjewe, D., Seigle-Murandi, F. 2004. Enhanced degradation of fluorene in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. *Chemosphere.* 56, 159–166.

Gharieb, M.M, Kierans, M., Gadd, G.M. 1999. Transformation and tolerance of tellurite by filamentous fungi: accumulation, reduction and volatilization. *Mycol. Res.* 103, 299–305.

Gill, P.E. 1996. Process for bioremediation of soils. United States Patent 5,525,139.

Gill, P.E. 1997. Spill clean-up process. United States Patent 5,609,668.

Goruntzy, T., Drzyzga, O., Kahl, M.W., Bruns-Nagel, D., Breitung, J., von Loew, E., Blotevogel, K.H. 1994. Microbial degradation of explosives and related compounds. *Crit. Rev. Microbiol.* 20, 265–284.

Gramss, G., Kirsche, B., Voigt, K.D., Gunther, T., Fritsche, W. 1999. Conversion rates of five polycyclic aromatic hydrocarbons in liquid cultures in fifty-eight fungi and the concomitant production of oxidative enzymes. *Mycological Research.* 103, 1009–1018.

Green, N.A., Meharg, A.A., Till, C., Troke, J., Nicholson, J.K. 1999. Degradation of 4-fluorobiphenyl by mycorrhizal fungi as determined by 19F nuclear magnetic resonance spectroscopy and $^{14}C$ radiolabelling analysis. *Applied and Environmental Microbiology.* 65, 4021–4027.

Griffiths, R.P. and Caldwell, B.A. Mycorrhizal mat communities in forest soils In: *Mycorrhizas in Ecosystems.* (Edited by D. J. Read, D. H. Lewis, A. H. Fitter, and I. J. Alexander ), pp 98-105. CAB International, Wallingford (1992).

Hatakka, A. 1990. Lignin-modifying enzymes from selected white rot fungi: Production and role in lignin degradation. *FEMS Microbiology Reviews.* 13, 125–135.

Higson, F.J. 1992. Microbial degradation of nitroaromatic compounds. *Adv.Appl. Microbiol.* 37, 1–19.

Hobman, J.L., Wilson, J.R., Brown, N.L. 2000. Microbial mercury reduction. In: Lovley, D.R. (Ed.), *Environmental Microbe – Metal Interactions.* Am. Soc. Microbiol, Washington, pp. 177– 197.

Hofrichter, M., Gunther, T., Fritsche, W. 1993. Metabolism of phenol, chloro- and nitrophenols by the *Penicillium* strain Bi 7/2 isolated from a contaminated soil. *Biodegradation*, 3, 415–421.

Hutzinger O, Safe S, Zitko V. *The chemistry of PCBs.* Cleveland, OH: CRC Press. 1974, 7–39.

INE. *Bifenilos policlorados.* Mexico, DF: Instituto Nacional de Ecologia, Secretary of the Environment and Natural Resources, 1995 in Spanish.

Juste, C. 1988. Appréciation de la mobilité et de la biodisponibilité des éléments en traces du sol. *Sci. Sol.* 26, 103–112.

Katayama, A. and Matsumura, F. 1991. Photochemically enhanced microbial degradation of environmental pollutants. *Environ. Sci. Technol.*, 25, 1329–1333.

Kerem, Z., Friesem, D., Hadar, Y. 1992. Lignocellulose degradation during soild-state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium. Applied Environmental Microbiology.* 58, 1121–1127.

Kotterman, M.J.J., Wasseveld, R.A., Field, J.A. 1996. Hydrogen peroxide production as a limiting factor in xenobiotic compound oxidation by nitrogen-sufficient cultures of *Bjerkandera* sp.. Strain BOS55 overproducing peroxidases. *Applied Environmental Microbiology.* 62, 880–885.

Kubatova, A., Erbanova, P., Homolka, L., Nerud, F., Sasek, V. 2001. PCB congener selective biodegradation by the white rot fungus *Pleurotus ostreatus* in contaminated soil. *Chemosphere.* 43, 207–215.

Kurek, E., Majewska, M. 2004. In vitro remobilization of Cd immobilized by fungal biomass. *Geoderma.* 122, 235–246.

Lamar, R.T., Evans, J.W., Glaser, J.A. 1993. Solid-phase treatment of a pentachlorophenol-contaminated soil using lignin-degrading fungi. *Environ. Sci. Technol.*, 27, 2566–2571.

Lamar, R.T. 1992. The role of fungal lignin-degrading enzymes in xenobiotic degradation. *Biotechnology.* 3, 261–266.

Lappin, H.M., Greaves, M.P., Slater, J.H. 1985. Degradation of the herbicide mecoprop (2-(2-methyl-4-chlorophenoxy) propionic acid) by a synergistic microbial community. *Applied and Environmental Microbiology.* 49, 429–433.

Leyval, C., Binet, P. 1998. Effect of polyaromatic hydrocarbons in soil on arbuscular mycorrhizal plants. *Journal of Environmental Quality.* 27, 402–407.

Leyval, C., Turnau, K., Haselwandter, K. 1997. Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza.* 7, 139–153.

Lisowska K, Dlugonski J. 1999. Removal of anthracene and phenanthrene by filamentous fungi capable of cortexolone 11-hydroxylation. *J. Basic Microbiol.* 39, 117–25.

Liu, G., Chambers, S.M., Cairney, J.W.G. 1998. Molecular diversity of ericoid mycorrhizal endophytes isolated from Woollsia pungens (Cav.) F. Muell. (Epacridaceae). *New Phytologist.* 140, 145–153.

Lovley, D.R. 2000. Fe(III) and Mn(IV) reduction. In: Lovley, D.R. (Ed.), *Environmental Microbe –Metal Interactions.* Am. Soc. Microbiol, Washington, pp. 3–30.

Lovley, D.R., Coates, J.D. 1997. Bioremediation of metal contamination. *Curr. Opin. Biotechnol.* 8, 285–289.

Masaphy, S., Levanon, D., Vaya, J., Henis, Y. 1993. Isolation and characterization of a novel atrazine metabolite produced by the fungus *Pleurotus pulmonarius*, 2-chloro-4-ethylamino-6-(1-hydroxyisopropyl)amino-1,3,5-triazine. *Appl. Environ. Microbiol.* 59, 4342–4346.

McFarland, M.J., Qiu, X.J., Sims, J.L. 1992. Remediation of petroleum impacted soils in fungal compost bioreactors. *Water Science Technology.* 25, 197–206.

Meharg, A.A., Cairney, J.W.G. 2000. Ectomycorrhizas – extending the capabilities of rhizosphere remediation? *Soil Biology & Biochemistry.* 32, 1475–1484.

Meharg, A.A., Wright, J., Dyke, H., Osborn, D. 1998. Polycyclic aromatic hydrocarbon (PAH) dispersion and deposition to vegetation and soil following large scale chemical fire. *Environmental Pollution.* 99, 29–36.

Meharg, A.A., Dennis, G.R., Cairney, J.W.G. 1997a. Biotransformation of 2,4,6-trinitrotoluene (TNT) by ectomycorrhizal basidiomycetes. *Chemosphere.* 35, 513–521.

Meharg, A.A., Cairney, J.W.G., Maguire, N. 1997b. Mineralisation of 2,4-dichlorophenol by ectomycorrhizal fungi in axenic culture and in symbiosis with pine. *Chemosphere.* 34, 2495–2504.

Meysami, P., Baheri, H. 2003. Pre-screening of fungi and bulking agents for contaminated soil bioremediation. *Advances in Environmental Research.* 7, 881–887

Michels, J., Gottshalk, G. 1994. Inhibition of lignin peroxidase of *Phanerochaete chrysosporium* by hydroxylamino-dinitrotoluene, an early intermediate in the degradation of 2,4,6-tfinitrotoluene. *Appl. Environ. Microbiol.* 60, 187–194.

Milstein, O., Trojanowski, J., Hiittermann, A., Gressel, J. 1988. Catabolism of single ring aromatic acids by four *Aspergillus* species. *Microbios.* 55, 7–16.

Mougin, C., Laugero, C., Asther, M., Dubroca, J., Frasse, P. 1994. Biotransformation of the herbicide atrazine by the white-rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 60, 705–708.

Nicolotti, G., Egli, S. 1998. Soil contamination by crude oil: impact on the mycorrhizosphere and on revegetation potential of forest tress. *Environmental Pollution.* 99, 37–43.

Novotny, C., Svobodova K., Erbanova P., Cajthaml, T., Kasinath A., Lang, E., Sasek, V. 2004. Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biology and Biochemistry.* 36, 1545–1551.

Novotny, C., Rawal, B., Bhatt, M., Patel, M., Sasek, V., Molitoris, H.P. 2001. Capacity of *Irpex lacteus* and *Pleurotus ostreatus* for decolorization of chemically different dyes. *Journal of Biotechnology.* 89, 113–122.

Novotny, C., Erbanova, P., Cajthaml, T., Rothschild N. Dosoretz, C., Sasek, V. 2000. *Irpex lacteus*, a white rot fungus applicable to water and soil bioremediation. *Appl. Microbiol. Biotechnol.* 54, 850–853.

Ousley, M.A., Lynch, J.M., Whipps, J.M. 1994. Potential of *Trichoderma* spp. as consistent plant-growth stimulators. *Biol Fertil Soils.* 17, 85–90.

Perotto, S., Actis-Perino, E., Perugini, J., Bonfante, P. 1996. Molecular diversity of fungi from ericoid mycorrhizal roots. *Molecular Ecology.* 5, 123–131.

Pointing, S.B. 2001. Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology.* 57, 20–33.

Potin, O., Veignie, E., Rafin, C. 2004a. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. *FEMS Microbiology Ecology.* 51, 71–78.

Potin, O., Rafin, C., Veignie, E. 2004b. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. *Int. Biodeterior. Biodegrad.* 54, 45–52.

Read, D.J. 1991. Mycorrhizas in ecosystems. *Experientia.* 47, 376–391.

Read, D.J. The mycorrhizal mycelium. In: *Mycorrhizal Functioning: an Integrated Plant-Fungal Process.* (Edited by M. J. Allen), pp 102-133. Chapman and Hall, New York 1992.

Rojas-Avelizapa, N.G., Rodriguez-Vazquez, R., Enriquez-Villanueva, F., Martinez-Cruz, J., Poggi-Varaldo, H.M. 1999. Transformer oil degradation by an indigenous microflora isolated from a contaminated soil. *Resources, Conservation and Recycling.* 27, 15–26.

Ryan, T.P., Bumpus, J.A. 1989. Biodegradation of 2,4,5-trichlorophenoxyacetic acid in liquid culture and in soil by the white rot fungus *Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol.* 31, 302–307.

Sack, U., Gunther, T. 1993. Metabolism of PAH by fungi and correlation with extraceilular enzymatic activities. *J. Basic Microbiol.* 33, 269–277.

Sack, U., Fritsche, W. 1997. Enhancement of pyrene mineralization in soil by wood-decaying fungi. *FEMS Microbiology Ecology.* 22, 77–83.

Saraswathy, A., Hallberg, R. 2002. Degradation of pyrene by indigenous fungi from a former gasworks site. *FEMS Microbiology Letters.* 210, 227–232.

Schinner, F., Burgstaller, W. 1989. Extraction of zinc from an industrial waste by a *Penicillium sp. Appl. Environ. Microbiol.* 55, 1153–1156.

Schutzendubel, A., Majcherczyk, A., Johannes, C., Huttermann, A. 1999. Degradation of fluorene, anthracene, phenanthrene, fluor-anthene, and pyrene lacks connection to the production of extracellular

enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. *International Biodeterioration and Biodegradation.* 43, 93–100.
Silver, S. 1996. Bacterial resistances to toxic metal ions—a review. *Gene.* 179, 9–19.
Silver, S. 1998. Genes for all metals – a bacterial view of the periodic table. *J. Ind. Microbiol. Biotech.* 20, 1–12.
Smith, S.E., Read, D.J. 1990. Mycorrhizal symbiosis, 2nd ed. Academic Press, San Diego, Calif.
Spiker, J.K., Crawford, D.L., Crawford, R.L. 1992. Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosives-contaminated soils by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58, 3199–3202.
Sreekrishnan, T.R., Tyagi, R.D. 1994. Heavy metal leaching from sewage sludges: a techno-economic evaluation of the process options. *Environ. Technol.* 15, 531–543.
Stahl, J.D., Aust, S.D. 1993a. Metabolism and detoxification of TNT by *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Comm.* 192, 477–482.
Stahl, J.D., Aust, S.D. 1993b. Plasma membrane dependent reduction of 2,4,6-trinitrotoluene by *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Comm.* 192, 471–476.
Tamaki, S., Frankenberger, W.T. 1992. Environmental biochemistry of arsenic. Rev. *Environ. Contam. Toxicol.* 124, 79–110.
Thurston, C.F. 1994. The structure and function of fungal laccases. *Microbiology.* 140, 19–26.
Twigg, L.E., Socha, L.V. 2001. Defluorination of sodium monofluoroacetate by soil microorganisms from central Australia. *Soil Biology & Biochemistry.* 33, 227–234.
Vachon, P.R., Tyagi, R. D., Auclair, J.C., Wilkinson, K.J. 1994. Chemical and biological leaching of aluminium from red mud. *Environ. Sci. Technol.* 28, 26–30.
VanAcken, B., Godefroid, L.M., Peres, C.M., Naveau, H., Agathos, S.N. 1999. Mineralization of 14C-U ring labeled 4-hydroxylamino-2,6-dinitrotoluene by manganese-dependent peroxidase of the white-rot basidiomycete *Phlebia radiata*. *Journal of Biotechnology.* 68, 159–169.
Verrecchia, E.P., Dumont, J.-L. 1996. A biogeochemical model for chalk alteration by fungi in semiarid environments. *Biogeochemical.* 35, 447– 470.
Vyas, B.R.M., Bakowski, S., Sasek, V., Matucha, M. 1994. Degradation of anthracene by selected white-rot fungi. *FEMS Microbiology Ecology.* 14, 65–70.
Waldner, R., Leisola, M.S.A., Fiechter, A. 1988. Comparison of ligninolytic activities of selected white-rot fungi. *Applied Microbiology and Biotechnology.* 29, 400–407.
Walker, R.T. 2003. Surfactant coated products and methods for their use in promoting plant growth and soil remediation. United States Patent 6,565,860.
White, C., Sayer, J.A., Gadd, G.M. 1997. Microbial solubilisation and immobilization of toxic metals: key biogeochemical processes for treatment of contamination. *FEMS Microbiol. Rev.* 20, 503– 516.
White, C., Sharman, A.K., Gadd, G.M. 1998. An integrated microbial process for the bioremediation of soil contaminated with toxic metals. *Nature Biotechnol.* 16, 572–575.
Yadav, J.S., Reddy, C.A. 1993. Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) and mixtures of 2,4-D and 2,4,5-trichlorophenoxyacetic acid by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59, 2904–2908.
Zheng, Z., Obbard, J.P. 2002. Oxidation of polycyclic aromatic hydrocarbons (PAH) by the white rot fungus, *Phanerochaete chrysosporium*. *Enzyme and Microbial Technology.* 31, 3–9.

## Acknowledgements

The authors are sincerely thankful to Natural Sciences and Engineering Research Council of Canada (Grants A4984, Canada Research Chair) for financial support. The views and opinions expressed in this article are those of authors.

# Part II

# Antagonistic Fungi, *Trichoderma* spp.: Panoply of Biological Control

**Mausam Verma[a], Satinder K. Brar[a], R. D. Tyagi[a,*], R. Y. Surampalli[b] and J. R. Valéro[a,c]**

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

[b]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

[c]Centre Canadien des Forêts, Centre Foresterie des Laurentides 1055 du P.E.P.S., P.O. Box 3800, Sainte-Foy, Québec Canada, G1V 4C7

**Biochemical Engineering Journal (under revision)**

## Les champignons antagonistes *Trichoderma* spp.: une panoplie d'applications en lutte biologique


### Résumé

*Trichoderma* spp. ont largement été utilisés comme agents fongiques antagonistes contre plusieurs organismes nuisibles et comme stimulateurs de croissance végétale. Des taux métaboliques plus rapides, des métabolites antimicrobiens et leur conformation physiologique sont des facteurs clés qui contribuent principalement à l'antagonisme de ces champignons. D'un autre côté, *Trichoderma* spp. ont également été utilisés pour la production d'une vaste gamme d'enzymes commerciales dont les cellulases, les protéases et les chitinases. L'utilisation des *Trichoderma* spp. antagonistes comme agents de lutte biologique pourrait maintenant présenter un avantage additionnel pour ces champignons, tel que discuté dans cette revue de littérature. Malgré que ces champignons soient très utilisés commercialement ainsi qu'à l'échelle pilote, seulement très peu d'information est disponible dans la littérature sur leur production dans un contexte d'antagonisme. La possibilité de les produire de façon économique et la grande variété de composés antagonistes produits par plusieurs *Trichoderma* spp. garantissent leur potentiel comme agents de lutte biologique. De plus, la pleine exploitation des agents de lutte biologique basés sur *Trichoderma* spp. pourrait joueur un rôle crucial dans la lutte biologique via leurs modes typiques d'action – par exemple, le mycoparasitisme, la compétition pour les nutriments et l'espace, l'antibiose par les enzymes et les métabolites secondaires, et la production de stimulateurs pour la croissance des plantes. Néanmoins, plus de recherches et de revue de littérature sur l'information concernant ces agents de lutte biologique sont nécessaires afin d'exploiter leur potentiel actuel, ce qui est l'objectif de la présente revue de littérature.


***Mots-clés:*** Antagonisme; agents de lutte biologique; sources microbiens; *Trichoderma* spp.; eaux usées; boues d'épuration.

**Abstract**

*Trichoderma* spp. have been widely used as antagonistic fungal agents against several pests as well as plant growth enhancers. Faster metabolic rates, anti-microbial metabolites, and physiological conformation are key factors which chiefly contribute to antagonism of these fungi. On the other hand, *Trichoderma* spp. have likewise been used in a wide range of commercial enzyme productions namely, cellulases, proteases, and chitinases. Meanwhile, use of antagonistic *Trichoderma* spp. as biocontrol agents could present an additional advantage of these fungi, as discussed in this review. Despite being extensively used commercially as well as in pilot scale, only scarce information is present in literature about their production from an antagonism point of view. An economically possible production and a wide array of antagonistic compounds produced by several *Trichoderma* spp. ensure their potential as biocontrol agent (BCA). Furthermore, full exploitation of *Trichoderma* spp. based BCAs could play a pivotal role in biocontrol via their typical modes – e.g., mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites, and production of plant growth hormones. Nevertheless, more research and review of the information regarding these biocontrol agents are needed to exploit their actual potential, which is the salient objective of this review.

***Keywords:*** Antagonism; Biocontrol agents; Microbial propagules; *Trichoderma* spp.; Wastewater; Wastewater sludge.

# 1. Introduction

## *1.1. Existence of Biological Control Agents (BCAs)*

Phytopathogenic microorganisms and insects have been co-existing with plants since the very beginning of agricultural evolution. Despite being a natural phenomenon, their mutual existence has adversely affected agriculture and forests from time to time. With the advent of technology, physico-chemical methods have been adopted to mitigate the phytopathogenic impacts on agriculture and forests. Use of crude (ash, raw extract of certain plants, lime) and chemical pesticides, physical traps for insects are some examples of these control methods [1]. However, later on, integration of biological control to pre-existing methods has revolutionized the agricultural and forest pest management.

## *1.2. Fungal BCAs*

Currently, the role of BCAs is a well established fact and has become increasingly crucial, and in several cases, complementary or even replaced their chemical counterparts where antagonistic fungi played an important part [2-4]. Fungal based BCAs have gained wide acceptance next to bacteria (mainly *Bacillus thuringiensis*), primarily because of their broader spectrum in terms of disease control and production yield [5]. In this context, *Trichoderma* spp. have been the cynosure of many researchers who have been contributing to biological control pursuit through use of fungi [6-16]. Furthermore, according to Whipps and Lumsden, *Trichoderma* spp. share almost 50% of fungal BCAs market, mostly as soil/growth enhancers and this makes them interesting candidates to investigate [4].

## *1.3. Status of Trichoderma spp.*

Although, *Trichoderma* spp. have been possibly known since 1865, [17], still their wide-spread application as BCAs has been exploited and reported only lately against several soil-borne phytopathogenic fungi [18,19]. Akin to most fungal BCAs, *Trichoderma* spp. could be efficiently used mainly as spores (especially, conidia), which are more tolerant to adverse environmental conditions during product formulation and field use, in contrast to their mycelial and chlamydospore forms as microbial propagules [20]. Nevertheless, the presence of a mycelial mass is also a key component for the production of antagonistic metabolites [7-21]. Conidia and mycelia can be produced in either a solid-state or liquid fermentation. Whilst, in general, liquid fermentation is more suitable method

over solid-state fermentation for large scale production, still special techniques are required for abundant conidia production.

*Trichoderma* fungi are cognizant for their antagonism against several soil-phytopathogens, involving fungi, invertebrates, and few anaerobic bacteria (Table 1). Their BCA activity is mainly attributable to various anti-microbial/antagonistic compounds they produce, in addition to their aggressive mode of growth and their physiology. Full exploitation of the BCA potential of *Trichoderma* spp. could easily provide growth enhancement of domestic plants, green house plants, and agricultural crops.

### *1.4. Constraints in Commercialization of Trichoderma spp. BCAs*

*Trichoderma* spp. are also preferred in bioremediation due to the production of metabolites that are rich in peroxidases, and laccase enzymes [22-23]. Despite all the acquired understanding about antagonistic action and growth promotion of *Trichoderma* spp., there are nevertheless some hurdles to their widespread success - (a) most of *Trichoderma* spp. based BCAs are unregistered and are being marketed simply as "soil enhancers", probably due to lack of "well defined" modes of action of these fungi and their underdeveloped bioassay methods (to ensure product quality) [4]; (b) the raw materials like, glucose, sucrose, corn steep liquor, wheat bran, soya meal, fish meal, used in culture media for production of the fungi are very costly [24-25]; (c) low efficacy; (d) low spore yield; and (e) difficulties in quantification of BCA activity. This has encouraged many researchers to investigate agricultural wastes [26], industrial wastes [13], and municipal wastes [27] as probable substrates for *Trichoderma* spp. production.

To recapitulate, the essential information regarding *Trichoderma* spp. as BCA is scarce as most of the current literature is focused mainly on its commercial enzyme production capacity. The aim of this review is to sum up the BCA activity potential of these fungi and to shed light on commercial production processes. In this review, mechanisms and/or processes for *Trichoderma* spp. are discussed regarding pest control, growth promotion, bioremediation, production processes and market values.

## 2. Pest Control

### *2.1. Fungi*

#### *2.1.1. Trichoderma spp. as biofungicides*

In general, the currently available literature indicates that *Trichoderma* spp. have been mostly used as biofungicide agents (Table 1). The documented first report on this subject was that of Coley-Smith et al. [28] who by means of microtome sections have shown that medulla of infected sclerotia of *Sclerotium delphinii* were completely replaced by hyphae and chlamydospores of *T. hamatum* on agar plates. Henis et al. likewise reported mycoparasitism (penetration and infection) of *Trichoderma* spp. against *Sclerotium rolfsii*, where chlamydospores were produced abundantly in contrast to conidia within the infected fungal sclerotia [29].

#### *2.1.2. Modes of action*

##### *2.1.2.1. Mycoparasitism*

According to Punja and Utkhede, [30] *Trichoderma* spp. are the most widely studied mycoparasitic fungi, but the importance of mycoparasitism has been difficult to demonstrate *in situ* until very recently due to technical difficulties in making *in situ* microscopic observations (e.g., fluorescence imaging and differential staining), such as at the soil-root interface. However, techniques involving antibodies, such as combined baiting-ELISA (enzyme linked immunosorbant assay) techniques to detect *Trichoderma* spp. in composts, would certainly increase our understanding of the mycoparasitic interaction of these fungi, which will subsequently help improving their BCA potential [31].

Previously, Cook classified the mycoparasitic interactions as (1) replacement (unilateral antagonism), (2) deadlock (mutual antagonism), and (3) intermingling (no antagonism), still explanation at microscopic level was absent [32]. However, more recently, the understanding of mycoparasitism has been improved/updated considerably which is reflected in studies reported by several authors [7,14,21,33-35]. Interestingly, the studies were carried out at both the genetic [35] and the microscopic levels [21,34]. However, a broader concept concerning living plants (with the exception of preservation of wood, where *Trichoderma* spp. alone kill pathogen fungi - discussed later), which comes to the fore is that after being treated with mycoparasites, plants induce defense mechanisms

themselves. Further, this phenomenon leads to production of fungal inhibitory compounds by plants along with *Trichoderma* spp. thereby, facilitating mycoparasitism. Additionally, Benhamou et al. successfully showed for a mycoparasite, *Pythium oligandrum* that mycoparasitism was host specific [34]. Therefore, it can be expected that a specific *Trichoderma* sp. might be required to control for a particular pathogenic fungus.

*2.1.2.2. Antibiosis*

A scrutiny of the available literature has shown that mycoparasitism is associated and followed by "antibiosis and competition". Moreover, antibiosis (secretion of anti-microbial compounds by *Trichoderma* spp.) and/or by plants through *Trichoderma* induced plant defense mechanism and competition for particular development and nutrient needs also play an important role (thus it could be referred to as complementary) in BCA activity of *Trichoderma* spp.

Menendez and Godeas, reported on a biocontrol study of *Trichoderma harzianum* against *Sclerotinia sclerotiorum* - a soilborne plant pathogen attacking many economically important crops, such as soybean [36]. The authors studied antibiosis of *Trichoderma harzianum* against the plant pathogen, assuming that the beneficial effect was due to concurrent mycoparasitism as reported earlier in a similar study [37]. Extracellular lytic enzymes produced by *Trichoderma harzianum* were mainly reported as possible antagonistic compounds.

In another example, despite close contact between hyphae of *Trichoderma* spp. and *Fusarium moniliforme*/*Aspergillus flavus* when co-cultured, hyphal penetration was absent, suggesting that mycoparasitism was not the sole cause for the observed inhibitory effects [11]. Therefore, metabolites elaborated by *Trichoderma* spp. (e.g. volatiles, extracellular enzymes and/or antibiotics) were considered to be the probable elements involved in antibiosis. However, Calistru et al. also discussed about morphological alterations in the hosts (*Fusarium moniliforme* and *Aspergillus flavus*) and did not consider them even as a partial cause of inhibition, and rather attributed it to the production of various enzymes as the sole factor [10,11]. Thus, considering the generally accepted significance of biological membranes for normal functioning of cellular organelles/cell, morphological alterations could be considered as a possible cause of total or partial inhibition. *Trichoderma* spp.

have also been effective in cases of a wide host range and in hindering the longevity of sclerotia of pathogenic fungi.

#### *2.1.2.3. Competition*

Celar [38], conducted a study on the forms of nutrients commonly available to phytopathogenic and antagonistic fungi, and reconfirmed the findings of Blakeman [39] that a shortage of easily accessible nutrients for microorganisms, especially of those living in soil and on plant surfaces, could result in explicit nutrient competition among microorganisms. Perhaps, these results explain the anomaly observed between laboratory and field experiments, where antagonistic fungi were inefficacious or required an additional substrate for producing an expected BCA activity [40,41]. Nevertheless, in the above example, the significance of competition for nutrient between antagonistic and pathogenic fungi was evident.

Several authors have highlighted on the significance of lytic enzymes in BCA activity and studied isolates of *Trichoderma* spp. with cellulose and chitin degradation characteristics [42-44]. Whilst others, Hutchinson [45] and Hanson and Howell [46] have reported on the significance of secondary metabolites (antibiotic activity) in antagonistic action of *Trichoderma* spp. against pathogenic fungi *Pythium ultimum* and *Rhizoctonia solani*. However, there seems to be a general consent on the combined synergistic effect of the two factors (enzymes and antibiotic compounds) [47-48].

### *2.1.3. Application in Wood Preservation*

#### *2.1.3.1. Hardwood*

Biological control studies to protect wooden distribution poles by *Trichoderma* spp. have also been carried out against the dry rot fungus, *Serpula lacrymans* [49] and brown rot fungi, namely, *Antrodia carbonica* and *Neolentinus lepideus* [50]. From these studies it was concluded that although *Trichoderma* spp. displayed a killing action against these fungi in *in vitro* tests, yet their *in situ* action was inefficacious as it was observed earlier [51]. However, further studies carried out by Bruce et al. showed that *in situ* BCA potency of *Trichoderma* spp. was still effective, and that in cases of lesser efficacy, a non uniform distribution or impoverishment of *Trichoderma* spp. and compartmentalization of

pathogenic fungi could be involved [52]. Therefore, suitable *in situ* application techniques should be of prime importance, for a successful application of BCAs in field tests.

Meanwhile, Score and Palfreyman, proposed another hypothesis to account for the inefficacy of these antagonistic fungi [49]. They postulated that the presence of varying nutrient concentrations in wood might have a marked effect on the nature of antagonistic interactions and which resulted in the registered anomaly between *in vitro* and *in situ* studies. As discussed earlier, Score and Palfreyman, successfully showed that varying nutrients in enriched media had a clear effect on hyphal extension rate of several *Trichoderma* spp. affecting their BCA activity [49]. However, determination of actual BCA activity would require extensive studies on the wood, like the possible interactions of various *Trichoderma* spp., metabolites with major wood components, namely, tannin, and lignin. Furthermore, it could be inferred that associated environmental factors affecting *Trichoderma* spp. interactions could readily be optimized to exploit their BCA potential.

#### *2.1.3.2. Cosmetic Woods*

In a similar study concerning wood preservation, Ejechia, investigated the ability of *Trichoderma viride* to inhibit the decay of obeche (*Triplochiton sceleroxylon*) wood by the decay fungi *Gloeophyllum* sp. and *G. sepiarium* under field conditions, over a period of 11 months, covering dry and wet season in tropical environment [12]. The results of *Trichoderma viride* use as a control agent were again encouraging, expressing total inhibition of the decay fungi by means of mycoparasitism and competition for nutrients by the antagonist, *Trichoderma viride*. A similar experiment performed with a *Penicillium* sp. showed that *Trichoderma* sp. was twice as more effective than the *Penicillium* sp. *Trichoderma* spp. are currently the most extensively investigated biocontrol fungi for forest product preservation, and, on a number of occasions, have successfully provided a protection against certain wood decay fungi [50-53]. Using modified versions of American [54] and European [55] standard test methods as well as a soil burial test system, Tucker et al. have shown that certain isolates of *Trichoderma* spp. were totally effective in protecting wood against certain basidiomycetes [56]. In the previous example the wood preservative action of *Trichoderma* spp. was based on the final inhibition effect on the pathogenic fungi and the mechanism of antagonism was not examined or discussed.

### *2.1.4. Application in Agriculture*

#### *2.1.4.1. Fruits and Vegetables*

Several *Trichoderma* spp. have also been used to protect commercially important fruits and vegetables, such as banana, apple, strawberries, mango, potato, and tomato during postharvest storage as enumerated in Table 1. Mortuza and Ilag utilized 10 isolates of *Trichoderma* spp. including *T. harzianum* and *T. viride* against the banana fruit rot pathogen, *Lasiodiplodia theobromae* [57]. Their investigation confirmed that mycoparasitism was associated with antibiosis and competition for substrate. The authors compared the cultural filtrates of *Trichoderma* spp. with a chemical fungicide, namely, Benomyl$^{TM}$, and concluded that *Trichoderma* spp. based fungicide could not be totally as effective as their chemical counterparts. However, the authors underestimated the role of formulations of *Trichoderma* spp. based fungicide for their field application, which might substantially enhance their BCA activity.

Biological control of mango stem-end rot using *T. viride* was studied by Moreno and Paningbatan, where they reported mycoparasitism and antagonism as major BCA activity factors [58]. Batta, examined invert emulsion formulation of *T. harzianum* Rifai against blue mold infection of apple to control postharvest fruit decay [59,60]. The author attributed the BCA activity of *Trichoderma* spp. to the necessary period of conidial humectation in order to germinate and penetrate into the pathogen fungi and reported invert-emulsion as better application mode for fungal BCAs like *Trichoderma* spp. Whereas, Brewer and Larkin compared the BCA activity of *Trichoderma* spp. with several other fungal BCAs and reported *Trichoderma* spp. to be a potential antagonist for stem disease of potato [61]. On the other hand, Hjeljord et al. reported the application of *Trichoderma* spp. on greenhouse strawberries could control postharvest rotting [62]. They emphasized that temperature and nutrients influenced on the BCA activity of *T. harzianum*. An interesting interpretation of a study carried out by Cooney and Lauren was significant effect on antagonistic metabolite production by a *Trichoderma* sp. (300-700% with respect to absence of plant pathogenic fungi, *Botrytis cinerea*, *Fusarium culmorum*, and *Rhizoctonia solani*) [63].

#### 2.1.4.2. Crops/Seeds

*Trichoderma* spp. are also well recognized fungal antagonists of crops/seeds pathogens. Biocontrol of brown blotch of cowpea caused by *Colletotrichum truncatum* by pre-treatment of cowpea seeds in *T. viride* spore suspension was considered to be due to both mycoparasitism and antibiosis [64]. Similarly, the application of *Trichoderma* spp. for the control of wheat [65] and sunflower [66] fungal diseases have been based on their mycoparasitic and antibiotic activities. However, particular factors triggering antagonism (molecular signal for mycoparasitism, as *Trichoderma* spp. are able to recognize their specific host) was not available. In another instance, the beneficial action of *T. virens* for pre-treatment of cotton seedlings has been reported to be due to plant phytoalexin induction and the inactivation metabolism of pathogen germination stimulants produced by germinating cotton seedlings [48].

Thus, it is evident that the process of mycoparasitism, antibiosis, and competition for substrates are well documented, as it was also exemplified in Figure 1. However, the exact phenomena involved in the overall antagonistic action of *Trichoderma* spp. are still not well understood. It is postulated by most researchers that once *Trichoderma* spp. succeed in antagonism via hyphal interactions, probable primal step in antagonism, subsequently, these antagonistic fungi kill the phytopathogenic fungi likely by means of toxins and later consume them using a combination of lysozymes [4].

On the other hand, it could be interpreted that the fungicidal activity of *Trichoderma* spp. is well known with respect to most of the fungal phytopathogens and almost all researchers agreed that the antagonists (*Trichoderma* spp.) control pathogens via interlinked synergistic complex strategies of mycoparasitism, antibiosis, and competition. Therefore, in order to exploit maximum potential of *Trichoderma* spp. against fungal pathogens, factors (e.g., distribution of inoculum at infected sites, concentration of inoculum, specificity towards pathogens, environmental conditions and enrichment medium - if any) affecting one or many of the antagonistic strategies should be optimized.

### 2.2. Bacteria

#### 2.2.1. Limited Application

In contrast to other fungi, *Trichoderma* spp. have been reported to have limited applications in biocontrol of pathogenic bacteria. An immediate explanation for this lies in

the fact that bacteria generally have a higher metabolic rate than fungi, thereby, antagonism via physical interaction such as mycoparasitism would be too slow to be effective from BCA point of view, where faster action is a must. However, if the time of application and the application of formulated metabolites from *Trichoderma* spp. were considered, the BCA potential of antagonist fungi would be considerably higher.

### *2.2.2. Potential - Future application*

In the context of limited studies on anti-bacterial action of *Trichoderma* spp. very few examples were reported in the literature; these have been listed in Table 1. Altogether, anti-bacterial action of *Trichoderma* spp. was reportedly based only on the action of the antibiotic compounds produced and no physical interaction between antagonist and pathogen was mentioned [46,67-69]. Therefore, in the present scenario, although, *Trichoderma* spp., possess antibacterial potential, yet their applicability cannot be advocated authentically and employed actually in field set-ups.

## *2.3. Invertebrates*

### *2.3.1. Application Potential*

*Trichoderma* spp. are basically soil-borne saprophytic fungi which have a symbiotic relationship with the plant rhizosphere [70]. In addition, they have innate ability to produce several chitin (a major component of cellular structure of invertebrates) degrading enzymes (endochitinases and exochitinases) in order to survive on and/or antagonize pathogen organism [43,71-73].

### *2.3.2. Rhizosphere - Nematodes*

Nematodes are mostly present in rhizosphere than in the bulk soil, therefore, their antagonist(s) should also be in sync with rhizosphere. Fungi like *Trichoderma* spp. fit very well in this category [74-80].

Results of most studies on nematodes concurred that the promising fungal antagonists - *Trichoderma* spp., had different and in fact multiple modes of action. For example, *Trichoderma virens* invaded/ramified/grooved/vacuolated the root-knot nematode eggs. Interestingly, Eapen et al. reported easy staining of eggs for microscopy due to the increased permeability of eggshell [80]. The antagonistic action of *Trichoderma* spp. was chiefly attributed to chitinolytic activity of the fungi on cellular structure of nematodes,

which is rich in chitin. Additionally, unlike bacteria, nematodes were antagonized mainly by parasitism and antibiosis akin to fungal pathogens.

### *2.3.3. Foliar Application - Aphids*

The insecticidal activity of two strains of *T. harzianum* against aphids has been reported by Ganassi et al. [81] Since, *T. harzianum* is currently considered as an important candidate as BCA, their insecticidal activity has further importance. The authors proposed that the action of toxins produced by *T. harzianum* strains was facilitated by cuticle degrading extracellular enzymes (proteases and chitinases) that enabled the insertion of the toxins inside cuticle.

Thus, many of the *Trichoderma* spp. are reported as occasional parasites of invertebrates in literature indicating that their actual potential in the natural suppression of insects affecting economical crops is still underrated and exacerbates further research on using *Trichoderma* spp. based BCAs to cope with insect related plant disorders.

## 3. Growth Promotion

### *3.1. Direct Effect*

### *3.1.1. Metabolite Production*

Literature is replete with many studies that account for growth promotional activity of *Trichoderma* spp. [4,30,33,82]. Many researchers have suggested production of growth hormones and enhanced transfer of minerals to rhizosphere as direct factors behind spectacular performance of *Trichoderma* spp. based BCAs as also reported in Table 2. Besides, *T. harzianum* has been reported to produce growth hormones that enhanced plant growth. This was in concordance with formation of micro-metabolites capable of solubilizing phosphates and enhanced micronutrient levels in plants treated with *T. harzianum* as reported by Yedidia et al. [21].

### *3.1.2. Participation with ectomycorrhizal sphere in growth promotion*

Lindsey and Baker, demonstrated that the symbiosis of *T. viride* in rhizosphere aided in growth of gnotobiotic (either, well defined or, no microflora present) tomato, thereby, showing growth promotional ability of this fungus [83]. Besides, physical presence of mycelial mass in rhizosphere in itself would serve as appendage to the normal rhizosphere

of plants, thereby, enhancing nutrient uptake. Further, the advantages of *Trichoderma* spp. based BCAs for growth promotion were magnified due to their symbiosis with ectomycorrhizal sphere [16,84,85]. *Trichoderma* spp. easily acquired nutrients from complex substrates like, protein-tannin, and glucosamine, in soil due to their ectomycorrhizal association. Further, the nutrients were easily utilized by the plant due to their mutual symbiotic relationship. In particular, *Trichoderma* spp. has been extensively utilized in waste composting [16,45,85], which ultimately ends up in agricultural land, consequently affecting plant yield. The positive role of *Trichoderma* spp. in ectomycorrhizal sphere has been elaborated by Wu et al. an indirect mode for their plant growth promotional activity [86].

### *3.2. Indirect Effect*

#### *3.2.1. Induction of Plant Defense Mechanism*

In addition to *Trichoderma* fungi's well-recognized mycoparasitic nature, induction of resistance against pathogens in plants has also been reported by Yedidia et al. [21], Benhamou and Picard [33], and Zeilinger et al. [35] as indirect growth promotion factors. The authors suggested that during association of *Trichoderma* with roots, several lytic enzymes induced by plant defense system were not only involved in destruction of the pathogen cell wall but also played crucial role by consuming them, thereby, providing nutrients to the plant as demonstrated in Figure 1b. A mycoparasitic interaction study in this context revealed that *ech42* (a chitinolytic enzyme encoding gene) transcription was induced prior to physical contact of *T. harzianum* with host plant [35]. The authors also expressed the possibility of different mechanisms of induction for each type of lytic enzyme and the necessity to characterize these enzymes and their relevance in mechanisms of growth promotion and biocontrol.

From the literature available, although the plant growth promotional activity of *Trichoderma* spp. has been acclaimed by various researchers and industries, still exact or quantitative BCA assessment was difficult due to multiple factors associated and/or occasional typical information about the same. Nevertheless, there are many *Trichoderma* spp. based commercial products in market which aim at greenhouse plants (mainly ornamental and garden vegetables), as depicted in Table 3.

## 4. Bioremediation

The concept of utilizing fungi for bioremediation of soil contaminated with certain pollutants is relatively older. There is ample evidence of various *Trichoderma* spp. contributing to polycyclic aromatic hydrocarbons (PAHs) degradation, whilst affecting native mycorrhizal fungi both positively and/or, negatively [87]. Saraswathy and Hallberg, reported a maximum of 75% removal for pyrene (4-ring PAH) at 50 mg $l^{-1}$ for axenic cultures of *Trichoderma* spp. where pyrene served as sole carbon source [88].

Katayama and Matsumura, also demonstrated degradation potential of rhizosphere-competent fungus *Trichoderma* sp. against several synthetic dyes, pentachlorophenol, endosulfan, and dichlorodiphenyl trichloroethane (DDT) [22]. Owing to these facts, *Trichoderma* spp. have found application in herbicide/pesticide laden soil bioremediation as sustainable approach. Review of present literature suggests that hydrolases, peroxidases, laccases and other lytic enzymes produced in abundance by *Trichoderma* spp. are probable factors aiding in degradation of these contaminants.

From BCA point of view, bioremediation potential of fungi like *Trichoderma* would be an additional advantage as this will aid in soil enhancement where excessive use of herbicides needs to be curtailed. However, this clean-up objective can be achieved, if and only if the contaminated soil is inoculated with *Trichoderma* spp. at regular defined intervals, i.e., inclusion of *Trichoderma* spp. in "integrated pest management" program. In the current context, the scientific community believes that use of herbicides could not be significantly reduced due to immediate concern of needs of agricultural commodities.

Therefore, consistent-simultaneous application of some "detoxifying" agents along with (*Trichoderma* spp. vs. harmful pesticides/herbicides; the former can tolerate and degrade application concentrations of several pesticides/herbicides) would provide an agreeable soil environment. This will assure not only the health of soil and plant but also a sustained crop yield protection. Future research must be streamlined for consistent-simultaneous use of *Trichoderma* spp. as BCA cum soil remediation agent along with obligatory pesticides/herbicides.

## 5. Production

### *5.1. Microbial Propagules*

#### *5.1.1. Spore/Conidia*

The eventual objective of any BCA lies in its feasibility of economical mass production which also holds true for *Trichoderma* spp. based BCAs. Further, from Table 3 and Figure 2, it is obvious that *Trichoderma* spp. based BCAs are commercially viable as their numerous commercial products exist in market, albeit a majority of them (not presented here) are "unsung" BCAs, rather being promoted solely as soil enhancer and/or growth promoter. Almost all available *Trichoderma* spp. based BCA products contain spores as active ingredients [4,30,59,60]. This could be attributed to the physiological aspects of their three microbial propagules, namely, mycelia, conidia, and chlamydospores [20,89,25], as, the three propagules possess distinct physiological characteristics in terms of production, stability and BCA activity. Therefore, it is imperative to select the best suitable form of *Trichoderma* spp. propagules in order to efficiently execute the previously mentioned modes of BCA action.

#### *5.1.2. Mycelium and Chlamydospore*

Although, mycelia have excellent BCA activity, unfortunately, they cannot survive down stream processing steps such as drying and hence are not useful [49]. On the other hand, chlamydospores require a period of 2-3 weeks for cultivation and likewise could not survive drying processes [25], albeit, they are more stable than mycelia. Meanwhile, as stated earlier, conidia are active as BCA, less susceptible to several environmental conditions and could be produced at higher rate (3-4 days) in abundance [27]. Thus, production of *Trichoderma* spp. as conidia would be the best option from its BCA application point of view. However, the presence of mycelia along with conidia in the production media could not be ruled out. In addition, simultaneous mycelial production would assure presence of various essential metabolites (e.g., antibiotics) for BCA activity [45,46]. Thus, production of *Trichoderma* spp. containing conidia as main propagules along with mycelia would be the best production strategy.

### 5.2. *Mass Scale Production Strategies*

#### 5.2.1. *Solid State Fermentation (SSF), and Liquid Fermentation (LF)*

Commercial success of *Trichoderma* spp. based BCAs would also require economically feasible mass scale production processes (as 35-40% costs of production depends on raw material). Of the two broad categories of production, namely, solid state fermentation (SSF) and liquid fermentation (LF), LF has been adopted by many researchers even though it normally yields lesser sporulation [90,91]. Labour, scale-up, process control, productivity, material handling (pumping, pressurized lines), compatibility with pre-existing large scale facilities are some stabilizing features of LF that encourage most researchers to pursue LF in lieu of SSF. However, several times SSF is often preferred to LF, when production scale is of a moderate range and labour force is cheap [92]. Additionally, recent advances in industrial automatization have encouraged major BCA producers to consider SSF as a viable mass scale option [93].

Unfortunately, accurate information regarding factors directing sporulation process in LF of fungi, especially, for *Trichoderma* spp. is scarce and incomplete. Therefore, production of spores in LF still remains a challenging task and warrants considerable research inputs.

#### 5.2.2. *Combined Process*

To overcome limitations of SSF and LF, many researchers also suggested strategies involving both SSF and LF as a combined process (discussed subsequently). Normally, LF is followed by SSF in many industrial production processes, however, relevant information on production techniques is so far evasive in current literature. In a typical *Trichoderma* spp. production process, 2-3 days old broth of LF is used as inoculum for solid substrates e.g., bran, rice, grain-husk etc. The solid substrates thus inoculated is incubated for further 2-8 days, followed by addition of formulation agents, e.g., carboxy methyl cellulose, silica, talc and moderate temperature (about 20 - 40°C) air-drying below 8-10% moisture content. However, labour-intensive nature of similar techniques certainly could not replace LF, moreover, the problems in LF will no doubt eventually be overcome.

### *5.3. Solutions to Improve LF*

#### *5.3.1. Inoculum Effect*

The ongoing research, also indicates that several techniques might help improving sporulation during LF. For example, fungal morphology and the general course of fermentation has been primarily affected by the amount, type (spore or vegetative) and age of the inoculum [94-96]. In general, studies carried out on fungi until now suggested that high spore concentrations in inoculum ($\geq 10^5$-$10^8$ spores/ml) were not good for sporulation. Therefore, any direct role of inoculum (in form of spores) in sporulation could be ruled out. Nevertheless, same study also suggested that inoculum could be important for production of metabolite(s) and mycelial mass responsible for the BCA activity. Furthermore, type, quantity, and age of inoculum should be explored for probable enhancement in sporulation.

#### *5.3.2. C:N Ratio*

Variation in values of carbon : nitrogen (C:N) ratio in medium could also be helpful in sporulation/conidiation for *Trichoderma* spp. Olsson et al. observed start-time of sporulation phase in *Trichoderma reesei* Rut-30 and remarked that decreasing C:N ratio, adversely affected sporulation [97]. Moreover, studies on other antagonistic fungi, namely, *Neurospora crassa* [98] and *Beauveria* sp. [99] also showed marked effect of C:N ratio on sporulation. Therefore, manipulation of C:N ratio remains a viable option for further investigation of sporulation process of *Trichoderma* spp.

As mentioned earlier, during antagonism study of *Trichoderma* spp. Score and Palfreyman, used a standard complex medium (malt extract agar, MEA) and a minimal essential medium (MEM) designed to mimic the C:N ratio in the pathogen infected wood [49]. Their findings revealed that *Trichoderma* spp. could be significantly antagonistic even at low nitrogen content. Therefore, any increase in C:N ratio owing to decrease in N would not affect the BCA activity of these fungi, in other words, substrates with higher C:N ratio could be beneficial for both sporulation and BCA activity.

#### *5.3.3. Nature of Carbon and Nitrogen*

Sporulation of *Trichoderma* spp. is greatly affected by the nature of carbon and nitrogen sources used as substrates. Pascual, observed that peptone, free amino acids (e.g.,

arginine), mannose, xylose, and fructose induced high levels of sporulation [100]. It could be inferred that exploring various carbon and nitrogen sources and their pre-treatments could also be potential means to induce sporulation.

### *5.3.4. Sporulation Inducer Compounds*

Introduction of some triggering agents has also been helpful in sporulation process. These agents might be metal ions (e.g., manganese ions, [101]), or complex organic compounds. Roncal et al. have isolated conidiogenol and coniodiogenone, tetracyclic diterpenes with potent and selective inducing activity for conidiogenisis in *Penicillium cyclopium* [102]. These findings are still at their natal stage and might be helpful in practical application at later satge. Nevertheless, it appears that introduction of some metal ions and/or some complex compounds to the growth medium could induce sporulation. Perhaps, various metals and complex compounds in renewable waste, could be helpful in achieving high spore concentration.

### *5.3.5. Physical –Chemical Production Parameters*

Environmental parameters like, temperature has been reported to be a less significant parameter for sporulation [103], whereas, pH of the medium plays an important role. Although, conidial fungi can grow over a wide range of pH, they grow and sporulate maximally near neutral pH [101]. Felse and Panda investigated the effect of agitation conditions on a *Trichoderma* sp., and inferred that extremely agitated conditions were not good for growth and sporulation [104]. Dissolved oxygen and carbon dioxide may also play an important role in inducing sporulation, probably by imposing mass transfer related stress conditions on the culture. The sporulation in fungi is also an important mode of reproduction, therefore, sporulation for *Trichoderma* spp. could also be correlated to their respiration quotient (RQ).

### *5.3.6. Fermentation Modes*

In general, there are no research reports on the studies regarding culture conditions like continuous and fed-batch culture in fermenter that allow finer control of substrate concentration (solids concentration, C:N ratio), biomass (mycelia and/or spores concentration), single/multiple nutrient addition (feeding strategy for complex and/or

simple substrates), and should therefore be examined and studied adequately to induce sporulation.

### *5.4. Selection of Raw Materials*

#### *5.4.1. Conventional/Semi-synthetic Substrates*

Several growth/production media for *Trichoderma* spp. spores production are represented in Table 4. Use of media based on molasses, D-glucose, cellulose, or soluble starch have been also reported by many authors [25,90,91]. In general, maximum sporulation of the order of $10^8$ spores per ml fermentation broth could be achieved, however, it was noteworthy that production of *Trichoderma* spp. spores for BCA use (application in soil), using these substrates could not be economical, owing to high cost of raw material and moderate sporulation. Meanwhile, a much higher spore concentration ($\approx 10^7$-$10^{13}$ CFU/ha application requirements will necessitate > $10^7$-$10^{10}$ CFU/g formulated product, consequently requiring $\geq 10^7$-$10^{10}$ CFU/ml fermented medium) will be required for their field application [62].

#### *5.4.2. Alternate/Recycled Substrates*

In order to obtain crude proteins, enzymes as mycelial mass, use of oil mill effluent as a substrate for producing *Trichoderma* spp. has been suggested by Karim and Kamil [105]. Nevertheless, the time of fermentation was longer ($\geq$ 2 weeks) and also the BCA potential of fermented products was never assessed by the authors. On the other hand, Felse and Panda [72,104] have reported the use of untreated crab shell and Olsson et al. used cellulose, raw and treated sugar beet pulp without additional nutrients and Mendel's medium components for the development of inoculum [97]. Remarkably, in the preceding examples, the authors were concerned only with the yields of cellulosic and hemicellulosic enzymes production and not spores. The sole presence of metabolites in *Trichoderma* spp. based BCA is not sufficient for its application viability, high spore concentration is equally important.

Meanwhile, studies on steam-pretreated willow (a lignocellulosic material) by Vlaev et al. [26] and Reczey et al. [106] suggested replacement of glucose and corn steep liquor in Tanaka media (Table 4) by Avicel (10 $gl^{-1}$), or by corn fibre dry mass (20-140 $gl^{-1}$). Several studies have been reported in the literature on use of alternative cheap raw

materials for *Trichoderma* spp. production processes [107-110]. However, most of the production studies focused mainly on metabolites (enzymes and/or antibiotics) production and not sporulation (an important factor for practical application of BCAs). In the present context, validation/assessment of commercial viability of these raw materials/processes from BCA point of view would require further investigations aimed at spore production and bioassay against commercial pests.

### *5.4.3. Wastewater and Wastewater Sludge*

Based on the preceding discussion for economical and efficient production of *Trichoderma* spp. based BCAs, a novel array of substrates would be needed to explore and overcome both low spore yields and higher medium cost. Akin to prior mentioned wastes, experimenting with wastewater and wastewater sludges (source of carbon, nitrogen, phosphorus, and other essential nutrients for many microbial processes) could provide probable viable solution to combat the raw material cost and enhance sporulation, as in the case of *Bacillus thuringiensis* [111]. Meanwhile, Verma et al. have reported production of *T. viride* on municipal wastewater from BCA point of view and advocated potential utilization of municipal wastewater sludge as shown in the mass balance flow chart (Figure 3) according to the proposed estimation of use of sludge [27]. Thus, *Trichoderma* spp. production on sludge would not just serve as potent BCA but also as a novel technique for sustainable sludge management.

Nevertheless, these wastes often contain certain pollutants like, pesticides, metals complexes, whilst *Trichoderma* spp. are quite efficient in degrading these pollutants (e.g., organochlorine pesticides; [112] as they can tolerate high metal content by chelating metal ions [113]. The aforestated fact strengthens and encourages the ideology of using these wastes as substrates for *Trichoderma* spp. based BCAs. Furthermore, wastewater and wastewater sludges contain various complex compounds along with the essential components, hence there are ample opportunities that one could encounter compound(s) that support(s)/ induce(s) sporulation, in addition to vegetative growth of the fungi.

Above all, huge requirements of *Trichoderma* spp. based BCAs (50% of fungal BCAs, [4]) and their subsequent demands for enormous amounts of wastes will serve two purposes; (1) sustainable *Trichoderma* spp. based BCAs production and (2) waste

management in a novel and environment-friendly way (reduced green house gases via incineration; reduced hazard during land spreading-*Trichoderma* treated).

### *5.5. Standard Bioassay for Quality Control*

Last but not the least, akin to any other biopesticides, standard bioassay technique for *Trichoderma* spp. is mandatory. However, this arena of *Trichoderma* spp. based BCAs is perhaps least considered by researchers. Bioassay protocols, simulating *in situ* environment and under *in vitro* conditions by best possible means (diet incorporation technique, drop-let test) for *Trichoderma*, as standard is still awaited. Meanwhile, the literature is replete with either, rudimentary protocols, such as, measurement of inhibition zone and viable spore count [15,46,62,59,60,114], lytic enzyme activity/metabolites [10,11] or, cumbersome greenhouse/field studies [62,115]. Consequently, there are several examples where *in vitro* high potential *Trichoderma* spp. failed under *in situ*. Fortunately, there are occasional reports on development of simpler bioassay techniques for *Trichoderma* spp., which could simulate true *in situ* conditions, such as, microdilution method based on infinite inhibition concentration [116-118]; tubular bioassay system to measure production of antagonistic metabolites at the antagonist-pathogen interface [63]. In all, there is a need to encourage and emphasize such research, thereby, facilitating standardization and commercialization of *Trichoderma* spp. based BCA.

## 6. Market Potential

Presently, *Trichoderma* spp. based products are considered as relatively novel type of BCAs. In comparison to *Bacillus thuringiensis* (Bt) biopesticide, their market size is quite small (Bt shares about 97% of overall biopesticides), they fall in remaining 3% bracket, which also comprises viral and nematode based biopesticides. The current status of *Trichoderma* spp. based BCAs is unlikely, especially, if we consider the positive features of *Trichoderma* spp. over bacteria as depicted in Table 5. In addition, their actual/true market size is vague and only scattered information could be obtained based on registered as well as non-registered biofungicides (Figure 2). However, a general consent is that *Trichoderma* spp. based BCAs share about 60% of all fungal based BCAs and an increasing number of *Trichoderma* spp. based BCAs products are registered regularly. Moreover, field application/trials throughout the world is already accepted and many

biopesticide companies are endorsing these products on regular basis (Table 3). The innate qualities (e.g., simultaneous biocontrol and growth promotion) of *Trichoderma* spp. based BCAs are the driving factors behind its steadily cumulating success.

## 7. Conclusions

*Trichoderma* spp. play major role in the arena of fungal biocontrol agents, owing to their capabilities of ameliorating crop-yields by multiple action, as biopesticide/bioherbicides and/or plant growth promotion. Mechanisms of antagonism of these fungi have been only well documented, and not yet understood completely. However, presently, a vast empirical knowledge about the modes of action of these fungi can be obtained from literature according to their target pathogen and/or application environment. In order to enhance marketability of these fungi as BCAs, feasible commercial production processes are of utmost importance. Search for cheaper substrates, and/or optimal operating parameters are in progress, and many encouraging results are being reported by researchers worldwide. Thus, it is expected that, in near future, exploitation of these interesting BCAs would be maximized.

## Acknowledgement

The authors are sincerely thankful to 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. Last but not the least, the authors would also like to thank Dr. Guillemond Ouellette (Histology, Host-pathogen Interaction – Natural Resources Canada) for his valuable suggestions to this work.

## References

[1] Vincent C, Hallman G, Panneton B, Fleurat-Lessard F. Management of agricultural insects with physical control methods. Annual Review of Entomology 2003; 48: 261-281.

[2] Templeton GE, Heiny DK. 1989. Improvement of fungi to enhance mycoherbicide potential. In: *Biotechnology of fungi for improving plant growth* (Whipps, J. M.; Lumsden, R. D. eds.). Cambridge University Press, UK. Chapter 6 pages 127–151.

[3] Chet I. 1993. *Biotechnology in Plant Disease Control.* John Wiley & Sons, New York.

[4] Whipps JM and Lumsden RD. 2001. Commercial use of fungi as plant disease biological control agents: status and prospects. In: *Fungal Biocontrol Agents: Progress;, Problems and Potential,* (eds T. Butt, C. Jackson & N. Magan), Wallingford: CABI Publishing, pp 9-22.

[5] Copping LG, Menn JJ. Biopesticides: their action, applications and efficacy – a review. Pest Management Science 2000; 56(8): 651–676.

[6] Ahmad JS and Baker R. Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutants of *Trichoderma harzianum*. Phytopathology 1987; **77:** 358-362.

[7] Benhamou N, Chet I. Hyphal interaction between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold chemistry of the mycoparasitic process. Phytopathology 1993; 83: 1062-1071.

[8] Aziz NH, El-Fouly MZ, El-Essawy AA, Khalaf MA. Influence of bean seedling root exudates on the rhizosphere colonization by *Trichoderma lignorum* for the control of *Rhizoctonia solani*. Bot. Bull. Acad. Sin 1997; 38: 33-39.

[9] Bonnarme P, Djian A, Latrasse A, Féron G, Giniès C, Durand A, Le Quéré JL. Production of 6-pentyl-α-pyrone by *Trichoderma* sp. from vegetable oils. Journal of Biotechnology 1997; 56: 143-150.

[10] Calistru C, McLean M, Berjak P. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. A study of the production of extracellular metabolites by *Trichoderma* species. Mycopathologia 1997a 137: 115-124.

[11] Calistru C, McLean M, Berjak P. *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species *1*. Macroscopical and microscopical observations of fungal interactions. Mycopathologia 1997b; 139: 115-121.

[12] Ejechia BO. Biological control of wood decay in an open tropical environment with *Penicillium* spp. and *Trichoderma viride*. International Biodeterioration and Biodegradation 1997; 39: 295-299.

[13] Zheng Z, Shetty K. Cranberry processing waste for solid state fungal inoculant production. Process Biochemistry 1998; 33(3): 323-329.

[14] Yedidia I, Benhamou N, Chet I. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Applied and Environmental Microbiology 1999; 65(3): 1061–1070.

[15] Ortiz A, Orduz S. In vitro evaluation in *Trichoderma* and *Gliocladium* antagonism against the symbiotic fungus of fungus growing ant *Atta cephalotes*. Mycopathologia 2001; 150: 53–60.

[16] Héraux FMG, Hallett, SG, Weller, SC. Combining *Trichoderma virens*-inoculated compost and a rye cover crop for weed control in transplanted vegetables. Biological Control 2005; 34: 21–26.

[17] Bisby GR. *Trichoderma viride* Pers. ex. Fries, and notes on *Hypocrea*. Transactions of the British Mycological Society, 1939; 33: 149-168.

[18] Baker KF and Cook RJ. 1974. Biological Control of Plant Pathogens. Freeman. San Francisco.

[19] Papavizas GC, Lewis JA, Abd-El Moity TH. Evaluation of new biotypes of *Trichoderma harzianum* for tolerance to benomyl and enhanced biocontrol capabilities. Phytopathology 1982; 72: 126-132.

[20] Amsellem Z, Zidack NK, Quimby Jr. PC, Gressel J. Long-term dry preservation of viable mycelia of two mycoherbicidal organisms. Crop Proctection 1999; 18: 643-649.

[21] Yedidia I, Benhamou N, Kapulnik Y, Chet I. Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. Plant Physiol. Biochem 2000; 38: 863-873.

[22] Katayama A and Matsumura F. Photochemically enhanced microbial degradation of environmental pollutants. Environ. Sci. Technol 1991; 25: 1329-1333.

[23] Karam J, Nicell, JA. Potential applications of enzymes in waste treatment. J. Chem. Tech. Biotechnol 1997; 69: 141-153.

[24] Mandels M, Weber J. Production of cellulases. Adv. Chem. Ser 1969; 95: 391-414.

[25] Lewis JA, Papavizas GC. Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. Soil Biol. Biochem 1983; 15(3): 351-357.

[26] Vlaev SD, Djejeva G, Raykovska V, Schugerl K. Cellulase production by *Trichoderma* sp. Grown on corn fibre substrate. Process Biochemistry 1997; 32: 561-565.

[27] Verma M, Brar SK, Tyagi RD, Valéro JR and Surampalli RY. Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. Water Research 2005; 39(15): 3587-3596.

[28] Coley-Smith JR, Ghaffar A, Javed ZUR. The effect of dry conditions on subsequent leakage and rotting of fungal sclerotia. Soil Biology and Biochemistry 1974; 6: 307-312.

[29] Henis Y, Adams PB, Papavizas GC, Lewis JA. Penetration of sclerotia of *Sclerotium rolfsii* by *Trichoderma* spp. Phytophathology 1982; 72: 707.

[30] Punja ZK, Utkhede RS. Using fungi and yeasts to manage vegetable crop diseases. Trends in Biotechnology 2003; 21: 400-407.

[31] Thornton CR. Production of a monoclonal antibody specific to the genus *Trichoderma* and closely related fungi, and its use to detect *Trichoderma* spp. in naturally infested composts. Microbiol. 2002; 148: 1263-1279.

[32] Cook RJ. Making greater use of introduced microorganisms for biological control of plant pathogens. Annu. Rev. Phytopathol 1993; 31: 53–80.

[33] Benhamou N and Picard K. La résistance induite : une nouvelle stratégie de défense des plantes contre les agents pathogènes. Phytoprotection 1999; 80(3): 137-168.

[34] Benhamou N, Rey P, Picard K, Tirilly Y. Ultrastructural and cytochemical aspects of the interaction between the mycoparasite, *Pythium oligandrum*, and soilborne pathogens. Phytopathology 1999; 89: 506–517.

[35] Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP. Chitinase Gene Expression during Mycoparasitic Interaction of *Trichoderma harzianum* with Its Host. Fungal Genetics and Biology 1999; 26: 131-140.

[36] Menendez AB, Godeas A. Biological control of *Sclerotinia sclerotiorum* attacking soybean plants. Degradation of the cell walls of this pathogen by *Trichoderma harzianum* (BAFC 742). Mycopathologia 1998; 142: 153-160.

[37] Inbar J, Menendez A, Chet I. Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. Soil Biol Biochem 1996; 28: 757-763.

[38] Celar F. Competition for ammonium and nitrate forms of nitrogen between some phytopathogenic and antagonistic soil fungi. Biological Control 2003; 28: 19–24.

[39] Blakeman JP. Microbial competition for nutrients and germination of fungal spores. Ann. Appl. Biol 1978; 89: 151-155.

[40] Sivan A, Chet I. Biological control of *Fusarium* spp. in cotton, wheat and muskmelon by *Trichoderma harzianum*. J. Phytopathol. 1986; 116: 39-47.

[41] Lewis JA, Papavizas GC. Biocontrol of damping-off caused by *Rhizoctonia solani* in the field with formulations of *Trichoderma* spp. and *Gliocladium virens*. Crop Protection 1991; 10: 396-402.

[42] Zaldívar M, Velásquez JC, Contreras I., Pérez LM. *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. Electronic Journal of Biotechnology 2001; 4(3): 160-168. (This article is available online at, http://www.ejb.org/content/vol4/issue3/full/7).

[43] Lisboa De Marco J, Valadares-Inglis MC, Felix CR. Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity against *Crinipellis perniciosa*, the causal agent of witches' broom of cocoa. Brazilian Journal of Microbiology 2003; 34: 33-38.

[44] Roberts DP, Lohrke SM, Meyer SLF, Buyer JS, Bowers JH, Baker CJ, Li W, de Souza JT, Lewis JA, Chung S. Biocontrol agents applied individually and in combination for suppression of soilborne diseases of cucumber. Crop Protection 2005; 24: 141-155.

[45] Hutchinson CM. *Trichoderma virens*-inoculated composted chicken manure for biological weed control. Biological Control 1999; 16: 217–222.

[46] Hanson LE, Howell CR. Biocontrol efficacy and other characteristics of protoplast fusants between *Trichoderma koningii* and *T. virens*. Mycol. Res 2002; 106(3): 321-328.

[47] Liu P, Yang Q. Identification of genes with a biocontrol function in *Trichoderma harzianum* mycelium using the expressed sequence tag approach. Research in Microbiology 2005; 156: 416–423.

[48] Howell CR, Puckhaber LS. A study of the characteristics of "P" and "Q" strains of *Trichoderma virens* to account for differences in biological control efficacy against cotton seedling diseases. Biological Control 2005; 33: 217–222.

[49] Score AJ, Palfreyman JW. Biological control of the dry rot fungus *Serpula lacrymans* by *Trichiderma* secies: The effects of complex and synthetic media on interaction and hyphal extension rates. International Biodeterioration and Biodegradation 1994; 33: 115-128.

[50] Highley TL, Ricard J. antagonism of *Trichoderma* spp. and *Gliocladium virens* against wood decay fungi. Material und Organismen 1988; 23: 157-169.

[51] Bruce A, King B. Biological control of decay in creosote treated distribution poles. II. Control of decay in poles by immunizing commensal fungi. Material und Organismen 1986; 21(3): 165-179.

[52] Bruce A, Smith GM, King B, Hainey SD, Evans PD. Soil-bed decay studies of softwood pole segments treated with CCA by sap-displacement. 1. Evaluation of soil bed exposure and assessment of soft rot decay. Wood Protection 1991; 1(1): 1-7.

[53] Bruce A, Highley T. Control of growth of wood decay basidiomycetes by *Trichoderma* spp. and other potentially antagonistic fungi. Forest Products Journal 1991; 41(2): 63-67.
[54] American Wood-Preservers' Association Standard M10-77, 1977. Standard method of testing wood preservatives by laboratory soil-block cultures. American Wood-Preservers' Association, 1-8.
[55] European Standard EN 113, 1980. Wood preservatives. Determination of the toxic values against wood destroying basidiomycetes cultured on an agar medium. CEN, European Committee for Standardization.
[56] Tucker EJB, Bruce A, Staines HJ. Application of modified international wood preservation chemical testing systems standards for assessment of biocontrol treatments. International Biodeterioration and Biodegradation 1997; 39(2-3): 189-197.
[57] Mortuza MG, Ilag LL. Potential for biocontrol of *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. in banana fruits by *Trichoderma* species. Biological Control 1999; 15: 235–240.
[58] Moreno LS, Paningbatan RA. Biological control of mango stem-end rot caused by *Diplodia natalensis* with *Trichoderma viride.* Philippine Phytopathol. 1995; 31: 103–116.
[59] Batta YA. Postharvest biological control of apple gray mold by T*richoderma harzianum Rifai* formulated in invert emulsion. Crop Prot. 2004a; 23: 19– 26.
[60] Batta YA. Effect of treatment with *Trichoderma harzianum Rifai* formulated in invert emulsion on post harvest decay of apple blue mold. International Journal of Food Microbiology 96; 2004b: 281–288.
[61] Brewer MT and Larkin RP. Efficacy of several potential biocontrol organisms against *Rhizoctonia solani* on potato. Crop Protection. (In Press)
[62] Hjeljord LG, Stensvand A, Tronsmo A. Effect of temperature and nutrient stress on the capacity of commercial *Trichoderma* products to control *Botrytis cinerea* and *Mucor piriformis* in greenhouse strawberries. Biological Control 2000; 19: 149–160.
[63] Cooney JM, Lauren DR. *Trichoderma*/pathogen interactions: measurement of antagonistic chemicals produced at the antagonist/pathogen interface during tubular bioassay. Letters in Applied Microbiol 1998; 27: 283-286.
[64] Bankole SA, Adebanjo A. Biocontrol of brown blotch of cowpea caused by *Colletotrichum truncatum* with *Trichoderma viride.* Crop Protection 1996; 15(7): 633-636.
[65] Perello A, Monaco C, Simon MR, Sisterna M, Dal Bello G. Biocontrol efficacy of *Trichoderma* isolates for tan spot of wheat in Argentina. Crop Protection 2003; 22: 1099-1106.
[66] Escande AR, Laich FS, Pedraza MV. Field testing of honeybee-dispersed *Trichoderma* spp. to manage sunflower head rot (*Sclerotinia sclerotiorum*). Plant Pathology 2002; 51: 346–351.
[67] Itoh Y, Kodama K, Furuya K, Takahashi S, Haneishi T, Takiguchi Y, Arai M. A new sesquiterpene antibiotic, heptelidic acid producing organisms, fermentation, isolation and characterization. J Antibiot (Tokyo) 1980; 33(5): 468-73.
[68] Stipanovic RD and Howell CR. The x-ray crystal structure determination, and biosynthetic studies of the antibiotic, heptelidic acid. Tetrahedron 1983; 39: 1103-1107.
[69] Liss SN, Brewer D, Taylor A, Jones GA. Antibiotic activity of an isocyanide metabolite of *Trichoderma hamatum* against rumen bacteria. Can J Microbiol. 1985, 31(9):767-72.
[70] Ousley MA, Lynch, JM Whipps, JM. Potential of *Trichoderma* spp. as consistent plant-growth stimulators. Biol Fertil Soils 1994; 17: 85–90.
[71] de la Cruz J, Llobell A. Purification and properties of a basic endo-β-1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum.* Eur. J. Biochem 1999; 265: 145-151.
[72] Felse PA, Panda T. Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. Proc Biochem 1999; 34: 563-566.
[73] Hoell IA, Klemsdal SS, Vaaje-Kolstad G, Horn SJ, Vincent GH, Eijsink, T. Overexpression and characterization of a novel chitinase from *Trichoderma atroviride* strain P1. Biochimica et Biophysica Acta 2005; 1748: 180–190.
[74] Kerry BR. Fungal parasites: a weapon against cyst nematodes. Plant Dis 1981; 65: 390-393.
[75] Windham GL, Windham MT, Williams WP. Effects of *Trichoderma* spp. on maize growth and *Meloidogyne arenaria* reproduction. Plant Dis. 1989; 73: 493–494.
[76] Santos MA, dos Ferraz S, Muchovej JJ. Evaluation of 20 species of fungi from Brazil for biocontrol of *Meloidogyne incognita* Race 3. Nematropica 1992; 22: 183-192.
[77] Saifullah, Thomas, BJ. Studies on the parasitism of *Globodera rostochiensis* by *Trichoderma harzianum* using low temperature scanning electron microscopy. Afro-Asian J. Nematol. 1996; 6: 117-122.

[78] Spiegel Y, Chet I. Evaluation of *Trichoderma* spp. as a biocontrol agent against soil-borne fungi and plant-parasitic nematodes in Israel. Integr. Pest Manag. Rev. 3, 1998; 169-175.

[79] Sharon E, Bar Eyal M, Chet I, Herrera Estrella A, Kleifeld O, Spiegel Y. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology 2001; 91: 687-693.

[80] Eapen SJ, Beena B, Ramana KV. Tropical soil microflora of spice-based cropping systems as potential antagonists of root-knot nematodes. Journal of Invertebrate Pathology 2005; 88: 218–225.

[81] Ganassi S, Moretti A, Stornelli C, Fratello B, Bonvicini Pagliai AM, Logrieco A, Sabatini MA. Effect of *Fusarium*, *Paecilomyces* and *Trichoderma* formulations against aphid *Schizaphis graminum*. Mycopathologia 2000 ; 151: 131-138.

[82] Harmann GE and Björjmann T. 1998. Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In *Trichoderma and Gliocladium Vol. 2.* (Harmann, G.E. and Kubicek, C.K., eds), pp 229–265, Taylor and Francis Ltd., London.

[83] Lindsey DL, Baker R. Effect of certain fungi on dwarf tomatoes grown under gnotobiotic conditions. Phytopathology 1967; **57:** 1262-1263.

[84] Bae YS, Knudsen GR. Soil microbial biomass influence on growth and biocontrol efficacy of *Trichoderma harzianum*. Biological Control 2005; 32: 236–242.

[85] Vargas-Garcia MC, Lopez MJ, Suarez F, Moreno J. Laboratory study of inocula production for composting processes. Bioresource Technology 2005; 96: 797-803.

[86] Wu T, Kabir Z, Koide RT. A possible role for saprotrophic microfungi in the N nutrition of ectomycorrhizal *Pinus resinosa*. Soil Biology & Biochemistry 2005; 37: 965–975.

[87] Azcbn-Aguilar C, Barea JM. Applying mycorrhiza biotechnology to horticulture: significance and potentials. Scientia Horticulturae 1997; 68: 1–24.

[88] Saraswathy A., Hallberg R. Degradation of pyrene by indigenous fungi from a former gasworks site. FEMS Microbiology Letters 2002; 210: 227-232.

[89] Pelczar Jr MJ, Chan, ECS, Kreig NR. *Microbiology*, 5th ed., Tata McGraw-Hill, Inc. New York, 1993, ISBN 0-07-049234-4.

[90] Saxena RK, Gupta R, Kuhad RC, Khurana N. Light independent conidiation in *Trichoderma* spp., a novel approach to microcycle conidiation. World Journal of Microbiology 1993; 9: 353-356.

[91] Gupta R, Saxena RK, Goel S. Short communication: Photo-induced sporulation in *Trichoderma harzianum* - an experimental approach to primary events. World Journal of Microbiology and Biotachnology 1997; 13: 249-250.

[92] Molla AH, Fakhru'l-Razia A, Hana MM, Alam MZ. Optimization of process factors for solid-state bioconversion of domestic wastewater sludge. International Biodeterioration & Biodegradation 2004; 53: 49-55.

[93] Jenkins NE, Heviefo G, Langewald J, Cherry AJ, Lomer CJ. Development of mass production technology for aerial conidia for use as mycopesticides. Biocontrol News and Information, 1998; 19(1): 21N-31N.

[94] Clark DS. Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of *Aspergillus niger*. Can J Microbiol 1961; 8: 133-136.

[95] van Suijdam JC, Kossen, NWF, Paul, PG. An inoculum technique for the production of fungal pellets. Eur J Appl Microbiol Biotechnol 1980; 10: 211-221.

[96] Domingues FC, Queiroz JA, Cabral JMS, Fonseca LP. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. Enzyme and Microbial Technology 2000; 26: 394-401.

[97] Olsson L, Christensen TMIE, Hansen KP, Palmqvist EA. Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C-30. Enzyme and Microbial Technology 2003; 33: 612-619.

[98] Maheshwari R. Microconidia of *Neurospora crassa*. Fungal Genetics and Biology 1999; 26(1): 1-18.

[99] Bosch A, Yantorno O. Microcycle conidiation in the entomopathogenic fungus *Beauveria bassiana* bals. (vuill.). Process Biochemistry 1999; 34: 707-716.

[100] Pascual S, Rico JR, De Cal A, Melgarejo P. Ecophysiological factors affecting growth, sporulation and survival of the biocontrol agent *Penicillium oxalicum*. Mycopathologia 1997; 139: 43-50.

[101] Papagianni M. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances 2004; 22: 189-259.

[102] Roncal T, Cordobés S, Ugalde U, He Y, Sterner O. Novel diterpenes with potent conidiation inducing activity. Tetrahedron Letters 2002; 43: 6799-6802.

[103] Carlsen M, Spohr AB, Nielsen J, Villadsen J. Morphology and physiology of a α-amylase producing strain of *Aspergillus oryzae* during batch cultivations. Biotechnol. Bioeng. 1995; 49: 266-76.
[104] Felse PA, Panda T. Submerged culture production of chitinase by *Trichoderma harzianum* in stirred tank bioreactors – the influence of agitator speed. Biochemical Engineering Journal 2000; 4: 115-120.
[105] Karim MIA, Kamil AQA. Biological treatment of palm oil mill effluent using *Trichoderma viride.* Biological Wastes 1989; 27: 143-152.
[106] Reczey K, Szengyel ZS, Eklund R, Zacchi G. Cellulase production by *T. reesei.* Bioresource Technology 1996; 57: 25-30.
[107] Xia L, Shen X. High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. Biores Technol 2004; 91: 259–62.
[108] Gutierrez-Correa M, Tengerdy RP. Production of cellulose on sugar cane bagasse by fungal mixed culture solid substrate fermentation. Biotechnol Lett 1997; 19: 665–7.
[109] Kansoh AL, Essam, SA, Zeinat AN. Biodegradation and utilization of bagasse with *Trichoderma reesie.* Polymer Degradation and Stability 1999; 63: 273-278.
[110] Sharma SK, Kalra KL, Grewal HS. Fermentation of enzymatically saccharified sunflower stalks for ethanol production and its scale up. Bioresource Technology 2002; 85: 31–33.
[111] Tirado-Montiel ML, Tyagi RD, Valéro JR. Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. Water Research 2001; 35: 3807-3816.
[112] Gadgil NJ, Daginawala HF, Chakrabarti T, Khanna P. Enhanced cellulase production by a mutant of *Trichoderma reesei.* Enzyme and Microbial Technology1995; 17: 942-946.
[113] Srinivasan U, Staines HJ, Bruce A. Influence of media type on antagonistic modes of *Trichoderma* spp. against wood decay basidiomycetes. Material and Organismen 1992; 27(4): 301-321.
[114] Roco A, Pérez LM. *In vitro* biocontrol activity of *Trichoderma harzianum* on *Alternaria alternata* in the presence of growth regulators. EJB Electronic Journal of Biotechnology 2001; 4(2): 68-73.
[115] Clarkson JP, Payne T, Mead A, Whipps JM. Selection of fungal biological control agents of *Sclerotium cepivorum* for control of white rot by sclerotial degradation in a UK soil. Plant Pathology 2002; 51: 735–745.
[116] Mischke, S. A quantitative bioassay for extracellular metabolites that antagonize growth of filamentous fungi, and its use with biocontrol fungi. Mycopathologia 1997; 137: 45–52.
[117] Zgoda, JR, Porter, JR. A convenient microdilution method for crude screening of natural products against bacteria and fungi. Pharmaceutical Biology 2001; 39(3): 221-225.
[118] Basappa PBS, Swamy SN, Rangappa KS. Synthesis and characterization of novel 6-fluoro-4-piperidinyl-1,2-benzisoxazole amides and 6-fluoro-chroman-2-carboxamides: antimicrobial studies. Bioorganic & Medicinal Chemistry 2005;13: 2623-2628.
[119] Askew DJ, Laing MD. The in vitro screening of 118 *Trichoderma* isolates for antagonism to *Rhizoctonia solani* and an evaluation of different environmental sites of *Trichoderma* as sources of aggressive strains. Plant Soil 1994; 159: 277–281.
[120] Shaban, GM, El-Komy, HMA. Survival and proliferation of alginate encapsulated *Trichoderma* spp. in Egyptian soil in comparison with allyl alcohol soil fumigation. Mycopathologia 2000;151: 139–146.
[121] Mukherjee PK, Raghu K. *Trichoderma* sp. as a microbial suppressive agent of *Sclerotium rolfsii* on vegetables. World Journal of Microbiology and Biotechnology 1997; 13: 497-499.
[122] Howell, CR, Hanson, LE, Stipanovic, RD, Puckhaber, LS, Wheeler, MH. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. Phytopathology 2000; 90: 248–252.
[123] Howell, CR. Cotton seedling pre-emergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. Phytopathology 2002; 92: 170–182.
[124] Brown HL, Bruce A. Assessment of the biocontrol potential of a *Trichoderma viride* isolate Part I: Establishment of field and fungal cellar trials. International Biodeterioration & Biodegradation 1999; 44: 219-223.
[125] Brown, HL, Bruce, A, Staines, HJ. Assessment of the biocontrol potential of a *Trichoderma viride* isolate. Part II: Protection against soft rot and basidiomycete decay. International Biodeterioration and Biodegadation 1999; 44: 225-231.
[126] Lewis, JA, Lumsden, RD. Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. Crop Prot 2001; 20: 49–56.

[127] Perello AM, Monaco C, Cordo C. Evaluation of *Trichoderma harzianum* and *Gliocladium roseum* in controlling leaf blotch of wheat (*Septoria tritici*) under in vitro and greenhouse conditions. Z Pflanzankr Pflanzanschutz 1997; 104: 588–598.

[128] Perello A, Simon MR, Arambarri AM. Interactions between foliar pathogens and the saprophytic mycoflora of the wheat (*Triticum aestivum* L.) phylloplane. J Phytopathol 2002; 150: 232–243.

[129] Kredics L, Antal Z, Manczinger L, Nagy E. Breeding of mycoparasitic *Trichoderma* strains for heavy metal resistance. Letters in Applied Microbiology 2001a; 33: 112-116.

[130] Kredics L, Antal Z, Doczi I, Manczinger L. Effect of heavy metals on growth and extracellular enzyme activities of mycoparasitic *Trichoderma* strains. Bulletin of Environmental Contamination and Toxicology 2001b; 66: 249-254.

[131] Kredics L, Manczinger L, Antal Z, Penzes A, Szekeres F, Kevei F, Nagy E. In vitro water activity and pH dependence of mycelial growth and extracellular enzyme activities of *Trichoderma* strains with biocontrol potential. Journal of Applied Microbiology 2004; 96: 491–498.

[132] Metcalf DA, Wilson CR. The process of antagonism of *Sclerotium cepivorum* in white rot affected onion roots by *Trichoderma koningii*. Plant Pathology 2001; 50: 249-257.

[133] Clarkson JP, Mead A, Payne T, Whipps JM. Effect of environmental factors and *Sclerotium cepivorum* isolate on sclerotial degradation and biological control of white rot by *Trichoderma*. Plant Pathology 2004; 53: 353–362.

[134] Prasad RD, Rangeshwaran R. Effect of soil application of a granular formulation of *Trichoderma harzianum* on seed rot and damping-off of chickpea incited by *Rhizoctonia solani*, saprophytic growth of the pathogen and bioagent proliferation. J Mycol Plant Pathol 2000; 30: 216–220.

[135] Prasad RD, Rangeshwaran R., Hegde SV, Anuroop CP. Effect of soil and seed application of *Trichoderma harzianum* on pigeonpea wilt caused by *Fusarium udum* under field conditions. Crop Protection 2002; 21: 293–297.

[136] Elad Y. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Protection 2000;19: 709-714.

[137] Pozo MJ, Baek J-M, Garcia JM, Kenerley CM. Functional analysis of tvsp1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genetics and Biology 2004; 41:336–348.

[138] Okigbo RN, Oikediugwu FE. Studies on biological control of postharvest rot in yams *Dioscorea* spp using *Trichoderma viride*. J Phytopathology 1999; 148: 351-355.

[139] Thangavelu R, Palaniswami A, Velazhahan R. Mass production of *Trichoderma harzianum* for managing fusarium wilt of banana. Agriculture, Ecosystems and Environment 2004; 103: 259–263.

[140] Cotxarrera L, Trillas-Gay, MI, Steinberg C, Alabouvette,C. Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. Soil Biology and Biochemistry 2002; 34 (4), 467-476.

[141] Grondona I, Hermosa R, Tejada M, Gomis, MD, Mateos, P F, Bridge PD, Monte E, Garcia-Acha I. Physiological and biochemical characterization of *Trichoderma harzianum*, a biological control agent against soilborne fungal plant pathogens. Applied and Environmental Microbiology 1997; 63(8): 3189–3198.

[142] Lopez E, Orduz S. *Metarhizium anisopliae* and *Trichoderma viride* for control of nests of the fungus-growing ant, *Atta cephalotes*. Biological Control 2003; 27: 194-200.

[143] Evans HC. The safe use of fungi for biological control of weeds. Phytoprotection (suppl.) 1998; 67-74.

[144] Koide RT, Suomi L, Stevens CM, McCormick L. Interactions between needles of Pinus resinosa and ectomycorrhizal fungi. New Phytologist 1998; 140: 539–547.

[145] Koide RT, Kabir Z. Nutrient economy of red pine is affected by interactions between Pisolithus tinctorius and other forest-floor microbes. New Phytologist 2001; 150: 129–188.

[146] Wu T, Sharda JN, Koide RT. Exploring interactions between saprotrophic microbes and ectomycorrhizal fungi using a protein– tannin complex as an N source by red pine (Pinus resinosa). New Phytologist 2003; 159: 131–139.

[147] Vestberg M, Cassells AC, Schubert A, Cordier C, Gianinazzi S. AMF and micropropagation of high value crops. In: Gianinazzi, S., Schüepp, H., Barea, J.M., Haselwandter, K. (Eds.), Mycorrhizal Technology in Agriculture. Birkhäuser Verlag, Basel, 2002; pp. 223–233.

[148] Vestberg M, Kukkonen S, Saari K, Parikka P, Huttunen J, Tainio L, Devos N, Weekers F, Keversd C, Thonart P, Lemoine M-C, Cordier C, Alabouvette C, Gianinazzi S. Microbial inoculation for improving the growth and health of micropropagated strawberry. Applied Soil Ecology 2004; 27: 243–258.

[149] Ezzi MI, Lynch JM. Biodegradation of cyanide by *Trichoderma* spp. and *Fusarium* spp. Enzyme and Microbial Technology 2005; 36: 849–854.

[150] Rudresh DL, Shivaprakash MK, Prasad RD. Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea (Cicer aritenium L.). Applied Soil Ecology 2005; 28: 139–146.

[151] Potin O, Veignie E, Rafin C Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. FEMS Microbiology Ecology 2004; 51, 71–78.

[152] Potin O, Rafin C, Veignie E. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. Int. Biodeterior. Biodegrad 2004b; 54, 45–52.

[153] Mandels M, Reese ET. Induction of cellulase in fungi by cellobiose. J. Bacteriol 1960; 79(6): 816–826.

[154] Tanaka M, Taniguchi M, Morinaga T, Matsuno R, Kamikubo T. Cellulase productivity of *Eupenicillium javanicum.* Journal of Fermentation Technology 1980; 58: 149-154.

[155] Giese EC, Covizzi LG, Borsato D, Dekker RFH, Corradi da Silva Maria de Lourdes BarbosaAM. Botryosphaeran, a new substrate for the production of β-1,3-glucanases by *Botryosphaeria rhodina* and *Trichoderma harzianum Rifai.* Process Biochemistry 2005 (In press)

[156] Kanauchi M, Bamforth CW. Growth of *Trichoderma viride* on crude cell wall preparations from barley. J Agric Food Chem 2001; 49: 883-887.

[157] Kanauchi M, Bamforth CW. Growth of *Trichoderma viride* on crude cell wall preparations from barley. J Agric Food Chem 2001; 49: 883-887.

**Table 1.** List of different *Trichoderma* spp. and respective BCA facts.

| Active agent | Antagonist against | Responsible Metabolites/Factors | Disease/ Epidemic control |
|---|---|---|---|
| **Fungi** | | | |
| *T. harzianum* 1051<br>*T. harzianum 39.1* | *Crinipellis perniciosa* | Chitinase, N-acetylglucosaminidase, β-1,3-glucanase, total cellulase, endoglucanase, aryl- β-glucosidase, β-glucosidase, protease and amylase | Witches' broom disease (*Marasmius perniciosus* Stahel) of Cocoa [43] |
| *T. lignorum/Trichoderma virens/Trichoderma hamatum Trichoderma harzianum and T. pseudokoningii (Rifai)* | *Rhizoctonia solani* | Unknown inhibitory substances; extracellular metabolites or antibiotics, or lytic enzyme action | Damping-off of bean [8,116,119,120] |
| *T. viride*<br>*T. harzianum* | *Aspergillus flavus* and *Fusarium moniliforme* | Lipolytic, proteolytic, pectinolytic and cellulolytic enzymes. Unknown (mycotoxins) antibiotic compounds (e.g. peptides, cyclic polypeptides) | Fungal – seed-associated; [10,11] |
| *T. harzianum* BAFC 742 | *Sclerotinia sclerotiorum* BAFC 2232 | 1,3-β-glucanase and chitinase | Fungal – soyabean plant [36] |
| *T. sp.* | *Sclerotium rolfsii* | Competitive inhibition | Rotting of common vegetables [121] |
| *T. harzianum* 25<br>*T. viride* | *Serpula lacrymans* | Antibiotic; anthraquinones | Fungal wood decay [49] |
| *T. harzianum* | *Alternaria alternata* | Endo-chitinase | Fungal plant disease [114] |
| *Trichoderma virens* "Q" strain | *Rhizopus oryzae/Pythium sp.* | Plant phytoalexin induction by antibiotic compound, gliovirin | Cotton seedling Disease [48,122,123] |

(Table 1 contd.)

| Active agent | Antagonist against | Responsible Metabolites/Factors | Disease/ Epidemic control |
|---|---|---|---|
| *Trichoderma viride isolate, T60* | Soft rot and basidiomycetes decay fungi, namely, *Coniophora puteana, Postia placenta* and *Serpula lacry-mans* | Volatile organic compounds, lytic enzymes and soluble antibiotics; nutrient competition | Sap stain discoloration of Pine and spruce trees [124,125] |
| *T. virens* isolates GL3 and GL21; *Trichoderma harzianum* T-203 | *Rhizoctonia solani, Pythium ultimum,* and *Meloidogyne incognita* | Antibiotics glioviren and gliotoxin, and other inhibitory metabolites | Damping-off of cucumber [14,44,126] |
| *Trichoderma harzianum Trichoderma aureoviride Trichoderma koningii* | *Pyrenophora tritici-repentis (Died) Drechs. (anamorph=Drechslera tritici-repentis (Died) Shoem.* | Lytic enzymes such as chitinases and proteases; antibiotics; mycoparasitism | Tan spot and leaf blotch of wheat [65,127,128] |
| *T. viride* | *Colletotrichum truncatum* | Volatile compounds and nonvolatile antibiotics, viridin with antifungal and antibacterial properties | Brown blotch disease of cowpea [64] |
| *Trichoderma aureoviride* T122 ; *T. harzianum* T66 and T334, and *T. viride* T124 and T228 | *Fusarium, Pythium* and *Rhizoctonia* strains | β-glucosidase, cellobiohydrolase; β -xylosidase and protease enzymes | General fungal plant disease [84,129-131] |
| *Trichoderma viride* and *Trichoderma pseudokoningii Trichoderma koningii* | *Sclerotium cepivorum* | Volatile organic compounds, lytic enzymes and soluble antibiotics | *Allium* (Onion seedlings) white rot [115,132,133] |
| *Trichoderma harzianum* | *Fusarium udum* | Lytic enzymes such as chitinases and proteases; antibiotics | Pigeonpea wilt [134,135] |
| *Trichoderma harzianum* | *Penicillium expansum* | Enzymes; antibiotics; mycoparastitism | Apple blue and gray mold [59,60] |

(Table 1 contd.)

| Active agent | Antagonist against | Responsible Metabolites/Factors | Disease/ Epidemic control |
|---|---|---|---|
| *Trichoderma virens* and *T. harzianum* | *Rhizoctonia solani* | Enzymes; antibiotics | Stem canker or black scurf of potato [61] |
| *Trichoderma harzianum* *Trichoderma koningii* | *Fusarium culmorum, Botrytis cineria* and *Rhizoctonia solani* | Anti-fungal compound: 6-n-pentyl-2H-pyran-2-one (6PAP); lytic enzymes, mainly, chitinases, glucanases, and proteases | Plant pathogens [63,136,137] |
| *Trichoderma koningii* *T. aureoviride* *T. longibrachiatum* | *Sclerotinia sclerotiorum* | Antagonism – enzymes; antibiotics and mycoparasitism | Sunflower head rot [66] |
| *Trichoderma harzianum* | *Alternaria alternata* | Fungal cell wall degrading enzymes | Fungal infection of leaves, stems, flowers and fruits of annual plants, especially vegetables and ornamentals [114] |
| *T. harzianum* and *T. viride* | *Lasiodiplodia theobroma; Diplodia natalensis; Botryodiplodia theobromae ; Fusarium moniliforme var sublutinans;Penicillium oxalicum Currie Penicillium sclerotienum Yamamoto; Aspergillus niger van Tiegh;Aspergillus tarmarii Kita;Rhizoctonia sp and Serratia sp* | Antifungal metabolites; non-volatile and volatile antibiotics | Fruit rot/wilt of banana; mango stem-end rot and post harvest rotting of yams [57,58,138,139] |
| *Trichoderma asperellum* | *Fusarium oxysporum* | Antibiosis, mycoparasitism and competition for nutrients | Wilt of tomato [140] |
| *Trichoderma harzianum* | *Aphanomyces cochlioides, Rhizoctonia solani, Phoma betae, Acremonium cucurbitacearum, and Fusarium oxysporum f. sp. radicis lycopersici* | Enzymes; antibiotics and anti-fungal properties | Common plant fungal diseases [141] |

(Table 1 contd.)

| Active agent | Antagonist against | Responsible Metabolites/Factors | Disease/ Epidemic control |
|---|---|---|---|
| *T. harzianum* | *Botrytis cinerea* and *Mucor piriformis.* | Antagonistic factors like enzymes and antibiotics | Post harvest rotting of strawberries [62] |
| **Bacteria** | | | |
| *T. viride* | Anaerobic bacteria - *Bacteroides fragilis* | Heptelidic acid – antibiotic action | [67,68] |
| *T. hamatum* | Rumen bacteria - *Escherichia coli, Megasphaera elsdenii, Streptococcus bovis, Bacteroides ruminicola, B. succinogenes, Succinivibrio dextrinosolvens, Ruminococcus albus, R. flavefaciens* | Antibiotic activity of an isocyanide metabolite - 3-(3-isocyanocyclopent-2-enylidene) propionic acid | Control of rumen bacteria [69] |
| *T. virens, T. koningii* | *Bacillus subtilis* | Gliotoxin, dimethylgliotoxin, heptelidic acid, viridiol and viridin | Biocontrol of plant diseases [46] |
| **Invertebrates (Insects and Nematodes)** | | | |
| *T. viride, T. koningii*<br>*T. longibrachiatum*<br>*T. hamatum, T. harzianum* | *Atta cephalotes** (a leaf-cutting, fungus growing ant) | 1,3-β-glucanase, chitinases, proteases, and lipases.<br>Unknown antibiotic compounds | Damage to plant leaves by ants. [15,146] |
| *T. harzianum* Rifai ITEM 908 and ITEM 910 | *Schizaphis graminum* (aphid) | Polysaccharide lyases, proteases, and lipases | Poisoning of cereal crops. [81] |
| *Trichoderma* spp. | *Caenorhabditis elegans; Meloidogyne incognita* | Enzymes; Antibiotics | Root-knot nematode of horticultural crops [80] |
| **Plants (Weeds)** | | | |
| *Trichoderma virens* (=*Gliocladium virens*) | *Setaria viridis* and *Amaranthus retroflexus* | Phytotoxins , including gliovirin, gliotoxin, viridian, and viridiol; allelochemicals | Broadleaf and grass weeds [16,45,122] |
| *T. viride* | *Lantana camara; Rottboellia cochinchinensis; Mikania micrantha* | Phytotoxins and lytic enzymes | Invasive weed [143] |

* Indirect antagonism: *Trichoderma* spp. inhibits the symbiotic fungus of *Atta cephalotes*, thereby, affecting the normal growth of the pest.

**Table 2.** Plant growth mechanism and bioremediation by different *Trichoderma* spp.

| **Trichoderma sp.** | **Plant name/Target compound** | **Growth promoters/Target compound concentration** | **Mode of growth promotion/bioremediation** | **Reference (s)** |
|---|---|---|---|---|
| *Trichoderma* sp. | *P. resinosa* Ait (red pine) | $NH_4^+$ - N; organic N (amino acid) | Saprotrophic fungus, *Trichoderma* sp. mobilized N from organic compounds via ectomycorrhizal fungi incapable of directly uptaking organic N from soil | [144-146] |
| *Trichoderma harzianum* DB11 | Strawberry, *Fragaria ananssa* | Rhizophere enhancers – N and P | Mycorrhizal fixation of phosphorus and nitrogen | [147,148] |
| *Trichoderma harzianum* (O90, O77, O82 and O80) and one *T. pseudokoningi* strain (O10) | Cynanide | Cyanide to ammonia | Co-metabolism of cyanide in the presence of glucose | [149] |
| *T. harzianum, T. viride* and *T. virens* | Chickpea | Nitrogen and phosphorus | Nutrient supplementation | [150] |
| *Trichoderma longibrachiatum, Trichoderma harzianum, Trichoderma viride*, and *Trichoderma koningii* | Pine | $NH_4^+$ - N and $NO_3^-$-N | Competitive inhibition | [38] |
| *Trichoderma* sp. | Polyaromatic hydrocarbons (PAHs) | 800–1100 mg PAHs /kg soil | Co-metabolism in the presence of glycerol | [151,152] |
| *Trichoderma hamatum, T. harzianum, T. koningii.* | Fluorene | 100 µg /g soil | Co-metabolism in the presence of glucose | [153] |

**Table 3.** List of *Trichoderma* fungi based BCAs and respective suppliers (source: www.google.com -internet search engine)

| Beneficial Organism | Trade Name | Manufacturers and Suppliers | Pests Controlled | Type of Action as per Manufacturer | Country Registered |
|---|---|---|---|---|---|
| *Gliocladium* spp.# | GlioMix™ | Kemira Agro Oy | Soil pathogens | | Finland |
| *Gliocladium virens*# | Soil Guard 12G™ | Certis | Soil pathogens that cause damping off and root rot, esp. *Rhizoctonia solani* & *Pythium* spp. | Antagonist | U.S. |
| *Trichoderma harzianum* | RootShield™ BioTrek 22G™ Supresivit™ T-22G™, T-22HB™ | BioWorks, Wilbur-Ellis, Borregaard | Soil pathogens - *Pythium, Rhicozoktonia, Verticillium, Sclerotium,* and others | Parasite, competitor | U.S., Europe |
| *T. harzianum* | Trichodex™ | Makhteshim | *Botritis cinerea* and others | Mycoparasite living on other fungi | Israel |
| *T. harzianum* and *T. polysporum* | Binab™ | Bio-Innovation | Tree-bound pathogens | Mycoparasite | U.K., Sweden |
| *T. harzianum* and *T. viride* | Trichopel™ Trichojet™ Trichodowels™ Trichoseal™ | Agrimm Technologies | *Armillaria, Botryoshaeria,* and other fungal diseases | Antagonism by enzymes and antibiotics | New Zealand |
| *Trichoderma spp.* | Promot™ *Trichoderma* 2000 Biofungus | J.H. Biotech Mycontrol, Ltd. De Ceuster | Growth promoter, *Rhizoctonia solani, Sclerotium rolfsii, Pythium* spp., *Fusarium* spp. on nursery and field crops | - | U.S. U.S. Belgium |
| *T. viride* | Trieco | Ecosense Labs | For management of *Rhizoctonia* spp., *Pythium* spp., *Fusarium* spp., root rot, seedling rot, collar rot, red rot, damping-off, *Fusarium* wilt on wide variety of crops | - | India |
| *Trichoderma harzianum* | *Trichoderma* 2000 | Mycontrol (EfA1)Ltd, | *Rhizoctonia solani, Sclerotium rolfsii, Pythium* | - | Israel |

#The genus *Gliocladium* have been reclassified and included in the more rapidly expanding genus *Trichoderma* [82].

**Table 4.** Growth/production media used for the production of *Trichoderma* spp. for BCA use**

| Media | Composition ($gl^{-1}$) | Remarks (spore yield) | Reference |
|---|---|---|---|
| Basal Salt Medium | Yeast extract, 0.5; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_20$, 0.3; $KH_2PO_4$, 1.36; and either cellulose powder or the holocellulose, 10. | Semi-synthetic (LF) [spore yield ≈ $10^6$-$10^7$ $ml^{-1}$] pH*- 4 – 7 Temperature*- 28 – 30°C Incubation time*- 4 – 21 d | [154] |
| Mendel's medium | Glucose, 10; urea, 0.3; $(NH_4)_2SO_4$, 1.4; $KH_2PO_4$, 2.0; $CaC1_2.2H_20$, 0.4; $MgSO_4.7H_20$, 0.3; peptone, 0.75; yeast extract, 0.25; $FeSO_4.7\ H_20$, 0.005; $MnSO_4.4H_20$, 0.0016; $ZnSO_4.7H_20$, 0.0014; $CoC1_2.6H_20$, 0.020. | | [124] |
| Tanaka medium | Glucose, 20; urea, 0.3; corn steep liquor, 10; $(NH_4)_2SO_4$, 1.4; $KH_2PO_4$, 2.0; $CaC1_2.2H_20$, 0.4; $MgSO_4.7H_20$, 0.3; proteose peptone (Difco), 1; $FeSO_4.7\ H_20$, 0.005; $MnSO_4.4H_20$, 0.0016; $ZnSO_4.7H_20$, 0.0016; $CoC1_2.6H_20$, 0.004. | | [155] |
| M-CSL<br>SN<br>GT | Glucose, 25; NaCl, 25; corn steep liquor, 5; molasses (blackstrap), 50.<br>Sucrose, 20; $NaNO_3$, 6; $KH_2PO_4$, 1.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 20.<br>Glucose, 20; $MgSO_4.7H_2O$, 1; $KH_2PO_4$, 2; ammonium tartrate, 1; $FeSO_4$, 0.001. | | [25] |
| Corn cobs<br>Wheat bran<br>Cornmeal | Quartz sand, 1000; corn cobs, 400. (in 1600 ml liquid)<br>Quartz sand, 1000; wheat bran, 400. (in 800 ml liquid)<br>Quartz sand, 1000; cornmeal, 40. (in 200 ml liquid) | Complex (SSF) (liquid; any of, M–CSL, SN, GT, or water) [spore yield ≈$10^7$-$10^8$ $g^{-1}$] | |
| Czapek dox agar medium | Sucrose, 20.0; $NaNO_3$, 2.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; agar, 20.0. | Synthetic (SSF) | [109] |
| PDA | Potato starch, ; dextrose, ; | Semi-synthetic (SSF) | |
| Oat agar | Oat flour, 30; agar, 15; $ZnSO_4{\cdot}7H_2O$, 0.01; $CuSO_4{\cdot}5H_2O$, 0.005. | | [97] |
| Botryosphaeran (exopolysaccharide (EPS) | Vogel minimal salts medium and 1.5 g/l of EPS as carbon substrate | Semi-synthetic (LF) | [156] |
| Cranberry pomace-based medium | Cranberry pomace, 10 g; $CaCO_3$, 0.5 g; water, 20 ml; $NH_4NO_3$, 0.5 g ,and or fish protein hydrolysate (FPH), 2 ml | Semi-synthetic (SSF) 5 mg/g pomace protein | [13] |
| Crude cell wall preparations from barley | (in %w/v) $KH_2PO_4$, 0.2; $(NH_4)_2SO_4$, 0.14; urea, 0.03; $MgSO_4.H_2O$, 0.03; $CaCl_2$, 0.03; peptone, 0.1; crude cell wall of fungi, 1.0; plus trace metal solution (1.0 mL) | Semi-synthetic (LF) | [157] |

(Table 4contd.)

| | | | |
|---|---|---|---|
| Organic substrate medium | Farm yard manure, rice chaffy grains, dried banana leaf, banana pseudostem and rice bran (500 g) and 100 ml of 30% molasses solution (v/v) | Complex (SSF) $4 \times 10^{15}$ to $30 \times 10^{32}$ CFU/g organic substrate | [139] |

* Valid for all media types

** This table contains different media used for *Trichoderma* spp. BCA production

**Table 5.** Comparison between Bacteria and *Trichoderma* spp. based BCAs

| Bacteria | Trichoderma sp. |
|---|---|
| Mode of action on pests through ingestion | Usually, mode of action on pests/weeds through contact |
| Narrow spectrum/pest specific biocontrol action | Broad spectrum biocontrol action |
| Mass production normally by liquid fermentation | Mass production normally by solid state fermentation |
| They have been used extensively in field | Limited reported uses and market exploitation |
| Require elaborate downstream processing | Relatively simple downstream processing |
| Very few have been reported as plant growth enhancers e.g., *Rhizobium* | Promote general plant growth and nutrition too |
| Limited use in bioremediation of recalcitrants | Widely exploited in bioremediation methods of recalcitrants |
| Examples; *Bacillus thuringiensis*, *Rhizobium* spp. | |

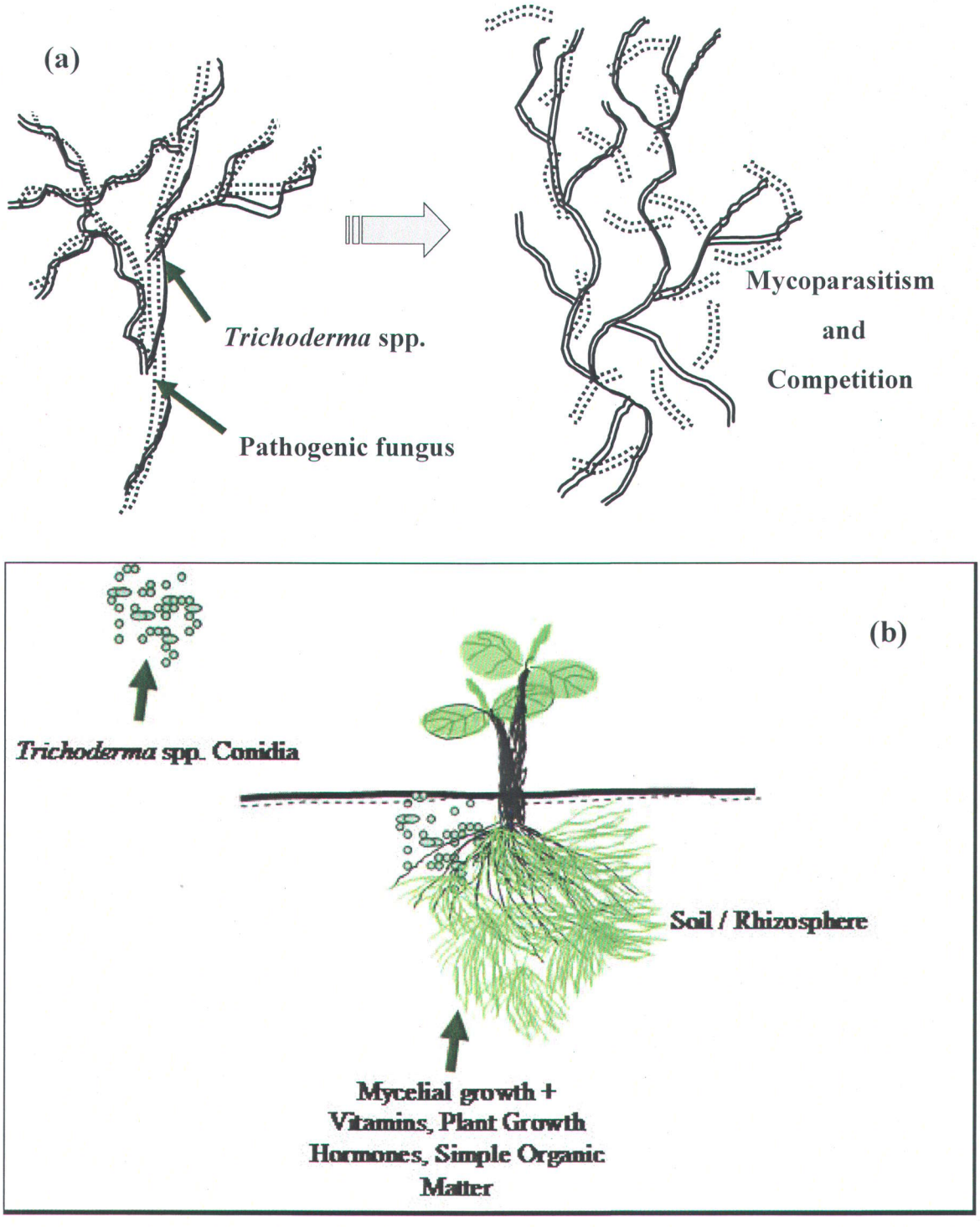



**Figure 1.** Growth promotional activity of *Trichoderma* spp.: a) Indirect, b) Direct.

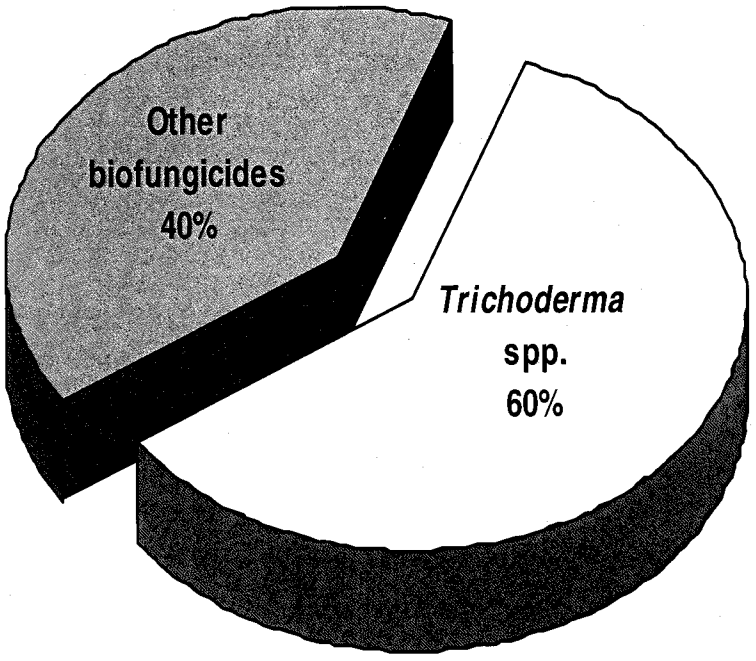



**Figure 2.** *Trichoderma* spp. based biofungicide market statistics. Other biofungicides include, bacteria, nematodes and virus.

Note: The market is based on scattered data of registered biofungicides.

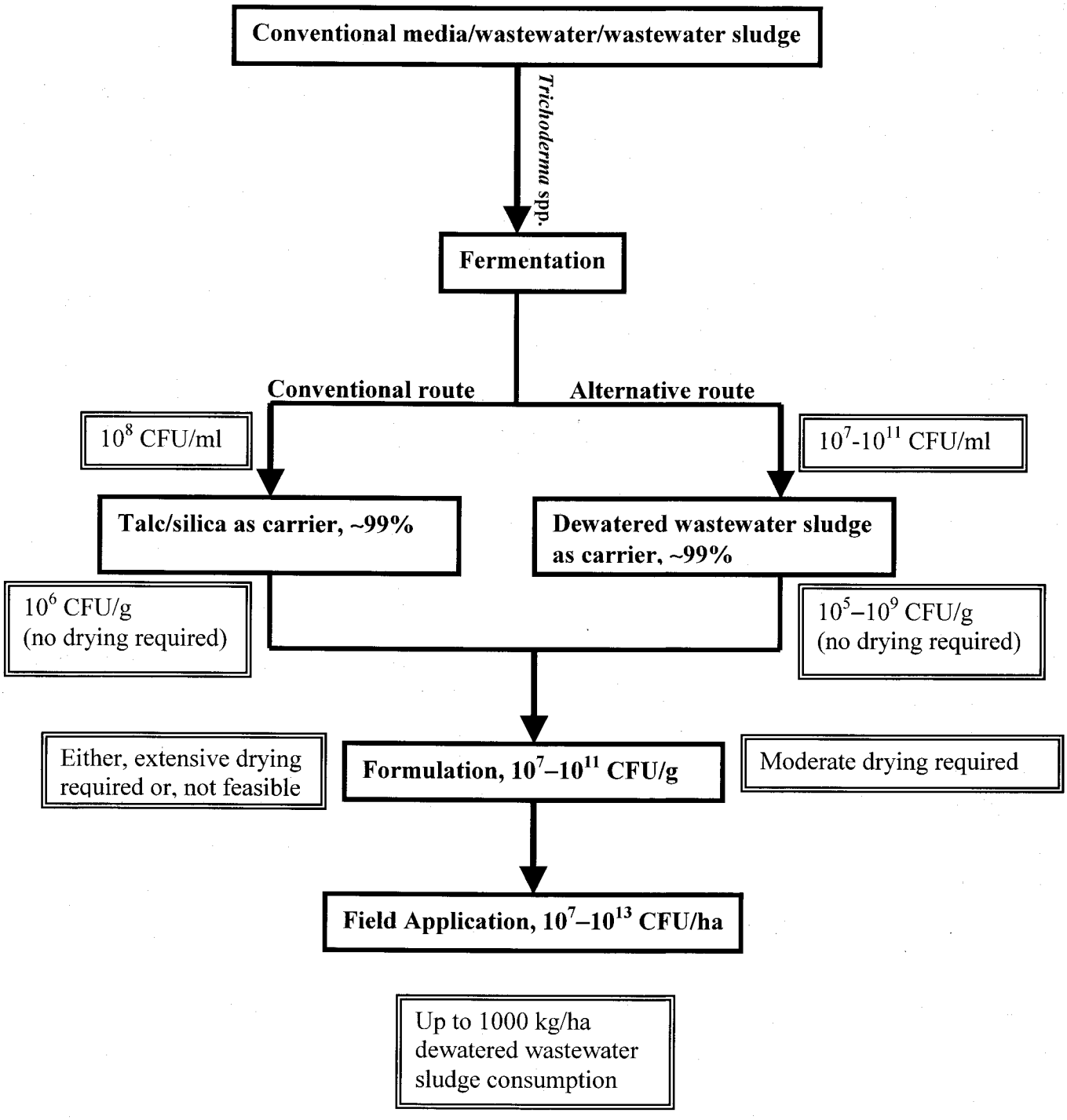



**Figure 3.** Schematic comparison of conidia requirements in conventional and alternative (non-conventional) routes of *Trichoderma* spp. BCAs.

# CHAPITRE 3.
# ÉTUDES DE BIODÉGRADABILITÉ DES BOUES D'ÉPURATION ET DE PRODUCTION DE *T. VIRIDE*

# Part I

# PRE-TREATMENT OF WASTEWATER SLUDGE – BIODEGRADABILITY AND RHEOLOGY STUDY

M. VERMA[1], SATINDER K. BRAR[1], A. R. RIOPEL[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]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

**Environmental Technology (28, xxx-xxx, in press, 2007)**

## Pré-traitement des boues d'épuration – Études de biodégradabilité et de rhéologie


### Résumé

Cette étude rapporte les changements dans la biodégradabilité, la rhéologie et la concentration en métal des boues d'épuration – non hydrolysées (brutes), stérilisées et hydrolysées (pré-traitement thermo-alcalin) à différents concentrations de solides entre 10 et 50 g/l pour déterminer la biodisponibilité des nutriments pour la fermentation subséquente. La concentration en solides dissout augmente linéairement avec les solides totaux. Indépendamment de la boue (brute ou pré-traitée), le pourcentage de biodégradabilité en terme de solides totaux (26.5–44.5%), de demande chimique en oxygène total (25.8–56.5%) et de solides dissout (41.9–66.9%) était maximal à une concentration en solides près de 20 g/l. Le comportement pseudoplastique de la boue diminue (l'index de consistance diminue de 895.1 à 5.2 et l'index du comportement d'écoulement augmente de 0.28 à 0.88 pour tous les types de boues) avec un pré-traitement et augmente avec la concentration en solides totaux. Les boues pré-traitées, soit les boues stérilisées et hydrolysées, ont permis d'obtenir une plus forte croissance microbienne (augmentation de 1 à 2 niveaux sur l'échelle logarithmique en comparaison avec les boues brutes), ce qui suggère une plus grande susceptibilité vis-à-vis la dégradation microbienne. Le ratio C:N diminue avec un pré-traitement (boues brutes > stérilisées > hydrolysées) durant la biodégradation. Bien que la concentration en métaux ait augmenté dans les boues hydrolysées incubées, leur concentration finale respectait les normes réglementaires pour leur application sur les terres agricoles. Ainsi, le pré-traitement des boues a permis d'augmenter la biodégradabilité, ce qui en fait un excellent support pour les produits fermentés à valeur ajoutée.


*Mots-clés:* biodégradable, boues municipales, hydrolyse thermo-alcaline, produits à valeur ajoutée, viscosité.

**Abstract**

This study investigates the changes in biodegradability, rheology and metal concentration of wastewater sludge – non-hydrolyzed (raw), sterilized, and hydrolyzed (thermal alkaline pre-treatment) at total solids concentration from 10-50 g $l^{-1}$ to ascertain the bioavailability of nutrients for subsequent fermentation. The dissolved solids concentration increased linearly with total solids. Irrespective of the wastewater sludge (raw or, pre-treated), percentage biodegradability in terms of total solids (26.5–44.5%), total COD (25.8–56.5%) and dissolved solids (41.9–66.9%) was maximum around 20 g $l^{-1}$ solids concentration. The pseudoplasticity of sludge decreased (consistency index decreased from 895.1 to 5.2 and flow behaviour index increased from 0.28 to 0.88, for all sludge types) with pre-treatment and increased with total solids concentration. The pre-treated sludge, namely, sterilized and hydrolyzed sludge showed higher microbial growth (1-2 log cycles increase in comparison to raw sludge) suggesting their susceptibility to microbial degradation. The C:N ratio decreased with pre-treatment (raw sludge > sterilized > hydrolyzed) during biodegradation. Although the metal concentration increased in incubated hydrolyzed sludge, the final concentration was within the regulatory norms for agriculture application. Thus, pre-treatment of sludge resulted in increase in biodegradability making it an excellent proponent for fermented value-added products.

*Keywords:* biodegradable, municipal sludge, thermal alkaline hydrolysis, value-added products, viscosity.

# INTRODUCTION

Municipal and industrial wastewater need extensive treatment before being discharged into the natural water bodies due to stringent environmental laws. In wastewater treatment plants, different treatment processes produce sludge which is required to be treated further before disposal. Wastewater sludge constitutes refractory, and difficult-to-biodegrade cellulosic compounds along with biodegradable organic matter, causing difficulties in sludge disposal. In order to degrade the refractory structure of wastewater sludge and increase its biodegradability, physico-chemical pretreatment methods are commonly utilized to transform the particulates into soluble compounds [1, 2]. While thermal or thermochemical pretreatment of sludge increases biodegradability, it consumes substantial amounts of energy in addition to chemicals. The biodegradability of wastewater sludge until now has been only considered from its aerobic and anaerobic digestion point of view [3]. However, the changing norms in wastewater treatment and sludge management methods have shifted the scenario towards environmentally benign disposal of wastewater sludge [4-6]. This involves reutilization of wastewater sludge as a raw material for diverse products, namely, adsorbants, bioflocculants, biosurfactants, biopesticides or biofertilizers [7].

Value addition to wastewater sludge by utilizing it as raw material for commercially important products like biopesticides [8-10], biofertilizers [11, 12], bioplastics [13], and enzymes [15] is a novel sustainable approach of sludge management. Nevertheless, the biodegradability and/or, bioavailability of wastewater sludge is a limiting factor in their efficient utilization in mass scale processes. The bioavailability of wastewater sludge has been explored by investigating various pre-treatment strategies [16-18]. However, none of the studies mentioned biodegradability, metal concentration and rheology of the wastewater sludge which will play an important role in the development of fermented value-added products.

Hence, present research is an effort to study the biodegradability of wastewater sludge – non-hydrolyzed (raw), sterilized, and hydrolyzed (thermal alkaline pre-treatment) to ascertain its bioavailability for different value-added products being studied in our laboratory, namely, biopesticides, enzymes and biofertilizers. This study is based on the hypothesis that in the presence of large amount of bioavailable substrate, the microbial

fermentation will be enhanced resulting in higher concentration of desired end products. In particular, a mixed consortium was selected as inoculum so that a generalization of the results could be carried out. Furthermore, rheology of wastewater sludge was determined to ascertain its mass scale suitability as a raw material and change in metal load was analyzed to establish the environmental safety during wastewater sludge application.

## MATERIALS AND METHODS

### Wastewater sludge

The wastewater sludge was obtained from CUQ (Communauté Urbaine de Québec, Québec) aerobic wastewater treatment facility as a secondary pollutant (cyclic backwash of installed BIODROF® biofilter to remove excess biofilm and/or, biomass chunks). The total solids (TS) of the procured sludge normally varied between 0.7–2.9 % (w/v) due to non-homogeneity of biofilm and/or, biomass chunks.

### Physico-chemical characterization of sludge

#### Solids and total COD

The sludge was subjected to physico-chemical characterization after adjusting TS concentration varying from 10 to 50 g $l^{-1}$. Different TS concentration was used to study the effect of pre-treatment on biodegradability. Table 1 shows physico-chemical characteristics of raw sludge (NH) at 10 g $l^{-1}$ TS only, since, at TS, 20–50 g $l^{-1}$, the metal concentration change was nominal (< ± 2-5% difference) in comparison to 10 g $l^{-1}$ TS, therefore, the data were not presented herein. The sludge followed the requirements of metal concentrations according to Québec Govt. guidelines for agricultural application [19]. Total solids, total volatile solids (TVS), suspended solids (SS), volatile suspended solids (VSS), dissolved solids (DS) and total chemical oxygen demand (TCOD) were determined as per Standard Methods [20].

*Solids amendment, sterilization and pre-treatment*

The TS of sludge was concentrated from ≅1.2–5% w/v (dry basis) to higher TS concentration by gravity sedimentation for 30-60 minutes followed by centrifugation at 7650 g for 15 minutes in a Sorvall RC 5C plus Macrocentrifuge (DuPont, USA). The supernatant was discarded and demineralized water was used for further dilutions. Maximum storage period of sludge was 1 week at 4 ± 1 °C to minimize microbial degradation (slow endogenous respiration). Raw sludge (TS concentrations of 10, 20, 30, 40, and 50 g $l^{-1}$) was obtained by suitably diluting 5% (w/v) sludge and homogenizing in a Waring$^{TM}$ blender for 30 seconds and adjusting the pH to 7.0 ± 0.1.

Sludge samples at all TS concentrations (200 ml sludges) were pre-adjusted to pH of 7.0 ± 0.1 with 4 N NaOH and/or, 4 N $H_2SO_4$ (500 ml Erlenmeyer flasks) and sterilized in an autoclave at 121 ± 1°C for 30 minutes. For pre-treatment, during sterilization step, the pH was pre-adjusted to 10.25±0.1. Subsequently, the pH was lowered to 7.0 ± 0.1. Afterwards, the sludge was sterilized at 121 ± 1°C for 15 minutes, designated as hydrolyzed sludge (to compare with earlier studies by using pure cultures, where similar method was followed for fermentation). Following sterilization, or, pre-treatment steps, the volume loss (normally up to 5%) due to evaporation during heating was compensated with demineralized water. Raw, sterilised, and hydrolyzed sludges will be henceforth referred to as NH, ST and TH sludges, respectively, meanwhile, the addition of superscripts 0 and 20 to these symbols would signify respectively, 0 and 20 d incubated sludge.

The significant difference of solids concentration and TCOD analyses were determined by using paired *t*-test for NH, ST and TH sludge sample triplicates at $P < 0.01$.

*Carbon and nitrogen*

Total C and N were determined by using Leco 932 CHNS analyzer (Carlo Erba Instruments, USA) as per Standard Methods [20].

*Metals*

The metal concentrations (Al, Ca, Cd, Cr, Cu, Fe, Mn, Na, Ni, Pb and Zn) were determined by adopting a slightly modified pre-digestion procedure with concentrated nitric acid, hydrofluoric acid and perchloric acid. For this, 25 ml of $HNO_3$ was added to

0.25 g dry sludge sample and left overnight at ambient temperature to avoid explosion during heating due to excess organic matter in sludge according to Standard Methods (APHA, 1998). The analysis was carried out by using inductively coupled plasma – atomic emission spectrophotometry (ICP-AES) (Varian vista AX CCD simultaneous ICP-AES with SPS 5 sample preparation system) according to the Method 305 of Standard Methods [20]. To achieve better reproducibility and quality, a control was prepared by using the same digestion process (PACS–2 and MESS–3, sediment samples donated by National Research Council of Canada, Ottawa, Ontario).

*Particle size and morphology*

The particle size analysis was carried out by using Dynamic particle size analyzer DPA 4100 (Brightwell Technologies Inc., Ontario, Canada). The glassware was cleaned thoroughly so that the impact of residual particles was statistically insignificant relative to the concentration of the sample under test. Each sample was tumbled and rolled ten times (by hand) and then introduced into the Brightwell cell system. The samples were diluted by a factor of 1 ml per 250 ml of particle free water (0.22 µm filter). Each sample was gently stirred upon a magnetic stir plate during analysis. The samples were stored at room temperature (~ 20°C), until analyzed. Size distribution comprised three runs per sample (1-400 µm size range with 0.25 µm measurement resolution) and image acquisition of particles was done at a magnification of 4.9 X. The samples were analyzed at the laboratory of Brightwell Inc., Ontario.

Biodegradability

For biodegradability assessment, fresh activated wastewater sludge was used as inoculum for 200 ml of NH, ST, and TH sludges. The inoculum contained, equivalent to $3.37 \times 10^4$ fungal and $9.2 \times 10^5$ bacterial CFU $ml^{-1}$. An inoculum of 2 % (v/v) activated sludge was used. The incubation was carried out at 25 ± 1°C at 150 rpm on a rotary shaker for 20 d. At the end of incubation, the volume loss due to evaporation during incubation was readjusted to 200 ml with demineralized water. The CFU plating was carried out automatically on tryptic soya agar medium using Whitley Automatic Spiral Plater 2, Fisher Scientific, Quebec, Canada.

The biodegradability of sludge in this study was defined as % concentration change of TS, TCOD and DS values after 20 d incubation period. Mathematically, it was defined as,

$$\frac{\text{Value at 0 d - Value at 20 d}}{\text{Value at 0 d}} \times 100$$

(i)

Rheological properties

Fresh samples were used for rheological analysis on a rotational viscometer (LV-DVII+, Brookfield, Middleboro, USA) with either ultralow (ULA, Brookfield) or small sample adapter cylindrical spindle (SC4 34, Brookfield). The gaps between spindle and respective sample chamber were 1.235 and 4.83 mm, respectively, for ultralow (viscosity range, 1-30 mPas), and small sample (viscosity range ≥30 mPas) adapter spindle to accommodate sludge flocs. The calibration and rheological testing procedure for each spindle was carried out as per instrument manual. For ultralow adapter spindle, ≈18.0 ml, and for small sample adapter spindle, ≈9.5 ml samples were taken. The viscosity data acquisition and analysis was carried out using Rheocalc V2.6 software (comprising commands, B.E.A.V.I.S. – Brookfield Engineering Advanced Viscometer Instruction Set, allowed the creation of programs to control connected instrumentation and manipulate data acquisition), Brookfield Engineering Labs, 1999.

In order to describe rheological characteristics of all samples, the following equations were tested: Ostwald-deWaele power law, $\tau = k\,\gamma^{n}$

(ii)

Bingham equation, $\tau = \tau_0 + \mu_P \gamma$

(iii)

and Casson equation, $\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{\mu_P \gamma}$

(iv)

Rheological indices for consistency ($K$), and flow behaviour ($n$) were also computed by the aforestated software.

## RESULTS AND DISCUSSION

### Solids and COD Profile

The effect of pre-treatment on dissolved solids concentration of $NH_0$, $ST_0$, and $TH_0$ sludges is shown in Figure 1. The DS change was linearly correlated with TS at all solid concentrations and the respective equations are presented in Figure 1a. The thermal effect on solubilization of organic matter of sludge was dominant factor in DS increase [21]. Any heat treatment (e.g., sterilization and thermal alkaline hydrolysis) can aid in release of dissolved organic matter due to thermal expansion of pores of microbial cell membranes, and expansion of sludge flocs. Meanwhile, the soluble organic matter present in sludge possessed multiplicity due to complex sludge composition [8, 16, 22]. For example, presence of microbial cells, debris and organic matter in sludge could yield dissolved matter on agitation, application of pressure, or osmotic shock. In addition, the heat treatment of sludge can cause disturbances at molecular level due to inter and intra-particle interactions resulting in cleavages of labile bonds (e.g., multiple bonds, hydrogen bonds, Van der Waal's interactions) [23]. Therefore, the increasing order of the slopes of DS change for $NH_0$, $ST_0$, and $TH_0$ sludges was anticipated; however, the difference between DS change for $ST_0$, and $TH_0$ sludges was smaller in comparison to $NH_0$. Additionally, the heat treatments could also result in an increase in the overall surface area of sludge flocs during disintegration and solubilization, thereby making them more susceptible to microbial action.

In order to assess the biodegradability, percent decrease (change in 0 d and 20 d samples) of TS, TCOD, and DS were measured (Figure 1b). For all sludges, the biodegradation (%) was maximum around 20 g $l^{-1}$ TS concentration which showed a declining profile towards higher solids concentration. Increase in substrate inhibition, mass transfer limitations, osmotic pressure difference, and rheological complexity (discussed later, Figure 1c) could be the major factors governing organic matter consumption and some of them have been already established by many researchers [22, 24]. The biodegradability (percent change of TS) increased with degree of pre-treatment. For NH, ST and TH sludges, the percent change of TS observed were 13.5–26.5%, 22.2–36%, and 29.7–44.5%, respectively. Meanwhile, the TCOD profile also suggested similar trend as

that of TS (Figure 1b), however, slight difference between TS and TCOD trends could be due to the presence of chemically non-oxidizable matter in sludge at all solids concentration. The change in DS concentration was comparable in ST and TH sludges due to presence of almost comparable DS at 0 d (Figure 1b). Furthermore, a significant increase in percent TS reduction (paired *t*-test, $P < 0.01$) in TH sludge was obtained on 20 d in comparison to ST sludge (Figure 1b). The TH sludge had higher overall surface area due to low particle size [22] as a result of disintegrated flocs. This helped in increased nutrient availability so that higher TS consumption was possible. Therefore, the TH pre-treatment was more efficient approach to increase biodegradability than ST pre-treatment.

Growth of microorganisms

The biodegradation of organic matter in terms of sludge flocs (Figures 2a and 2b) must result in an increase in population of the mixed consortia; therefore, the incubated samples (20 d old) were enumerated for CFU including, fungal spores, and bacteria (Figure 2c). Irrespective of sludge type and TS concentration, approximately $1.08 \times 10^6$ CFU/ml were inoculated (equivalent of 2% v/v inoculum) to initiate biodegradation of sludge organic matter. The highest CFU concentrations were observed ($2.8 \times 10^6$ to $2.34 \times 10^7$ CFU/ml) at 20-30 g $l^{-1}$ TS concentration in $ST_{20}$ and $TH_{20}$ sludges. This established 20-30 g $l^{-1}$ TS concentration to be the optimal TS range for pre-treated sludges. An overall decline in CFU was observed for $NH_{20}$ sludge at all TS, $ST_{20}$ sludges declined at 40–50 g $l^{-1}$ TS, and $TH_{20}$ sludge showed decrease at 50 g $l^{-1}$ TS concentration. Endogenous decay of microbial consortia due to deficiency of available nutrients could be one of the possible reasons for the above decline. However, considering period of incubation, elimination of susceptible microbes (less resistant to adverse growth conditions) could not be ruled out. Notably, the fungal spp. was eliminated during the course of incubation (Figure 2c, Inset; larger colonies belong to *Bacillus* spp. and smaller one to coliforms). This could be due to longer incubation time as well as lower initial ratio of fungal to bacterial CFU in the activated wastewater sludge inoculum. Therefore, it was concluded that pre-treated sludges could be more susceptible to microbial degradation. In fact, the inoculum culture also

showed possible presence of some pathogenic microbes (dark green, red, yellow colonies), which were absent in 20 d old culture (small white colonies and *Bacillus* spp.).

Rheological parameters

*Viscosity*

Viscosity is a measure of resistance due to movement between two adjacent layers of a fluid with no transfer of matter from one layer to another. It involves weak physical interactions e.g., London forces, Vander Wal's interaction and hydrogen bonds. Physically, it is measured as a ratio of shear stress to shear rate observed for any fluid and which depends upon the type of fluid (Newtonian and non-Newtonian). In complex media like sludge, presence of particulate matter (flocs in secondary wastewater sludge, Figure 2b) and multiplicity of fluid characteristics, macro interactions like flow hindrance often impart the observed viscosity. This is commonly termed as "apparent viscosity". Moreover, nature of sludge particulates or, flocs (size and shape) and extra cellular polymers (ECP) concentration do play a crucial role in determining apparent viscosity of the fluid [25]. Thus, viscosity of wastewater sludge is the most important rheological parameter for determining mixing, and transportation limitations for wastewater sludge as already reported [26].

The pre-treatment and incubation effects on apparent viscosity at all solids concentrations of ST and TH sludges are shown in Figures 3a and 3b, respectively. Irrespective of the type of pre-treatment (ST and TH), the apparent viscosity of sludge showed exponential increase with TS concentration (Equations in box, Figure 3), which was in agreement with earlier viscosity studies on *B. thuringiensis* fermentation of wastewater sludge at 10-40 g $l^{-1}$ solids concentration [22]. Moreover, the constants of exponential relationships in this research were pre-treatment characteristics of processes and could be used for sludge handling (estimation and control of mixing and pumping requirements) at conditions stated herein. At 0 d (Figure 3a), the apparent viscosity ranged from 3.18–218.95, 2.98–169.99, and 2.76–125.97 mPa.s, respectively for $NH_0$, $ST_0$, $TH_0$ sludges at 10–50 g $l^{-1}$ TS concentration. Hence, the apparent viscosity was highly labile to pre-treatments. The pre-treatment effect on viscosity was amplified with TS increment, possibly due to disruption of sludge flocs into smaller particulates and hydrolysis of

proteinaceous matter. Moreover, increase in DS concentration as explained earlier resulted in attenuation of flow hindrance, thereby, decreasing apparent viscosity.

The change in apparent viscosity after 20 d incubation followed slightly different pattern in comparison to 0 d samples (Figure 3b). In this case, the apparent viscosity ranged from 2.86–157.99, 2.36–70.66, and 2.22–39.99 mPa.s, respectively, for $NH_{20}$, $ST_{20}$, $TH_{20}$ sludges for 10–50 g $l^{-1}$ TS concentration. Meanwhile, the dependency of viscosity on TS concentration followed similar exponential profile as that of 0 d sludge samples (Equations in box, Figure 3). However, the relative decrease in apparent viscosity was higher with respect to 0 d sludge samples. Therefore, it was concluded that the microbial degradation of sludge organic matter and constant agitation at incubation conditions were more effective for pre-treated sludge causing reduction of viscosity in contrast to NH sludge. As stated earlier, release of dissolved solids and destruction of organic matter during aerobic respiration of microorganisms were probable factors in viscosity reduction after 20 d incubation. Thus, pre-treatment of sludges at all TS aided in an immediate decrease in apparent viscosity (Figure 3a), which enhanced consumption of organic matter by the microbial consortia. Furthermore, the organic matter consumption decreased the apparent viscosity (Figure 3b).

*Shear stress and shear rate*

Furthermore, the shear stress vs. shear rate profiles of all sludges at 0 and 20 d are shown in Figure 4. The profiles clearly indicated that the non-Newtonian characteristics (non-linearity) of all sludges shifted towards more Newtonian behaviour (increased linearity) as the sludge was subjected to pre-treatment (NH to ST to TH sludges). Meanwhile, the sludges also showed a less non-linear behaviour on incubation (0 d vs. 20 d samples). However, the shear stress of sludge after incubation was less pronounced as compared to 0 d samples which may arise due to decrease in ECP which might have been consumed during endogenous decay due to prolonged incubation (20 d) as well as decrease in floc size resulting in lower viscosity (Figure 3). Therefore, lower the shear stress, higher the biodegradability as the nutrient assimilation by microbes increase due to improved rheology (decreased non-Newtonian behaviour). The rheological classification of wastewater sludge is crucial owing to extreme variability of its viscosity with respect to dynamic (shear rate e.g., mixing, transportation) and static (time e.g., storage) conditions.

In general, most wastewater sludges showed non-Newtonian characteristics at TS concentrations ≥ 10 g $l^{-1}$ [27].

*Consistency and flow indices*

The shear stress and shear rate data for different sludge samples were further analyzed according to Power law which resulted in 68–95 % confidence of fit. As stated earlier, Figure 1c shows rheological parameters, i.e., *K* (consistency index) and *n* (flow behaviour index) profiles at all TS concentrations for all sludges at 0 and 20 d. The consistency index decreased from 18.1–5.2 to 3139–1186.3, from 24.2–4.2 to 850.3–437.6, and from 18.4–3.2 to 895.1–346 mPa.$s^n$, respectively, for $NH_0$ to $NH_{20}$, $ST_0$ to $ST_{20}$, and $TH_0$ to $TH_{20}$ sludges from 10 to 50 g $l^{-1}$ TS. On the other hand, the respective flow behaviour index increased from 0.52–0.62 to 0.28–0.33, from 0.45–0.63 to 0.41–0.45, and from 0.53–0.88 to 0.45–0.46, respectively. The variation in Power law constants clearly showed decreasing pseudoplasticity of wastewater sludge due to increase in degree of pre-treatment as well as increase in pseudoplasticity with TS concentration. The decrease in pseudoplasticity would play an important role in improving mass and heat transfer [24]. In addition, it was observed that in all sludge types, the rheological complexity (pseudoplasticity) increased at ≥ 30 g $l^{-1}$ TS (Figure 1c), rendering, ≤30 g $l^{-1}$ TS as more preferable medium for microbial processes.

Biodegradability and correlations to axenic fermentations

The data obtained in our earlier studies on *Bacillus thuringiensis* and *Trichoderma viride* based biopesticides production [10, 22] were reanalyzed in relation to biodegradability (Figure 5). It was observed that optimal TS concentration to achieve higher conidium concentration and entomotoxicity for *Trichoderma* spp. (Figure 5a) was about 30 g $l^{-1}$ for ST sludge. In case of *B. thuringiensis* (Figure 5b) the optimal TS concentration was about 25 g $l^{-1}$ for ST sludge. However, an optimal TS concentration of about 30 g $l^{-1}$ was observed for both microorganisms in case of TH sludge. The difference in optimal TS concentration between *B. thuringiensis* and *Trichoderma* for ST sludge may

arise due to their individual ability to produce enzymatic system to assimilate sludge organic matter resulting in variable growth patterns.

In case of ST as well as TH sludge, the amount of TS degraded increased up to 30 g $l^{-1}$ and remained almost constant from 30 to 40 g $l^{-1}$. The maximum biodegradability at 30 g $l^{-1}$ for ST as well as TH sludges corresponded to maximum conidia concentration and entomotoxicity (Tx) value for *Trichoderma* spp. for ST and TH sludges. The maximum value of spore count and Tx observed in case of *B. thuringiensis* for TH sludge at 30 g $l^{-1}$ was also in agreement with TS degraded. However, in case of ST sludge, the spore count and Tx value did not increase concomitantly with increased biodegradability from 25 to 30 g $l^{-1}$ which could be attributed to substrate inhibition, increased viscosity and reduced mass transfer [10, 22]. Another point that must also be emphasized is that the maximum TS reduction (at 30 g $l^{-1}$ solids) increased in TH sludge in comparison to ST sludge (from 3.95 to 7.4 and from 3.92 to 6.45, respectively for *Trichoderma* spp. and *B. thuringiensis*). This resulted in higher conidium/spore concentration and Tx value for *Trichoderma* spp. (Figure 5a) as well as *B. thuringiensis* (Figure 5b). Thus, the biodegradability of sludge irrespective of type of microorganism or the type of pre-treatment, plays an important role to achieve high product yield (conidium/spore and entomotoxicity in present case).

Carbon and nitrogen

In general, carbon and nitrogen are basic elements required for microbial growth. Therefore, in order to measure consumption of total carbon and nitrogen after 20 d incubation, the $NH_0$ (Table 1) and $TH_{20}$ (Table 2) sludges were analyzed for total carbon and nitrogen. The C:N ratio varied between 7.59–9.19 (at 10–50 g $l^{-1}$ TS concentration) in 0 d sludge ($NH_0$), which decreased slightly to 7.27–8.72 after 20 d incubation ($TH_{20}$). The decrease in C:N ratio was mainly due to $CO_2$ generation during biodegradation of sludge organic matter.

Metals

Metal composition of wastewater sludge plays a deterministic role in its safe disposal in the environment [19]. Therefore, it warrants characterization of metal

composition in wastewater sludge. Foregoing sections suggested that thermal alkaline pre-treatment gave maximum biodegradability. Therefore, it could be used for the production of various value added products, especially, biopesticides and biofertilizers as stated earlier [5, 8, 10-12, 16, 22]. Therefore, metal composition of $TH_{20}$ sludges (10–50 g $l^{-1}$) must be known. Tables 1 and 2 represent physico-chemical characterization of $NH_0$ and $TH_{20}$ sludges respectively. Physico-chemical characterization of $NH_0$ sludges was carried out to assess the relative change in composition of $TH_{20}$ sludges (10–50 g $l^{-1}$). It was observed that at all TS concentrations, $NH_0$ sludge was within the prescribed limits of regulated metals concentration [19]. The final concentration of all metals in $TH_{20}$ sludges (10–50 g $l^{-1}$) after 20 d incubation was increased due to decrease in TS concentration as a consequence of release of gases during incubation. However, the metals concentration in $TH_{20}$ was still lower than prescribed limits [19], except for increase in Cd and Pb in $TH_{20}$ (10 g $l^{-1}$) and Cu in $TH_{20}$ (20–50 g $l^{-1}$). However, the metals concentration of $TH_{20}$ sludge could be further controlled by mixing the NH sludge with agricultural or industrial sludge with lesser or, nominal metal concentration. Therefore, the increase in metal load of sludge at the end of 20 d was mainly due to decrease in TS concentration. Meanwhile, the decrease in TS concentration during growth of *Trichoderma* spp. (<24.6% for all sludge, Figure 5a) and *B. thuringiensis* (<21.5% for all sludge, Figure 5b) was lesser than 20 d incubated sludge (26 to 44.5% for all sludge). Hence, the possible increase in metal concentration would be insignificant so that the microbial fermentation of sludge would cause only nominal increase in metal concentration.

## CONCLUSIONS

The study investigated the biodegradability of wastewater sludge in three forms – raw; sterilized and hydrolyzed, which led to following conclusions:

1) Irrespective of sludge type, the dissolved solids concentration increased linearly with total solids concentration and the order of biodegradability was: raw < sterilized < hydrolyzed.
2) Highest biodegradability was observed at TS 20–25 g $l^{-1}$ irrespective of treatment type (raw, sterilized and hydrolyzed).

3) The sterilized and hydrolyzed wastewater sludge showed higher microbial growth (approximately 1-2 log cycles increase with respect to raw sludge) suggesting possible microbial degradation of these substrates.
4) Pre-treatment helped in decreasing viscosity and pseudoplasticity (consistency index decrease – between 895.1 to 5.2 and flow behaviour index increase between 0.28 to 0.88) at all total solids concentration.
5) The C:N ratio decreased slightly after biodegradation due to higher relative loss of carbon as $CO_2$ during aerobic microbial process.
6) Pre-treated wastewater sludge, namely, sterilized and hydrolyzed demonstrated higher biodegradability making them suitable raw materials for value-added products.

**Nomenclature**

| *Scientific Notations* | |
|---|---|
| $\tau$ | shear stress (mPa) |
| $\tau_0$ | yield stress (shear stress at 0 rpm of spindle, mPa) |
| $\gamma$ | shear rate ($s^{-1}$) |
| $K$ | consistency index ($mPa.s^n$) |
| $n$ | flow behaviour index (dimensionless) |
| $\mu_P$ | plastic viscosity (mPa.s) |
| Abbreviations | |
| CFU | Colony forming units |
| DS | Dissolved solids ($g\ l^{-1}$) |
| ECP | Extracellular polymers |
| NH | Non-hydrolyzed sludge |
| SBU/µl | Spruce budworm units $\mu l^{-1}$ |
| SC | Spore count ($CFU\ ml^{-1}$) |
| ST | Sterilized sludge |
| TC | Total cells ($CFU\ ml^{-1}$) |
| TCOD | Total chemical oxygen demand ($mg\ l^{-1}$) |
| TH | Hydrolyzed sludge |
| TS | Total solids ($g\ l^{-1}$) |
| TVS | Total volatile solids ($g\ l^{-1}$) |
| Tx | Entomotoxicity (SBU $\mu l^{-1}$) |

## ACKNOWLEDGEMENTS

The authors are sincerely thankful to 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 would like to extend our thanks to Mr. Sean Russell of Brightwell Technologies Inc., Ottawa who provided the image analysis of the wastewater sludge samples.

## REFERENCES

1. Lin, J.G., Ma, Y.S., Hung, C.C., Alkaline hydrolysis of the sludge generated from a high-strength, nitrogenous-wastewater biological treatment process. Bioresource Technology, 65, 35–42 (1998).
2. Lin, J.G., Ma, Y.S., Chao, A.C., Huang, C.L., BMP test on chemically pretreated sludge. Bioresource Technology, 68, 187–192 (1999).
3. Metcalf and Eddy., Wastewater engineering – Treatment and reuse. 4th Edition, Tchobanog lous, G., Burton, F.L. and Stensel, H.D. (Eds.), Mc Graw Hill Inc., New York, N.Y., pp. 888–893 (2003).
4. Cameron, M.D., Timofeevski, S., Aust, S.D., Enzymology of *Phanerochaete chrysosporium* with respect to the degradation of recalcitrant compounds and xenobiotics. *Applied Microbiology and Biotechnology*, **54**, 751 (2000).
5. Tirado-Montiel, M.L., Tyagi, R.D., Valéro, J.R., Wastewater treatment sludge as a raw material for the production of *Bacillus thuringiensis* based biopesticides. *Water Research*, **35**, 3807-3816 (2001).
6. Molla, A.H., Fakhru'l-Razi, A. Abd-Aziz, S., Hanafi, M.M., Roychoudhury, P.K., Alam, M.Z., A potential resource for bioconversion of domestic wastewater sludge. *Bioresource Technology*, **85**, 263-272 (2002).
7. Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Value addition of sewage sludge - a clean management strategy. *Practice Periodical of Hazardous, Toxic & Radioactive Waste Management* (Submitted) (2005a).
8. Tirado-Montiel, M.L., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Production biopesticides using wastewater sludge as a raw material: Effect of process parameters. *Water Science and Technology*, **48**, 239-246, (2003).
9. Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Banerji, S., Development of sludge based stable aqueous *Bacillus thuringiensis* formulations.*Water Science and Technology*, **50**, 229-236 (2004).
10. Verma, M., Brar, S.K., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. *Water Research*, **39**, 3587-3596 (2005).
11. Rebah, F.B., Tyagi, R. D., Prévost, D., Surampalli, R.Y., Wastewater Sludge as a new medium for rhizobial growth. *Water Quality Research Journal of Canada*, **37**, 353-370 (2002a).
12. Rebah, F.B., Prévost, D., Tyagi, R.D., 2002b. Growth of Alfalfa in sludge-amended soils and inoculated with Rhizobia produced in sludge. *Journal of Environmental Quality*, **31**, 1339-1348 (2002b).
13. Molla, A.H., Fakhru'l-Razia, A., Hana, M.M., Alam, M.Z., Optimization of process factors for solid-state bioconversion of domestic wastewater sludge. *International Biodeterioration and Biodegradation*, **53**, 49-55 (2004).
14. Nakasaki, K., Akakura, N., Adachi, T., Akiyama, T., Use of Wastewater Sludge as a Raw Material for Production of L-Lactic Acid. *Environmeantal Science and Technology*, **33**, 198-200 (1999).
15. Tyagi, R.D., Foko, V.S., Barnabe, S., Vidyarthi, A.S., Valero, J.R., 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 (2001).

16. Barnabé, S., Verma, M., Tyagi, R.D., Valéro, J.R., 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 (2005).
17. Neyens, E., Baeyens, J., Weemaes, M., De Heyder, B., Advanced biosolids treatment using $H_2O_2$ oxidation. *Environmental Engineering Science*, **19**, 27–35 (2002).
18. Vlyssides, A.G., Karlis, P.K., Thermal-alkaline solubilization of waste activated sludge as a pre-treatment stage for anaerobic digestion. *Bioresource Technology*, **91**, 201–206 (2004).
19. MENV., Guide sur la valorisation des matières résiduelles fertilisantes: Critères de références et normes rég lementaires. Direction du milieu rural, environnement Québec, Canada, 138 pages (2004).
20. APHA, AWWA, WPCF., Standard Methods for Examination of Water and Wastewaters. 20$^{th}$ edn. (Clesceri, L.S., Greenberg, A.E. and Eaton, A.D. eds.), American Public Health Association, Washington, DC, USA (1998).
21. Chu, C.P., Lin, W.W., Lee, D.J., Chang, B., Peng, X.F., Thermal treatment of waste activated sludge using liquid boiling. *Journal of Environmental Engineering*, **128**, 1100–1103 (2002).
22. Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., Surampalli, R.Y., Sludge based *Bacillus thuringiensis* biopesticides: Viscosity Impacts. *Water Research*, **39**, 3001-3011 (2005b).
23. Erdincler, A., Vesilind, P.A., Effect of sludge cell disruption on compactability of biological sludges. *Water Science and Technology*, **42**, 119-126 (2000).
24. Li, G.Q., Qiu, H.W., Zheng, Z.M., Cai, Z.L., Yang, S.Z., Effect of fluid properties on mass transfer in a bioreactor. *Journal of Chemical Technology and Biotechnology,* **62**, 385-391 (1995).
25. Dentel, S.K., Evaluation and role of rheological properties in sludge management. *Water Science and Technology*, **36**, 1-8 (1997).
26. Lotito, V., Spinosa, L., Mininni, G., Antonacci, R., The rheology of sewage sludge at different steps of treatment. *Water Science and Technology*, **36**, 79-85 (1997).
27. Guibaud, G., Dollet, P. Tixier, N. Dagot, C., Baudu M., Characterisation of the evolution of activated sludges using rheological measurements. *Process Biochemistry,* **39**, 1803–1810 (2004).

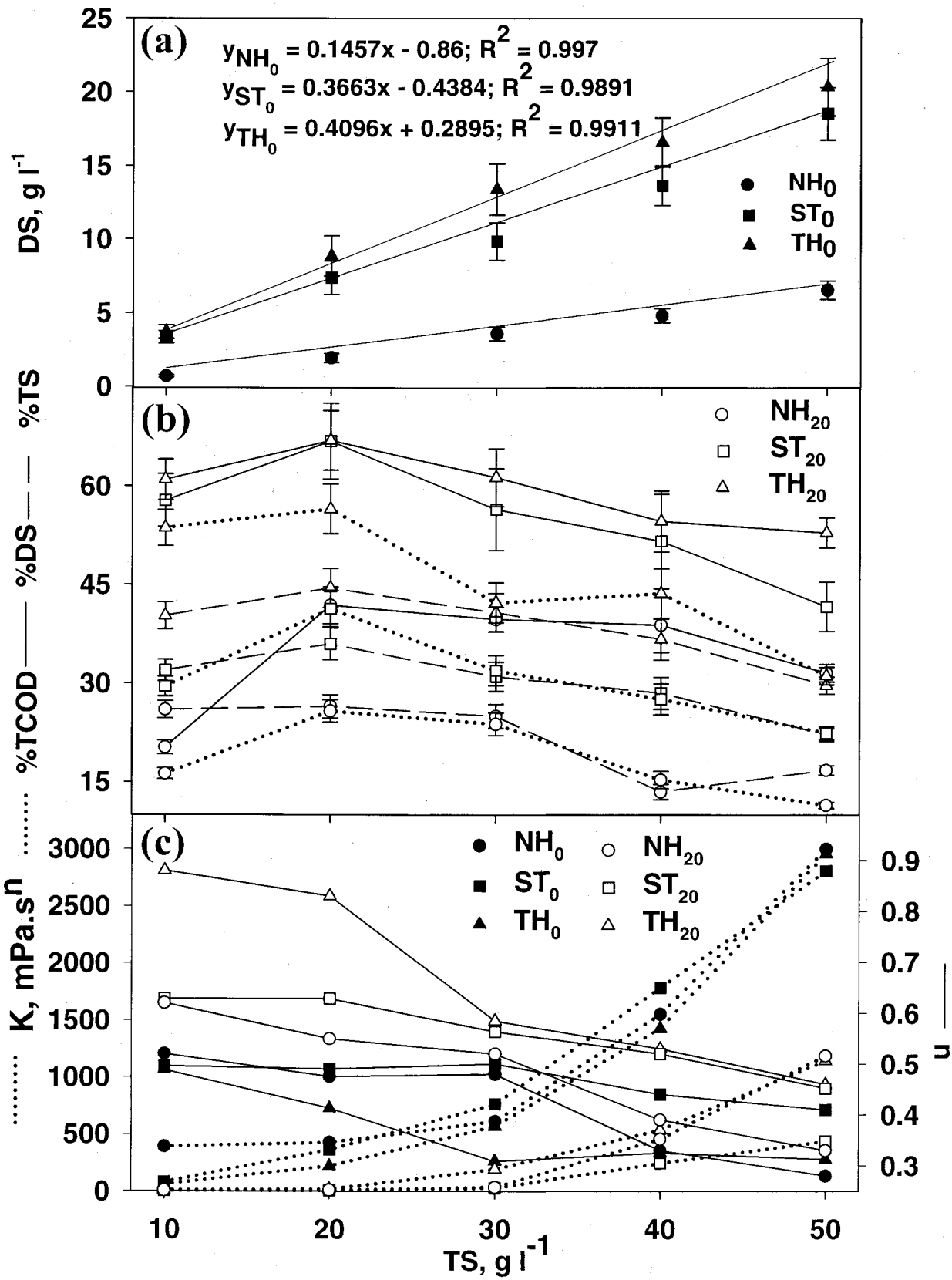
**Table 1.** Physico-chemical characterization of $NH_0$ sludge at 10 g $l^{-1}$ TS concentration

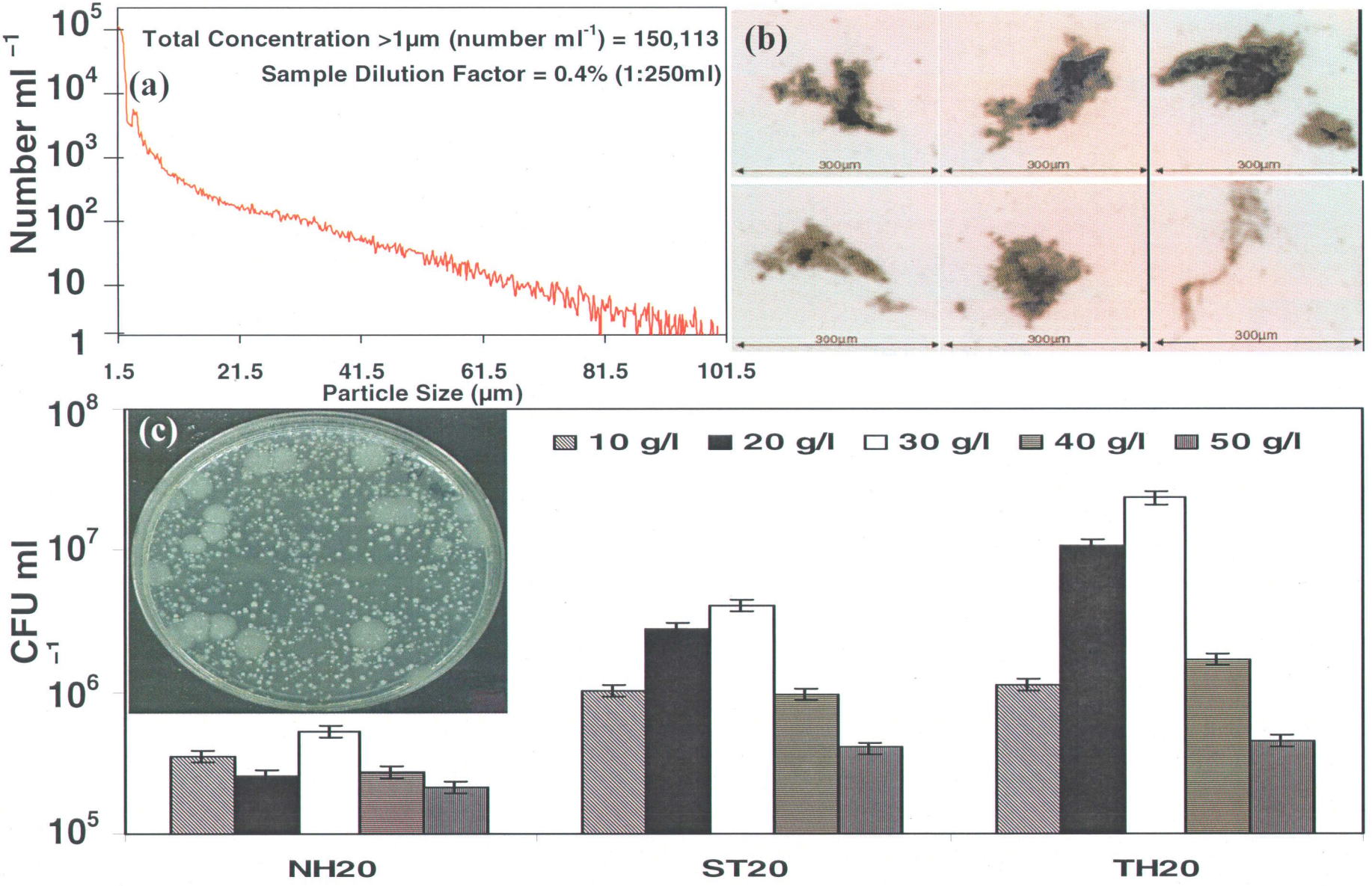
| **Concentration (mg $kg^{-1}$; unless stated)** | **$NH_0$(10)** |
|---|---|
| Total solids (g $l^{-1}$) | 10.0 ± 0.33 |
| Total volatile solids (g $l^{-1}$) | 9.2 ± 0.38 |
| Suspended solids (g $l^{-1}$) | 8.6 ± 0.27 |
| Volatile suspended solids (g $l^{-1}$) | 8.0 ± 0.34 |
| Total carbon | 371000 ± 8000 |
| Total nitrogen | 46200 ± 800 |
| C:N ratio | 8.03 |
| Total phosphorus | 1481 ± 43 |
| $N\text{-}NH_3$ | 7433 ± 217 |
| $N\text{-}NO_2^-$ , $N\text{-}NO_3^-$ | 98 ± 4.6 |
| $P\text{-}PO_4^{3-}$ | 708 ± 20 |
| Al | 2858 ± 62 |
| Ca | 27500 ± 800 |
| Cd | 7 ± 0.1 |
| Cr | 125 ± 2.6 |
| Cu | 238 ± 13 |
| Ni | 70 ± 0.8 |
| Fe | 14800 ± 500 |
| Pb | 39.4 ± 2.2 |
| Mn | 126 ± 4.2 |
| Zn | 101 ± 1.5 |
| Na | 520 ± 18 |

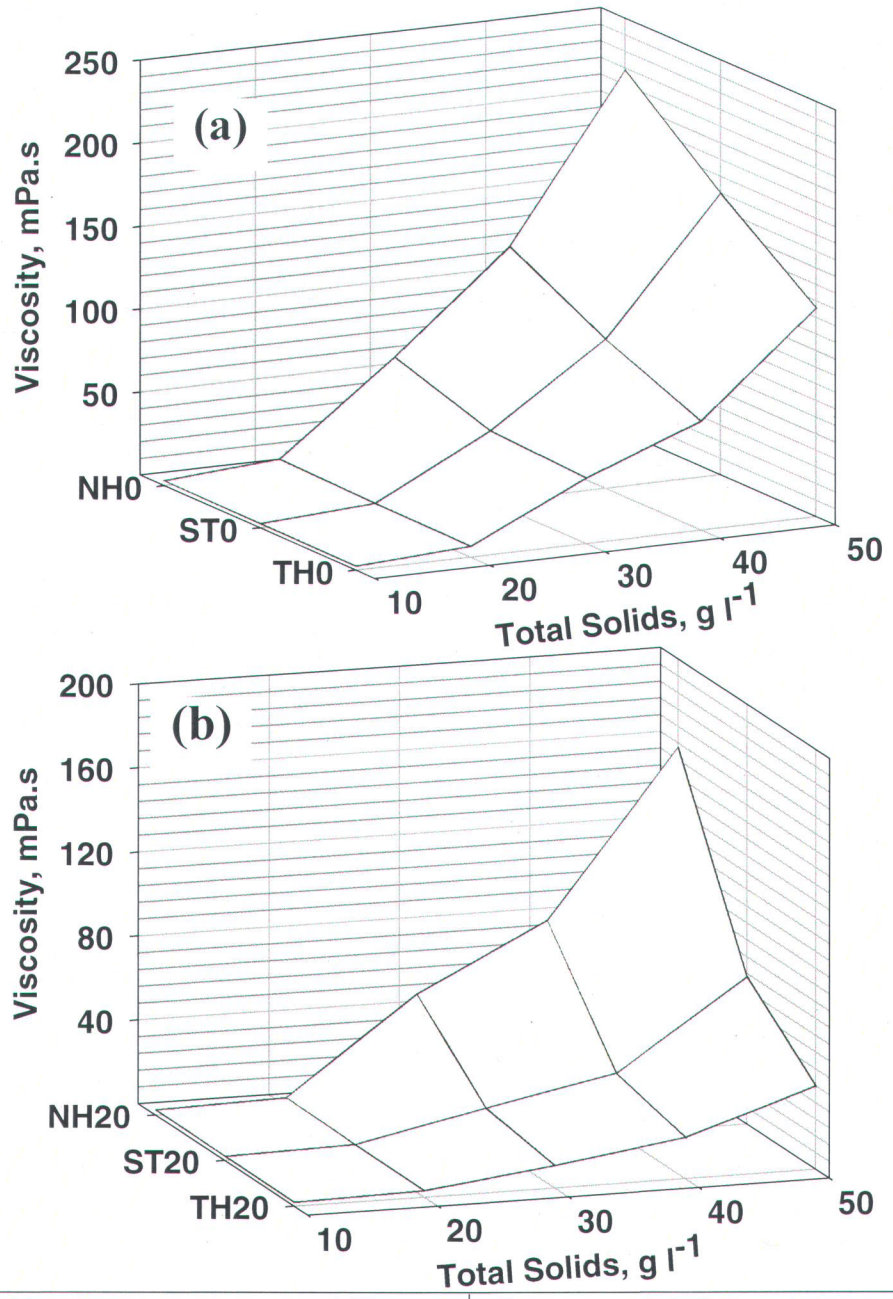
**Table 2.** Physico-chemical characterization of $TH_{20}$ sludge at different TS concentration (values indicated in parentheses)

| Concentration (mg $kg^{-1}$; unless stated) | $TH_{20}$(10) | $TH_{20}$(20) | $TH_{20}$(30) | $TH_{20}$(40) | $TH_{20}$(50) |
|---|---|---|---|---|---|
| Total solids (g $l^{-1}$) | 6.0 ± 0.38 | 11.1 ± 0.55 | 17.8 ± 0.42 | 25.3 ± 1.20 | 35.2 ± 1.78 |
| Total volatile solids (g $l^{-1}$) | 4.8 ± 0.28 | 10.3 ± 0.61 | 15.1 ± 0.69 | 22.4 ± 0.71 | 33.2 ± 1.51 |
| Suspended solids (g $l^{-1}$) | 4.1 ± 0.25 | 5.2 ± 0.26 | 7.4 ± 0.25 | 10.3 ± 0.39 | 16.0 ± 0.66 |
| Suspended volatile solids (g $l^{-1}$) | 2.8 ± 0.05 | 4.7 ± 0.27 | 6.7 ± 0.25 | 8.1 ± 0.30 | 14.5 ± 0.38 |
| Total carbon | 355000 ± 17000 | 435000 ± 20000 | 463000 ± 10000 | 377000 ± 21000 | 404000 ± 22000 |
| Total nitrogen | 46000 ± 2400 | 54300 ± 2100 | 63800 ± 1900 | 43200 ± 1800 | 50700 ± 2900 |
| C:N ratio | 7.72 | 8.02 | 7.27 | 8.73 | 7.96 |
| Total phosphorus | 1802 ± 23 | 1940 ± 65 | 1636 ± 41 | 2020 ± 45 | 1880 ± 46 |
| $N\text{-}NH_3$ | 325 ± 14 | 423 ± 16 | 840 ± 29 | 595 ± 47 | 355 ± 10 |
| $N\text{-}NO_2^-$ , $N\text{-}NO_3^-$ | 21 ± 0.2 | 8 ± 1 | 9 ± 0.2 | 10 ± 0.2 | 8 ± 0.2 |
| $P\text{-}PO_4^{3-}$ | 52 ± 2 | 65 ± 2 | 87 ± 3 | 64 ± 3 | 57 ± 2 |
| Al | 4555 ± 232 | 5271 ± 130 | 4020 ± 182 | 3436 ± 66 | 3412 ± 78 |
| Ca | 47500 ± 2100 | 27200 ± 1400 | 29200 ± 1000 | 31100 ± 1100 | 26400 ± 900 |
| Cd | 12 ± 0.5 | 6 ± 0.4 | 8 ± 0.3 | 8 ± 0.3 | 8 ± 0.1 |
| Cr | 196 ± 9 | 133 ± 6 | 90 ± 3 | 68 ± 3 | 55 ± 2 |
| Cu | 376 ± 7 | 705 ± 10 | 572 ± 11 | 561 ± 27 | 423 ± 13 |
| Ni | 115 ± 0.3 | 117 ± 5 | 103 ± 1 | 80 ± 3 | 112 ± 5 |
| Fe | 24700 ± 600 | 15400 ± 800 | 15900 ± 600 | 12700 ± 700 | 15200 ± 200 |
| Pb | 64.2 ± 1.2 | 51.2 ± 2 | 32.2 ± 3 | 36.1 ± 1 | 44.6 ± 1 |
| Mn | 155 ± 6 | 154 ± 8 | 78 ± 3 | 84 ± 1 | 97 ± 2 |
| Zn | 166 ± 4 | 173 ± 6 | 99 ± 5 | 106 ± 5 | 130 ± 7 |
| Na | 865 ± 22 | 1354 ± 4 | 929 ± 52 | 939 ± 32 | 1036 ± 36 |

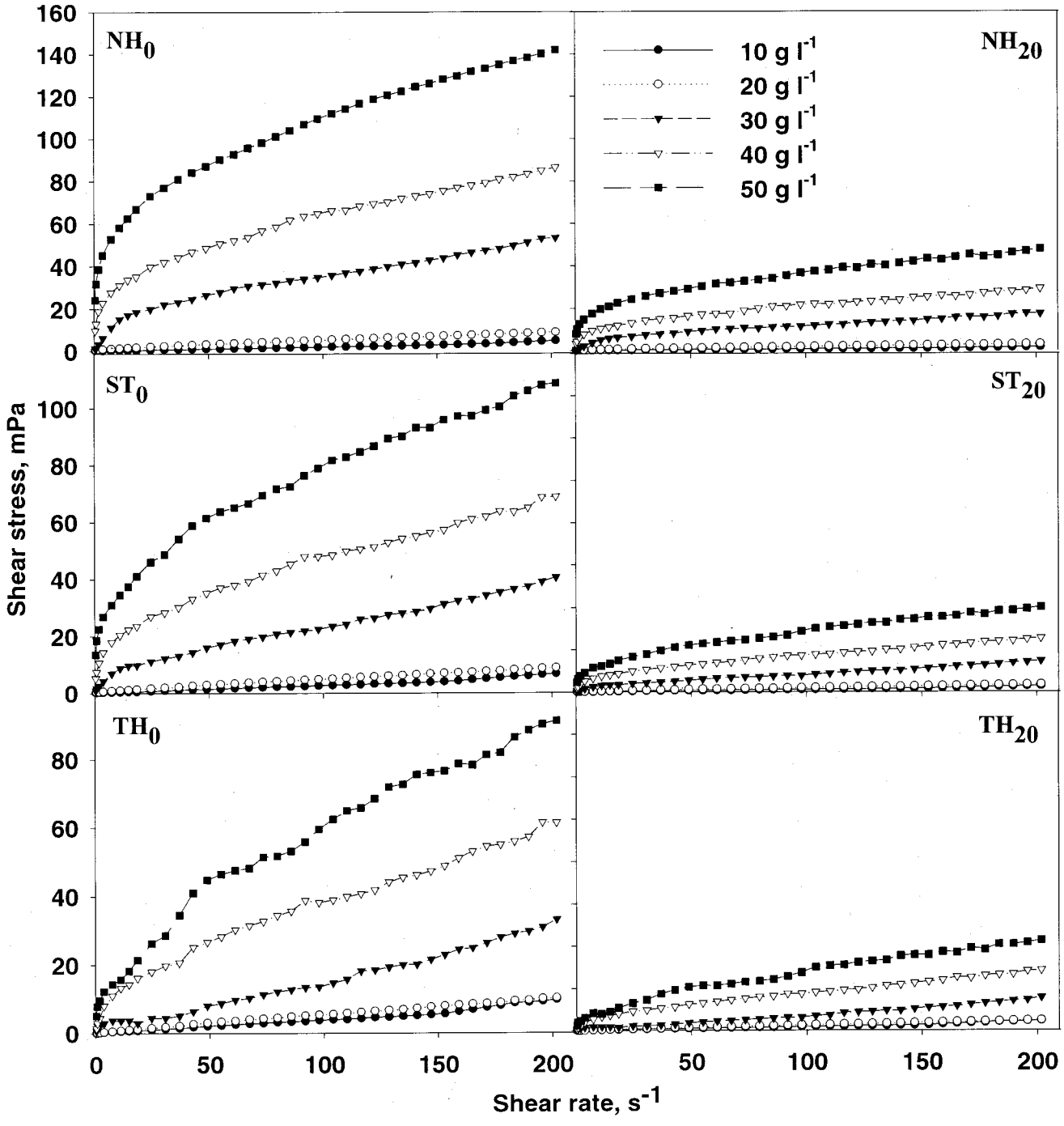
## LIST OF FIGURES

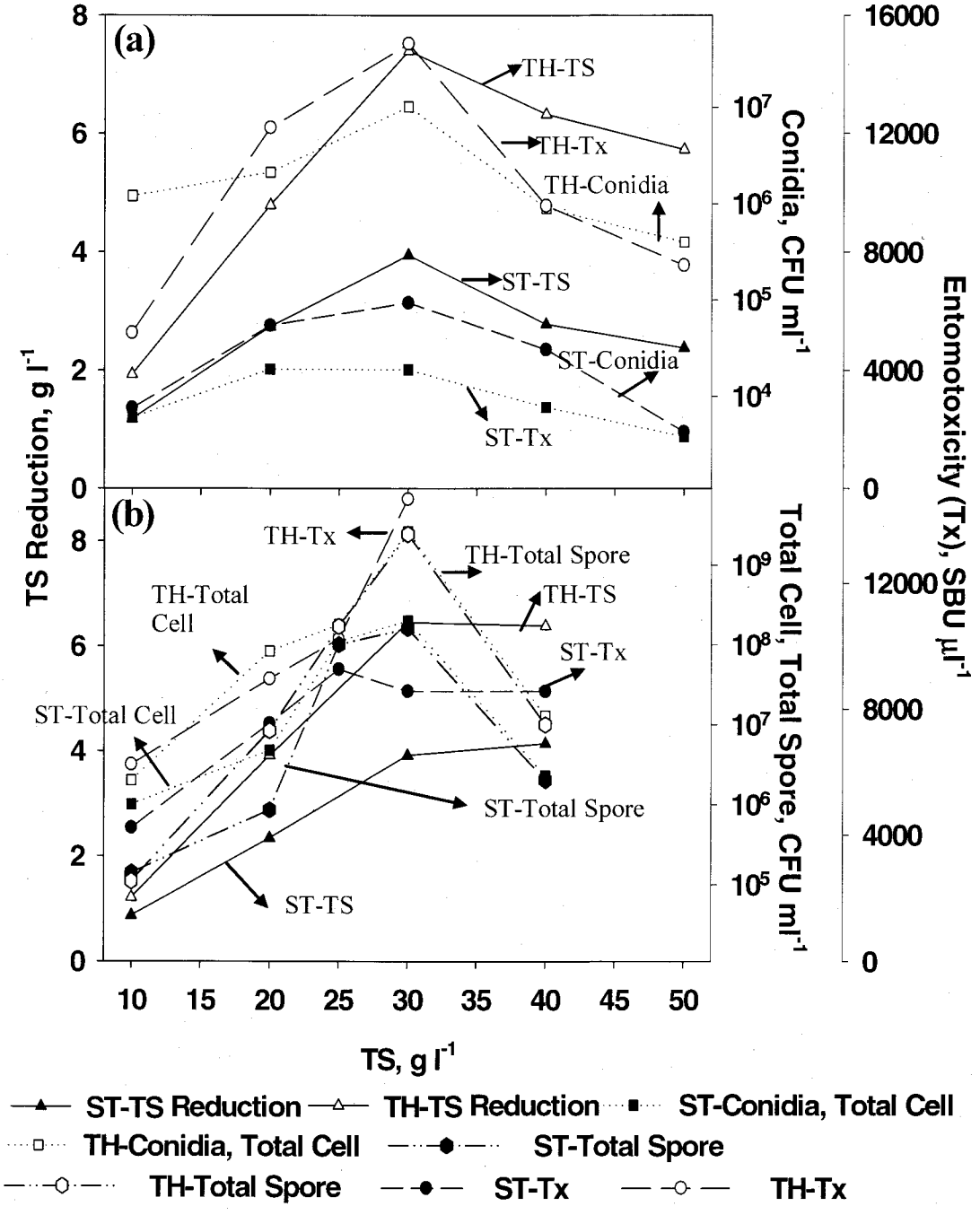

(a)
$y_{NH_0}$ = 0.1457x - 0.86; $R^2$ = 0.997
$y_{ST_0}$ = 0.3663x - 0.4384; $R^2$ = 0.9891
$y_{TH_0}$ = 0.4096x + 0.2895; $R^2$ = 0.9911
NH0
ST0
TH0
DS, g l-1
(b)
NH20
ST20
TH20
%TCOD —— %DS —— %TS
(c)
NH0
ST0
TH0
NH20
ST20
TH20
K, mPa.sn
n
TS, g l-1


Total Concentration >1µm (number ml-1) = 150,113
Sample Dilution Factor = 0.4% (1:250ml)
(a)
Number ml -1
Particle Size (µm)
(b)
300µm
(c)
CFU ml -1
10 g/l
20 g/l
30 g/l
40 g/l
50 g/l
NH20
ST20
TH20




| | |
|---|---|
| $\mu_{NH_0} = 1.2042\ e^{0.1112(TS)}$; $R^2 = 0.9528$ | $\mu_{NH_{20}} = 0.9131\ e^{0.1091(TS)}$; $R^2 = 0.9241$ |
| $\mu_{ST_0} = 1.1269\ e^{0.1059(TS)}$; $R^2 = 0.9672$ | $\mu_{ST_{20}} = 0.8714\ e^{0.0887(TS)}$; $R^2 = 0.9721$ |
| $\mu_{TH_0} = 1.1813\ e^{0.0989(TS)}$; $R^2 = 0.9560$ | $\mu_{TH_0} = 0.947\ e^{0.0757(TS)}$; $R^2 = 0.9814$ |


NH0
NH20
ST0
ST20
TH0
TH20
10 g l-1
20 g l-1
30 g l-1
40 g l-1
50 g l-1
Shear stress, mPa
Shear rate, s-1
0
20
40
60
80
100
120
140
160
0
50
100
150
200


(a)
TH-TS
TH-Tx
TH-Conidia
ST-TS
ST-Conidia
ST-Tx
(b)
TH-Tx
TH-Total Spore
TH-Total Cell
TH-TS
ST-Tx
ST-Total Cell
ST-Total Spore
ST-TS
TS Reduction, g l-1
Conidia, CFU ml-1
Total Cell, Total Spore, CFU ml-1
Entomotoxicity (Tx), SBU µl-1
TS, g l-1
ST-TS Reduction
TH-TS Reduction
ST-Conidia, Total Cell
TH-Conidia, Total Cell
ST-Total Spore
TH-Total Spore
ST-Tx
TH-Tx

## Part II

# Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration

**Mausam Verma[1], Satinder K. Brar[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]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

**(2005) Water Research, 39: 3587–3596.**

**Les boues d'épuration comme matières premières potentielles pour les champignons antagonistes (*Trichoderma* sp.): rôle du pré-traitement et de la concentration en solides**

**Résumé**

La faisabilité de produire les conidies des champignons antagonistes *Trichoderma* sp. en utilisant des boues d'épuration à différentes concentrations en solides comme matière première a été étudiée en fioles. Le compte maximal de spores conidiales obtenu dans les boues brutes était $1.98 \times 10^4$ UFC/ml, alors qu'il est plus élevé après le pré-traitement des boues (alcaline et thermo-alcaline). Des comptes de spores conidiales entre $1.3 \times 10^6$ et $2.8 \times 10^7$ UFC/ml ont été observées avec les boues ayant subies un pré-traitement alcalin et thermo-alcalin. La concentration optimale de solides en suspension était 30 g/L ($10^7$ UFC/ml) alors que des concentrations plus faibles (< 20 g/L) et plus élevées (> 30 g/L) étaient moins efficaces. Une croissance diauxique a été observée dans les boues ayant subi un pré-traitement thermo-alcalin en raison de la multiplicité de la biodégradabilité des boues. Une technique simple et modifiée de filtration des UFC, a aussi été développée pour évaluer les spores fongiques dans les boues. Des bioessais faits sur des boues fermentées contre des larves de la tordeuse des bourgeons de l'épinette ont révélé une entomotoxicité (15036 SBU/µl) comparable aux biopesticides à base de *Bacillus thuringiensis*. Cette étude a démontré avec succès le potentiel des boues d'épuration comme matière première pour l'obtention de produits à valeur ajoutée, contribuant ainsi à la gestion des boues et à l'essor d'agents de lutte biologique écologique et économique.

*Mots-clés*: Agent de lutte biologique, conidies, entomotoxicité, pré-traitement, solides, *Trichoderma viride*, boues d'épuration.

**Abstract**

Feasibility of production of antagonistic *Trichoderma* sp. conidial spores using wastewater sludge as a raw material employing different suspended solids concentration (10-50 g/L) was investigated in shake flasks. Maximum conidial spore count obtained for raw sludge was $1.98 \times 10^4$ CFU/ml, which was enhanced by sludge pre-treatments (alkaline and thermal alkaline). Conidial spore count ranging from $1.3 \times 10^6$ to $2.8 \times 10^7$ CFU/ml was observed for alkaline and thermal alkaline treated sludges. Optimal suspended solids concentration was 30 g/L ($10^7$ CFU/ml) whereas, lower (< 20 g/L) and higher (> 30 g/L) solids concentration were less efficient. Thermal alkaline pre-treated sludge showed diauxic growth due to multiplicity of sludge biodegradability. A simple, modified CFU filtration technique was also developed for fungal spore assessment in sludge. Bioassay of fermented sludge against spruce budworm larvae showed entomotoxicity (15036 SBU/µl), on par with *Bacillus thuringiensis* biopesticides. This study successfully demonstrated potential of wastewater sludge as a raw material for production of value added product, aiding in sludge management and proliferation of eco-friendly and economical biocontrol agents.

*Keywords*: Biocontrol agent, conidia, entomotoxicity, pre-treatment, solids, *Trichoderma viride*, wastewater sludge

## 1. Introduction

*Trichoderma* based biocontrol agents (BCAs) pose added advantage of plant growth promotion and soil remediation activities compared to their counterparts (virus, bacteria, nematodes, and protozoa) (Harman et al., 1993; Esposito et al., 1998). Their capability to synthesize antagonistic compounds (proteins, enzymes, and antibiotics) and micro nutrients (vitamins, hormones and minerals) enhance their biocontrol activity.

Akin to other fungal BCAs, conidial mass of *Trichoderma* is most proficient propagule, which can tolerate downstream processing (e.g. air drying) (Amsellem et al., 1999). Despite the advantages, mass production of *Trichoderma* BCAs is less prevalent owing to high cost raw materials like Mendel's medium, molasses-corn steep liquor, sodium tartrate ($10^6$–$10^7$ CFU/ml or, CFU/g inoculum medium), and/or year round availability of raw material (Lewis and Papavizas, 1983; Zheng and Shetty, 1998; Saxena et al., 2001).

At this crux, a cheaper raw material of ubiquitous nature comprising essential nutrients for growth of *Trichoderma* is necessary. Wastewater sludge is most befitting due to its successful use as a raw material for bacterial (*Bacillus thuringiensis* - Bt) biopesticide production (Tirado-Montiel et al., 2001; Brar et al., 2005). Additionally, variation of carbon to nitrogen (C:N) ratio, medium complexity (Maheshwari, 1999), triggering agents (certain metal ions; Papagianni, 2004) and complex organic compounds (Roncal et al., 2002) can enhance conidiation in several fungi. Further, the bioavailability of carbon, nitrogen (effective C:N ratio) and other nutrients present in sludge could be increased through sludge pre-treatment (Barnabé, 2004). Thus, this phenomenon could enhance or induce conidiation in *Trichoderma* spp. in sludge.

On the other hand, accurate and rapid quantification of conidia for BCA evaluation of *Trichoderma* is a difficult task. Various conventionally employed methods viz. haemocytometer for total spore count; common CFU plating (filtration through cheese cloth, requiring larger sample volume); indirect measurement of CFUs through dehydrogenase activity (Stentelaire et al., 2001) encompass certain disadvantages. Furthermore, assessing fungal spores in wastewater or wastewater sludge possesses accentuated difficulties due to complex rheology warranting a suitably modified method.

In toto, objective of the present study was to explore wastewater sludge as a potential economical raw material for *Trichoderma* spp. production incorporating sludge pre-treatment and solids concentration.

## 2. Materials and Methods

### *2.1 Sludge*

The sludge was procured as periodic backwash of BIODROF® biofilter installed at CUQ (Communauté Urbaine de Québec, Québec) wastewater treatment facility. The suspended solids (SS) in the backwash stream varies normally between ~0.5 % – ~2.5 % (w/v). The SS concentration in sampled sludge was 16 g/l (Table 1). The sludge was subjected to physico-chemical characterization (Table 1) as per Standard Methods (APHA, 1998). The sludge meets the requirements for metal concentrations according to Québec guidelines for agricultural application (MENV, 2004).

The SS concentration was increased to 5% w/v (dry basis) by gravity clarification for 30 minutes followed by centrifugation at 7650 g for 15 minutes. Sludge supernatant was stored at 4 ± 1 °C and used for dilution of the concentrated sludge when required. Total storage period of sludge was 1 week at 4 ± 1 °C to minimize microbial degradation. Raw sludge (SS concentration 10, 20, 30, 40, and 50 $gl^{-1}$) was obtained by adequately diluting 5% (w/v) sludge and homogenizing in a blender.

### *2.2 Sludge pre-treatments*

The raw sludge was subjected to two pre-treatments types, (a) alkaline hydrolysis - pH of amended (respective SS) sludge (150 ml in 500ml Erlenmeyer flask) was raised to 10.25 ± 0.01, and incubated in a rotary shaker at 30 ± 1°C, 200 ± 5 rpm for 24 h. (b) thermal alkaline hydrolysis - pH of the amended sludge was raised to 10.25 ± 0.01 followed by microwave heating in a sample preparation system (Perkin Elmer Multiwave™, Model-Paar Physica) at 140 ± 1°C and 30 bar pressure for 30 minutes. These pre-treatment conditions were found to be optimum for sludge (Barnabé, 2004). The microwave device was equipped with a set of 6 cylindrical Teflon vessels (working volume, 50 ml each). These vessels can be subjected to a maximum of 300 °C and 75 bar.

The pH of raw and pre-treated sludges was adjusted to 6 ± 0.01 and were sterilized at 121 ± 1 °C for 30 minutes. In this study, sterilized raw, sterilized alkaline hydrolysed and sterilized thermal alkaline hydrolysed sludges will be referred as NH (non-hydrolysed), AH and TAH respectively.

*2.3 Starter Culture and Inoculum*

A commercial strain of *Trichoderma viride* was used. This strain was isolated from soil and was found to be active against phytopathogenic fungi, *Fusarium* and *Rhizoctonia* and insect spruce budworm (SB) larvae as tested in our laboratory. Microscopic examinations of mycelial mass and spores suggested greater morphological similarity to existing *Trichoderma viride* strains.

The culture was inoculated on potato dextrose agar (PDA) plates and incubated for 4-7 days under dark at 28 ± 1 °C (35 ± 2% relative humidity) and subsequently maintained at 4 ± 1 °C and subcultured monthly. The starter culture consisted of ≈ ½" x ½" scraped piece of 32-36 h old mycelial mat of subculture. In order to prepare inoculum for sludge, a single piece of starter culture was aseptically homogenized with a Micro Tissue Grinder® (VWR, Canada) and inoculated into 500 ml Erlenmeyer flask containing 150 ml sterile (121 ± 1 °C for 15 minutes) tryptic soya broth (TSB, Difco) at pH 6.0 ± 0.01 (optimum pH for the *Trichoderma viride* strain; unpublished data) for fermentation. The Erlenmeyer flasks were incubated in a rotary shaker at 28 ± 1°C and 250 ± 5 rpm for 48 h.

For preparation of conidial suspension of known concentration, surface of 6–7 days old PDA culture was washed with sterile saline solution, followed by CFU plating. The conidial suspension thus obtained was stored at 4 ± 1 °C and was ready–to–use once CFU was determined.

*2.4 Fermentation*

Erlenmeyer flasks (500 ml) containing 135 ml of NH, AH and TAH sludges were inoculated with 15 ml TSB inoculum and incubated in a rotary shaker at 28 ± 1°C and 250 ± 5 rpm for 96 h. The samples were drawn aseptically at regular intervals and stored at 4 ± 1°C for subsequent analyses.

*2.5 Modified CFU filtration and plating method*

A simple modification was done in conventional CFU plating method making it more practical for fungal spore count. It involved physical separation of conidia from mycelia and sludge agglomerates in absence of any external agent (chemical or biological), shown schematically in Figure 1a. To do so, an autoclavable small filtration tip was made from cut-pieces of two pipette tips (40-200 µl and 100-1000 µl) and filter cloth (Kimberly Clark Profession – Teri® reinforced wipers; average pore size ≈ 10 µm) shown in Figure 1. Meanwhile, the filter components could be equally substituted with similar materials as per availability.

For CFU plating, fermented sample was diluted 10× in saline solution (0.85% NaCl) and eventually filtered with aforementioned filtration tip. The filtration tip was detachable to the pipette tip, which was removed for obtaining filtered broth from the pipette tip. A new filtration tip was used for each sample. Afterwards, appropriately diluted 100 µl samples were plated on tryptic soya agar (TSA) plates and incubated at 28 ± 1 °C and 35 ± 2% relative humidity (RH) for 30-36 h in dark. Triplicates for three different dilutions were used for enumeration. Standard deviation for CFU count was 8-10 %.

*2.6 Microscopy*

Fresh 100 µl sample was taken for smear preparation. Morphology of mycelia and conidia, and contamination, if any was examined using a computer coupled optical microscope (Zeiss Axiolab) equipped with a digital camera (Axiocam HRC Zeiss).

*2.7 Protease activity (PA)*

The samples were centrifuged at 7650 g for 20 min at 4 ± 1°C. The supernatant was appropriately diluted in borate buffer at pH 8.2 ± 0.01 and used as enzyme aliquot. Modified Kunitz (1947) method was employed for PA determination. The standard deviation was 7-9% , based on triplicate samples of two fermentation runs.

*2.8 Soluble Chemical Oxygen Demand (SCOD) and Dissolved Solids (DS)*

The SCOD and DS were measured by closed reflux method as per Standard Methods (APHA, 1998). The standard deviation was 6-8%, based on triplicate samples of two fermentation runs.

*2.9 Insect Bioassay*

The entomotoxicity (Tx) of samples was determined by using eastern spruce budworm (SB) larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae), provided by Natural Resources Canada (Sault Ste-Marie, Ontario). Bioassays were conducted using diet incorporation method (Beegle, 1990). Sample preparation and diet protocol was carried out as per method described by Brar et al. (2005). Industry standard contained spores and crystals of Bt at a potency of 20.1 x $10^9$ IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni*). On comparison of Tx of *Trichoderma* fermented sludge, SBU reported in this study was 20-25 % higher than IU. Tx of preparations was expressed in SB units/µl (SBU/µl) with 8-10 % of standard deviation.

## 3. Results and Discussion

*3.1 Consistency of CFU filtration technique*

Validation of CFU plating technique was carried out by examining fermentation samples of different SS (10, 20, 30, 40, and 50 $gl^{-1}$) for NH, AH, and TAH sludges. Accordingly, number of filtering layers were varied in filter tip. The variation of CFU between single and double filtering layers was nominal ($p > 0.05 - 0.20$, using paired *t*-test for 0–96 h samples). Additionally, filtered samples when examined microscopically, showed no mycelial mass. Besides, consistency tests performed for starch industry wastewater, pulp and paper industry wastewater and dewatered sludge yielded similar results (data unreported).

For verification of CFU recovery through the filter tip, autoclaved sludge samples of different SS concentration (10, 20, 30, 40 and 50 g/l) were used to prepare a conidia suspension of $10^6$ CFU/ml by appropriately diluting 1 ml of $10^8$ CFU/ml. Immediate CFU

plating was carried out for all sludges using samples filtered through the filtration tip. The recovery efficiency varied within ± 6% of the actual value ($10^6$ CFU/ml).

Alternatively, 96 h samples were also enumerated by conventional filtration (cheese cloth filtration) followed by CFU plating. The conidial counts of samples filtered through conventional and modified filtration methods showed nominal difference ($p > 0.10$, using paired *t*-test). Hence, consistency of this technique was validated and single filtering layer was adopted subsequently throughout the study.

### *3.2 Suitability of sludge for growth*

From the sludge composition presented in Table 1, C:N ratio was found to be ≈ 8. This is in concordance with reported ratio suitable for normal growth of *Trichoderma* sp. (Xia and Shen, 2004). Effective or, bioavailable C:N could be different from its actual value, and currently, no method exists for this measurement. Nevertheless, pre-treatments can enhance the biodegradability through change in medium conformation and enhancement of dissolved solids (DS). Changes in DS during incubation of NH, AH and TAH sludges with and without *Trichoderma* inoculation are presented in Table 2 (discussed later). Additionally, presence of other components like, $P\text{-}PO_4^{3-}$, Ca, Fe, $N\text{-}NH_3$, $N\text{-}NO_2$, $N\text{-}NO_3^-$, and Zn rendered sludge as a suitable growth medium for *Trichoderma.* Papagianni (2004) has extensively reviewed role of these nutrients for fungal growth, therefore, sludge could be considered as an adequate raw material for *Trichoderma* growth.

### *3.3 Fermentation*

#### *3.3.1 Vegetative Growth and Conidiation*

*Trichoderma* showed sustained vegetative growth at all SS as observed microscopically. This was possibly due to enzymatic hydrolysis of sludge particulates by extracellular enzymes of *Trichoderma*. Whilst, a quantitative estimation of vegetative growth was intricate due to superfluous sludge flocs. *Trichoderma* spp. are known to produce several lytic enzymes on diverse substrates (Donzelli et al., 2003).

Maximum CFU concentration of NH, AH and TAH sludges (Table 3) increased until 30 g/l and decreased thereafter (>30 g/l). The maximum CFU attained for various sludges are presented in Table 3. Accordingly, optimum solids concentration to achieve maximum CFU was 20-30 for NH and 30 g/L for AH and TAH. Increase in SS (10-30 g/L) resulted in enhanced DS (Table 2), thereby, supported CFU production. In spite of higher DS at SS concentration >30 g/L, higher osmotic pressure and mass transfer limitations (Papagianni, 2004) possibly resulted in decreased overall growth (degeneration of mycelial mass, observed microscopically) and hence decreased CFU concentration.

The sludge pre-treatments enhanced the maximum CFU by 1 to 3 logarithmic cycles (Table 3). Thus, AH and TAH processes improved sludge biodegradability by improving medium conformation (smaller sludge flocs, observed microscopically) and hence boosted conidiation. The organic matter in activated sludge could be defined as easily biodegradable, difficult to degrade and non-biodegradable as it principally contains cell debris and cellular materials, which were possibly hydrolyzed during alkaline or, thermal alkaline treatments and hence increased growth and conidiation. Particularly, hydrolysis of proteins, carbohydrates and lipids into simpler polymeric units are expected. Moreover, alkaline and thermal pre-treatments of wastewater sludge were reported to significantly enhance biodegradability of sludge organic matter and improve rheology (sludge complexity) of the medium, enhancing mass transfer (Vlyssides and Karlis, 2004).

In TAH sludge, a stepwise increase in conidiation (12–22 h and 46–96 h) indicated diauxic growth pattern and could be attributed to the multiplicity of raw material.

Maximum CFU achieved in NH and AH sludges were significantly lower ($1.98 \times 10^4 - 4.9 \times 10^5$ CFU/ml at 30 g/L SS, Table 3) in comparison to conventional media ($10^6$ CFU/ml; Lewis and Papavizas, 1983). TAH sludge resulted in higher CFU production ($1.2 \times 10^7$ CFU/ml) owing to better combination of thermal and alkaline treatment leading to more efficient solubilization of organic matter.

### *3.3.2 SCOD and DS*

An initial increase in SCOD (0-12 h) of NH, AH and TAH sludges at all SS was observed, followed by a decrease until 96 h owing to growth requirements of *Trichoderma*

(Figure 3). The exact reason for initial increase in SCOD was not clear, however, it is possible that initially DS consumption by *Trichoderma* was very low and some dissolved matter could be released into the liquid phase from the sludge particulate due to diffusion resulting from agitation of shake flask. The change in DS with respect to initial SS concentration in sludges incubated without *Trichoderma* supports DS diffusion (Table 2).

Further, the DS change (without *Trichoderma* inoculation), increased in the order, raw<NH<AH<TAH, justifying AH and TAH treatments (Table 2). Moreover, the DS change increased with SS concentration 10–20 g/l due to generation of more solubilized matter aided by the increase in solids concentration. However, the DS decreased with SS concentration 20–50 g/l probably due to restrained inter and intra-particle interactions caused by poor rheology of the medium. On the other hand, DS increase with pre-treatments (NH, AH and TAH) could be simply due to increase in degree (elevated pH and/or, temperature) of different treatment processes.

### *3.3.3 Protease Activity*

Protease activity (PA) increased from 12–28 h of fermentation followed by stabilization (28–48 h) and a constant decrease (48–84 h) irrespective of solids concentration and type of sludge pre-treatment (Figure 4). The maximum PA value followed the order, TAH>AH>NH, showing dependence on nutrient availability (Table 3). In case of NH, the maximum PA increased with SS; probably more protease activity was required to hydrolyse increased complex matter (Harman et al., 1993). Meanwhile, in case of AH and TAH sludges, maximum PA increased until 40 g/l and then decreased at 50 g/l SS. This decrease in PA could be attributed to relatively more adverse growth conditions (inhibition due to high solids, mass transfer and osmotic pressure, mentioned earlier) at 50 g/l in comparison to 40 g/l SS. The inconsistent behaviour of PA with respect to SS in NH and pre-treated sludges (AH and TAH) could not be explained. However, it is possible that pre-treatments were less effective at 50 g/l with respect to 40 g/l, thereby, causing this non-proportionate change of PA.

It would be noteworthy to mention that protease production in the medium could be either due to constitutive or, inducible mechanism. In case of constitutive nature, PA will be proportional to vegetative/mycelial growth. Unfortunately, measurement of mycelium

in the sludge is difficult, therefore, mechanism of protease production associated with vegetative growth cannot be authentically assessed. A second mechanism could be release of enzyme in the medium during conidiation (Ueno et al., 1987). As mentioned earlier, CFU concentration kept increasing (after 28 h) irrespective of decrease in PA at all SS (Figure 2). Thus, enzyme production was not proportional to conidia concentration in this case.

Another fact worth mentioning was that between 28–48 h of fermentation, rate of enzyme production could be equal to the rate of enzyme inactivation which stabilized the PA during this period. Moreover, the decrease in PA could also be due to degradation of protease by lytic enzymes produced by *Trichoderma*. It is known that the lytic enzymes secreted by bacteria degrade proteases produced by the same bacteria (Chu et al., 1992).

*3.4 BCA Activity*

Entomotoxicity (Tx) against SB larvae for *Trichoderma* fermented sludge; NH, AH, and TAH at 30 $gl^{-1}$ SS was 6278, 10112, and 15036 SBU/µl respectively. The Tx value for control sludge (i.e., sterilized and not fermented by *Trichoderma*) was zero. The Tx for TAH fermented sludge was comparable to earlier studies reported on *Bacillus thuringiensis* (Bt) fermented TAH sludge (Barnabé, 2004). It is important to mention that no research study till date has been reported using *Trichoderma* spp. Expression of higher CFU and protease activity in sludge (Fig. 4) could have contributed to Tx of *Trichoderma* against SB larvae. Further, biological control of certain insects by *Trichoderma* spp. has been reported due to production of extracellular degradative proteolytic enzymes (Lopez and Orduz, 2003). Moreover, several other lytic enzymes (e.g. chitinase) and antibiotics (Zheng and Shetty, 1998; Yedidia et al., 1999; Papagianni, 2004), might have accumulated in fermented sludge (not analyzed in this study) and possibly contributed towards higher Tx.

Thus, higher Tx of *Trichoderma* in fermented sludge established *Trichoderma* to be a potent BCA. This will aid in entry of a broad spectrum BCAs in market with an additional ability of plant growth promotion. However, detailed mode of action of *Trichoderma* on SB larvae is completely unknown and warrants in depth research.

Furthermore, many *Trichoderma* preparations are used in granular and powder form (Batta, 2004). Being saprophytic fungi, sludge could also serve as potential natural habitat for *Trichoderma*. In this respect, fermented sludge could be mixed with dewatered sludge to prepare powder formulations. Further, a sludge based powder formulation of *Trichoderma* at $10^{13}$ CFU/hectare application rate (Vega et al., 2003), would require approximately 5-6 tons/hectare of dewatered sludge (based on dewatered sludge formulation, unpublished data), which is within the prescribed limits for agricultural application guidelines of municipal sludge (MENV, 2004). In this way, substantial amount of sludge could be utilized to prepare powder formulation of *Trichoderma*, besides production of many other products using wastewater sludge as a raw material, namely, Bt based biopesticides (Brar et al., 2005), biofertilizers (Ben Rebah et al. 2001) and industrial enzymes (Tyagi et al., 2001).

## 4. Conclusions

To summarize, findings of the present study suggest the following:

1. A novel process for economical production of *Trichoderma* sp. based BCA is feasible.
2. An ameliorated CFU plating method was developed which would aid in enumeration of conidial mass of fungi (e.g.*Trichoderma* sp.) in complex medium like sludge.
3. Pre-treatments of sludge increased DS through solubilization of organic matter, enhancing nutrient availability for *Trichoderma viride*.
4. Thermal alkaline hydrolyzed sludge (spore-yield, $1.19 \times 10^7$ CFU/ml at 30 g/L SS; about 604 times increase with respect to NH sludge at 30 g/L SS) showed immense potential as a pre-treatment of choice for *Trichoderma* sp. growth.
5. Protease activity was in congruence with suspended solids and pre-treatments of sludge.
6. Bioassay of *T. viride* fermented sludge showed significant Tx (≈15000 SBU/µl) vis-à-vis Bt, thereby, rendering them as potential BCAs.

## Acknowledgements

The authors are sincerely thankful to 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.

## References

Amsellem, Z., Zidack, N.K., Quimby Jr., P.C. and Gressel, J. (1999) Long-term dry preservation of viable mycelia of two mycoherbicidal organisms. Crop Protection **18**, 643–649.

APHA-AWWA-WPCF (1998) Standard Methods for Examination of Water and Wastewaters. 20th edn. (Eds. Clesceri, L.S., Greenberg, A.E. and Eaton, A.D.), 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 335.

Batta, Y.A. (2004) Effect of treatment with *Trichoderma harzianum* Rifai formulated in invert emulsion on postharvest decay of apple blue mold. International Journal of Food Microbiology **96**, 281–288.

Beegle, C.C. (1990) Bioassay methods for quantification of *Bacillus thuringiensis* δ-endotoxin, Analytical Chemistry of *Bacillus thuringiensis*, In: Analytical Chemistry of *Bacillus thuringiensis* (Eds. Hickle L.A. and Fitch W.L.), American Chemical Society, pp. 14–21.

Ben, R.F., Tyagi, R.D. and Prévost, D. (2001) Acid and alkaline treatments for enhancing the growth of rhizobia in sludge. Canadian Journal of Microbiology **47**, 467–474.

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 (in Press).

Chu, I.M., Lee, C. and Li, T.S. (1992) Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. Enzyme and Microbial Technology **14**, 755–761.

Donzelli, B.G.G., Ostroff, G. and Harman, G.E. (2003) Enhanced enzymatic hydrolysis of langostino shell chitin with mixtures of enzymes from bacterial and fungal sources. Carbohydrate Research **338**, 1823–1833.

Esposito, E. and da Silva, M. (1998) Systematics and Environmental Application of the Genus *Trichoderma.* Critical Reviews in Microbiology **24**, 89–98.

Harman, G.E., Haynes, C.K., Lorito, M., Broadway, R.M., di Pietro, A., Peterbauer, C. and Tronsmo, A. (1993) Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. Phytopathology **83**, 313–318.

Kunitz, M. (1947) Crystalline soybean trypsin inhibitor. Journal of General Physiology **30**, 291–310.

Lewis, J.A. and Papavizas, G.C. (1983) Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. Soil Biology and Biochemistry **15**, 351–357.

Lopez, E. and Orduz, S. (2003) *Metarhizium anisopliae* and *Trichoderma viride* for control of nests of the fungus-growing ant, *Atta cephalotes*. Biological Control **27,** 194–200.

Maheshwari, R. (1999) Microconidia of *Neurospora crassa*. Fungal Genetics and Biology **26**, 1–18.

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.

Papagianni, M. (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances **22**, 189–259.

Roncal, T., Cordobés, S., Ugalde, U., He, Y. and Sterner, O. (2002) Novel diterpenes with potent conidiation inducing activity. Tetrahedron Letters **43**, 6799–6802.

Saxena, R.K., Sangeetha, L., Vohra, A., Gupta, R. and Gulati, R. (2001) Induction and mass sporulation in lignin degrading fungus *Ceriporiopsis subvermispora* for its potential usage in pulp and paper industry. Current Science **81**, 591–594.

Stentelaire, C., Antoine, N., Cabrol, C., Feron, G. and Durand, A. (2001) Development of rapid and highly sensitive biochemical method for the measurement of fungal spore viability. An alternative to the CFU method. Enzyme and Microbial Technology **29**, 560–566.

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**, 3807–3816.

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 Science and Technology **46**, 247–254.

Ueno, S., Miyama, M., Ohashi, Y., Izumiya, M., Kusaka, I. (1987) Secretory enzyme production and conidiation of *Aspergillus oryzae* in submerged liquid culture. Appl. Microbiol. Biotechnol. **26**, 273–276.

Vega, F.E., Jackson, M.A., Mercadier, G. and Poprawski, T.J. (2003) The impact of nutrition on spore yields for various fungal entomopathogens in liquid culture. World Journal of Microbiology and Biotechnology **19**, 363–368.

Vlyssides, A.G. and Karlis, P.K. (2004) Thermal-alkaline solubilization of waste activated sludge as a pre-treatment stage for anaerobic digestion. Bioresearch Technology **91**, 201–206.

Xia L. and Shen X. (2004) High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. Bioresource Technology **91**, 259–262.

Yedidia, I., Benhamou, N. and Chet, I. (1999) Induction of defense responses in cucumber by the biocontrol agent *Trichoderma harzianum*. Applied Environmental Microbiology **65**, 1061–1070.

Zheng, Z. and Shetty, K. (1998) Cranberry processing waste for solid state fungal inoculant production. Process Biochemistry **3**, 323–329.

Table 1
Characteristics of raw wastewater sludge

| Parameter | Concentration (mg/kg; unless stated) |
|---|---|
| Total solids (g/l) | 23.0 |
| Total volatile solids (g/l) | 18.0 |
| Suspended solids (g/l) | 16.0 |
| Suspended volatile solids (g/l) | 14.0 |
| pH | 5.22 |
| Total carbon | 404000 |
| Total nitrogen | 53000 |
| Total phosphorus | 1219 |
| $N\text{-}NH_3$ | 9913 |
| $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ | 74.8 |
| $P\text{-}PO_4^{3-}$ | 661 |
| Al | 1738 |
| Ca | 15517 |
| Cd | 4.0 |
| Cr | 28.4 |
| Cu | 359.3 |
| Ni | 56.3 |
| Fe | 9239 |
| K | 2416 |
| Pb | 58.7 |
| Mn | 72.8 |
| S | 700 |
| Zn | 585 |
| Na | 3233 |

Table 2

DS of various sludges before and after 4 days incubation (with and without *Trichoderma*)

| SS, g/l | NH | | | AH | | | TAH | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 96 h | Change | 0 h | 96 h | Change | 0 h | 96 h | Change |
| With | | | | | | | | | |
| 10 (0.30) | 0.46 | 0.40 | −0.06 | 2.18 | 1.53 | −0.65 | 5.27 | 3.15 | −2.12 |
| 20 (0.74) | 1.20 | 0.87 | −0.33 | 3.96 | 2.50 | −1.46 | 9.60 | 5.29 | −4.31 |
| 30 (1.16) | 2.11 | 1.48 | −0.63 | 4.08 | 1.91 | −2.17 | 12.20 | 5.55 | −6.65 |
| 40 (1.52) | 2.86 | 2.13 | −0.73 | 6.68 | 4.39 | −2.29 | 17.27 | 10.51 | −6.76 |
| 50 (2.04) | 3.67 | 3.55 | −0.12 | 7.42 | 6.91 | −0.51 | 20.73 | 18.83 | −1.90 |
| Without | | | | | | | | | |
| 10 (0.33) | 0.54 | 0.77 | 0.23 | 2.38 | 3.28 | 0.90 | 5.84 | 7.86 | 2.02 |
| 20 (0.84) | 1.40 | 1.84 | 0.44 | 4.42 | 5.76 | 1.34 | 9.48 | 11.62 | 2.14 |
| 30 (1.10) | 2.08 | 2.38 | 0.30 | 5.04 | 6.15 | 1.11 | 12.99 | 14.43 | 1.44 |
| 40 (1.40) | 2.60 | 2.87 | 0.27 | 6.46 | 6.95 | 0.49 | 17.56 | 18.72 | 1.16 |
| 50 (1.92) | 3.38 | 3.64 | 0.26 | 7.33 | 7.59 | 0.26 | 21.23 | 21.77 | 0.54 |

( ) DS in raw (non-sterilized) sludge

Change corresponds to increase or, decrease in DS at 96 h with respect to 0 h.

With − sludges inoculated with *Trichoderma* and incubated for 96 h

Without − sludges without *Trichoderma* inoculation and incubated for 96 h (control)

Table 3

Summary of fermentation parameters of sludges

| NH | | | | | AH | | | | TAH | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SS (g/L) | Final pH | Total DS consumed (g/L) | Maximum CFU/ ml (X $10^4$) | Maximum Protease activity (U/ml) | Final pH | Total DS consumed (g/L) | Maximum CFU/ ml (X $10^5$) | Maximum Protease activity (U/ml) | Final pH | Total DS consumed (g/L) | Maximum CFU/ ml (X $10^6$) | Maximum Protease activity (U/ml) |
| 10 | 8.44 | 0.29 (37.6) | 0.75 [71] | 0.73 [28] | 8.42 | 1.54 (47.0) | 0.67 [71] | 1.38 [46] | 8.35 | 4.14 (52.7) | 1.2 [94] | 2.73 [54] |
| 20 | 8.17 | 0.77 (42.1) | 1.91 [46] | 0.80 [28] | 8.37 | 2.81 (48.7) | 2.91 [94] | 1.55 [28] | 8.08 | 6.45 (55.5) | 2.1 [94] | 3.57 [54] |
| 30 | 8.00 | 0.93 (38.9) | 1.98 [82] | 2.41 [28] | 8.29 | 3.28 (53.3) | 4.9 [94] | 3.29 [28] | 7.91 | 8.09 (56.1) | 12.2 [82] | 6.11 [46] |
| 40 | 8.07 | 1.0 (34.9) | 1.03 [82] | 3.12 [28] | 8.11 | 2.78 (40.1) | 1.7 [94] | 5.59 [28] | 7.98 | 7.92 (42.3) | 12.0 [82] | 7.97 [46] |
| 50 | 8.11 | 0.38 (10.3) | 0.41 [82] | 4.02 [28] | 7.89 | 0.77 (10.1) | 0.41 [82] | 4.44 [46] | 8.02 | 2.44 (11.2) | 0.43 [82] | 7.02 [46] |

[ ] Occurrence time ≡ h

( ) % DS consumed – based on DS at 96 h in control study (without *Trichoderma* inoculation, Table 2)

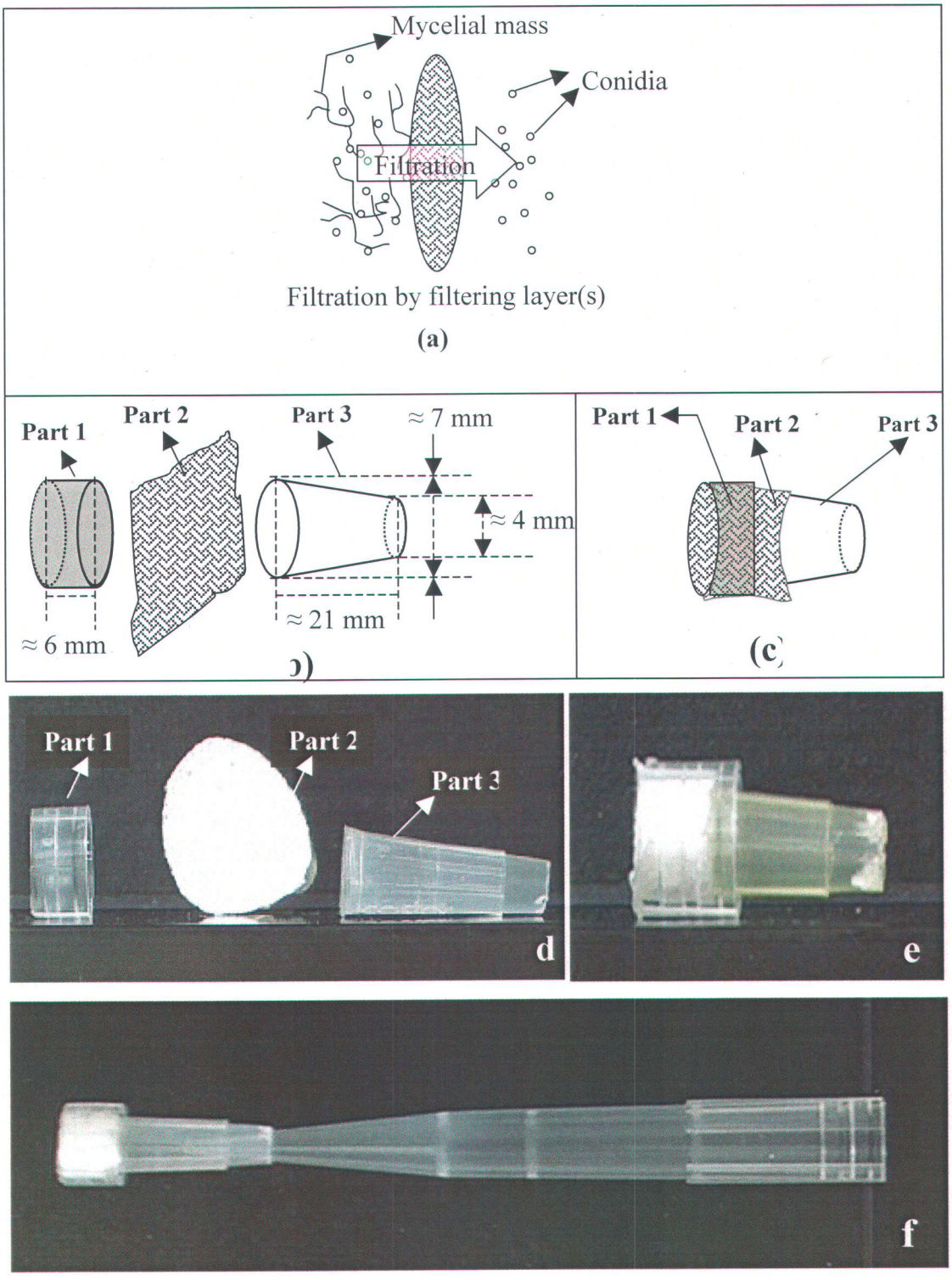



Fig. 1. a) Schematic of conidia separation from mycelia and sludge agglomerates; b) Schematic of conidia separation filtration tip components; c) assembled arrangement of the filtration tip; d) actual components of the filtration tip; e) assembled filtration tip; f) 1 ml pipette tip, equipped with conidia filtration tip.

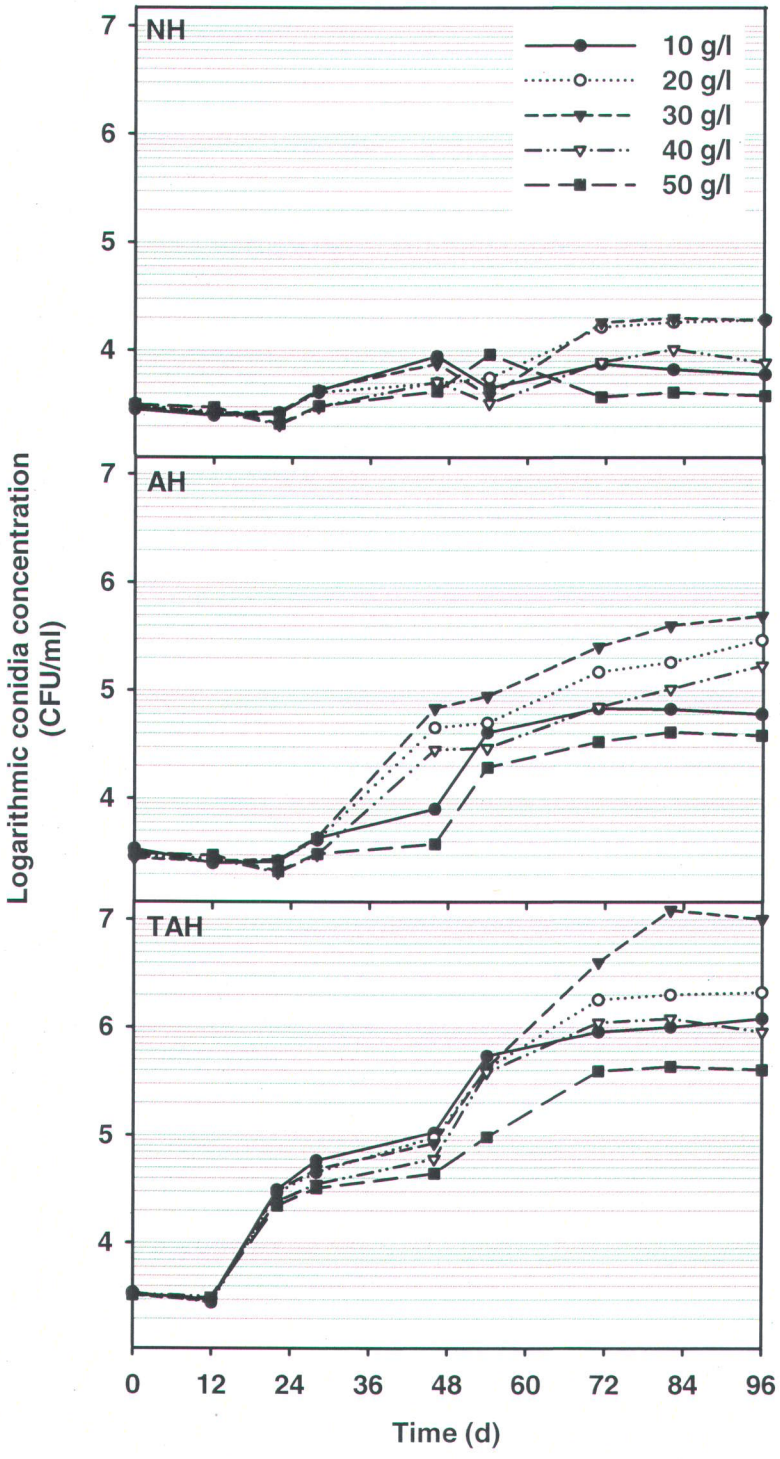



Fig. 2. CFU profile of *T. viride* in NH, AH and TAH sludges.

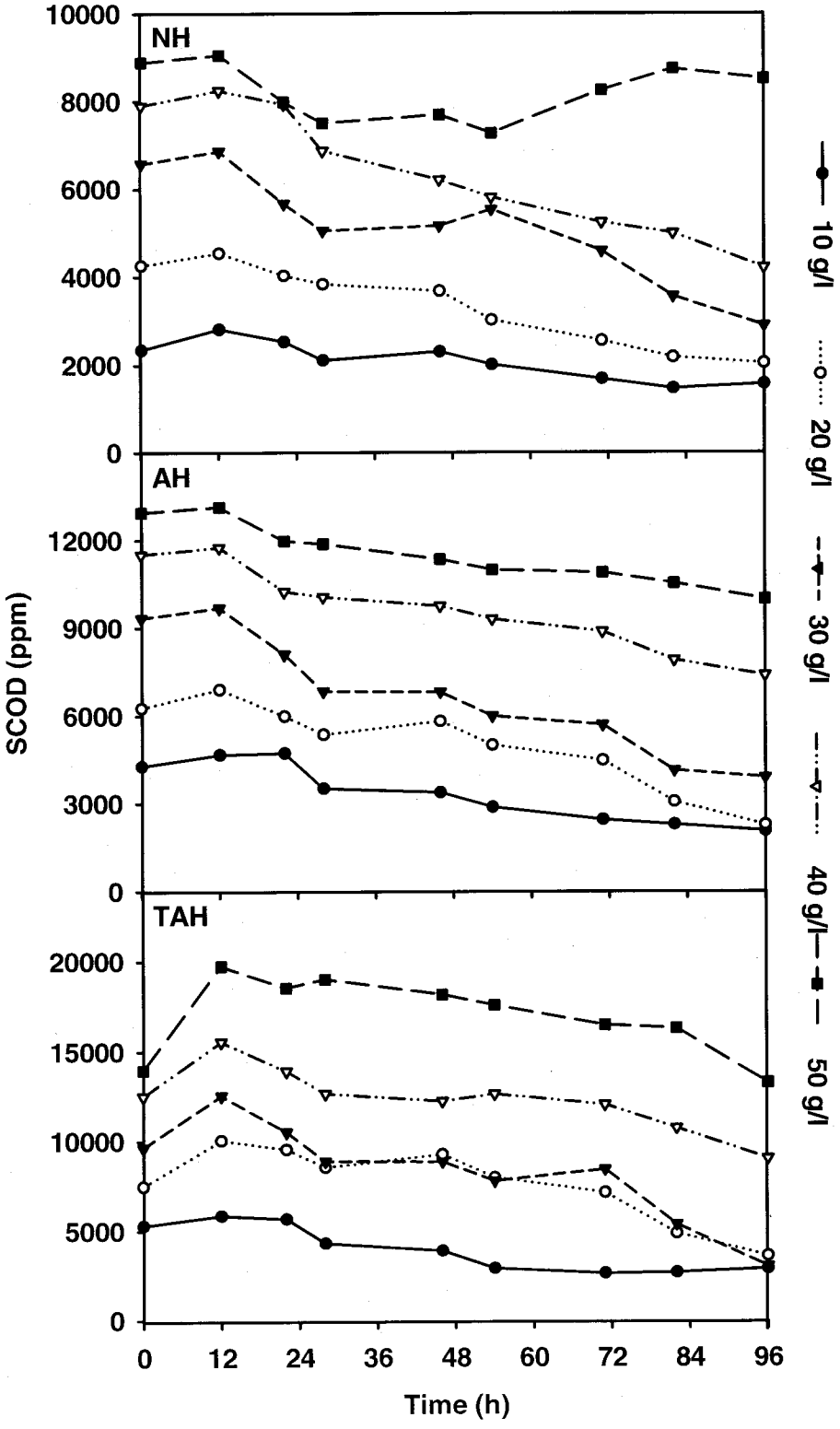



Fig. 3. SCOD profile of *Trichoderma viride* in NH, AH and TAH sludges.

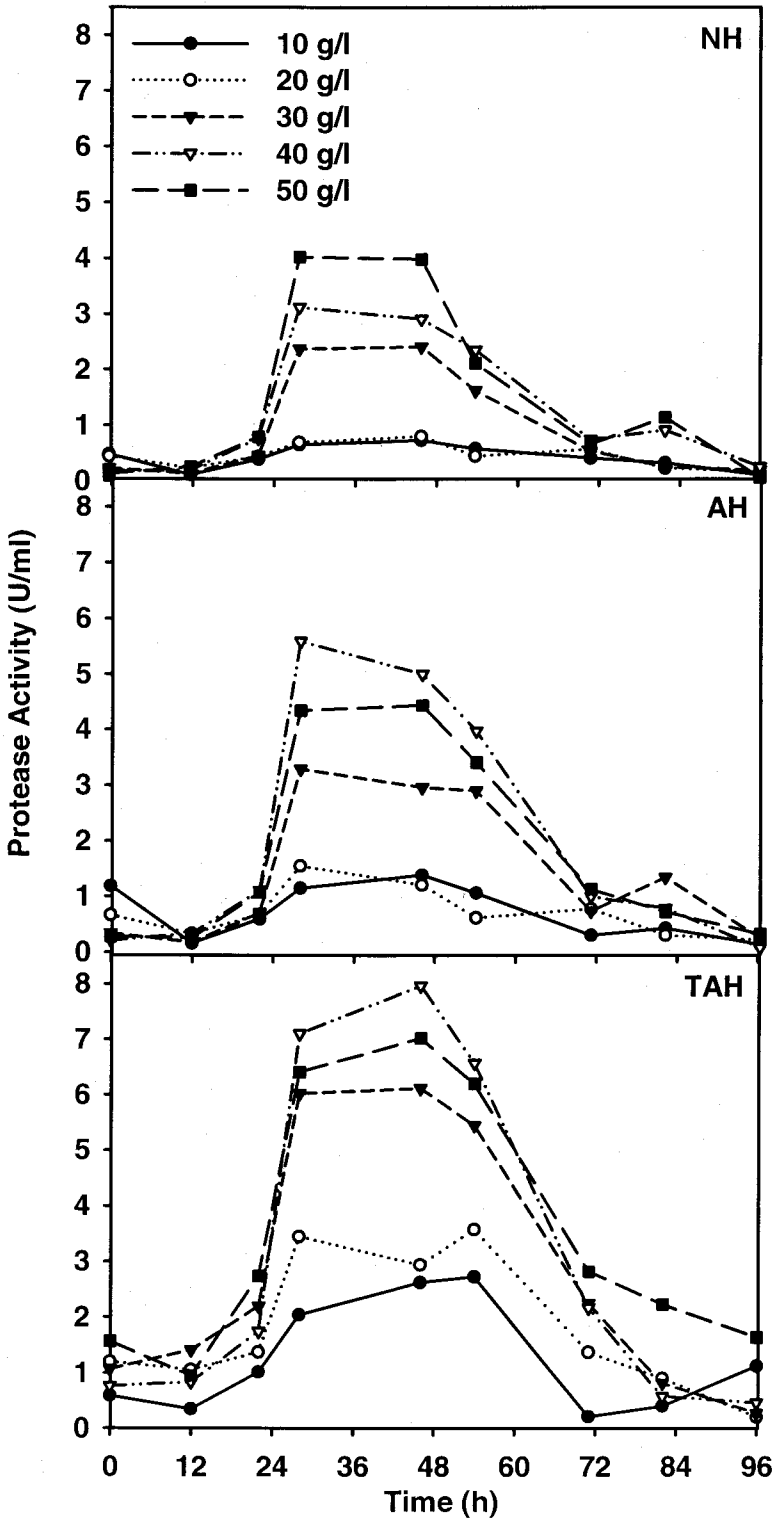



Fig. 4. Proteolytic activity of *T. viride* for NH, AH, and TAH sludges.

## Part III

# Bench-scale fermentation of *Trichoderma viride* on wastewater sludge: rheology, lytic enzymes and biocontrol activity

**Mausam Verma[1], Satinder K. Brar[1], R. D. Tyagi[1,*], D. Prévost[2], J. R. Valéro[1] and R. Y. Surampalli[3]**

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

[2]CRDSGC, Agriculture et Agro-alimentaire Canada, 2560 boul. Hochelaga, Québec, Canada G1V 2J3

[3]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

**Enzyme and Microbial Technology (Submitted)**

**Fermentation de *Trichoderma viride* dans des boues d'épuration à échelle de laboratoire: rhéologie, enzymes lytiques et activités de lutte biologique**

**Résumé**

La conidiation et la production d'enzymes lytiques par *Trichoderma viride* en utilisant des boues d'épuration comme une matière première ont été étudiées sous des conditions contrôlées (pH, température et oxygène dissout) pour évaluer le potentiel de lutte biologique du champignon. Différentes concentrations en solides de boues d'épuration municipales pré-traitées (ayant subies une hydrolyse thermo-alcaline) ont été étudiées pour accroître l'activité de lutte biologique dans un fermenteur de 15 litres à une concentration en oxygène dissout de ≥ 30%. La concentration maximale de conidies (5.94 × UFC $ml^{-1}$ à 96 h) a été obtenue à 30 g $L^{-1}$ de matières en suspension dans les boues d'épuration. Les enzymes lytiques analysées quantitativement étaient les protéases, les amylases, les cellulases, les laccases et les chitinases. Les activités enzymatiques maximales se situaient aux environs de 12–30 h, soit plus tôt par rapport aux cultures en fioles. Le potentiel de lutte biologique des boues fermentées ainsi obtenues a été évalué par des bioessais sur des champignons, des insectes et des plantes. Les bioessais contre le phytopathogène fongique *Fusarium* sp. ont montré une activité maximale à environ 96 h et 30 g $L^{-1}$ de solides en suspension. L'entomotoxicité contre des larves de la tordeuse des bourgeons de l'épinette ont montré une activité maximale de ≈ 17290 SBU $\mu l^{-1}$ à 30 g $L^{-1}$ de solides en suspension à la fin de la fermentation (96 h). Les bioessais sur des plantes ont montré une action double de *T. viride*, soit la prévention des maladies et la stimulation de la croissance. Les analyses rhéologiques des boues fermentées ont montré un comportement pseudoplastique. En comparaison avec une concentration de solides en suspension de 30 g $L^{-1}$, les besoins en agitation et en aération ont augmenté significativement à 35 g $L^{-1}$. Le taux d'assimilation de l'oxygène et le coefficient volumétrique de transfert de l'oxygène ($k_La$ à 35 g $L^{-1}$) ne pouvaient pas être augmentés en comparaison avec une concentration de 30 g $L^{-1}$ en raison de la complexité rhéologique du bouillon durant la fermentation. Ainsi, le succès d'une opération de fermentation du champignon de lutte biologique *T. viride* est une indication rationnelle de son potentiel

pour la production de masse à grande échelle dans les secteurs agricoles et forestiers comme agent de lutte biologique à large spectre.

**Mots-clés:** Bioessai, agent de lutte biologique, entomotoxicité, fermentation, enzyme lytique, rhéologie, *Trichoderma viride*.

**Abstract**

Conidiation and lytic enzyme production by *Trichoderma viride* with wastewater sludge as a raw material were investigated under controlled conditions (pH, temperature and dissolved oxygen) to assess the biocontrol potential of the fungus. Different solids concentration of thermal alkaline pre-treated municipal wastewater sludge was examined to enhance biocontrol activity in 15 L fermenter at ≥ 30% dissolved oxygen. The maximum conidia concentration ($5.94 \times 10^7$ CFU $ml^{-1}$ at 96 h) was obtained at 30 g $L^{-1}$ suspended solids of wastewater sludge. The lytic enzymes analyzed quantitatively were protease, amylase, cellulase, laccase and chitinase. The enzyme activities maxima achieved were around 12–30 h, which was earlier than in the case of shake flask culture. The biocontrol potential of the fermented sludge thus obtained was assessed by fungal, insect and plant bioassays. Fungal bioassay against a fungal phytopathogen, *Fusarium* sp. showed maximum activity around 96 h at 30 g $L^{-1}$ suspended solids concentration. Entomotoxicity against spruce budworm larvae showed maximum activity ≈ 17290 SBU $\mu l^{-1}$ at 30 g $L^{-1}$ suspended solids concentration at the end of fermentation (96 h). Plant bioassay showed dual action of *T. viride*, i.e., disease prevention and growth promotion. The rheological analyses of fermentation sludges showed the pseudoplastic behaviour. Relative to 30 g $L^{-1}$ suspended solids concentration, the requirements of agitation and aeration increased significantly at 35 g $L^{-1}$. The oxygen uptake rate and volumetric oxygen mass transfer coefficient, $k_La$ at 35 g $L^{-1}$ could not be increased in comparison to 30 g $L^{-1}$ due to rheological complexity of the broth during fermentation. Thus, the successful fermentation operation of the biocontrol fungus *T. viride* is a rational indication of its potential for mass scale production for agriculture and forest sector as a broad-spectrum biocontrol agent.

**Keywords:** Bioassay, biocontrol agent, entomotoxicity, fermentation, lytic enzyme, rheology, *Trichoderma viride*.

## Introduction

Mass scale production of biocontrol fungi has been a great challenge in their commercial success. The literature is replete with fungal biocontrol agents, which show great potential at laboratory scale (Batta, 2004; Harman 2004), but their mass scale production could be meagerly cited (Jenkins et al., 1998). Moreover, several factors like raw material cost, production technique and biocontrol efficacy have been major constraints in the commercialization of fungal biocontrol agents (Thangavelu, 2004). In order to overcome these shortcomings, several researchers have explored negative cost raw materials like agricultural residues (Howard et al., 2003) and wastewater sludges (Verma et al., 2005) by using *Trichoderma* spp. liquid fermentation. Nevertheless, the production of these biocontrol agents using wastewater sludge as a raw material at fermenter scale, a key to mass scale production, under controlled condition of pH, temperature, dissolved oxygen in fermenter has not been investigated so far. Consequently, fermenter study of *T. viride* production process on wastewater sludge was essential.

Although, *T. viride* has been reported to be antagonistic against several phytopathogens, namely, *Fusarium* sp., *Rhizoctonia* sp. (Harman, 2004) and spruce budworm larvae (Verma et al., 2005), still the mode of action of *T. viride* is unclear. Many authors have suggested that several lytic enzymes produced during fermentation could be a major factor behind antagonistic characteristics of *T. viride* (Mischke, 1997). In fact, several researchers have successfully demonstrated the antagonistic action of lytic enzymes (Yedidia et al, 1999). Hence, it was expected that ensuring higher production of lytic enzymes during fermentation process of *T. viride* could be advantageous from biocontrol point of view. Besides, *T. viride* is a saprophytic fungus and is known to be easily colonizing in plant rhizosphere, therefore, it could also help in plant growth promotion (Harman, 2004). Thus, a detailed analysis of types and quantities of lytic enzymes produced by *T. viride* fermentation on wastewater sludge was required. Further, although *in vitro* bioassay could provide first hand assessment of biocontrol potential of any antagonist organism, yet only *in situ* bioassay would ensure eventual success of the antagonistic action. Several researchers have reported *in vitro* biocontrol assessment of *T.*

*viride* (Batta, 2004; Mischke, 1997). However, *in situ* bioassay has been scarcely reported. Thus, plant bioassay under actual field conditions was very crucial step in order to develop a feasible process for the production of *T. viride* based biocontrol agent, which has been realized in the present study.

Despite the ease of control and other advantages in liquid fermentation process some shortcomings like rheological complexity can significantly affect the microbial growth and product formation (Verma et al., 2006; Bhargava et al., 2003). As the wastewater sludge has been previously investigated only in shake flasks, the rheological characteristics are unknown. Thus the rheology should be dealt in depth at small scale so that the mass scale fermentation could be feasible (Jenkins et al., 1997). For example, different rheological states of the wastewater sludge should be examined (e.g., different total solids concentration of wastewater sludge). Moreover, detailed understanding of rheology would be eventually helpful in downstream processing (centrifugation, pumping operation) of fermented sludge. Therefore, the present study was aimed at investigating best possible strategy for the bench-scale production of *T. viride* based biocontrol agent using wastewater sludge as substrate. Eventually, this study will help in scale-up of the process to pilot scale and finally translating the results into a large scale production. In addition, utilization of wastewater sludge for the production of a value-added product (biocontrol agent, *T. viride*) will serve dual purpose, a) environment friendly phytopathogen control, b) reduction of secondary pollution of wastewater treatment facilities. Thus, objectives of the present study comprised: 1) submerged fermentation process development of the antagonist fungus *T. viride* and 2) assessment of extracellular lytic enzymes, antagonism and plant growth promotion bioassays of *T. viride* produced utilizing wastewater sludge as a raw material.

## Materials and Methods

*Chemicals*

The chemicals used as reagents for enzyme activity measurements were of analytical grade, obtained from EM Sciences (Ontario, Canada).

*Wastewater sludge and solids amendments*

The wastewater sludge was obtained from a local wastewater treatment facility, Communauté Urbaine de Québec (CUQ), Québec. The physico-chemical characteristics of sludge were determined as per Standard Methods (APHA, 1998) and is presented in Table 1. The sampled sludge was assessed to meet the metal concentrations guidelines according to Québec norms for agricultural application of sludge (MENV, 2004).

The sludge suspended solids (SS) concentration was adjusted to 4–4.5% (w $v^{-1}$, dry basis) primarily by gravity settling for about 30 minutes followed by centrifugation at 7650 g for 15 minutes. Demineralized water was used for solids adjustments or dilution purposes. The amended raw wastewater sludge was kept refrigerated at 4 °C to minimize microbial degradation for a maximum period of 1 week.

*Direct-steam hydrolysis and sterilization*

Amended raw wastewater sludge was subjected to thermal alkaline hydrolysis in a 15 l (working volume, 7–10 l) custom-built superheated-steam hydrolyser equipped with variable agitation (marine propeller type) system (0–100 rpm). For this, the pH of the raw sludge was adjusted to 10.25 ± 0.01 by adding NaOH pellets and the sludge was transferred to the hydrolyzer. The temperature of the raw wastewater sludge was increased up to 110 ± 2 °C by passing superheated-steam (160 ± 2 °C @ ≈6 psia) through heating coils inside the hydrolyzer. Furthermore, the raw wastewater sludge was heated up to 140 ± 2 °C @ ≈3.5 psia by direct steam injection by using superheated-steam (160 ± 2 °C @ ≈6 psia). The raw sludge was maintained at 140 ± 2 °C @ ≈3.5 psia for 30 minutes followed by rapid cooling down to room temperature by circulation of cold water. Meanwhile, in order to avoid settling, the agitation was kept constant at 80 rpm during the entire hydrolysis process. The direct steam injection resulted in dilution of ≈1.2×. The desired SS concentrations (30 and 35 g $L^{-1}$) were achieved by further dilution with demineralized water. Afterwards, the hydrolyzed sludge was transferred to fermenter and the pH was adjusted to 6 ± 0.01 by using 4 N $H_2SO_4$ and was sterilized at 121 °C for 30 minutes. The 30 and 35 g $L^{-1}$ sterilized alkaline hydrolyzed sludges will be henceforth referred to as TH30 sludge and TH35 sludge, respectively, throughout the text.

*Trichoderma viride and phytopathogenic fungus inocula*

For biocontrol experiment, an antagonist fungal strain of *T. viride* was utilized in this study (Verma et al., 2006). The *T. viride* fungus maintenance and preparation of inoculum for the shake flask starter culture are described elsewhere (Verma et al., 2006). In brief, Erlenmeyer flasks containing 150 ml sterile (121 °C for 15 minutes in autoclave) tryptic soya broth (TSB, Difco) at pH 6.0 were inoculated with *T. viride* and incubated in a rotary shaker at 28 °C and 250 rpm for 48 h. An inoculum prepared in TSB medium of 10% (v $v^{-1}$) was employed for sludge fermentation purpose. The shake flask starter culture inoculum of phytopathogenic fungus (*Fusarium* sp., Verma et al., 2006) was also prepared in similar way as *T. viride*, except, at pH 7.0, 25 °C and incubated for 24 h. Furthermore, an inoculum of 5% (v $v^{-1}$) was employed for fermenter scale production.

The *T. viride* inocula for 15 l (10 l, working volume) fermenters were prepared in a 7.5 l (3 l, working volume) fermenter (Verma et al., 2006) using tryptic soya broth (TSB) as substrate. The operating pH, temperature and DO were maintained, respectively, at 6.0, 28 °C and ≥ 50%. The inoculum was transferred aseptically to the 15 l fermenter after 48 h of incubation.

*Fermenter operation*

The fermentation study of *T. viride* on hydrolyzed wastewater sludges and the production of spores phytopathogenic fungus (*Fusarium* sp.) were accomplished in a 15 l (10 l, working volume) fermenter (BIOGENIE, Quebec). The pH, agitation, anti-foam addition, dissolved oxygen, temperature and air flow rate were automatically controlled. The data acquisition was linked to a process control software (iFix 3.5, Intellution, USA) and was utilized for process automation. The aeration system was equipped with a tubular sparger, three equally spaced Rushton turbine-type impellers and four baffles. The TH25, TH30 and TH35 sludges for *T. viride* were sterilized inside the fermenter for 30 min at 121 °C. Likewise, TSB medium for the production of spores of *Fusarium* sp. was sterilized only for 15 min at 121 °C. The dissolved oxygen (DO) level was maintained at ≥ 30% for *T. viride* and at ≥ 50% to avoid oxygen limitation on growth by varying the agitation speed and aeration rate via PID control. The calibration methods of pH and DO probes and the measurements of $k_La$, oxygen uptake rate (OUR) are explained in (Brar et al., 2005).

Fermentation broth samples (100-150 ml) were drawn aseptically at regular intervals for subsequent analyses. The duplicate set of fermentations were carried out for significance tests of the data.

*Rheological measurements*

The rheology of the fresh fermentation media was evaluated by viscosity, shear stress vs. shear rate and surface tension profiles. The viscosity and shear stress vs. shear rate were measured on a rotational viscometer (DVII+, Brookfield) equipped with small sample adapter spindle (SC4 34, Brookfield). The details of the viscometer are explained elsewhere (Verma et al., 2006). The surface tension was measured using a semi-automatic tensiometer (Fisher Scientific Ltd., Canada). Water was used for the calibration of the tensiometer.

*CFU measurement*

The conidia production was assessed in terms of CFU measurement as described earlier (Verma et al., 2005). In brief, the conidia was filtered from fermentation broth by using a small autoclavable filtration unit. Subsequently, the CFU plating was conducted automatically on tryptic soya agar medium using Whitley Automatic Spiral Plater 2, Fisher Scientific, Quebec, Canada. For statistical significance, five replicates of three different dilutions of two fermentation runs were employed for ANOVA enumeration.

*Microscopy*

Samples of approximately 50 µl were examined under microscope for detection of mycelia and conidia, and contamination, if any. A computer coupled optical microscope (Zeiss Axiolab) equipped with a digital camera (Axiocam HRC Zeiss) was used for this purpose.

*Protein content and enzyme activities*

Protein content for fermentation broths was determined using BCA$^{TM}$ Protein Assay Kit - 23225 (PIERCE, Rockford, USA). All enzyme activity measurements were carried out in soluble phase. For this, *T. viride* fermented sludge samples were centrifuged

at 7650 g for 20 min at 4 ± 1°C and the supernatant was used as an enzyme aliquot for subsequent assays.

Protease

Protease activity was measured by UV spectrometry (at 275 nm) of tyrosine catalyzed from casein. The enzyme activity was determined in IU $ml^{-1}$ as per modified Kunitz (1947) method. The supernatant was appropriately diluted in borate buffer at pH 8.2 and used for enzymatic reaction.

Amylase

A slightly modified method of Bergmeyer and Bernt (1974) was used for amylase activity determination. The reaction mixture consisted of 40 µl of 0.5 M sodium acetate buffer at pH 6.0, 60 µl of enzyme solution, 100 µl of 0.5% (w $v^{-1}$) soluble starch, and ultrapure water to make up the volume to 200 µl. The reaction was allowed to proceed at 50 °C for 30 min and was stopped by heating at 96 °C for 5 min as described in Campos and Felix (1995). The appearance of glucose in the reaction mixture was determined using glucose assay kit from Sigma-Aldrich Chemicals (GAGO20-1KT). The amylase activity units were defined in terms of the amount of enzyme that releases 1 µmol of glucose per min from soluble starch.

Cellulase

Cellulase activity measurement was carried out as per method described in Wang et al. (2002). In brief, 0.1 ml of enzyme solution was mixed with 0.4 ml of substrate solutions, which contained 1.25% (w $v^{-1}$) carboxy methyl cellulose (CMC) in an acetate buffer solution (125 mM, pH 5). Subsequently, the reaction mixture was maintained at 37 ± 1 °C for 10 min. The reaction mixture was then centrifuged, and the amount of reducing sugar produced in the supernatant was measured by the dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of cellulase activity was defined as the release of 1 µmol of reducing sugar per minute at 37 °C and a pH of 5.

Laccase

The laccase activity was measured as per method utilized by Silva et al., (2005). In this method, the oxidation of *o*-dianisidine was detected by spectrophotometry at 525 nm ($^{TM}525$ = 65,000 $M^{-1}cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme necessary to oxidize 1 µmol of *o*-dianisidine per minute.

Chitinase

For chitinase assay, colloidal chitin was used as a substrate and *N*-acetylglucosamine as a final product with slight modification in method as described in Ueda and Arai, (1992). The colloidal chitin was prepared by adding 1 g of cob shell chitin in 100 ml of 0.01M acetate buffer, pH 5.0 and stirring at 50 rpm overnight at 30 °C on a rotary shaker (Roy et al., 2003). The enzymatic mixture containing, 0.5 ml of enzyme solution appropriately diluted in 0.01M acetate buffer, pH 5.0 and 1 ml of colloidal chitin was incubated at 40 °C for 30 min. The chitinase activity was measured in terms of the amount of reducing ends (*N*-acetylglucosamine) produced in the reaction mixture by spectrophotometry at 420 nm as per the method of Imoto and Yagishita (1971). One unit of chitinase activity was defined as the amount of the enzyme that produced 1µmol of *N*-acetylglucosamine per minute.

For all enzyme activity measurements, the standard deviation was between 5–8%, based on triplicate samples of two fermentation runs.

*Biocontrol Activity*

Insect bioassay

The entomotoxicity (Tx) of samples was determined by using eastern spruce budworm (SB) larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) as per method described by Brar et al. (2005). Entomotoxicity of *T. viride* fermented samples was expressed in SB units $\mu l^{-1}$ (SBU $\mu l^{-1}$). For statistical significance, twenty replicates at three different dilutions of two fermentation runs were employed for ANOVA enumeration.

Fungal bioassay

A slightly modified scored response bioassay (Verma et al., 2006) as originally described in Mischke (1997) was employed. The phytopathogen used for fungal bioassay

was a spruce tree pathogen *Fusarium* sp. procured from the Laurentian Forestry Centre (LFC, Quebec, Canada). The fermentation broth was centrifuged at 12000 g for 15 min at 4 °C followed by filtration through ϕ 0.45 μm pore-size glass fiber filter (Whatman paper 934-AH) for bioassay. In this procedure, serial dilutions of 1/2, 1/4, 1/8 and 1/16 in ultrapure water were used in 24-well tissue culture plates. The scoring time and the scale of inhibition were similar to those of the original study (Mischke, 1997).

Plant bioassay

Plant bioassay comprised of growth promotion and disease prevention assessment of *T. viride* fermented sludge (96 h) in soil on three commercially important plants, namely, green pepper, tomato and soya under greenhouse conditions. The soil sample was unfortified and was used in four combinations; (1) without any treatment, (2) mixed with *T. viride* conidia at $10^6$ CFU (g soil) $^{-1}$ (conidia concentration used in soil studies, Yedidia et al., 1999), (3) mixed with spores of *Fusarium* sp. at $10^6$ CFU (g soil) $^{-1}$ soil, and (4) mixed with *T. viride* conidia and *Fusarium* sp., each at $10^6$ CFU (g soil) $^{-1}$. For growth promotion effect, dry weight of whole plant (excluding root section) was measured by drying in incubator at 50 °C for 7 days. For statistical significance of data, for each plant, the bioassay was carried out by using randomized blocks design containing a total of 20 plastic pots as represented in Table 2. About 7–9 seeds of each plant were sown in separate pots containing 1.2 kg soil at 0 d and after 2 weeks period, only 3 germinated plants per pot were retained for bioassay purpose. Analysis of variance (ANOVA) test was carried out at the level of $p < 0.05$ to determine the significance difference within as well as between treatments.

**Results and Discussion**

*Fermenter operation*

Solids concentration, % DO control and fermentation broth rheology

The influence of wastewater sludge solids concentration has already been studied earlier in shake flask culture of *T. viride* (Verma et al., 2005). However, it was expected that under controlled environmental conditions the solids concentration in fermenter might vary from shake flask. Therefore, in the present study, in addition to the optimal solids

concentration for conidia and biocontrol activity as obtained for shake flask culture of *T. viride*, i.e., 30 g $L^{-1}$ SS (Verma et al., 2005), two SS concentrations (25 and 35 g $L^{-1}$) were also examined. The fermentation operation parameters, namely, % DO, agitation and aeration and volumetric mass transfer coefficient ($k_La$) for TH30 and TH35 sludges are shown in Figure 1 and Figure 2, respectively. The results of TH25 are not presented due to very poor conidia production and biocontrol activities (significant at $P < 0.01$) in comparison to TH30 and TH35 sludges. The agitation and aeration profiles for TH30 sludge show that at around 6 h of fermentation, the agitation and aeration requirements started increasing and continued until about 20 h. Afterwards, the agitation and aeration requirement decreased up to about 40 h to maintain required DO ($\geq$ 30%) concentration. The incubation period between 6 to 40 h concurred with vegetative growth phase (observed under microscope) and conidiation, thereby, higher demand for agitation and aeration was observed. The DO control after 40 h up to about 86 h was relatively difficult mainly due to the persistent generation but manageable foaming. The effect of agitation on mycelial mass of *Trichoderma* spp. was reported to be only nominally adverse (Domingues et al., 2000; Felse and Panda, 2000). Therefore, the adverse effect of agitation used in the present study was assumed to be negligible. On the other hand, the DO requirement in TH35 sludge was lesser in comparison to TH30 and was experienced from about 14 h until 36 h (Figure 2A). The lower agitation and aeration requirement was mainly due to decreased mycelial growth, also observed under microscope. The production of foam was also observed throughout the fermentation affecting DO control to a lesser extent as in the case of TH30 sludge. Thus, relatively lower mycelial (biomass) activity in TH35 sludge helped in slightly higher DO control than TH30. Nevertheless, lower mycelial (biomass) activity was a major drawback for TH35 sludge from *T. viride* mass scale conidia production point of view.

The oxygen uptake rates of TH30 and TH35 sludges are shown in Figure 3. High OUR values between 14 – 42 h for TH30 sludge and between 14 – 54 h for TH35 sludge suggest the periods of dominant vegetative growth, which was also concurrent with variation of viscosity (discussed later). Therefore, the evident larger area under OUR curve of TH30 sludge with respect to TH35 sludge also support the presence of higher mycelial biomass and oxygen requirement of TH30 sludge.

The importance of rheological parameters on fungal fermentation have been reported by several researchers (Bhargava et al., 2002; Pollard et al., 2002). The effect of fermentation broth viscosity and surface tension on $k_La$ and product formation have been studied earlier for other fungi, however, the results of all studies are specific to the fungus examined as well as the fermentation medium (Pollard et al., 2002). Hence, detailed rheological analysis was a pre-requisite for *T. viride* grown on TH30 and TH35 sludges. The changes in viscosity and $k_La$ during *T. viride* fermentation on TH30 and TH35 sludges show inverse relationship between viscosity and $k_La$ throughout the fermentation, except from 54 – 84 h in TH30 (Figure 1B) and from 72 – 96 h in TH35 (Figure 2B). This was due to several factors like variation in agitation to maintain ≥ 30% DO in the fermentation broth, foam control (intermittent addition of polypropylene glycol as anti-foam), pH control (addition of acid or alkaline) and physiological changes of *T. viride* (observed as breakage of mycelial mass). Nevertheless, only a small change in surface tension was observed at all SS concentration (Figures 1B and 2B) during the incubation period. Therefore, surface tension was apparently a less important parameter at present, however, the data could be essential during scale-up of the fermentation process (Zlokarnik, 1998). Furthermore, the present data of viscosity and $k_La$ would be decisive in design of agitation and aeration units of large scale fermenter as well as development of feed forward process control strategies.

The consistency and flow behaviour indices profiles, obtained from shear stress vs. shear rate data of TH30 and TH35 sludges (Figure 4) indicates the varying pseudoplastic characteristics of both broths during fermentation. It was observed that the both broths became more pseudoplastic at the end of fermentation, which will have further implications on both mass and heat transfer (Charles, 1978).

*Growth kinetics: conidia and enzyme*

The mass scale feasibility of fungal biocontrol agents (e.g., *Trichoderma* fungus) necessitates to achieve higher conidia concentrations in the fermented broth (Thangavelu et al., 2004; Jenkins et al., 1998). Therefore, production of conidia was measured during the fermentation of TH30 and TH35 sludges (Figures 1B and 2B). In comparison to shake flask culture (Verma et al., 2005), significant increase (up to ≈0.47 log cycle) in the maximum conidia concentration was observed in fermenter, yet the optimum SS concentration for conidia production remained 30 g $L^{-1}$ irrespective of scale of operation.

Meanwhile, the increase in maximum conidia concentration of TH35 sludge in fermenter was also higher than (≈0.54 log cycle) shake flask culture at higher solids (≥ 40 g $L^{-1}$). However, the conidia kinetics suggested that the vegetative growth (mycelial mass formation, seen under microscope) was predominant during first 24 h, followed by conidia formation. Irrespective of SS concentration, the conidia production kinetics demonstrated that in fermenter, the maximum conidia concentration was achievable in lesser incubation period (about 42 h) as compared to shake flask culture (about 72 h). The increase in conidia concentration and lesser time requirement to achieve maximum conidia concentration was mainly due to the controlled pH, DO and enhanced mixing in fermenter in comparison to shake flask. However, the biological activity of *T. viride* (discussed later) could attain maximum near 96 h, thereby, rendering the batch time of 96h as in the case of shake flask.

The antagonistic activities of *Trichoderma* spp. have also been attributed to the lytic enzymes production during growth on suitable substrates (Harman et al., 2004; Yedidia et al., 1999). As wastewater sludge comprises of substrates of multiple origins (e.g., cellulosic; fungal and insect debris – chitin; wood materials – laccase; and others, Verma et al., 2005), it was pertinent to carry out quantitative estimation of lytic enzymes produced by *T. viride* grown on wastewater sludge (Figure 5). The enzyme production kinetics was different from that of the shake flask culture (Verma et al., 2005). Irrespective of the enzyme type, the final (96 h) enzyme activity was always higher than that of at 0 h, meanwhile, the enzyme activities were higher in TH30 sludge in comparison to TH35 sludge. In case of protease, the maximum activity was obtained around 48 h and was followed by a sharp decline during 54 – 60 h (Figure 5A) due to the increased agitation requirement and also probably due to addition of anti-foam (Pappagianni, 2004). In addition, the protease activity at 96 h was much higher than that obtained for shake flask culture in earlier studies. Amylase activity was relatively constant after 24 h in TH30 sludge (Figure 5B) but a decline of amylase activity in TH35 sludge was observed mainly due to adverse effect of high aeration and agitation rate (Figure 2B) and addition of antifoam on mycelial growth of *T. viride*. Cellulase activity maxima were achieved by 36 h of incubation for TH30 and around 60 h for TH35 (Figure 5C). Laccase activity increase was continuous until the end of the fermentation and adverse effect of SS concentration was more pronounced than other enzymes (Figure 5D). Furthermore, the chitinase activity

maxima of both sludges were achieved earlier than rest of the enzymes (Figure 5E). The presence of these lytic enzymes in considerable quantity was due to multiplicity of wastewater sludge as well as the physiological characteristics of *T. viride* (Yedidia et al., 1999). On the other hand, the protein content as an indirect indicator of enzyme production could not be measured because of complexity of sludge, in fact, the total protein content decreased at the end of incubation (Figures 1B and 2B). As the wastewater sludge is a rich source of microbial protein, it was possible that during fermentation of TH30 and TH35 sludges, only a part of the hydrolyzed form of protein (amino acids and polypeptide fragments) could be utilized by *T. viride* for the production of enzymes. Therefore, it was shown that *T. viride* was capable of producing several extracellular lytic enzymes in significant quantities on wastewater sludge.

*Biocontrol activities*

Antagonism

The fungal and insect bioassays showed significant activities by the *T. viride* fermented wastewater sludge. The inhibition index bioassay (Figure 6A) and insect bioassay (Figure 6B) both showed continuous increase in antagonistic activities until the end of the fermentation. The final Tx obtained in the case of TH30 sludge (17290 SBU $\mu l^{-1}$) was even higher than that of shake flask culture (15036 SBU $\mu l^{-1}$) probably due to higher conidia, enhanced production and stability of enzymes. However, the Tx obtained for TH35 was much lower than that of TH30 and could be attributed to the relatively lower conidia production and enzyme activities. Several researchers (Harman et al., 2004; Felse and Panda, 2000; Verma et al., 2005) have reported that the antagonistic activity of *Trichoderma* spp. was due to extracellular lytic enzymes (e.g., discussed here) and other metabolites, e.g., antibiotics and antimicrobial compounds. Therefore, it should be interpreted that the antagonistic activity of *T. viride* observed in this study was due to combined effect of lytic enzymes and fungal metabolites. The antagonism of *T. viride* against a fungal pathogen, *Fusarium* sp. (Figure 7A) is shown by growth inhibition of *Fusarium* sp. caused by *T. viride* (Figure 7B) on Petri. Figure 7C shows typical spots on leaves of soya plant, infected by *Fusarium* sp. Further, it was observed that the dry weight of plants infected by *Fusarium* sp. were minimum among all types of treatments in plant

bioassay. Thus, it was concluded that *T. viride* was capable of suppressing the growth of *Fusarium* sp. even under soil conditions.

Growth promotion

The plant bioassay was also aimed at evaluating the growth promotion capability of *T. viride* on commercially important plants, described earlier. The results of soil treated with and without *T. viride* as well as *Fusarium* sp. (Figure 7D) indicated that under green house conditions, *T. viride* was capable of enhancing overall growth of tomato and pepper plants. Furthermore, the *Fusarium* sp. was found to be pathogenic to all plants but was suppressed by *T. viride* at $10^7$ CFU $ml^{-1}$ conidia concentration. Moreover, the tomato and pepper plants with only *T. viride* treatment showed significant increase in overall plant dry weight. The ineffectiveness of *T. viride* treatment on soya plant for growth promotion was maily due to seed quality and existing ideal environmental conditions for growth (Harman et al., 2004). Thus the qualitative plant growth promotion assessment showed that *T. viride* grown in wastewater sludge was a feasible biocontrol agent for application in soil under green house conditions. Meanwhile, the conidia concentration used in this study was ≤ recommended dose of Yedidia et al. (1999), hence, a further decrease in requirement of *T. viride* conidia concentration is expected after a quantitative (varying conidia concentration) plant growth promotion bioassay.

Thus, the successful bench scale study of *T. viride* fermentation on pre-treated wastewater sludge demonstrates that this process could be a plausible option for the production of a broad spectrum biocontrol agent and could find application in forest and agriculture sector.

**Conclusions**

The fermenter operation of pre-treated wastewater sludge resulted in following important findings.

1. At non-limiting oxygen concentration, the solids concentration optimal for biological activity of *Trichoderma viride* was 30 g $L^{-1}$ suspended solids irrespective of scale of operation (shake flask to fermenter).

2. Rheological measurements of fermentation broth showed significantly varying non-Newtonian characteristics during incubation and the final fermented broth was more pseudoplastic with respect to pre-treated wastewater sludge.
3. Antagonism bioassays suggested that the conidia as well as fermentation metabolites of *T. viride* were effective against fungal as well as insect pathogens.
4. *T. viride* was capable of producing several lytic enzymes in wastewater sludge that possibly play important role in its broad spectrum biocontrol activity.
5. The plant growth promotion bioassay showed that *T. viride* was active even in soil, therefore, it possesses characteristics of a potential commercial biocontrol agent.

**Acknowledgements**

The authors are thankful to 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.

**References**

1. Batta, YA. 2004. Effect of treatment with *Trichoderma harzianum Rifai* formulated in invert emulsion on post harvest decay of apple blue mold. International Journal of Food Microbiology 96: 281– 288.
2. Bergmeyer, H. U., and E. Bernt. 1974. Determination off glucose with oxidase and peroxidase, p. 1205–1215. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 3. Verlag-Chimie-Academic Press, New York.
3. Bhargava S, Nandakumar MP, Roy A, Wenger KS, Marten MR (2003) Pulsed feeding during fed-batch fungal fermentation leads to reduced viscosity without detrimentally affecting protein expression. Biotechnol Bioeng 81(3):341–347. DOI: 10.1002/bit.10481.
4. 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.
5. Campos L, Felix CR (1995) Purification and Characterization of a Glucoamylase from *Humicola grisea.* Applied and Environmental Microbiology. 61(1): 2436–2438.
6. Charles, M. 1978. Technical aspects of the rheological properties of microbial cultures. Advances in Biochemical Engineering 8: 1–59.
7. Chatfield, C. 1983. The design and analysis of experiments: 1. Comparative experiments, pp. 224–256. *In* Statistics for Technology, 3rd ed. Chapman & Hall, Bristol, United Kingdom.
8. Domingues FC, Queiroz JA, Cabral JMS, Fonseca LP. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. Enzyme and Microbial Technology 2000; 26: 394-401.
9. Felse PA, Panda T. Submerged culture production of chitinase by *Trichoderma harzianum* in stirred tank bioreactors – the influence of agitator speed. Biochemical Engineering Journal 2000; 4: 115-120.
10. Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M. 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2: 43–56.
11. Howard, R.L., Abotsi, E., Jansen van Rensburg, E.L., Howard, S. 2003. Lignocellulose biotechnology: issues of bioconversion and enzyme production. African Journal of Biotechnology 2: 602–619.

12. Imoto T, Yagishita K 1971. A simple activity measurement by lysozyme. Agric Biol Chem 35, 1154–1156.
13. Jenkins NE, Heviefo G, Langewald J, Cherry AJ, Lomer CJ. Development of mass production technology for aerial conidia for use as mycopesticides. Biocontrol News and Information, 1998; 19(1): 21N-31N.
14. Kunitz, M. (1947) Crystalline soybean trypsin inhibitor. Journal of General Physiology 30, 291–310.
15. Miller GL (1959) Use of dinitrosalycylic acid reagent for determination of reducing sugars. Anal Chem. 32: 426–428.
16. Mischke S (1997) A quantitative bioassay for extracellular metabolites that antagonize growth of filamentous fungi, and its use with biocontrol fungi. Mycopathologia, 137: 45–52.
17. Pollard, D.J., Hunt, G., Kirschner, T.K., Salmon, P.M. 2002. Rheological characterization of a fungal fermentation for the production of pneumocandins. Bioprocess Biosyst Eng 24: 373–383. DOI 10.1007/s004490100260.
18. Roy I, Sardar M, Gupta MN 2003. Hydrolysis of chitin by Pectinex$^{TM}$. Enzyme and Microbial Technology 32, 582–588.
19. Silva CMMS, Melo IS, Oliveira PR 2005. Ligninolytic enzyme production by *Ganoderma* spp. Enzyme and Microbial Technology 37, 324–329.
20. Thangavelu R, Palaniswami A, Velazhahan R. Mass production of *Trichoderma harzianum* for managing fusarium wilt of banana. Agriculture, Ecosystems and Environment 2004; 103: 259–263.
21. Ueda M, Arai M (1992). Purification and some properties of chitinase from *Aeromonas* sp. No. 10S-24. Biosci Biotechnol Biochem 56: 460–464.
22. Verma M, Brar SK, Tyagi RD, Valéro JR, Surampalli RY (2005) Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. Water Research, 39(15), 3587-3596. doi:10.1016/j.watres.2005.07.001
23. 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. *Journal of Industrial Microbiology & Biotechnology*, 33, 941–952. DOI 10.1007/s10295-006-0164-6.
24. Wang SL, Yen YH, Tsiao WJ, Chang WT, Wang CL (2002) Production of antimicrobial compounds by *Monascus purpureus* CCRC31499 using shrimp and crab shell powder as a carbon source. Enzyme and Microbial Technology 31: 337–344.
25. Yedidia, I., Benhamou, N., Chet, I. 1999. Induction of defense responses in cucumber by the biocontrol agent *Trichoderma harzianum. Appl. Environ. Microbiol.,* **65:** 1061-1070.
26. Zlokarnik, M. 1998. Problems in the application of dimensional analysis and scale–up of mixing operations. Chemical Engineering Science, **53**(17) 3023–3030.

**Table 1** Characteristics of raw wastewater sludge

| Parameter | Concentration (mg/kg; unless stated) |
|---|---|
| Total solids (g/l) | 15.4 |
| Total volatile solids (g/l) | 13.3 |
| Suspended solids (g/l) | 12.1 |
| Suspended volatile solids (g/l) | 11.7 |
| pH | 5.41 |
| Total carbon | 461000 |
| Total nitrogen | 53400 |
| Total phosphorus | 13500 |
| $N\text{-}NH_3$ | 7133 |
| $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ | 48.3 |
| $P\text{-}PO_4^{3-}$ | 7200 |
| Al | 1540 |
| Ca | 18300 |
| Cd | 6.3 |
| Cr | 20.1 |
| Cu | 417 |
| Ni | 45.0 |
| Fe | 11100 |
| K | 3210 |
| Pb | 35.4 |
| Mn | 81.9 |
| S | 419 |
| Zn | 601 |
| Na | 4110 |

**Table 2.** Randomized blocks design for plant bioassay.

| Plant | Control | T1 | T1+ *Fusarium* | *Fusarium* |
|---|---|---|---|---|
| Tomato | 5 | 5 | 5 | 5 |
| Green Pepper | 5 | 5 | 5 | 5 |
| Soya | 5 | 5 | 5 | 5 |
| Total | 15 | 15 | 15 | 15 |

T1 *T. viride*

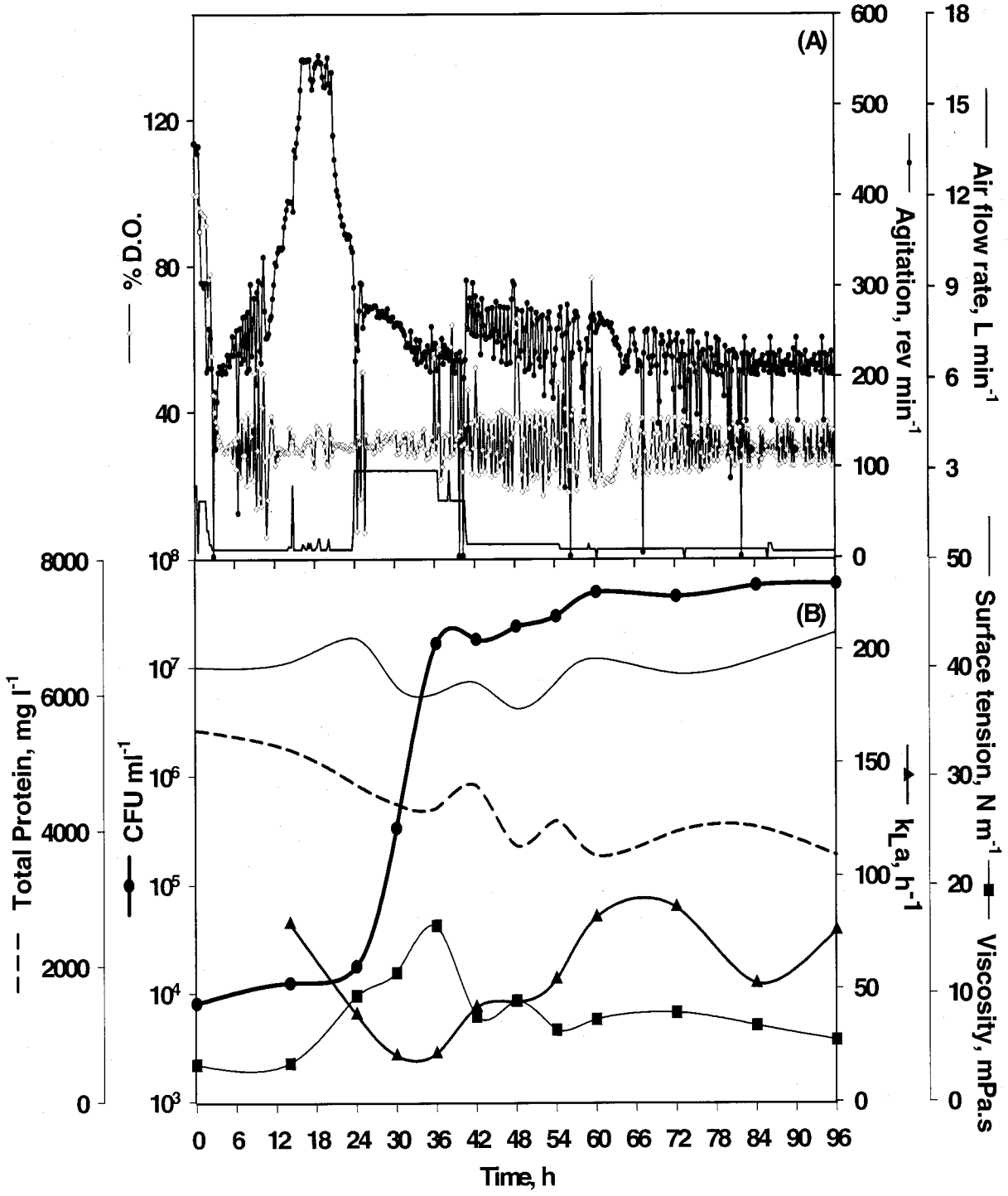



**Figure 1.** Profiles of; (A) fermentation operational parameters, (B) conidia production and medium rheology of *T. viride* inoculated TH30 sludge.

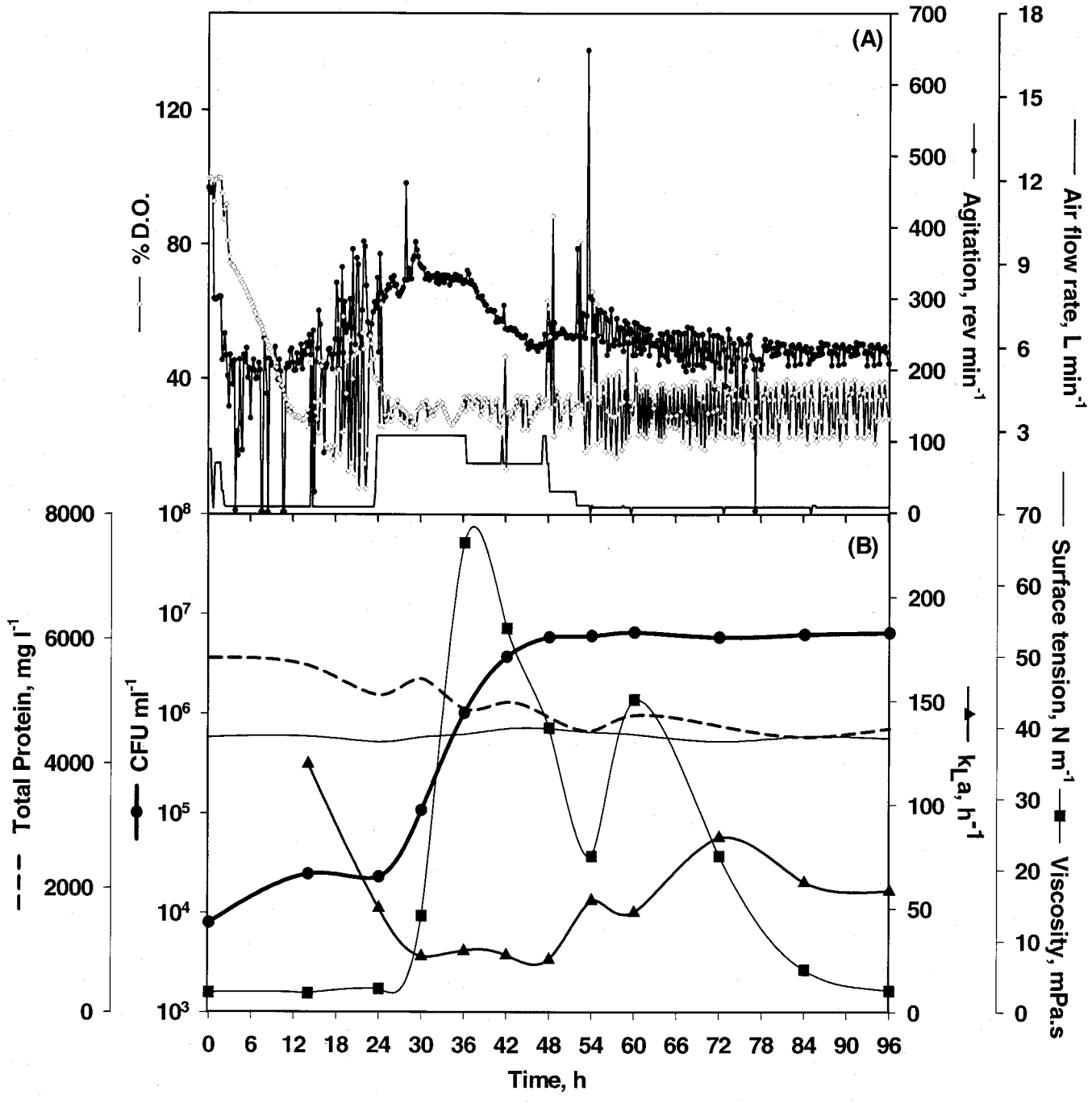



**Figure 2.** Profiles of; (A) fermentation operational parameters, (B) conidia production and medium rheology of *T. viride* inoculated TH35 sludge.

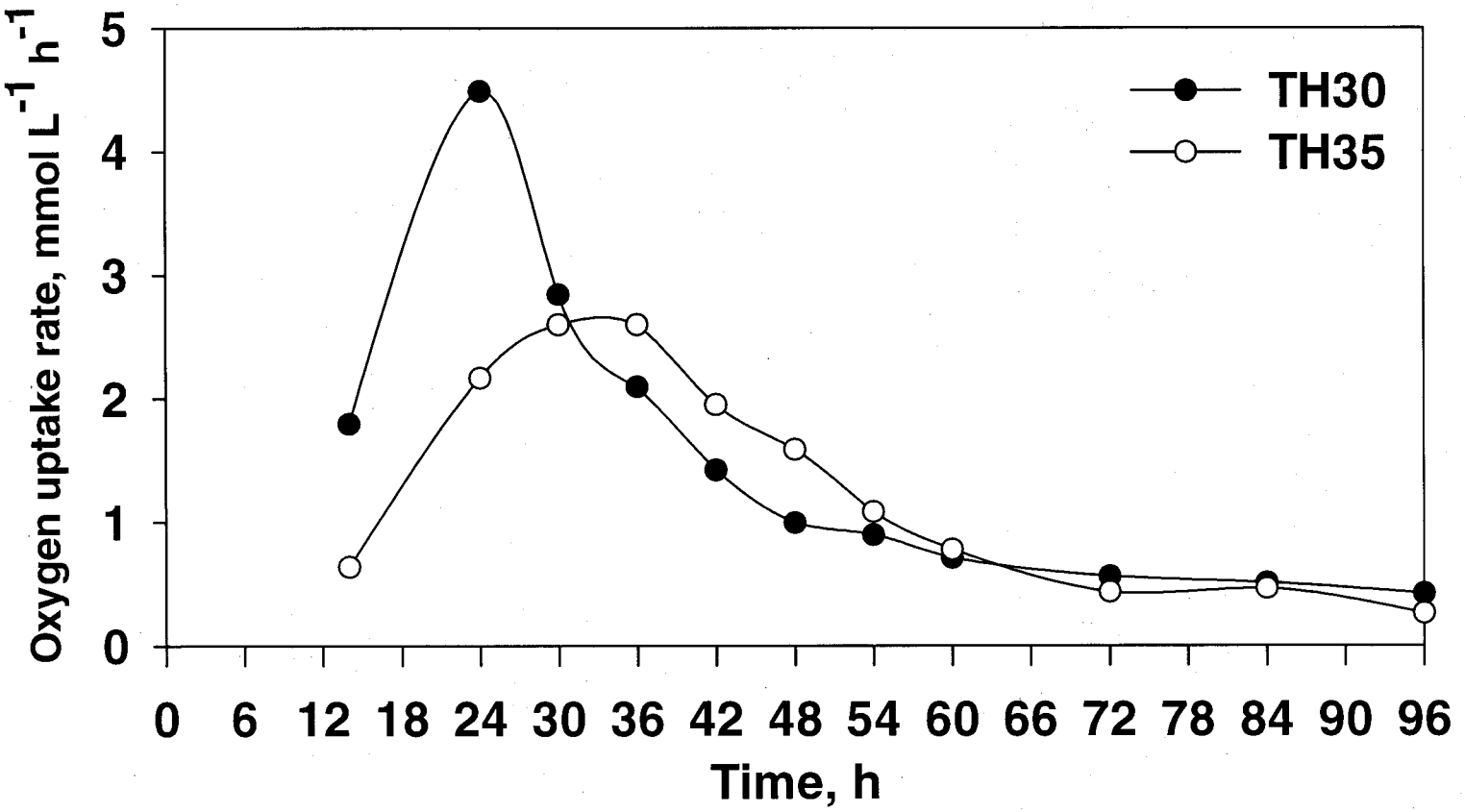



**Figure 3.** Oxygen uptake rate variations in TH30 and TH35 sludges during *T. viride* fermentation.

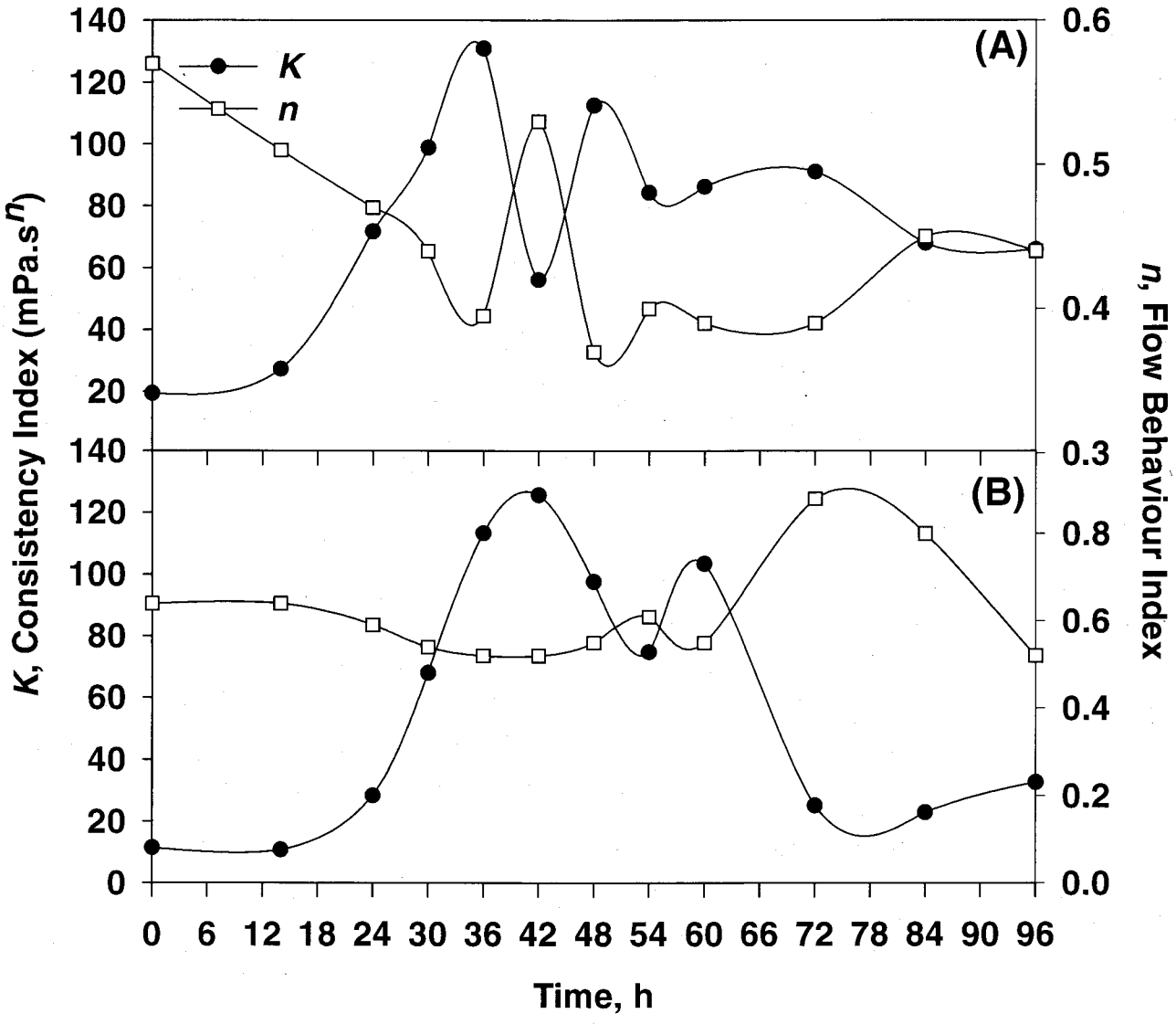



**Figure 4.** Rheological profiles of: (A) TH30 and (B) TH35 sludges.

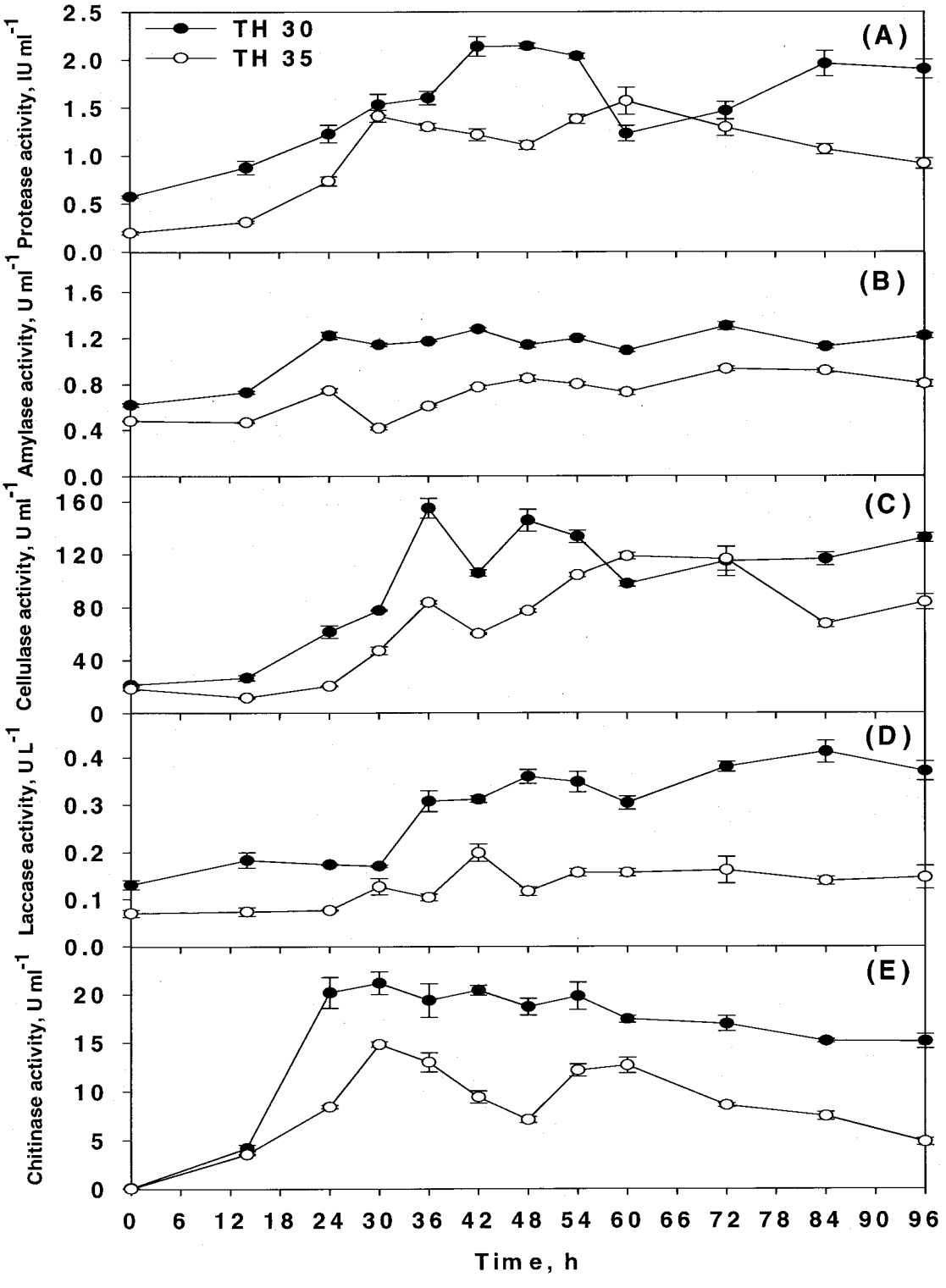



**Figure 5.** Enzyme production kinetics of TH30 and TH35 sludges; (A) protease, (B) amylase, (C) cellulase, (D) laccase and (E) chitinase.

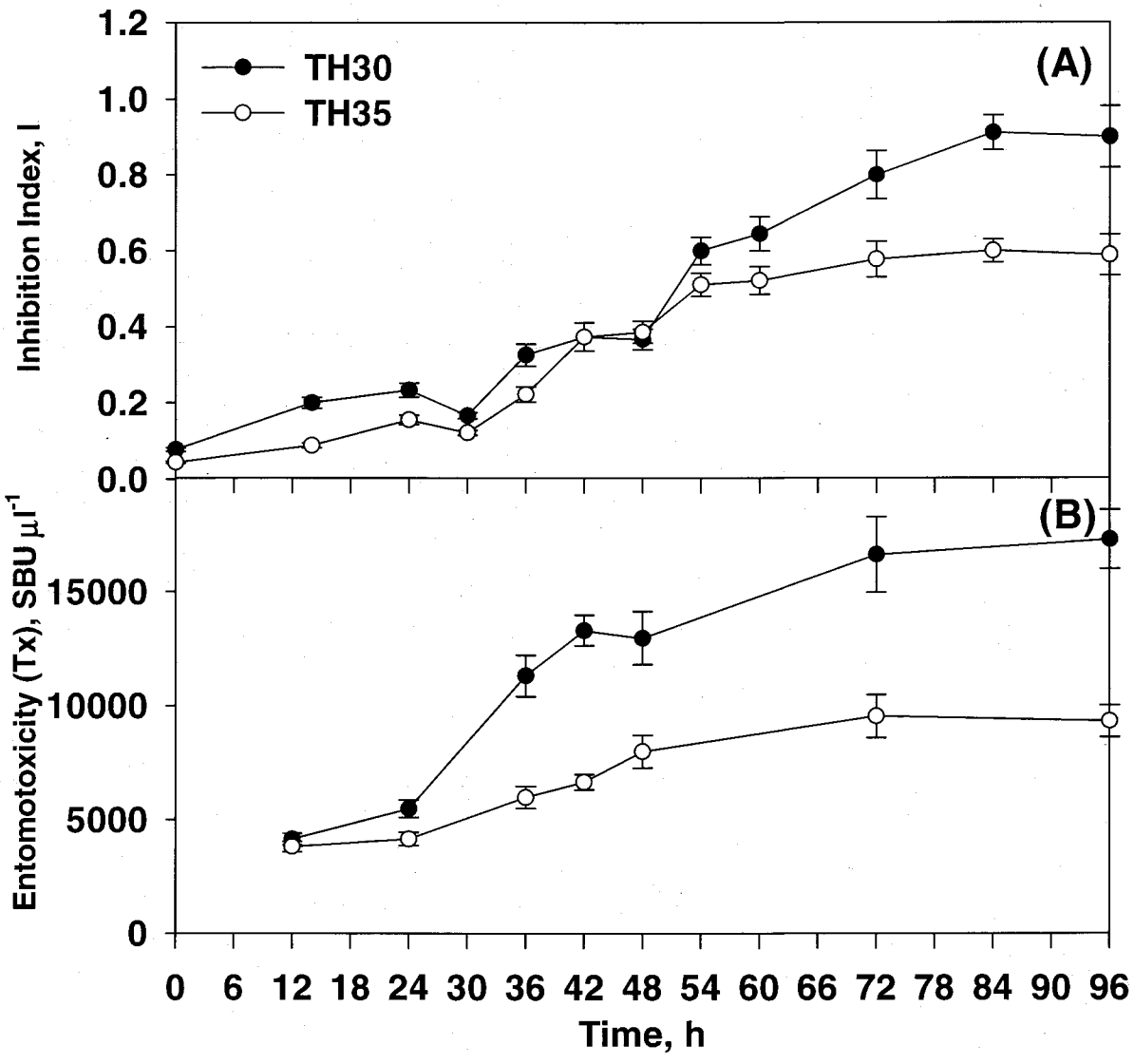
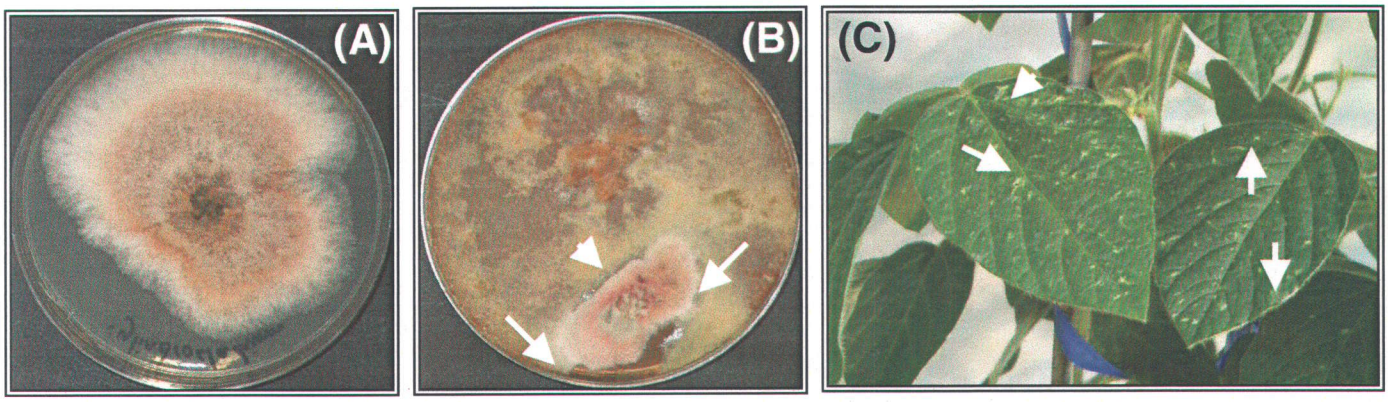



**Figure 6.** Antagonism of *T. viride* against, (A) *Fusarium* sp. (inhibition index) (B) spruce budworm larvae (entomotoxicity).



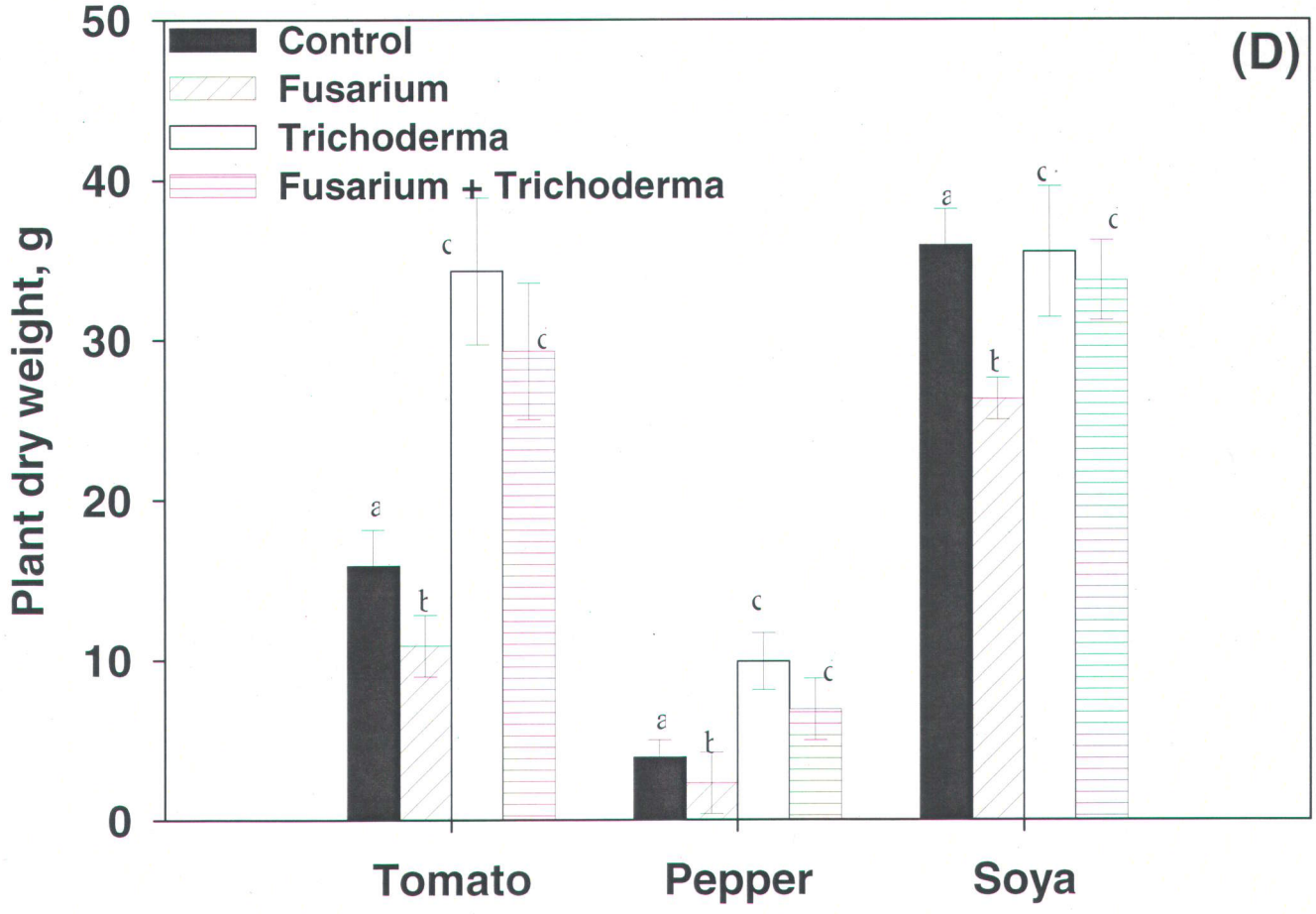




**Figure 7.** Effect of *T. viride* conidia on, (A) normal growth of *Fusarium* sp. on Petri (B) growth inhibition of *Fusarium* sp. by *T. viride* on Petri (marked by arrows), (C) *Fusarium* sp. infected soya leaves (marked by arrows) and (D) overall growth of plants.

# CHAPITRE 4.

# LES EAUX USÉES INDUSTRIELLES POUR LA PRODUCTION ET LA FORMULATION DE *T. VIRIDE*

## Part I

# Industrial wastewaters and dewatered sludge: rich nutrient source for production and formulation of biocontrol agent, *Trichoderma viride*

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

**World Journal of Microbiology and Biotechnology**

**(2007, DOI 10.1007/s11274-007-9417-4)**

**Les eaux usées industrielles et les boues déshydratées : source riche de nutriments pour la production et la formulation de l'agents de lutte biologique *Trichoderma viride***

**Résumé**

En quête d'une matière première durable pour obtenir une forte activité de lutte biologique, la culture axénique du champignon de lutte biologique *Trichoderma viride* a été réalisée dans un milieu de culture synthétique et dans différentes eaux usées et boues d'épuration en fioles. Plus particulièrement, un milieu synthétique basé sur de l'amidon soluble, des boues municipales déshydratées, des eaux usées de fromagerie, des eaux usées de papetière (pré-traitées ou non) et des eaux usées d'abattoir ont été testés pour la production de conidies et de protéases de *Trichoderma* spp.. La production maximale de conidies suivait cet ordre : milieu constitué d'amidon soluble (> $10^9$ UFC/ml), eaux usées de papetière non pré-traitées ($4.9\times10^7$ UFC/ml) > eaux usées de fromagerie ($1.88\times10^7$ UFC/ml) ≈ eaux usées d'abattoir ($1.63\times10^7$ UFC/ml) > boues municipales déshydratées ($3.5\times10^6$ UFC/ml) > eaux usées de papetière pré-traitées ($1.55\times10^6$ UFC/ml). L'activité protéolytique de *Trichoderma* spp. était particulièrement plus élevée dans les eaux usées d'abattoir (2.14 UI/ml) et les boues municipales déshydratées (1.94 UI/ml). L'entomotoxicité obtenue dans le milieu constitué d'amidon soluble était la plus basse (≈ 6090 SBU/µl) en opposition avec les autres matières premières. L'entomotoxicité baissait inversement avec le ratio carbone:azote dans le milieu de culture, alors que la concentration de conidies et l'activité protéolytique contribuaient aussi à l'entomotoxicité. Finalement, la formulation sèche de conidies de *Trichoderma* sp. (jusqu'à 19.2 kg de boues municipales déshydratées par litre de bouillon fermenté) était viable en termes de UFC résiduels (environ 70% viabilité après 1 mois à 25±1°C et environ 90% après 6 mois à 4±1°C). Ainsi, la production de conidies de *Trichoderma* sp. pourrait aider de façon appréciable à la commercialisation de biopesticides à coût moindre obtenus à partir de boues, ainsi qu'à la réduction de la charge polluante.

*Mots-clés*: Lutte biologique; Conidies; Entomotoxicité; *Trichoderma* spp.; Eaux usées; Boues.

**Summary**

In search of a suitable raw material to achieve high biocontrol activity, axenic cultivation of biocontrol fungus *Trichoderma viride* was conducted on a synthetic medium and different wastewaters and wastewater sludges in shake flasks. In particular, a soluble starch based synthetic medium, dewatered municipal sludge, cheese industry wastewater sludge, pre-treated and untreated pulp and paper industry wastewater and slaughter house wastewater were tested for *Trichoderma* spp. conidia and protease enzyme production. The maximum conidia production followed the order, soluble starch medium (> $10^9$ CFU/ml), untreated pulp and paper industry wastewater ($4.9\times10^7$ CFU/ml) > cheese industry wastewater ($1.88\times10^7$ CFU/ml) ≈ slaughter house wastewater ($1.63\times10^7$ CFU/ml) > dewatered municipal sludge ($3.5\times10^6$ CFU/ml) > pre-treated pulp and paper industry wastewater ($1.55\times10^6$ CFU/ml). The protease activity of *Trichoderma* spp. was particularly higher in slaughterhouse wastewater (2.14 IU/ml) and dewatered municipal sludge (1.94 IU/ml). The entomotoxicity obtained for soluble starch based synthetic medium was lower (≈ 6090 SBU/µl) in contrast to other raw materials. The entomotoxicity decreased inversely with carbon to nitrogen ratio in the growth medium and the conidia concentration and protease activity also contributed to the entomotoxicity. Finally, the dry formulation of *Trichoderma* sp. conidia (up to 19.2 kg dewatered municipal sludge per liter of fermented broth) was viable in terms of residual CFU (about 70% viability after 1 month at 25±1°C and about 90% after 6 months at 4±1°C). Thus, production of *Trichoderma* sp. conidia could appreciably help in marketability of low cost biopesticide from the sludge and safe reduction of pollution load.

*Keywords*: Biocontrol; Conidia; Entomotoxicity; *Trichoderma* spp.; Wastewater; Sludge.

## Introduction

Industrial wastewater treatment and in particular, sludge management is becoming an important environmental concern due to stricter regulations on sludge disposal. In this light, prevailing sludge treatment and disposal options, namely, aerobic and anaerobic biological treatment, incineration and land-filling are loosing ground due to secondary pollution issues and environmental and human health risks.

Meanwhile, infestation of forests and agriculture sector by several phytopathogens and consequential use or, overuse of harmful chemicals (herbicides, pesticides, fertilizers) is a major concern of present day consumers. This trend demands use of natural products like biocontrol agents and/or biopesticides. However, high production cost, lower product efficacy and stability have been major pitfalls limiting growth of biopesticides market. In fact, the biopesticides have captured a scant (about 1.4%) of overall world pesticides market (Dinham, 2005).

In this regard, utilisation of wastewater (Verma et al., 2005a) and wastewater sludge (Verma et al., 2005b) as potential raw materials for production of biocontrol fungi is a needful and eco-friendly venture. The literature also suggest that the fungi are important microorganisms due to their multi-utility (diverse) action (pest-control as well as plant growth promotion), which is inherently possessed by *Trichoderma* spp. (Herman et al., 1993). The *Trichoderma* spp. belongs to the class of saprophytes with the ability to survive on waste debris (Pelczar et al., 1993). Furthermore, lytic enzymes (e.g. protease, chitinase, cellulase, amylase, glucanase, Whipps and Lumsden, 1991;) and antimicrobial compounds (e.g. tricholin, trichodermin, 6-pentyl-α-pyrone, gliovirin, gliotoxin, viridian, and viridiol, Worasatit et al., 1994; Howell, 1982) produced by *Trichoderma* spp. are well documented by several researchers and play a major role in biocontrol and plant growth promotion (Lindsey and Baker, 1967). The *Trichoderma* sp. was also found to be active against commercial forest pest, spruce budworm (*Choristonuera fumiferana,* Lepidoptera: Tortricidae) (Verma et al., 2005b) .

The multi-utility and saprophytic nature of *Trichoderma* spp. and low cost or negative cost of raw materials were the basis of the hypotheses in the present study, whereby, cheese processing wastewater sludge (CH), slaughter house wastewater (SHW), treated pulp and paper industry wastewater (PPT), untreated pulp and paper industry wastewater

(PPN) and dewatered municipal sludge (DS) were investigated as potential raw materials for the growth of antagonist *Trichoderma viride*. Finally, formulation feasibility study was also conducted to confirm their commercialization potential.

## Material and methods

*Wastewaters, wastewater sludges and soluble starch medium*

Dewatered municipal sludge (DS) was procured from Black Lake, (Quebec, Canada) municipal wastewater treatment facility which consisted of secondary sludge from an aerobic biofilter reactor at ≈ 30% moisture and ≈ 2–2.5% filter aid. Cheese industry wastewater sludge (CH) was collected from an anaerobic reactor as spent biomass. Slaughterhouse wastewater (SHW) was obtained directly as the washing stream containing mainly low concentration of animal blood in water. The two types of pulp and paper industry wastewaters were taken from inlet (untreated – PPN) and outlet (treated – PPT) of a wastewater clarifier from a pulp and paper industry wastewater treatment site.

The viscosity of all wastewaters and wastewater sludges was measured by using a rotational viscometer (DVII+, Brookfield) equipped with small sample adapter spindle (SC4 34, Brookfield). DS, CH, PPN, PPT and SHW were used as sole nutrient source for *Trichoderma viride* and the physical characterization (Table 1) was determined as per standard methods (APHA, 1998). The conidia production potential of all wastewater and wastewater sludges was compared with a control consisting of soluble starch based synthetic medium (SSM) containing (g/l): soluble starch 30; $KH_2PO_4$ 2.5; $NH_4Cl$ 1.023; Urea 0.37; $MgSO_4.7H_2O$ 0.4; $CaCl_2.H_2O$ 0.3; and corn syrup 1.0.

*Solids amendment*

In the case of SSM, soluble starch concentration and for all wastewaters and wastewater sludges total solids (TS) were optimized to achieve maximum conidia concentration and biological activity. Most of the required nutrients are in general embedded in solids contained in wastewater or wastewater sludges. The TS concentration of DS, CH, PPN, PPT and SHW and soluble starch concentration of SSM were therefore varied in the range of 10–50, 3.8–20, 1.7–15, 1.3–15, 10–50 and 3.2–20 g/l respectively.

For solids amendments, the samples were centrifuged at 7650 g for 15 min at 20 ± 1°C. The supernatant was discarded and demineralized water was added to achieve appropriate concentrations.

*Microorganisms*

A commercial antagonist fungus, *Trichoderma viride* was used (Verma et al., 2005). The fungal strain was found to be antagonistic against phytopathogenic fungi, *Fusarium* sp. and *Cylindrocladium floridanum*, and spruce budworm (SB) insect larvae as examined in our laboratory.

*Starter culture, inoculum, sterilization and fermentation*

*Trichoderma viride* strain was maintained on potato dextrose agar (PDA) plates for 4-7 days under dark at 28 ± 1 °C (35 ± 2% relative humidity) as mother culture, subsequently maintained at 4 ± 1 °C and subcultured monthly. For starter culture, ≈ ½" x ½" scraped piece of 32-36 h old mycelial mat of subculture was used. The mycelial mat was aseptically homogenized in 4 ml sterile tryptic soya broth (TSB, Difco) medium with a Micro Tissue Grinder® (VWR, Canada). The grinder was submerged in ice bath in order to prevent heat build up. Afterwards, the homogenized mycelial mat was transferred to a 500 ml Erlenmeyer flask containing 150 ml sterile TSB (121 ± 1 °C for 15 minutes) medium at pH 6.0 ± 0.01. The shake flasks were incubated in a rotary shaker at 28 ± 1°C and 250 ± 5 rpm for 48 h and used freshly as inoculum for all wastewater and wastewater sludges.

All wastewater and wastewater sludge samples were taken in 500 ml shake flasks containing 150 ml sample volume. The pH of the samples was adjusted to 6.0 ± 0.1 and were autoclaved for 30 minutes at 121 ± 1 °C. After cooling to 28 ± 1 °C, the shake flasks were inoculated with 10% v/v tryptic soya broth (TSB) grown culture. Fermentation studies were carried out for 96 h at 28 ± 1°C and 250 ± 5 rpm and samples were collected at about 6–12 h intervals to measure conidia production (CFU/ml), protease enzyme activity, and insect bioassay.

*Microscopy*

All fermented samples were examined under a computer coupled optical microscope (Zeiss Axiolab) equipped with a digital camera (Axiocam HRC Zeiss). Moreover, contamination, if any, was verified by qualitative observation of all samples.

*Conidia counts*

The conidia concentration was measured in terms of CFU/ml fermented broth, using a modified conventional conidia assessment method (Verma et al., 2005). In this method, filtration of conidia is carried out through a single layer of an autoclavable fabric (≈ 10 µm pore size) so as to separate them from mycelial mass. The consistency of the modified technique was estimated by statistical analysis (Student's paired *t*-test, $P>0.20$).

*Protease activity*

The protease enzyme activity (PA) was measured in the centrifuged supernatant (7650 g for 20 min at 4 ± 1°C) of fermented broth. The supernatant was diluted 2× in borate buffer at pH 8.2 ± 0.01 and used as an enzyme aliquot. Modified method of Kunitz (1947) was employed for PA calculation in terms of IU/ml. The standard deviations were up to 7–8%, based on triplicate samples of two fermentation runs.

*Biological activity*

For the assessment of biocontrol potential of *Trichoderma* spp. fermented wastes, insect (Beegle, 1990) and fungal (Mischke 1997) bioassays were conducted. In fungal bioassay, the effect of extracellular metabolites of *Trichoderma* sp. on growth inhibition of pathogenic fungus is evaluated in terms of formation of mycelial mat by the test fungus. The fungal bioassay protocol was followed as per the method described by Verma et al. (2006). In this procedure, serially diluted ( 1/2, 1/4, 1/8 and 1/16) filtered (pore-size, 0.45 µm) fermented broth was used in 24-well tissue culture plates. The standard times for scoring, the ordinal scale of inhibition and the calculation equation were similar to those of the original study (Mischke 1997). The phytopathogen used in this study was *Fusarium* sp.

The entomotoxicity (Tx) test of the fermented samples was conducted on third instar eastern spruce budworm (SB) larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae),

provided by Natural Resources Canada (Sault Ste-Marie, Ontario). Sample preparation and diet protocol was carried out as per the method described by Brar et al. (2005). Industry standard contained spores and crystals of Bt at a potency of 20.1 x $10^9$ IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni*). On comparison of Tx of *Trichoderma* fermented sludge, SBU reported in this study was 20-25 % higher than IU. Tx of preparations was expressed in SB units/µl (SBU/µl) with 7–10 % of standard deviation.

Due to relative simplicity of fungal bioassay over insect bioassay, in this study, the former was used to decide optimal total solids concentration of fermentation medium.

*Formulation preparation and shelf-life*

The conidia formulation was prepared (5–40% final moisture content) by directly mixing *Trichoderma* sp. fermented broths of each wastewater and wastewater sludge with sterile lyophilized dry DS powder. For powder preparation, the DS was sterilized twice at 121 ± 1 °C for 45 minutes on two consecutive days and was examined under microscope for contamination before lyophilization. In order to minimize loss of conidia, the air drying process could be circumvented by manipulating the volume of fermented broths and initial moisture content of the sterile lyophilized dry DS. The desired final moisture content and volume of fermented broths were calculated as per Equations 1 and 2,

$$V = \frac{C_{Formulation}}{C_{Broth}} \text{ ml/g} \quad (1)$$

$$MC_{Initial} = MC_{Desired} \times (1 + V\rho_{FB}) - V(\rho_{FB} - s) \times 100\ \% \quad (2)$$

Where, $V$ is volume (ml) of fermented broth required per g of formulated powder; $C_{Formulation}$ (CFU/g), and $C_{Broth}$ (CFU/ml) are conidia concentrations, respectively, in formulated dry powder, and fermented broth; $MC_{Initial}$, and $MC_{Desired}$ are % moisture contents, respectively, of initial, and desired dry DS powder; $\rho_{FB}$ is density of fermented broth (g/ml), and $s$ is solids concentration (dry basis, g/ml).

In order to study shelf-life of formulation, polypropylene bottles containing formulated powder were kept at 4 and 25 ± 1°C and the formulated powder was cultured monthly on TSA petri plates for conidial viability and biological activity.

**Results and discussion**

Wastewater and wastewater sludge and soluble starch medium

The composition of various wastewaters and wastewater sludges are represented in Table 1. Irrespective of type of raw material, viscosity was higher (> 20 mPa.s) only in the case of TS > 20g/l, whereas, it was < 10 mPa.s for TS < 20g/l. Thus, DS (maximum conidia concentration at 30 g/l TS, Figure 1) and SSM (maximum conidia concentration at ≥ 30 g/l soluble starch in the fermentation medium, Figure 1) remained challenging media with respect to fermentation rheology and mass transfer.

The metal contents of CH, DS, PPN, and PPT media provided various trace metals known to be essential for growth of *Trichoderma* spp. (Papagianni, 2004). In general, C:N ratio is considered as most important for growth of microorganisms and their product formation capacity. Importantly, the C:N ratio varied in a wider range (3.0 to 21.36) for all wastewaters and wastewater sludges (Table 1). The C:N ratio of SHW was lowest amongst all wastewater and wastewater sludges (≈ 3.1 at 3.17 g/l TS concentration). Thus conidia production and biocontrol activity was also expected to be significantly different for all wastewater and wastewater sludges (discussed later).

Conidia production

All wastewater and wastewater sludges were suitable for cultivation of *Trichoderma* spp. as qualitatively (Figure 2) and quantitatively (Figure 1) shown by their vegetative growth and conidia production profiles at different TS concentrations, respectively. The microscopic observation showed active growth in an alternative medium (CH wastewater sludge) (Figure 2a) and a synthetic medium (SSM) (Figure 2b). The active phialides (conidia producing organelles of mycelia) in all wastewater and wastewater sludges were observed around 30–60 h (Figure 2c) of fermentation. Saprophytic nature of *Trichoderma* spp. (Pelczar et al., 1993) and production of lytic enzymes (discussed later) in abundance (Harman et al., 1993) were evident causes behind this phenomenon.

In order to obtain maximum conidiation, the conidia production was optimized with respect to TS concentration of all wastewaters and wastewater sludges (Figure 1). The conidia production of *Trichoderma viride* increased exponentially during 12 to 72 h, irrespective of the wastewater and wastewater sludge type (Figure 1). The exponential increase in fungal mycelia has been first demonstrated by Trinci (1974) and is a well

documented phenomenon. However, the current literature is scarce in kinetics of fungal conidia formation in submerged fermentation processes (Verma et al., 2005b), which is an important parameter for mass production of fungal biocontrol agents (Jenkins et al., 1998). The maximum conidia concentration (CFU/ml) in different media and TS concentration (soluble starch concentration, in case of SSM) at which maximum conidia occurred were in the order, SSM ($2.5 \times 10^{10}$ at 30 g/l soluble starch) > PPN ($1.92 \times 10^{8}$ at 5 g/l TS) > CH ($1.88 \times 10^{7}$ at 10 g/l TS) ≈ SHW ($1.63 \times 10^{7}$ at 10 g/l TS) > DS ($3.5 \times 10^{6}$ at 30 g/l TS) > PPT ($1.55 \times 10^{6}$ at 10 g/l TS). Although the conidia production was highest in SSM, yet the medium cost was high whereas, the biocontrol activity (discussed later) was low. Thus, the feasibility of SSM would need techno-economic evaluation study. In particular, kinetics of conidia formation of biocontrol fungi *Trichoderma* spp. suggested that when grown on raw material of cellulose origin (PPN and PPT), the conidia production increased ($\approx 10^{6}$ CFU/ml in PPT and $\approx 10^{8}$ CFU/ml in PPN) with C:N ratios 15.8 and 21.4, respectively (Figure 1 and Table 1). However, the vice-versa (conidia concentration decrease with C:N ratio) was observed on protein-rich wastes (CH, DS, and SHW). Thus, the origin of C and N (e.g., cellulose or, protein source) could be an important factor in deciding C:N ratio of a particular raw material for maximum conidia production.

Conidia concentrations > $10^{7}$ CFU/ml in literature are recommended for *Trichoderma* spp. mycopesticides (Batta, 2004). Therefore, the maximum conidia concentrations obtained in all wastewater and wastewater sludges suggested that PPN, CH, and SHW (> $10^{7}$ CFU/ml) could be directly used for *Trichoderma viride* conidia production for dry powder or, liquid formulation (containing ≈ $10^{7}$ CFU/g or, CFU/ml of *Trichoderma viride* conidia). Except DS and PPT, the other raw materials (CH, PPN, SSM, SHW) gave comparable conidia concentration (> $10^{7}$ CFU/ml) with that of pre-treated secondary wastewater sludge (Verma et al., 2005b). Meanwhile, DS could be used as an inert material for dry formulation development and PPT should be omitted as a raw material due to low conidia production (< $10^{6}$ CFU/ml). However, rest of the raw materials could be considered as suitable options for mass scale production.

Protease activity

The protease activity (PA) was measured at optimal TS concentration (soluble starch concentration, in case of SSM) for conidia production of all wastewater and wastewater sludges and SSM (Figure 3). The rest of TS concentration (soluble starch in case of SSM) were not analyzed for PA, as the PA at optimal solids concentration was always ≥ the rest of the solids concentration for conidia production as reported in the earlier study on *T. viride* conidia production (Verma et al., 2005). Except for PPN, and PPT, the PA reached maximum during 30 to 48 h of incubation in all media followed by continuous decrease until 96 h (Figure 1). Thus, the maximum PA was obtained during the exponential phase of conidiation. However, the decrease in protease activity (after reaching the maximum) until the end of fermentation could be due to lysis of enzyme under nutrient exhaustion (Tao et al., 1997), oxygen transfer limitation (Hoq et al., 1994; Purkarthofer et al., 1993a; Schafner & Toledo, 1992) and uncontrolled pH (Purkarthofer et al., 1993b).

Similar protease production profiles were obtained by Verma et al. (2005), while growing *Trichoderma viride* on secondary wastewater sludge in shake flasks. In this study, the protease activities (1.27 to 2.17 IU/ml for different media; Figure 3) were relatively higher than the value reported by Verma et al. (2005b; 0.26 to 0.79 IU/ml for pre-treated sludge) except for PPN and PPT. This could be due to the possibility that in case of DS, the protease enzyme was produced due to induction to overcome nutrient stress. Furthermore, for PPN and PPT the cellulose origin probably could not cause protease production. However, for rest of the raw materials, proteinacious origin could have induced protease enzyme production. Moreover, in the case of SSM, presence of abundant quantity of micronutrients might have caused higher protease production than secondary sludge studied by Verma et al. (2005b). Meanwhile, the protease activity was higher for wastewater and wastewater sludge with lower C:N ratio, i.e., SHW (3.13) > CH (5.76) ≈ DS (6.32) > SSM (30.32) > PPN (21.36) ≈ PPT (15.76). The protease activity was subdued towards the end of the fermentation process possibly due to the hydrolysis of the protease enzyme (could be due to the formation of smaller polypeptide units) (Harman et al., 1993).

Biocontrol activity

The mode of action of *Trichoderma viride* on spruce budworm larvae has never been studied to the best of author's knowledge. In the present study, the biocontrol activity of

*Trichoderma viride* was dependent on conidia concentration, C:N ratio of the medium, and maximum protease enzyme activity achieved for all wastewaters and wastewater sludges. Furthermore, in addition to conidia, action of lytic enzymes and antimicrobial chemicals produced by *Trichoderma* spp. could be postulated as probable factors. Moreover, the hydrolysis/degradation products of protease enzyme could also serve in synergizing the biocontrol activity. As discussed before, the hydrolysis products of protease as well as other enzymes (e.g., amylase, cellulose and chitinase) would result in polypeptides units, which have toxic (certain fungal toxins) and antibiotic properties (Lehninger et al., 1993; Creighton, 1984). It was also possible that the protease enzyme were temporarily inactivated during fermentation due to formation of inhibitory compounds, which later on became active during insect bioassay and played a key role in biocontrol activity. Therefore, it was expected that higher the maximum PA achieved, more hydrolysis/degradation products (polypeptides units/antibiotic compounds) were formed at the end of fermentation. This in turn could result in higher Tx with concomitant contribution by conidia concentration (Table 2).

In order to determine optimal TS concentration (soluble starch concentration, in case of SSM) with respect to biocontrol activity, fungal bioassay was performed (Figure 4). Based upon maximum inhibition index obtained in fungal bioassay, the optimal TS concentration (soluble starch concentration, in case of SSM) was selected for insect bioassay. The entomotoxicity (Tx) results on insect, spruce budworm larvae are shown in Table 2, which show SHW as most suitable raw material from antagonism point of view. The Tx in SHW (9806 SBU/µl) was lower than the pre-treated (thermal alkaline hydrolysis) secondary wastewater sludge (15036 SBU/µl) but was significantly > raw secondary wastewater sludge (6278 SBU/µl) (Verma et al., 2005b). However, the reduction in process step (e.g., hydrolysis step) could be helpful in overall process economy. In other words, use of untreated raw materials in this study in contrast to pre-treated secondary wastewater sludge would not require thermal alkaline hydrolysis step. Nevertheless, their biocontrol activity in terms of entomotoxicity was far lower (≈20–50%) than raw and pre-treated secondary wastewater sludges. Thus, as far as sludge is concerned, secondary wastewater sludge could be considered as a better option for biocontrol. From Table 2, it is evident that the Tx decreased with C:N increase (Table 1) with exceptions of SSM and PPN media. Notably,

SSM had very high conidia concentration and comparatively lower Tx, whereas, PPN had lower protease activity and competitive Tx. Therefore, in order to achieve higher biocontrol activity, a medium supporting higher conidia concentration with lower C:N ratio should be preferred.

Formulation efficacy

The storage feasibility of *Trichoderma* spp. produced by the present fermentation process was assessed by measuring the viability of *Trichoderma viride* conidia in formulated phase under different storage conditions (Batta, 2004). In order to make powder formulation, the fermented broth of CH, PPN, SSM and SHW (conidia > $10^7$ CFU/ml) were added in appropriate quantity to sterile lyophilized DS (at ≈ 5% $MC_{Initial}$) as per Equations 1 and 2 to $MC_{Desired}$ (up to 30 %). Thus, in order to adjust final moisture content the drying step of formulation was circumvented so that the loss in viability should be minimized. The formulated powder of all wastewater and wastewater sludge were stored at 25 and 4 ± 1 °C, for ≈ 6 months. The percentage viability of conidia on PDA culture for 25 and 4 ± 1 °C during the storage period is shown in Figure 5. The conidial viability decreased to 27 and 45%, respectively, at 25 and 4 ± 1 °C at the end of 6 months. Nevertheless, the formulation could be amended with stabilizing agents for better storage stability (Lisansky et al., 1993, Burges, 1998). Based on Equations 1 and 2, it was estimated that for a formulation (used in green house application) of about $10^7$ CFU/g dewatered sludge powder (≈ 40% moisture content), the amount of dewatered sludge required will be ≈ 1.63 − 19.2 kg per liter of fermented broths of all investigated media (SSM, CH, SHW, PPN, PPT, DS).

Thus, this study sets the base for screening of different alternative raw materials for the production of a broad-spectrum biopesticide, namely, *Trichoderma viride*. Furthermore, production of *Trichoderma* based biocontrol agents will serve as precedent for development of similar alternative/reusable raw material based viable technologies. In fact, the proposal of wastewater sludge as one of the major ingredients (used in large quantities) during drying and formulation makes the production technology more promising as it would affect overall economics and also create green products for a cleaner planet. Globally, value-addition of wastewater and/or wastewater sludge will aid in

sustainable sludge management, sequestration of carbon (mitigate greenhouse gas emissions), proliferation of eco-friendly pest control options (biocontrol agents) and promote social responsibility.

**Conclusions**

The explored wastewaters and wastewater sludges were suitable for *Trichoderma viride* conidia production by submerged fermentation process. In particular, pulp and paper industry wastewater–untreated, cheese industry wastewater, and slaughter house wastewater were suitable for conidia production $\geq 10^8$ CFU/ml and higher entomotoxicity (6265–9806 SBU/µl). On the other hand, pulp and paper industry wastewater-treated and dewatered municipal sludge cannot be used on the basis of poor conidia production ($3.5 \times 10^6$ CFU/ml) and entomotoxicity (5563 SBU/µl). However, dewatered municipal sludge proved to be an efficient inert medium for dry powder formulation. Meanwhile, higher conidia production but lower entomotoxicity of soluble starch medium rendered its feasibility questionable due to its high cost. The optimal total solids concentration (soluble starch concentration, in case of soluble starch medium) of wastewater and wastewater sludges with respect to conidia production were dependent on type of raw material. Thus, production of *Trichoderma viride* conidia in wastewater and wastewater sludge followed by their formulation in dewatered sludge could appreciably help in safe reduction of pollution load.

**Abbreviations:** CH, cheese processing wastewater; SHW, slaughter house wastewater; PPT, treated pulp and paper industry wastewater, PPN; untreated pulp and paper industry wastewater; DS, dewatered municipal sludge; SSM, soluble starch medium; TS, total solids; SB, spruce budworm; PDA, potato dextrose agar; TSB, tryptic soya broth; TSA, tryptic soya agar; CFU, colony forming unit; PA, protease activity; Tx, entomotoxicity; IU, international unit; SBU, spruce budworm unit;

## ACKNOWLEDGEMENTS

The authors are thankful to 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.

## REFERENCES

APHA–AWWA–WPCF 1998. Standard Methods for Examination of Water and Wastewaters. 20th edn. (Eds. Clesceri, L.S., Greenberg, A.E. and Eaton, A.D.), American Public Health Association, Washington, DC, USA.

Batta, Y.A. 2004. Effect of treatment with *Trichoderma harzianum* Rifai formulated in invert emulsion on postharvest decay of apple blue mold. *International Journal of Food Microbiology* **96**, 281–288.

Beegle, C.C. 1990. Bioassay methods for quantification of *Bacillus thuringiensis* δ–endotoxin, Analytical Chemistry of *Bacillus thuringiensis*, In: *Analytical Chemistry of Bacillus thuringiensis* (Eds. Hickle L.A. and Fitch W.L.), American Chemical Society, pp. 14–21.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R. & Surampalli, R.Y. 2005. Starch Industry Wastewater-Based Stable Bacillus thuringiensis Liquid Formulations. *Journal of Economic Entomology* **98,** 1890–1898.

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

Creighton, T.E. 1984. Proteins: Structures and Molecular Properties, W.H. Freeman and Company, New York.

Dinham, B. 2005. Agrochemical markets soar – pest pressures or corporate design? *Pesticides News* **68,** 9–11.

Harman, G.E., Haynes, C.K., Lorito, M., Broadway, R.M., di Pietro, A., Peterbauer, C. & Tronsmo, A. 1993. Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase. *Phytopathology* **83,** 313–318.

Hoq, M.M., Hempel, C. & Deckwer, W.D. 1994. Cellulase-free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration, and medium components on production. *Journal of Biotechnology* **37,** 49-58.

Howell, C.R. 1982. Effect of *Gliocladium virens* on *Pythium ultimum*, *Rhizoctonia solani*, and damping–off of cotton seeds. *Phytopathology* **72,** 496–498.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J. & Lomer, C.J. 1998. Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information* 19(1), 21N-31N.

Kunitz, M. 1947. Crystalline soybean trypsin inhibitor. *Journal of General Physiology* **30,** 291–310.

Lehninger, A.L., Nelson, D.L. & Cox, M.M. 1993. Principles of biochemistry. 2 ed. New York, NY, Worth Publishers, pp. 126–130.

Lindsey, D.L. & Baker, R. 1967. Effect of certain fungi on dwarf tomatoes grown under gnotobiotic conditions. *Phytopathology* **57,** 1262–1263.

Lisansky, S.G., Quinlan, R.J., & Tassoni, G. 1993. The *Bacillus thuringiensis* Production Handbook, CPL Press, Newbury, pp.124.

Papagianni, M. 2004. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances* **22**, 189–259.

Pelczar Jr., M.J., Chan, E.C.S. & Kreig, N.R. 1993. In: *Microbiology*, 5th ed., Tata McGraw–Hill, Inc. New York, ISBN 0–07–049234–4.

Purkarthofer, H., Sinner, M. & Steiner, W. 1993a. Cellulase-free xylanase from *Thermomyces lanuginosus*: Optimization of production in submerged and solid-state culture. *Enzyme and Microbial Technology* **15**, 677-682.

Purkarthofer, H., Sinner, M. & Steiner, W. 1993b. Effect of shear rate and culture pH on the production of xylanase by *Thermomyces lanuginosus*. *Biotechnology Letters* **15,** 405-410.

Schaffner, D.W. & Toledo, R.T. 1992. Cellulase production in continuous culture by *Trichoderma reesei* on xylose-based media. *Biotechnology and Bioengineering* **39**, 865-869.

Tao, S., Beihui, L. & Zuohu, L. 1997. Enhanced Cellulase Production in Fed-Batch Solid State Fermentation of *Trichoderma viride* SL-1. *Journal of Chemical Technology & Biotechnology* **69**(4), 429–432.

Trinci, A.P.J. 1974. A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. *Journal of General and applied Micrbiology* **81**, 225–236.

Verma, M., Brar, S.K., Tyagi, R.D., Valéro, J.R. & Surampalli, R.Y. 2005. Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. *Water Research* **39**(15), 3587–3596.

Whipps, J.M. & Lumsden, R.D. 2001. Commercial use of fungi as plant disease biological control agents: status and prospects. In: *Fungal Biocontrol Agents: Progress;, Problems and Potential,* (eds T. Butt, C. Jackson & N. Magan), Wallingford: CABI Publishing, pp 9–22.

Worasatit, N., Sivasithamparam, K., Ghisalberti, E.L. & Rowland, C. 1994. Variation in pyrone production, lytic enzymes and control of *Rhizoctonia* root rot of wheat among single–spore isolates of *Trichoderma koningii*. *Mycological Research* **98,** 1357–1363.

*Table 1.* Wastewater and wastewater sludge composition

| Parameter | Concentration (mg/kg; unless stated, otherwise) | | | | |
|---|---|---|---|---|---|
| | **DS** | **CH** | **PPN** | **PPT** | **SHW** |
| Total solids (g/l) | 30.0 | 3.8 | 1.7 | 1.3 | 3.2 |
| Total volatile solids (g/l) | 12.9 | 2.3 | 1.2 | 1.2 | 2.6 |
| Suspended solids (g/l) | 28.9 | 1.8 | 0.6 | 0.1 | 1.4 |
| Suspended volatile solids (g/l) | 12.3 | 0.5 | 0.3 | 0.1 | 0.9 |
| Viscosity (mPa.s) | 50.4 | 4.8 | 1.0 | 1.0 | 1.5 |
| Total carbon | 303000 | 219000 | 235000 | 63000 | 273000 |
| Total nitrogen | 48000 | 38000 | 11000 | 4000 | 87300 |
| C:N ratio | 6.3 | 5.8 | 21.4 | 15.8 | 3.1 |
| Total phosphorus | 7272 | 5317 | 510 | 559 | 1514 |
| $N\text{-}NH_3$ | 444 | 1506 | 164 | 92 | 115 |
| $N\text{-}NO_2$, $N\text{-}NO_3^-$ | 7.9 | 119.8 | 233.3 | 214.7 | 177.8 |
| $P\text{-}PO_4^{3-}$ | 132 | 2059 | 383 | 453 | 389 |
| Al | 1837 | 36787 | 10584 | 6143 | 3802 |
| Ca | 62000 | 7900 | 341000 | 554000 | 371000 |
| Cd | 2.7 | 16.4 | 34.4 | 66.6 | 52.5 |
| Cr | 26.5 | 98.6 | 28.8 | 44.0 | 49.0 |
| Cu | 83.2 | 139.4 | 291.8 | 386.4 | 309.0 |
| Ni | 96.0 | 409.9 | 122.4 | 175.4 | 154.9 |
| Fe | 11333 | 12592 | 9773 | 8739 | 5623 |
| K | 274.0 | 517.0 | 237.3 | 457.9 | 354.8 |
| Pb | 14.09 | 36.35 | 44.54 | 56.94 | 48.98 |
| Mn | 258.3 | 46.7 | 278.2 | 3790.5 | 3020.0 |
| S | 300 | 6600 | 7000 | 16900 | 9550 |
| Zn | 991 | 4980 | 8850 | 10623 | 3802 |
| Na | 587 | 2700 | 4070 | 14597 | 5623 |

*Table 2.* Maximum conidia concentration, maximum PA and Tx (96 h) of *T. viride* in different raw materials.

| Raw Material (TS concentration) | Maximum conidia ($10^7$ CFU/ml) | Maximum PA (IU/ml) | Tx (SBU/µl) |
|---|---|---|---|
| **DS (30)** | 0.35 | 1.98 | 5563 |
| **CH (10)** | 1.88 | 1.57 | 7541 |
| **PPT (10)** | 19.20 | 0.39 | 4045 |
| **PPN (5)** | 0.16 | 0.44 | 6265 |
| **SSM (30*)** | 2500.00 | 1.31 | 6090 |
| **SHW (10)** | 1.63 | 2.17 | 9806 |

* soluble starch concentration, in case of SSM

**List of Figures**

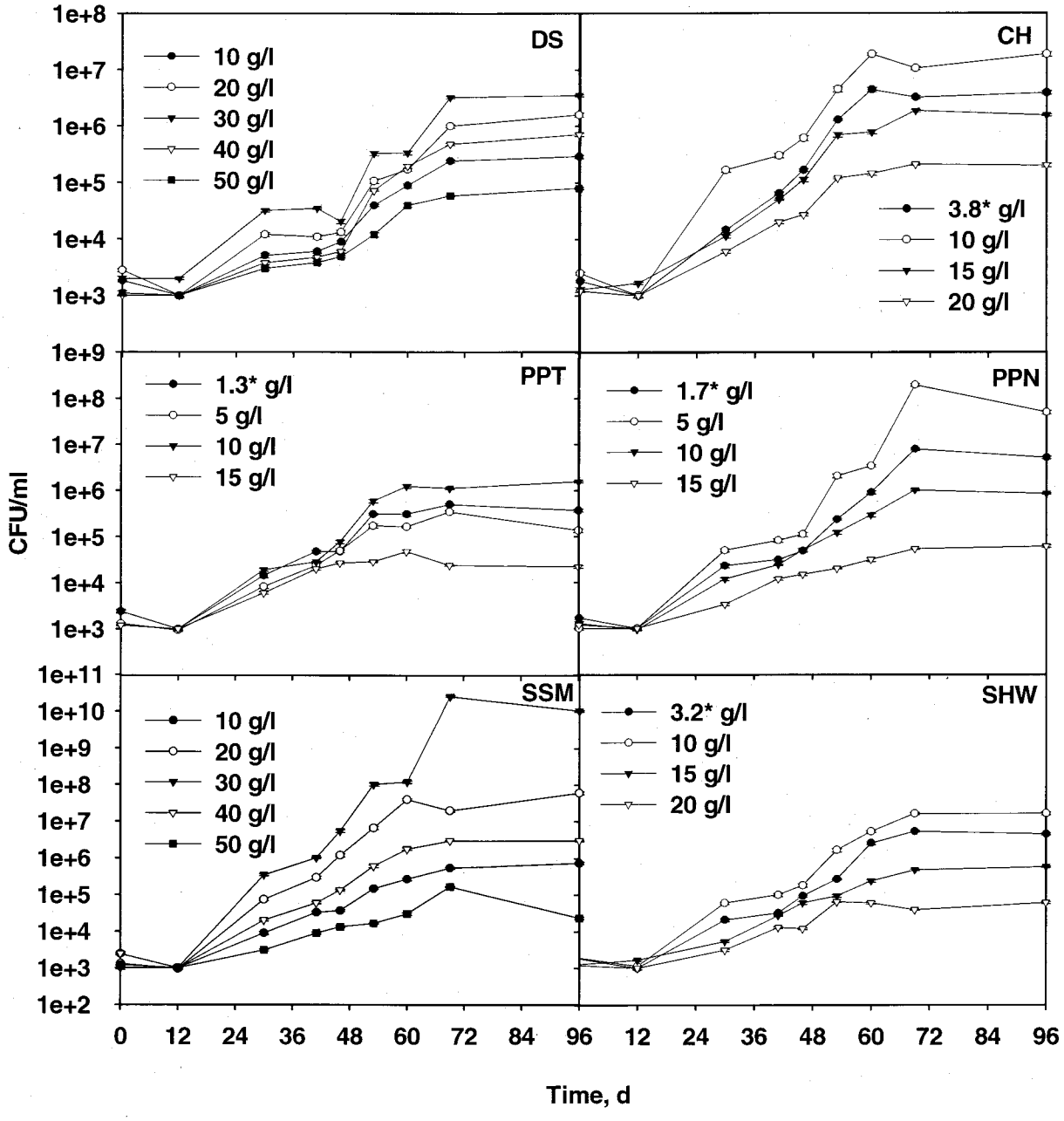



*Figure 1.* Optimization of TS concentration (soluble starch concentration, in case of SSM) for *Trichoderma viride* conidia production in different raw materials. (* TS concentration of the sample collected)

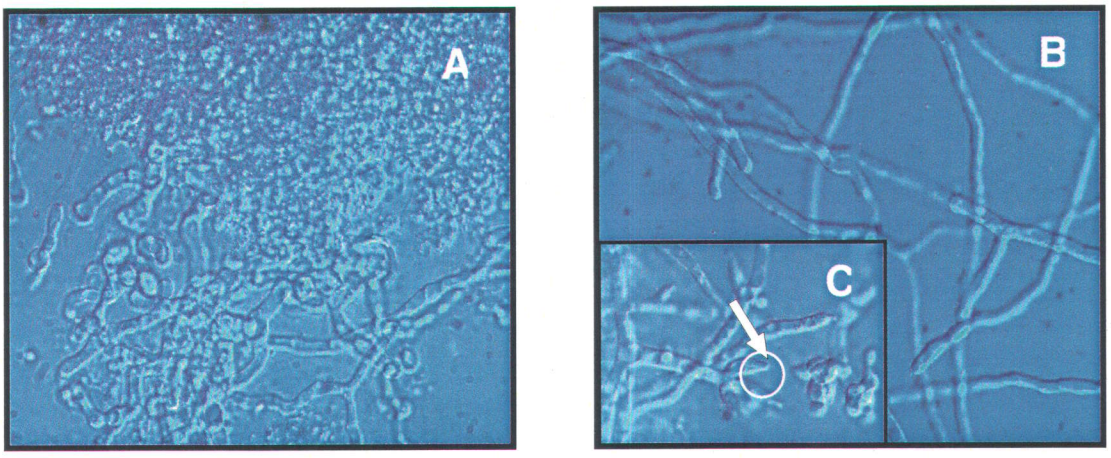



*Figure 2. Trichoderma viride* growth in: (A) alternative medium (e.g., cheese industry wastewater) and; (B) soluble starch medium (synthetic medium, control). Inset (C), shows conidia producing organelles, "phialides" – the arrow indicates a conidia forming at the tip of a phialide.

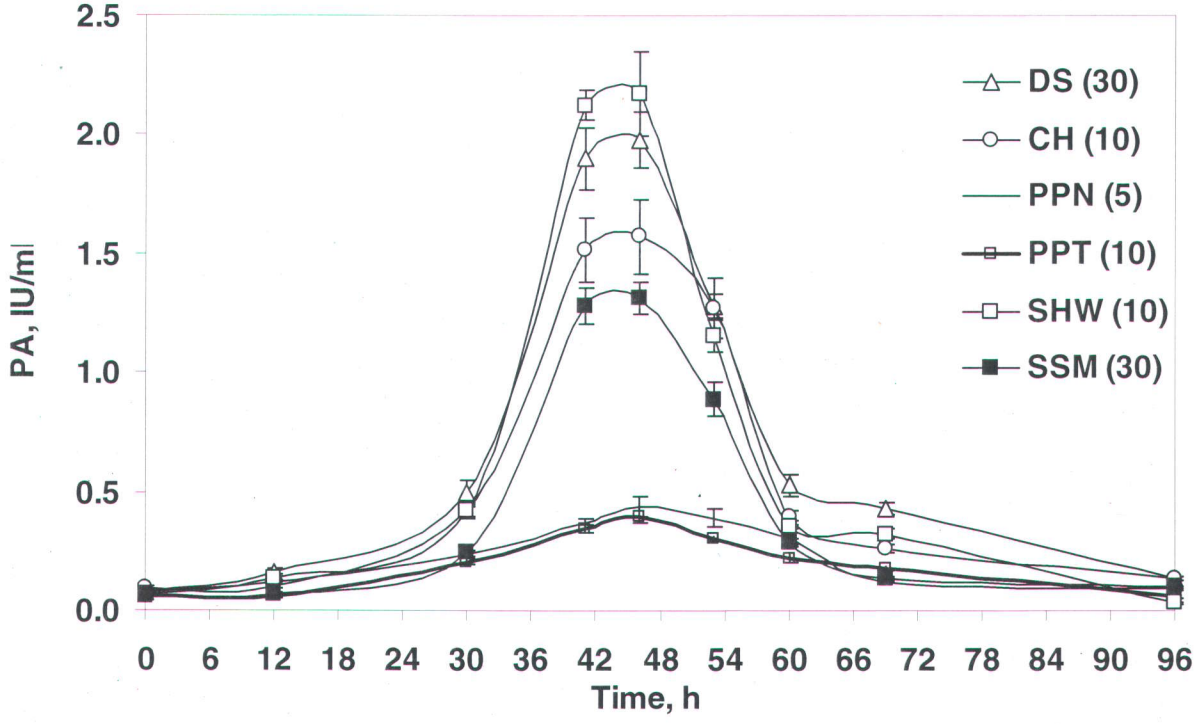



*Figure 3.* Protease activity of *Trichoderma viride* in different raw materials. Values in parentheses show TS concentration (soluble starch concentration, in case of SSM).

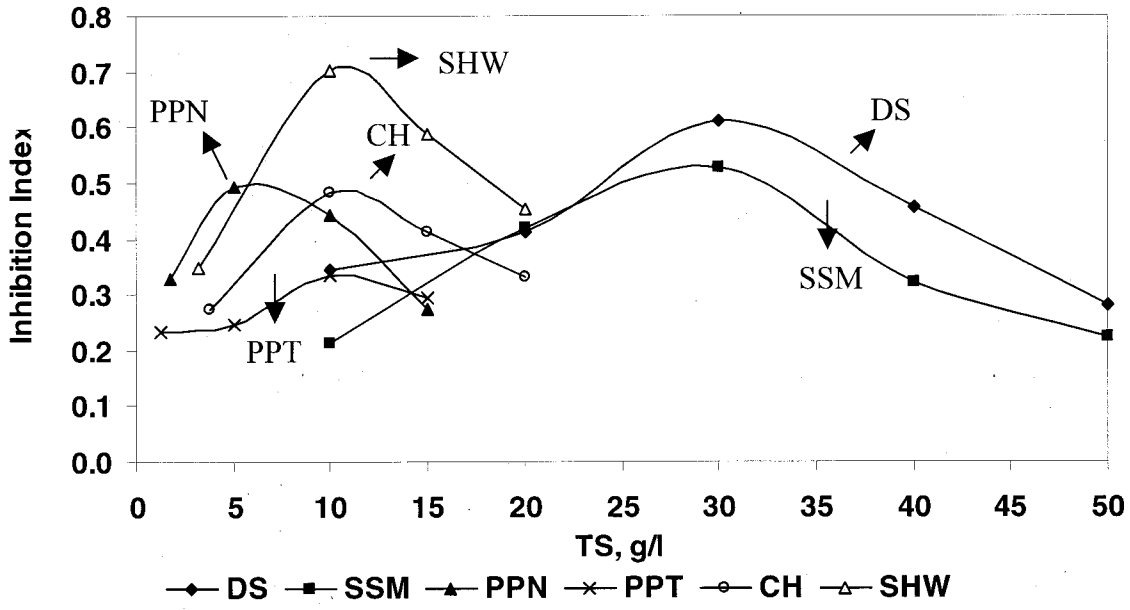



*Figure 4.* Optimal TS concentration (soluble starch concentration, in case of SSM) with respect to inhibition index of 96 h *Trichoderma viride* fermented samples of all raw materials.

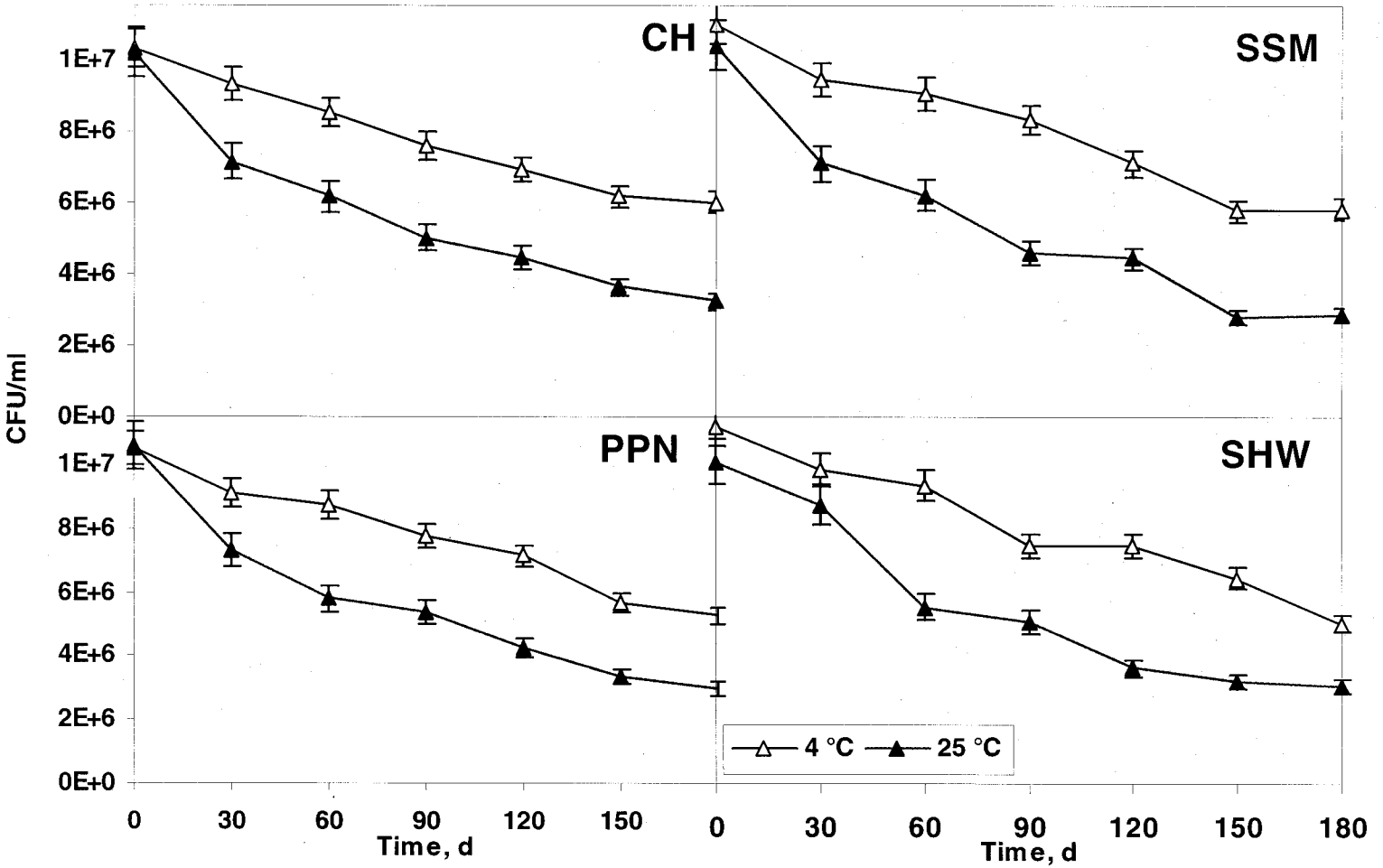



*Figure 5.* Shelf-life stability of *Trichoderma viride* conidia in the DS sludge formulation of different raw materials.

## Part II

# Starch industry wastewater as a substrate for antagonist, *Trichoderma viride* production

**M. Verma[1], Satinder K. Brar[1], R. D. Tyagi[1,*], R. Y. Surampalli[2], and J. R. Valéro[1,3]**

[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

[3]Centre Canadien des Forêts, Centre Foresterie des Laurentides 1055 du P.E.P.S.,

P.O. Box 3800, Sainte-Foy, Québec Canada, G1V 4C7

**Bioresource Technology (2006, 98, 2154–2162. DOI:10.1016/j.biortech.2006.08.032)**

## Les eaux usées de l'industrie de l'amidon comme un substrat pour la production de l'antagoniste *Trichoderma viride*

**Résumé**

Les eaux usées de l'industrie de l'amidon ont été étudiées pour évaluer et améliorer leur potentiel comme matière première pour la production de conidies du champignon *Trichoderma viride* utilisé en lutte biologique. Les eaux usées ont été testées avec et sans supplément de glucose, d'amidon soluble, de peptone de viande et d'inducteurs chimiques probables de la conidiation dans des cultures en fioles. L'addition d'une source de carbone complexe (amidon soluble, 1 et 2% p/v) a permis d'obtenir le maximum de conidies (≈ 3.02 et $4.2 \times 10^{10}$ UFC/ml, respectivement). D'un autre côté, l'addition de glucose comme une simple source de carbone était soit inefficace ou réduisait la production de conidies (de $1.6 \times 10^{8}$ dans le contrôle à $3.0 \times 10^{7}$ CFU/ml avec un supplément de glucose de 5% p/v). Le supplément de sources d'azote a donné une faible augmentation de la concentration en conidies. Les acides humique, propionique et maléique, l'EDTA, la pyridine, le glycérol et le $CaCO_3$ ont été étudiés comme des inducteurs probables de la conidiation et ont eu seulement des effets sur le taux initial de conidiation sans augmenter la concentration finale de conidies. La corrélation de l'ATP intra et extra-cellulaire avec la production de spores dépendait des milieux de culture et de la concentration de conidies à la fin de la fermentation. L'addition de sources de carbone et d'azote a permis d'augmenter l'activité protéolytique (de 0.4985 to 2.43 UI/ml) et l'entomotoxicité (de 10448 à 12335 SBU/µl). L'entomotoxicité a été améliorée de 11% en fermenteur par rapport aux fioles lorsque les eaux usées de l'industrie de l'amidon étaient additionnées de peptone de viande.

*Mots-clés:* Agent de lutte biologique, conidies, entomotoxicité, activité protéolytique, eaux usées de l'industrie de l'amidon, *Trichoderma* sp.

## Starch industry wastewater as a substrate for antagonist, *Trichoderma viride* production


### Abstract

Starch industry wastewater was investigated to assess and improve its potential as a raw material for the conidia production of biocontrol fungi, *Trichoderma viride*. The wastewater was tested with and without supplements of glucose, soluble starch, meat peptone and probable conidiation inducer chemicals in shake flask culture. Addition of complex carbon source (soluble starch, 1 and 2% w/v) produced maximum conidia (≈ 3.02 and $4.2 \times 10^{10}$ CFU/ml, respectively). On the other hand, glucose addition as a simpler carbon source was either ineffective or, reduced conidia production (from $1.6 \times 10^{8}$ in control to $3.0 \times 10^{7}$ CFU/ml in 5% w/v glucose supplement). Supplement of nitrogen source showed a small increase of conidia concentration. Propionic, maleic and humic acids, EDTA, pyridine, glycerol and $CaCO_3$ were examined as probable conidiation inducers and showed effect only on initial rate of conidiation with no increase in final conidia concentration. Intra and extracellular ATP correlation with spore production showed dependence on growth media used and conidia concentration at the end of fermentation. Addition of carbon and nitrogen sources showed an increase in protease activity (from 0.4985 to 2.43 IU/ml) and entomotoxicity (from 10448 to 12335 spruce budworm unit (SBU)/µl). Entomotoxicity was improved by 11% in fermenter over shake flask when starch industry wastewater was supplemented with meat peptone.


*Keywords:* Biocontrol agent, conidia, entomotoxicity, protease activity, starch industry wastewater, *Trichoderma* sp.

## 1. Introduction

*Trichoderma* spp. have gained wide acceptance as effective biological control agents (BCAs) against several commercial phytopathogens (Whipps and Lumsden, 2001). These antagonistic fungi are most common among fungal biocontrol agents due to their multiple BCA characteristics, namely, antagonism and plant growth stimulation (Punja and Utkhede, 2003). Thus, mass scale production of *Trichoderma* spp. would have great potential for commercial use. Micropropagules of *Trichoderma* spp. in the form of conidia are preferred over chlamydospores and mycelial biomass due to viability and stability in field application (Amsellem et al., 1999). Therefore, there are several BCA products of *Trichoderma* spp. in market containing conidia of *Trichoderma* spp. as active ingredients. Multiple BCA action renders the production of *Trichoderma* spp. conidia of commercial and environmental interest.

There is abundant literature on use of conventional synthetic media like, glucose, cellulose, soluble starch, molasses for production of *Trichoderma* spp. (Lewis and Papavizas, 1983; Gupta et al., 1997). However, conventionally used costly raw materials for commercial production of BCAs is one of the major limitations behind the restricted use. In order to overcome the cost limitation, many researchers have successfully used substrates like corn fibre dry mass (Vlaev et al., 1997), sewage sludge compost (Cotxarrera et al., 2002), and cranberry processing waste (Zheng and Shetty, 1998). Despite the use of these alternate sources, the cost of production was still high as these raw materials were required to be supplemented with other nutrients. In one of our earlier studies (Verma et al., 2005), we have already investigated the use of wastewater sludge (raw and pre-treated) for the growth of *Trichoderma* spp. where the spore production was of the order of $10^7$ CFU/ml. Still, even though wastewater sludge is a good source of nutrients for *Trichoderma* sp. production, use of alternative carbohydrate rich waste material like starch industry wastewater (SIW) could further broaden this approach.

To the best of our knowledge, there is no study reported so far on utilization of SIW for *Trichoderma* spp. conidia production. This study thus entails an environment friendly way to simultaneously reduce pollution load and lead to value-addition of wastes. In this light, objectives of the present study comprised investigation of starch industry wastewater as a raw material for *Trichoderma* sp. production with and without fortification

of nutrients (C and N sources) to augment spore concentration and biocontrol activity. In addition, total and dissolved ATP were also measured in an attempt to correlate the ATP concentration with spore concentration during fermentation.

Furthermore, many researchers have mentioned different class of chemicals as sporulation inducers, such as, organic acids, metal ions, and antimicrobial agents (Pascual, 1997; Roncal, 2002; Papagianni, 2004). Therefore, we examined some chemicals such as organic acids (propionic, maleic, and humic acids), metal ion (as $CaCO_3$), antimicrobial agents (EDTA, pyridine), and higher alcohol (glycerol) as probable supplement chemical agents to improve induction of conidiation in SIW.

## 2. Methods

### *2.1 Chemicals*

All chemicals used for analyses were of analytical grade, obtained from Sigma-Aldrich or BDH. Chemicals used as media additives were of laboratory grade.

### *2.2 Raw Material*

Starch industry wastewater was procured from a local starch industry (ADM Ogilvie, Candiac, Quebec) based on wheat starch processing. The physiochemical characteristics of SIW are presented in Table 1, which has metal concentrations in accordance with Québec Govt. guidelines for agricultural application (MENV, 2004). The total solids (TS) and dissolved solids (DS) concentration in sampled SIW were 17.3 g/l and 14.9 g/l, respectively, measured as per APHA (1998). The SIW was stored for a maximum of 3 weeks at $4 \pm 1$°C to minimize microbial growth.

In order to increase conidia concentration, SIW was fortified with different carbon (glucose and soluble starch) and nitrogen (meat peptone) sources. In addition, 1 g/l of each chemical agent one at a time (glucose, soluble starch, meat-peptone, $CaCO_3$, humic acid, glycerol, propionic acid, EDTA, maleic acid and pyridine) was added to SIW to induce sporulation in separate treatments. The medium pH was adjusted to 6.0 before sterilization.

*2.3 Starter Culture and Inoculum*

A commercial strain of *Trichoderma viride* (Verma et al., 2005) was used. The strain was isolated from soil and was found to be active against phytopathogenic fungi, *Fusarium* sp. and *Cylindrocaldium floridanum* and spruce budworm (SB) insect larvae as tested in our laboratory. The starter culture and the inoculum were similar to our previous study (Verma et al., 2005).

*2.4 Fermentation (Shake Flask and Bench Top Fermenter)*

Erlenmeyer flasks (500 ml) containing 135 ml of SIW (with, without supplements) were inoculated with 15 ml *T. viride* inocula prepared in tryptic soya broth (TSB), (Difco Inc., Ontario). The flasks were then incubated in a rotary shaker at 28 ± 1°C and 250 ± 5 rpm for 96 h. The samples were drawn aseptically at regular intervals and stored at 4 ± 1°C for subsequent analyses.

Fermenter studies were carried out on 1% meat peptone supplemented SIW in a bench top fermenter (Labfors III, Infors, Switzerland). The fermenter was equipped with automated programmable logic control for pH, agitation, anti-foam, dissolved oxygen, and temperature integrated with data acquisition software (IRIS version 5.01; Infors, Switzerland). During fermentation, similar operational parameters were maintained as in flask scale studies (inoculation age and volume, pH and temperature). Additionally, the dissolved oxygen was maintained at ≥ 30% throughout the experiment based on earlier studies (Verma et al., 2006) to meet the oxygen requirement.

*2.5 Direct Spore Assessment*

The fungal spores were separated from wastewater particulates and mycelial biomass and quantified as per the method described earlier (Verma et al., 2005). Five replicates for three different dilutions were used for enumeration by using an automatic Petri plater (Whitley Automatic Spiral Plater 2, Fisher Scientific, Quebec, Canada). Standard deviation for CFU count was 8-10 %.

*2.6 Indirect Spore Assessment – Total and Soluble ATP Measurement*

Test of extracellular and intracellular ATP measurements could provide relationship between the cellular ATP and the fungal spore concentrations (Eiland, 1983; Gikas and Livingston, 1993). Hence, dissolved and total ATP measurements were carried out in fermentation samples. This analysis is based on luciferin-luciferase enzyme reaction as a measurement of the light produced when an ATP solution is mixed with luciferase and placed in a luminometer (LKB Wallac 1251; LKB, Turku, Finland). For this, a protocol supplied by LuminUltra Technologies Ltd., Fredericton, NB, Canada was followed. The supplier provided a complete measurement kit for total and dissolved ATP, including following reagents; luciferin-luciferase mixture, buffer to release ATP from biomass, buffer to dilute ATP extracts of biomass, dissolved ATP stabilizing agent, and ATP standard solution (1 ng ATP/ml).

*2.7 Microscopy*

Approximately 10-50 µl samples were taken for smear preparation. Observation of mycelia and conidia, and contamination, if any, was carried out using a computer coupled optical microscope (Zeiss Axiolab) equipped with a digital camera (Axiocam HRC Zeiss).

*2.8 Protease activity (PA)*

Supernatant of samples were obtained by centrifugation at 7650 g for 20 min at 4 ± 1°C. The supernatants were used as enzyme aliquots, appropriately diluted in borate buffer at pH 8.2 ± 0.01. Modified Kunitz (1947) method was employed for PA determination. The protease activity was measured in terms of international units (IU), which is defined as the amount of enzyme which releases 1 mmol of tyrosin per minute from casein as substrate. The standard deviation was 8–9%, based on triplicate samples of two fermentation runs.

*2.9 Soluble Chemical Oxygen Demand (SCOD)*

The SCOD of fermentation samples was measured by closed reflux method as per Standard Methods (APHA, 1998). The standard deviation was 5–8%, based on triplicate samples of two fermentation runs.

*2.10 Insect Bioassay*

The insect bioassay was conducted on eastern spruce budworm (SB) larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae), provided by Natural Resources Canada (Sault Ste-Marie, Ontario). The entomotoxicity (Tx) of samples was determined by diet incorporation method (Beegle, 1990). Sample preparation and diet protocol was carried out as per the method described earlier by Brar et al. (2005). An industrial standard *Bacillus thuringiensis* based formulation, equivalent to a potency of 20.1 x $10^9$ IU/l (International Unit/l) was measured against cabbage looper larvae (*Trichoplusia ni*). The Tx of *T. viride* fermented sludge reported in this study was measured against spruce budworm larvae due to availability and compared with cabbage looper larvae. The Tx as spruce budworm unit (SBU) was observed to be 20-25 % higher than Tx as cabbage looper larvae unit (IU). The Tx of fermented broths were expressed in SBU/µl with 9-10 % of standard deviation. Twenty replicates of three different dilutions of samples in two fermentations runs were analyzed.

*2.11 Statistical Analysis*

The data obtained for direct spore and Tx measurements were analyzed in Microsoft Excel 2003 worksheet by using one-way analysis of variance (ANOVA) at $P = 0.05$ (Chatfield 1983). In case of ATP, PA and SCOD measurements significant difference between two samples was measured by using Student's paired *t*-test at $P < 0.05$ in Microsoft Excel 2003.

## 3. Results and Discussion

*3.1 SIW and Glucose-Supplemented SIW*

The maximum CFU concentration in SIW without any supplement was of the order of $\approx 10^8$ CFU/ml fermented broth (Figure 1). There are several evidences that glucose is easily metabolized carbon source for *Trichoderma* spp. (Harmann and Björjmann, 1998). Additionally, variation in C:N ratio has been reported by many researchers in enhancing conidiation in fungi (Papagianni, 2004). Therefore, SIW was supplemented with glucose concentrations (5 – 50 g/L) to vary C:N ratio (approximately, 12 to 61) to assess effect on conidiation (Figure 1). At 5 g/L glucose supplement, there was no significant effect on conidiation, which might be due to very small increment in carbon source for *T. viride*. It

was found that at up to 20 g/L glucose-supplement, conidiation was only retarded (low conidiation, between 0-60 h) with no net change in final conidia concentration (at 96 h). At 50 g/L glucose-supplement, final conidia concentration was adversely affected. It exhibited a net decrease in CFU ($\approx 10^7$ CFU/ml) with respect to non-supplemented SIW ($\approx 10^8$ CFU/ml) ($F = 0.526$; df = 2, 12; $P < 0.05$). The decrease in conidiation with addition of glucose could be owing to enhanced vegetative growth as shown in micrographs (Figure 2). In addition to micrographs, the addition of glucose resulted in lowering of final (96 h) pH with glucose ($\approx$ 6.2–6.9 $\pm$ 0.01) than without glucose (8.42 $\pm$ 0.01). Thus, sufficient availability of carbon leads to favorable condition for vegetative growth and lesser spore production (Figure 1a). Soluble COD consumption profile and residual COD at 96 h also supported the fact that nutrients (carbon) were present in abundance (Figure 3). Further, since no micro nutrients were supplemented along with glucose (essential trace minerals, Table 1) to SIW, it was possible that in order to build up mycelial biomass (during vegetative growth), the pre-existing micro nutrients in SIW (Table 1) exhausted. Consequently, the conidia concentration diminished (Figure 1).

*3.2 Meat Peptone-Supplemented SIW*

In order to examine another strategy for enhancing conidiation, SIW was further supplemented with meat peptone (C:N $\approx$ 3), thereby, changing C:N ratio as well as source of N as suggested in many studies for *Trichoderma* spp. and fungi in general (Celar, 2003 – C:N ratio $\cong$ 14.28; Olsson et al., 2003 – cellulose, sugar beat pulp, alkaline extracted sugar beat pulp; Pascual, 1997 – glucose, xylose, fructose, amino acids; Score and Palfreyman, 1994 – C:N ratio $\cong$ 8, 158, 800). Maximum conidia concentration in meat peptone supplemented SIW (up to 5 g/L meat peptone, corresponding to C:N $\approx$ 5.01 in growth medium) remained $\leq$ non-supplemented SIW (Figure 1). An increase of $\approx$ 4.68 times in conidia (from $1.6 \times 10^8$ to $7.5 \times 10^8$ CFU/ml; $F = 0.578$ and 0.316; df = 2, 12; $P < 0.05$) was observed at 10 g/L (C:N $\approx$ 4.37) meat peptone supplement. Here, it is important to add that owing to cost implications of meat peptone and its moderate (< 1 log increment; $F = 0.198$; df = 2, 12; $P < 0.05$) effects observed on conidia concentration increase, the meat peptone concentration as medium supplement was not further increased. Nevertheless, various other nitrogen source supplements, namely, amino acids, and

common nitrogen salts (Papagianni, 2004) remain to be tested as supplements to SIW for their feasibility for conidia concentration increase.

*3.3 Soluble Starch-Supplemented SIW*

It was very much logical to investigate, whether the starch concentration present in the SIW was sufficient or superfluous. Therefore, soluble starch was supplemented to SIW at concentrations of 10 and 20 g/L. The maximum CFU concentration (conidiation) obtained for 10 and 20 g/L soluble starch was similar (maximum concentrations, 3.02 to $4.2 \times 10^{10}$ CFU/ml; $F = 0.461$ and 0.273; df = 2, 12; $P < 0.05$) (Figure 1). This could be attributed to exhaustion of micro-nutrients available in SIW, as explained earlier, thereby, exhibiting no significant increase in CFU. Thus, 10 g/L soluble starch supplement was considered sufficient. Consequently, it was established that starch concentration in SIW was limiting and should be supplemented to enhance conidiation.

*3.4 Chemicals Examined as Probable Conidiation Inducers*

Based on past studies (Pascual, 1997; Roncal, 2002; Papagianni, 2004), organic acids, higher alcohols, metal ions and anti-microbial agents were investigated to induce conidiation in SIW. Effect on CFU production by the chemicals added to SIW as probable conidiation inducers are shown in Figure 4. Interestingly, the organic acids and glycerol supported initial increase in conidiation (higher CFU concentration at 24 h, with respect to SIW without inducer). However, no significant effect was observed in final CFU concentration with respect to non-supplemented SIW. On the other hand, $CaCO_3$ and EDTA addition retarded conidiation in the beginning of the fermentation process. Addition of $CaCO_3$ resulted final (96 h) CFU concentration similar to the fermentation process without $CaCO_3$. In the case of EDTA, CFU concentration was decreased by almost 2 log cycles at the end of the fermentation. Similarly, glycerol and pyridine decreased final CFU concentration (96 h) by almost 5 and 10 times, respectively. Although it was difficult to figure out a general conclusion based upon these examples, yet it was evident that for the concentrations of the chemical agents used, the organic acids influenced early conidiation. Moreover, chelating compounds like, EDTA had overall negative effect on conidiation and $Ca^{++}$ as metal ion essentially did not affect conidiation.

Furthermore, based on these studies any cause and effect explanation would be difficult. However, a recommendation could be made for future studies that changing the time of addition of these compounds might affect final CFU concentration.

*3.5 Indirect Spore Assessment*

ATP measurement has been advocated as an indicator of microbial growth (Eiland, 1983). Therefore, total and dissolved ATP of *T. viride* fermented samples were measured for glucose, soluble starch and meat-peptone supplements in SIW (Figure 5a and 5b). It was observed that in all cases, dissolved ATP was very low (0.092 to 30.35 ng/ml) compared to total ATP (0.709 to 8608 ng/ml) ($P < 0.05$).

Total ATP concentration decreased with increase inglucose concentrations (Figures 5a). On the other hand, the total ATP concentration increased with soluble starch (Figures 5a) and meat peptone (Figures 5b) additions. For meat peptone as a nitrogen source, ATP concentration reached maximum between 30-60 h. Later on, the ATP decreased monotonically towards the end of fermentation (Figure 5b). Thus, fortification of soluble starch and meat peptone to SIW increased CFU and ATP concentrations. Therefore, there seems to be a relationship between ATP and CFU (Figures 5c). However, the correlation coefficient ($R^2$) was low for soluble starch (0.53-0.58) and moderate for glucose supplements (0.72-0.87). In addition, the gradients of linearity were also different for each case.

ATP is fairly associated with biomass activity (synthesis of metabolites, sporulation/reproduction, maintenance of cellular conformation within living cells, Holm-Hansen and Booth, 1966). It is likely that once biomass growth is completed, ATP concentration is expected to decrease because ATP would be produced mainly for cellular maintenance. Moreover, decrease in ATP in the growth medium has been reported (Pitt and Bull, 1982) owing to cellular maintenance. In cases of glucose and soluble starch supplementation, ATP concentration kept on increasing until the end of fermentation (96 h) indicating the production of vegetative biomass and metabolites. Conversely, in the case of meat peptone, near maximum conidia concentration (≅8 log cycles; $F = 0.075$; df = 2, 12; $P > 0.05$) was reached during 48-54 h. Thus, *T. viride* generated less ATP during 54-96 h where there was a comparatively slower increase in conidia concentration.

A unique correlation between CFU and total ATP production was impossible, as ATP and CFU variation was also dependant on type of fermentation medium (Figure 5c). Several authors (Holm-Hansen and Booth, 1966; Neito et al., 1997) have suggested that the ATP concentration was directly dependent on overall biomass activity and environmental conditions (type of medium, in this case). Therefore, in the present study, the inexplicable variations in ATP could be attributed to complex phenomena involved during vegetative growth, sporulation and substrate-*T. viride* interactions. In fact, the ATP change might indicate biomass activity (*T. viride*), still, it cannot be compared with direct growth measurements like CFU counting.

*3.6 Protease Production Profile*

Protease activity was analyzed for experiments conducted on fortification of SIW with glucose, soluble starch and meat-peptone (Figure 6). The production of lytic enzymes has been reported by many researchers as representation of BCA potential of *Trichoderma* spp. (Chet, 1987; Benhamou and Chet, 1993; Calistru et al., 1997). Although, direct inference was difficult to incur from the present data, two common facts were observed in all cases of C and N fortification. Firstly, PA was noticeable between ≈12-60 h of fermentation. Secondly, PA increased with availability of nutrients and was maximum for the supplement concentration having highest CFU in each category, namely, glucose, soluble starch and meat-peptone. Besides, it was noticed that PA was higher in case of simple C source (glucose) with respect to complex C source (soluble starch) (Figure 6a and 6c). Further, addition of meat-peptone (nitrogen source) was effective in enhancing PA and Tx (discussed later). Therefore, this fact was further examined in fermenter under controlled conditions (constant pH and DO) in order to assess the feasibility of this process (Figure 6b). The maximum PA (peak value) observed in fermenter was lower, however, the final PA was higher than the shake flasks. The lower peak PA activity in fermenter was possibly because of zero DO during 24-42 h, as the oxygen demand of culture could not be accomplished even at maximum aeration and agitation (2.25 vvm and 1100 rpm) capacity of the fermenter (Figure 6b). Meanwhile, DO was subsequently controlled at ≥ 30% (42-96 h).

*3.7 Biocontrol Activity*

In order to assess BCA potential of *T. viride*, bioassays against spruce budworm larvae were conducted for *T. viride* grown SIW with and without C and/or, N supplementation. The maximum Tx of 12335 SBU/µl was obtained ($F = 1.148$; df = 2, 57; $P > 0.05$) for 96 h sample of 1% meat peptone supplemented SIW fermented broth (Figure 7). It was observed that Tx gradually decreased with increase in C concentration, either complex or simple. On the other hand, the Tx increased with N supplement, thereby, suggesting feasibility of fortification of SIW with meat peptone. Moreover, samples harvested at 96 h of growth from fermenter showed further increment in Tx (13670 SBU/µl; $F = 2.231$; df = 2, 57; $P > 0.05$). The explanation to the increase in Tx from shake flask to fermenter may be partially due to increase in CFU and PA (Figure 7), under controlled environmental conditions (pH and dissolved oxygen concentration). However, a quantification of Tx with respect to CFU and PA is not possible because in addition to CFU and PA, Tx possibly also depends on several other factors. These factors include, lytic enzymes (protease, amylase, chitinase), antibiotics and other insecticidal proteins which are produced during growth and sporulation process of *Trichoderma* and act synergistically with CFU (Harmann and Björjmann, 1998; Ganassi et al, 2000). For glucose supplement, as CFU decreased from $6.2 \times 10^7$ to $3 \times 10^7$ CFU/ml ($F = 0.300$ and 0.834; df = 2, 12; $P < 0.05$) when PA was almost constant (0.263 to 0.294 IU/ml; $P > 0.05$), Tx was practically unchanged. However, when meat peptone concentration was increased, Tx increased with CFU decrease and PA increase. The Tx of 96 h fermenter sample was associated with increase in both CFU ($3.47 \times 10^9$ CFU/ml; $F = 0.390$; df = 2, 12; $P > 0.05$) and PA (0.976 IU/ml; $P > 0.05$). However, in cases where SIW was supplemented with soluble starch, Tx change was largely independent of CFU and PA. This strengthens the contention that besides, PA and CFU, other metabolites produced by *T. viride* would have contributed to Tx.

Furthermore, a comparison of Tx of *T. viride* fermented SIW (with, 13670 SBU/µl or without, 10448 SBU/µl supplement) obtained in this study to that of wastewater sludge (non-treated - 6278 SBU/µl; treated - 15036 SBU/µl; Verma et al., 2005) suggested that SIW as a substrate was competitive to wastewater sludge with and without pre-treatments. Besides, higher CFU obtained in SIW as compared to wastewater sludge is an additional

feature of SIW. During application of *T. viride* in soil as BCA, higher CFU would insure faster colonization by *T. viride* in plant rhizosphere, thereby, providing plant growth promoting hormones and nutrients and antagonizing phytopathogens. These characteristics are commendable for BCA formulation development and future field application.

## 4. Conclusion

This study entails crucial information on conidiation of antagonistic fungi, *Trichoderma viride* from biocontrol point of view. Starch industry wastewater was reported for the first time for *T. viride* production with exceptionally higher CFU ($\geq 10^8$ CFU/ml). Study of effects of C:N ratio and source of C and N helped in determining the best possible strategies to further enhance conidia concentration ($\geq 10^{10}$ CFU/ml - 1 % soluble starch) and entomotoxicity against spruce budworm larvae (13670 SBU/µl in fermenter - 1% meat peptone). Supplementation of starch industry wastewater with different chemicals (probable conidiation inducers) induced early conidiation. Total and dissolved ATP as indirect growth measurement parameters could not be correlated to *T. viride* CFU, thereby, rendering CFU counting as the most reliable method.

## Acknowledgements

The authors are sincerely thankful to 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.

## References

Amsellem, Z., Zidack, N.K., Quimby Jr., P.C. and Gressel, J. (1999) Long-term dry preservation of viable mycelia of two mycoherbicidal organisms. Crop Protection **18**, 643–649.

APHA-AWWA-WPCF (1998) Standard Methods for Examination of Water and Wastewaters. 20th edn. (Eds. Clesceri, L.S., Greenberg, A.E. and Eaton, A.D.), American Public Health Association, Washington, DC, USA.

Beegle, C.C. (1990) Bioassay methods for quantification of *Bacillus thuringiensis* δ-endotoxin, Analytical Chemistry of *Bacillus thuringiensis*, In: Analytical Chemistry of *Bacillus thuringiensis* (Eds. Hickle L.A. and Fitch W.L.), American Chemical Society, pp. 14–21.

Benhamou, N., Chet, I. (1993) Hyphal interaction between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold chemistry of the mycoparasitic process. Phytopathology **83,** 1062–1071.

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

Calistru, C., McLean, M., Berjak, P. (1997). *In vitro* studies on the potential for biological control of *Aspergillus flavus* and *Fusarium moniliforme* by *Trichoderma* species. *A study of the production of extracellular metabolites by Trichoderma species.* Mycopathologia **137,** 115–124.

Celar, F. (2003) Competition for ammonium and nitrate forms of nitrogen between some phytopathogenic and antagonistic soil fungi. Biological Control **28,** 19–24.

Chatfield, C. (1983) The design and analysis of experiments: 1. Comparative experiments, pp. 224-256. *In* Statistics for technology, 3rd ed., Chapman & Hall., Bristol, United Kingdom.

Chet, I. (1987) *Trichoderma*-Applications, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. Pages 137-160 in: Innovative Approaches to Plant Disease Control. I. Chet, ed. John Wiley & Sons, New York.

Cotxarrera, L., Trillas-Gay, M.I., Steinberg, C. and Alabouvette, C. (2002) Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. Soil Biology and Biochemistry **34,** 467–476.

Eiland, F. (1983) A simple method for quantitative determination of ATP in soil. Soil Biology and Biochemistry **15,** 665–670.

Ganassi, S., Moretti, A., Stornelli, C., Fratello, B., Bonvicini, P.A.M., Logrieco, A., Sabatini, M.A. (2000) Effect of *Fusarium*, *Paecilomyces* and *Trichoderma* formulations against aphid *Schizaphis graminum*. Mycopathologia **151**, 131–138.

Gikas, P., Livingston, A.G. (1993) Use of ATP to characterize biomass viability in freely suspended and immobilized cell bioreactors. Biotechnology and Bioengineering **42,** 1337–1351.

Gupta, R., Saxena, R.K., Goel, S. (1997) Short communication: Photo-induced sporulation in *Trichoderma harzianum* - an experimental approach to primary events. World Journal of Microbiology and Biotechnology **13,** 249–250.

Harmann, G.E. and Björjmann, T. (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In *Trichoderma and Gliocladium Vol. 2.* (Harmann, G.E. and Kubicek, C.K., eds), pp 229–265, Taylor and Francis Ltd., London.

Holm-Hansen, O., Booth, C. R. (1966) The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnology and Oceanography **11,** 510–519.

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor. J. Gen. Physiol.30, 291–310.

Lewis, J.A. and Papavizas, G.C. (1983) Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. Soil Biology and Biochemistry **15**, 351–357.

MENV, 2004.Guide sur la valorisation des matières résiduelles fertilisantes: Critères de références et normes réglementaires. Direction du milieu rural, environnement Québec, Canada, 138pp.

Neito, F.E., Lee, J.J., Koestler, R.J. (1997) Assessing biodeterioration in wood using ATP photometry: Part II. Calculating a conversion factor for *Phanerochaete chrysosporium* using ATP and adenylate energy charge. International Biodeterioration and Biodegradation **39,** 159–164.

Olsson, L., Christensen, T.M.I.E., Hansen, K.P., Palmqvist, E.A. (2003) Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C-30. Enzyme and Microbial Technology **33,** 612–619.

Papagianni, M. (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances **22,** 189–259.

Pascual, S., Rico, J.R., De Cal, A., Melgarejo, P. (1997) Ecophysiological factors affecting growth, sporulation and survival of the biocontrol agent *Penicillium oxalicum.* Mycopathologia **139,** 43–50.

Pitt, D.E., Bull, A.T. (1982) The adenine nucleotide composition of growing and stressed cultures of *Trichoderma aureoviride*. Experimental Mycology **6,** 41–51.

Punja, Z.K., Utkhede, R.S. (2003) Using fungi and yeasts to manage vegetable crop diseases. Trends in Biotechnology **21,** 400–407.

Roncal, T., Cordobés, S., Ugalde, U., He, Y., Sterner, O. (2002) Novel diterpenes with potent conidiation inducing activity. Tetrahedron Letters **43,** 6799–6802.

Score, A.J., Palfreyman, J.W. (1994) Biological control of the dry rot fungus *Serpula lacrymans* by *Trichoderma* species: The effects of complex and synthetic media on interaction and hyphal extension rates. International Biodeterioration and Biodegradation **33,** 115–128.

Verma, M., Brar, S.K., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2006) Dissolved oxygen as principal parameter for conidia production of biocontrol fungi *Trichoderma viride* in non-Newtonian wastewater. Journal of Industrial Microbiology and Biotechnology, (DOI:10.1007/s10295-006-0164-6; in press).

Verma, M., Brar, S.K., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. (2005) Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. Water Research, **39**, 3587-3596.

Vlaev, S.D., Djejeva, G., Raykovska, V., Schugerl, K. (1997) Cellulase production by *Trichoderma* sp. Grown on corn fibre substrate. Process Biochemistry **32,** 561–565.

Whipps, J.M. and Lumsden, R.D. (2001) Commercial use of fungi as plant disease biological control agents: status and prospects. In: *Fungal Biocontrol Agents: Progress;, Problems and Potential,* (eds T. Butt, C. Jackson & N. Magan), Wallingford: CABI Publishing, pp 9-22.

Zheng, Z. and Shetty, K. (1998) Cranberry processing waste for solid state fungal inoculant production. Process Biochemistry **3**, 323–329.

**Table 1.** Characteristics of starch industry wastewater

| Parameter | Concentration (mg/kg; unless stated otherwise) |
|---|---|
| Total solids (g/l) | 17.3 |
| Total volatile solids (g/l) | 14.2 |
| Suspended solids (g/l) | 2.4 |
| Suspended volatile solids (g/l) | 2.4 |
| pH | 3.8 |
| Total carbon | 366000 |
| Total nitrogen | 54000 |
| Total phosphorus | 11600 |
| $N\text{-}NH_3$ | 619 |
| $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ | 36.6 |
| $P\text{-}PO_4^{3-}$ | 812 |
| Al | 210.5 |
| Ca | 24700 |
| Cd | 1.3 |
| Cr | 13.5 |
| Cu | 96.5 |
| Ni | 22.5 |
| Fe | 4455 |
| K | 1529 |
| Pb | 35.8 |
| Mn | 46.7 |
| S | 800 |
| Zn | 523 |
| Na | 5300 |

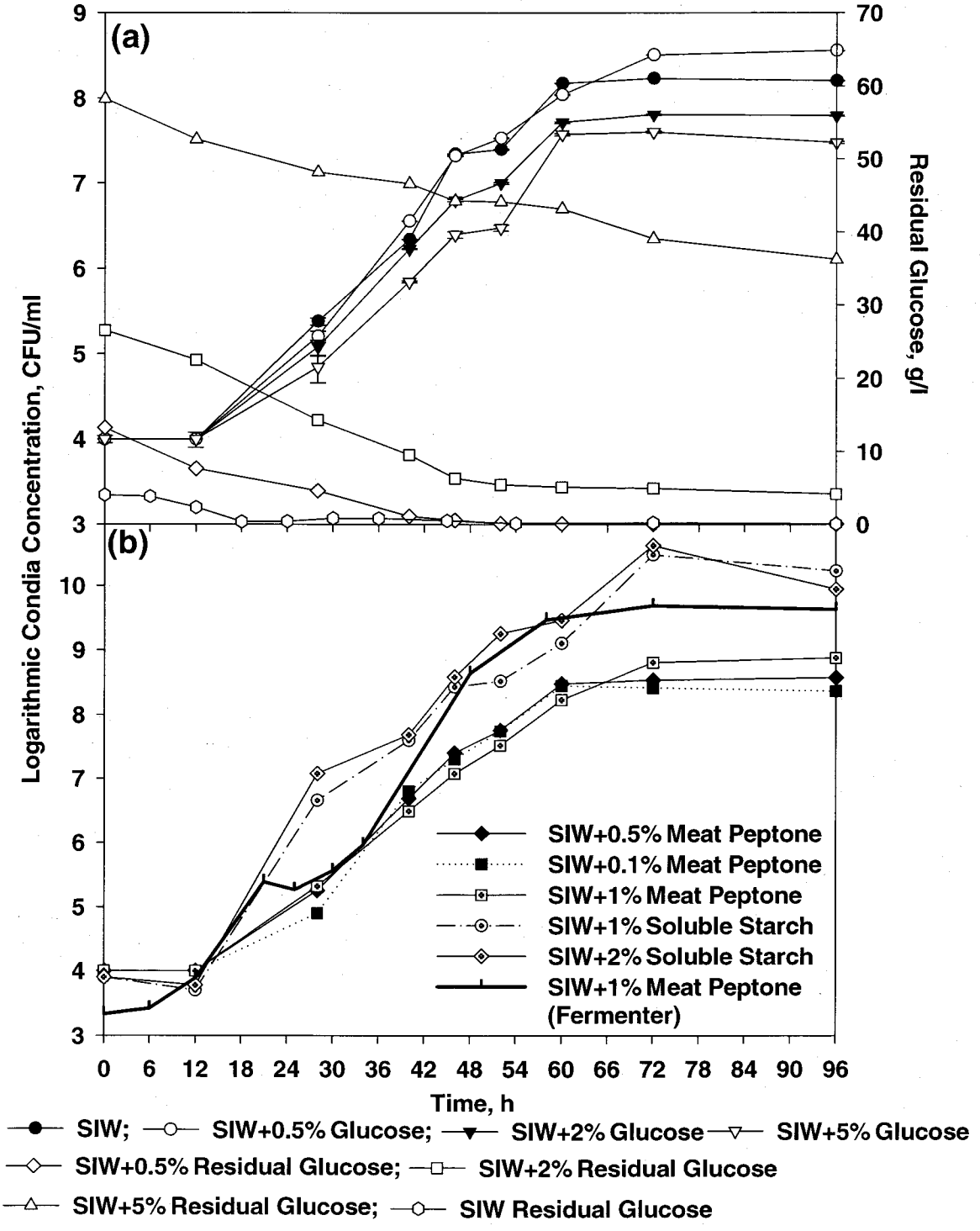



**Figure 1.** CFU and residual glucose profile of *T. viride* in SIW with, (a) varying glucose supplement, (b) varying N-source and soluble starch supplements.

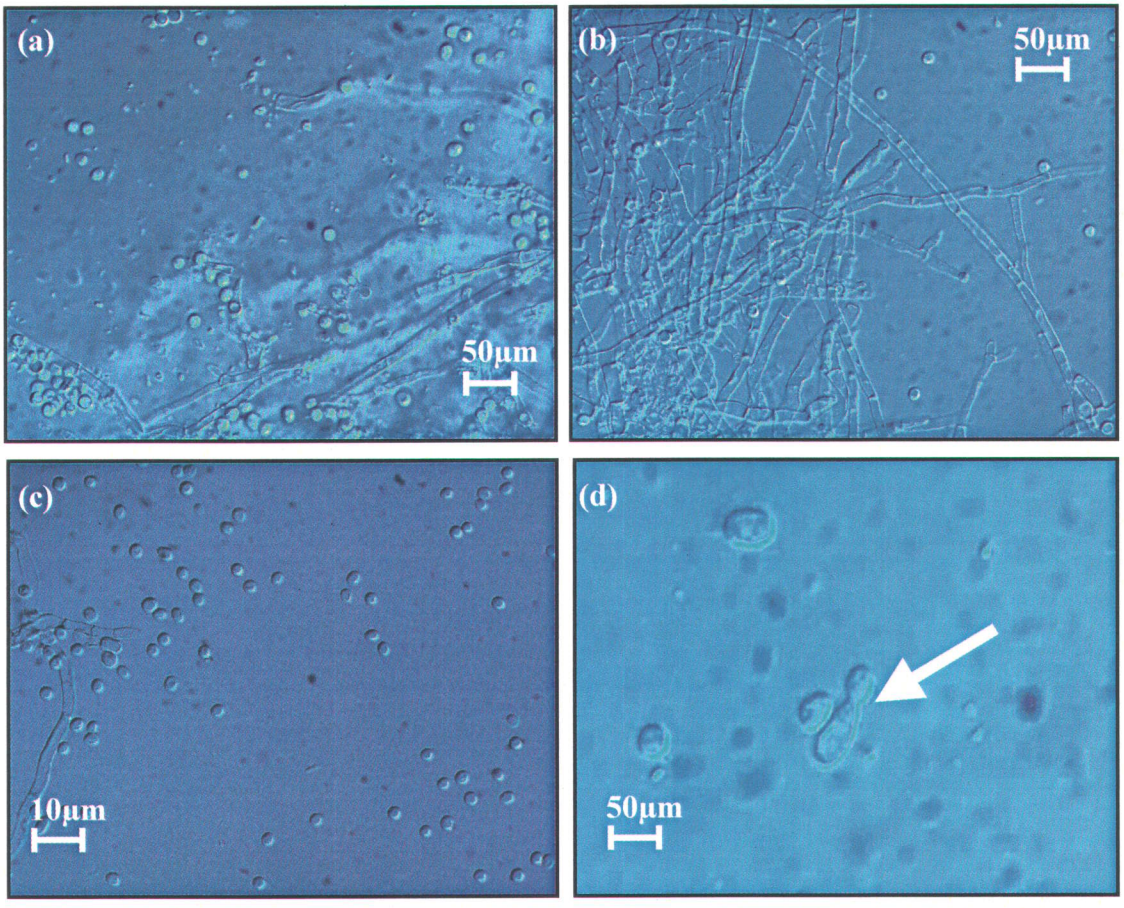



**Figure 2.** Micrographs of *T. viride* at 48 h, (a) SIW, (b) SIW with 2% glucose supplement, and (c) SIW with 1% soluble starch supplement (d) germination of conidia in SIW with 2% glucose supplement.

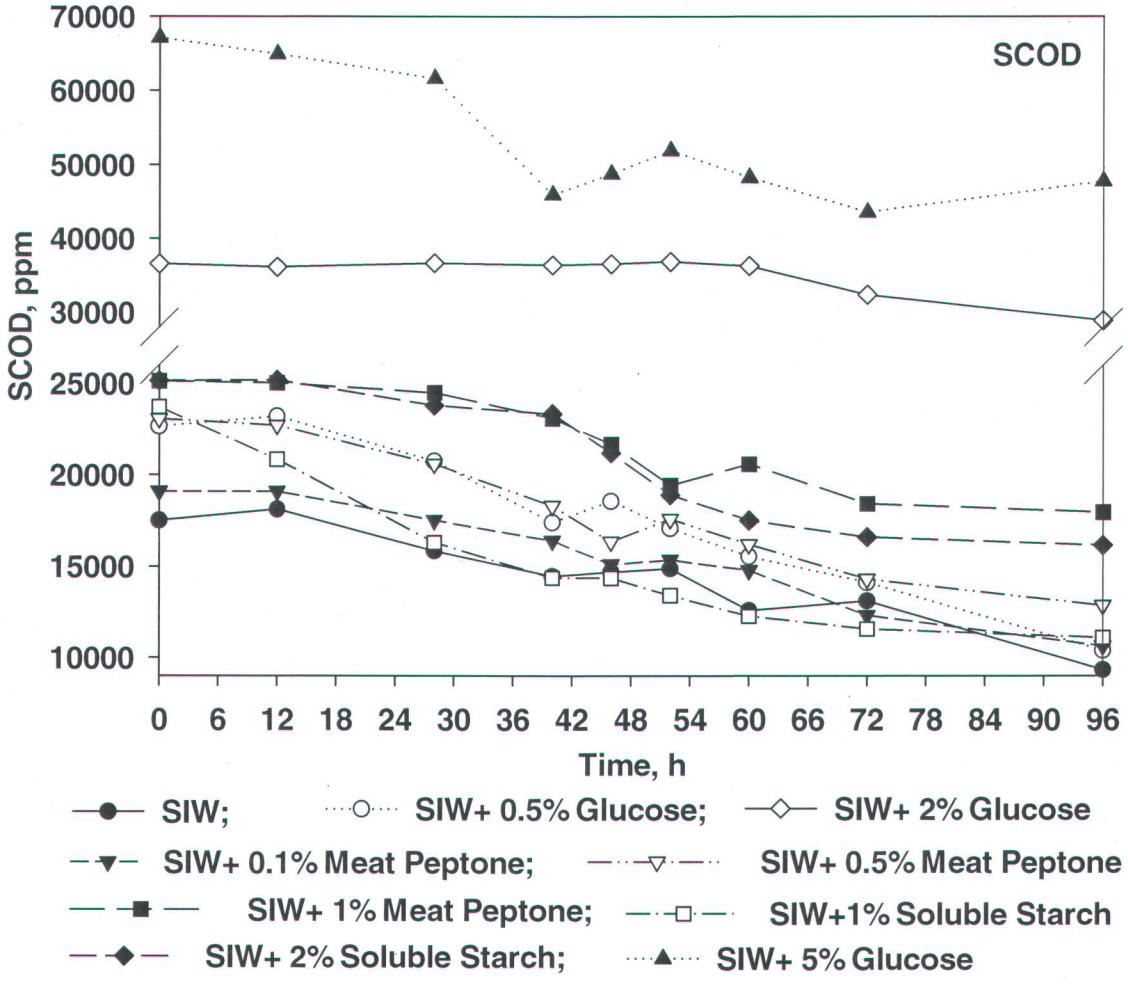



**Figure 3.** Soluble COD consumption of *T. viride* in SIW with varying supplements.

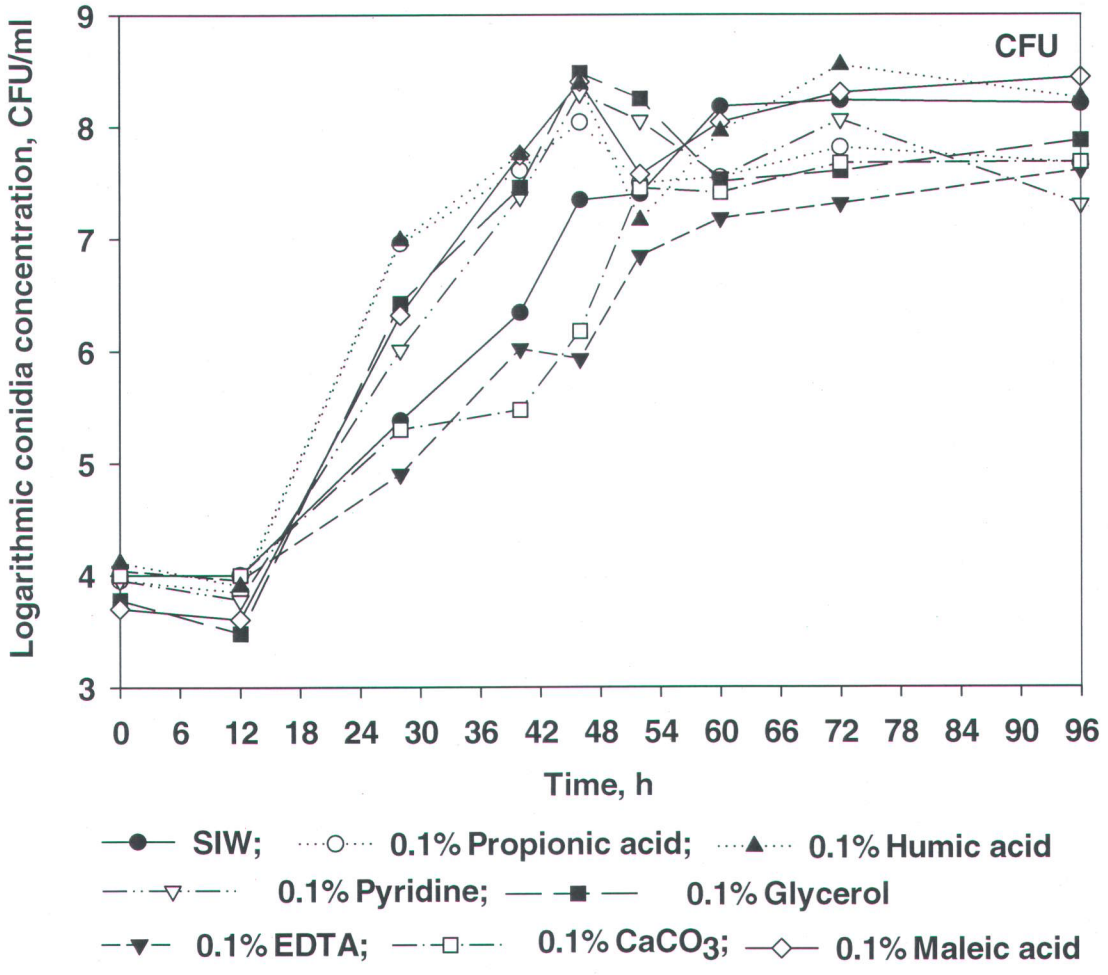



**Figure 4.** Effect of probable conidiation inducers on CFU production of *T. viride* in SIW.

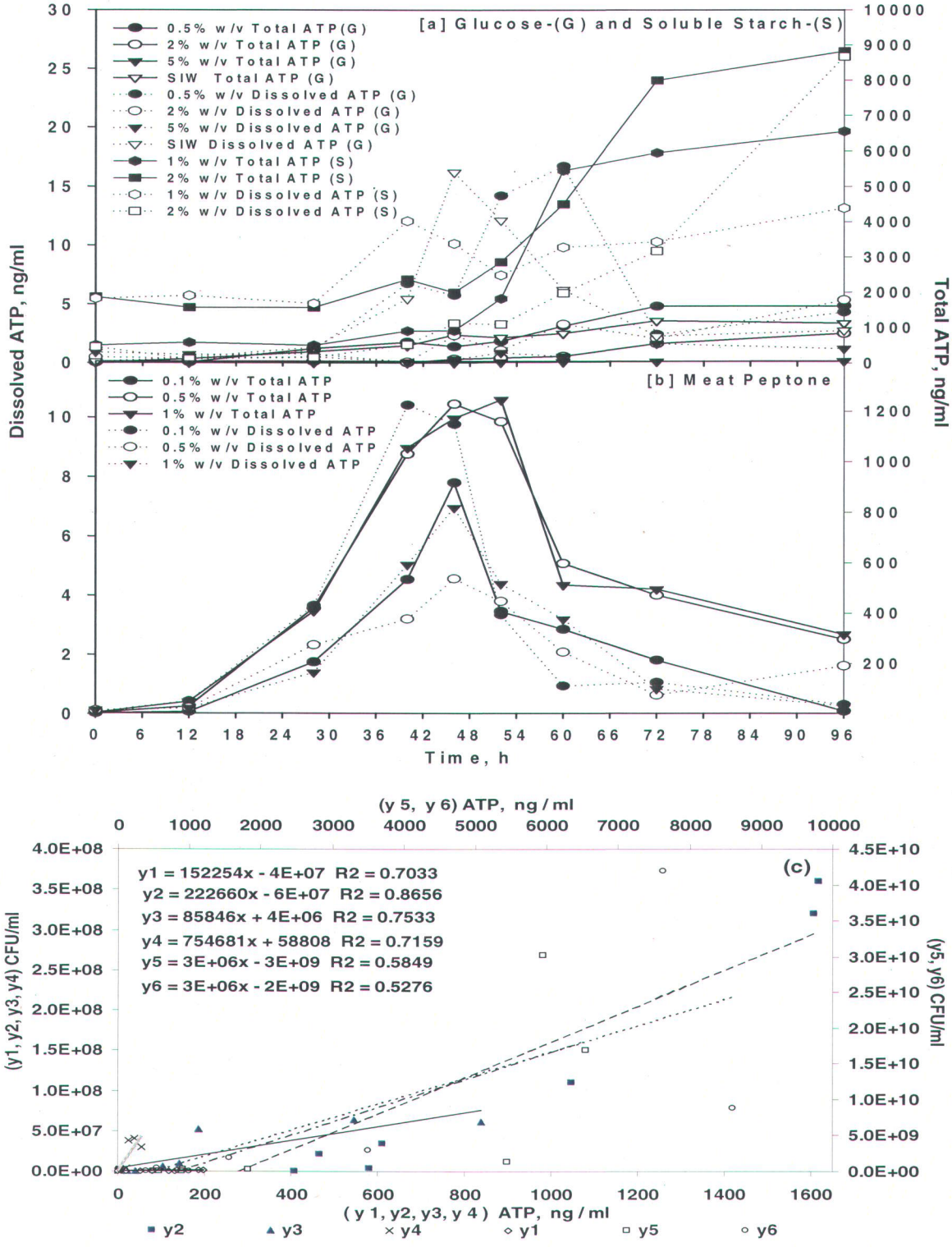



**Figure 5.** Total ATP vs. CFU correlation of *T. viride* in – y1, SIW; y2 SIW + 0.5% glucose; y3 SIW + 2% glucose; y4 SIW + 5% glucose; y5 SIW + 1% soluble starch; y6 SIW + 2% soluble starch.

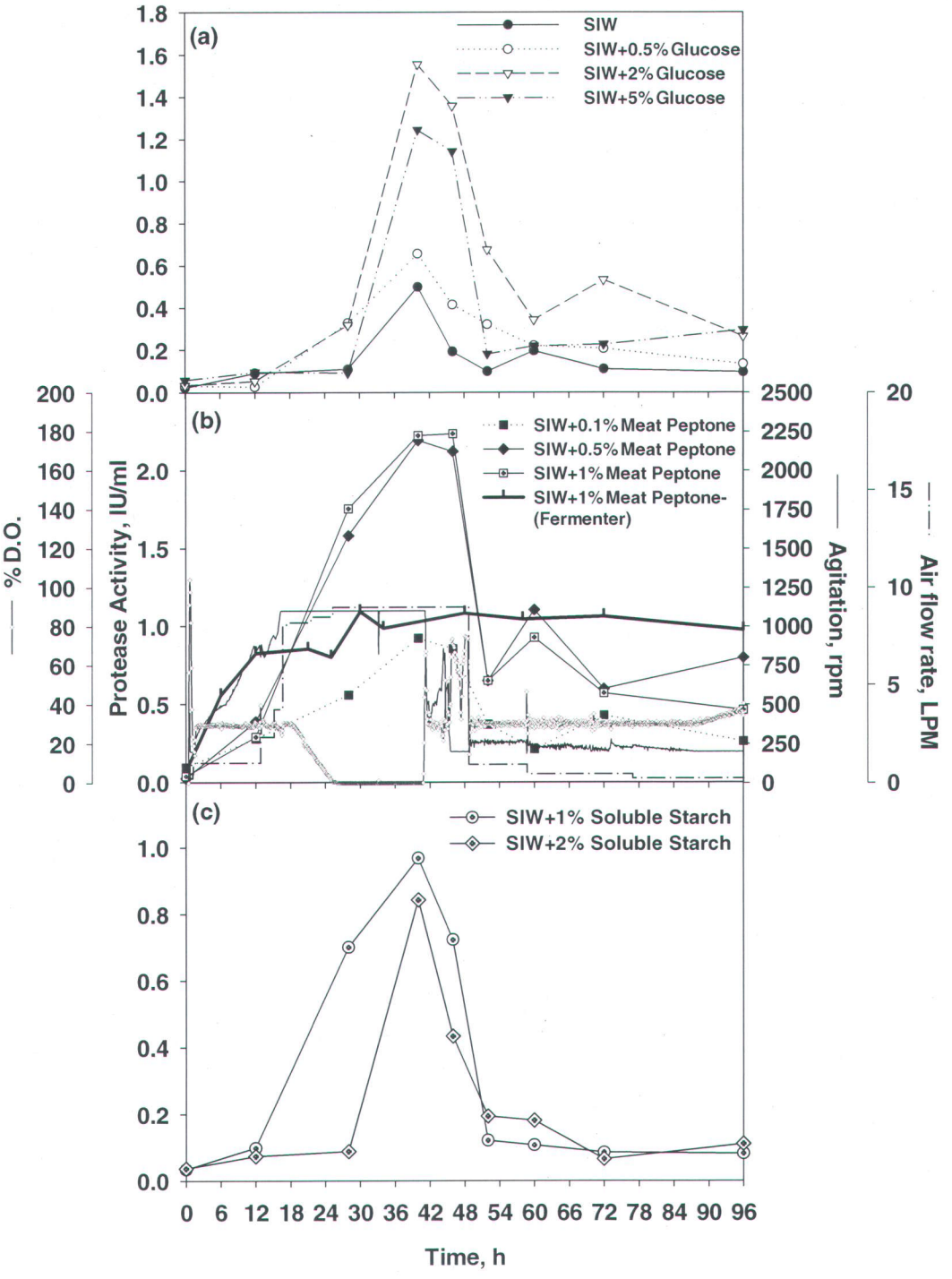



**Figure 6.** Protease activity of *T. viride* in SIW with, (a) varying glucose supplement, (b) varying N-source supplement, and (c) varying soluble starch supplement.

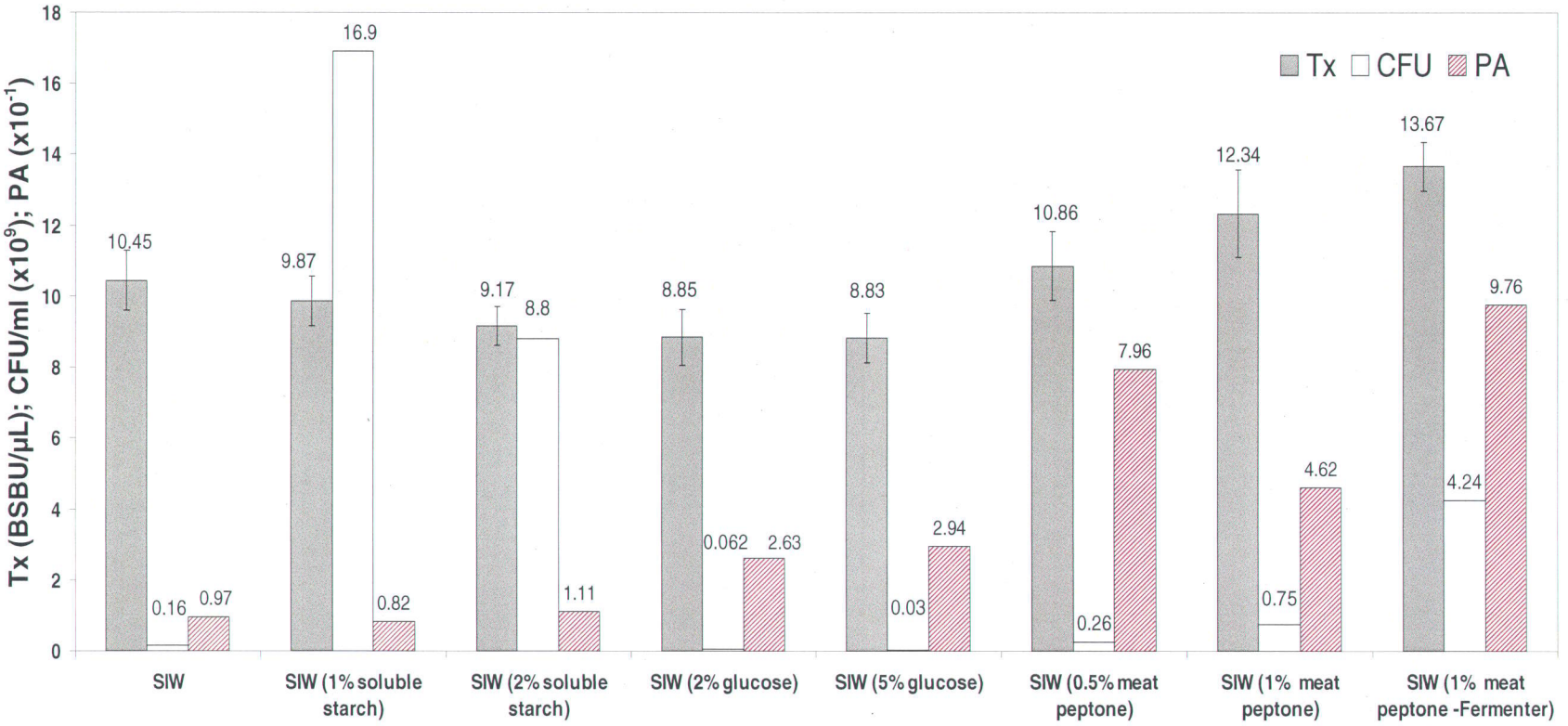



**Figure 7.** Effect of nutritional supplements on entomotoxicity of *T. viride* and fermenter scale feasibility.

## Part III

# Dissolved oxygen as principal parameter for conidia production of biocontrol fungi *Trichoderma viride* in non-Newtonian wastewater

**M. Verma[1], Satinder K. Brar[1], R. D. Tyagi[1,*], R. Y. Surampalli[2], and J. R. Valéro[1,3]**

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

[2]US EPA, P.O. Box-17-2141, Kansas City, KS 66117, U.S.A.

[3]NRCan Canadian Forestry Services, Laurentian Forestery Centre des 1055 du P.E.P.S., P.O. Box 3800, Sainte-Foy, Québec Canada, G1V 4C7

**Journal of Industrial Microbiology and Biotechnology, (2006, 33: 941–952)**

## L'oxygène dissout comme paramètre principale pour la production des conidies du champignon de lutte biologique *Trichoderma viride* dans des eaux usées non Newtoniennes

**Résumé**

La concentration en oxygène dissout (OD) a été sélectionnée comme un paramètre principal pour reproduire les résultats de fermentation de *Trichoderma viride* (un champignon utilisé pour la lutte biologique) en fioles à une échelle de fermenteur. Toutes les fermentations ont été menées dans un fermenteur automatisé de 7.5 litres et un volume de travail de 4 litres. Les paramètres de performance pour la fermentation tels que le coefficient volumétrique de transfert de l'oxygène ($k_La$), le taux d'assimilation de l'oxygène (OUR), la rhéologie, la concentration en conidies, la consommation du glucose, la demande chimique en oxygène (soluble), l'entomotoxicité et l'indice d'inhibition ont été mesurés. La concentration en conidies, l'entomotoxicité et l'indice d'inhibition étaient soit stables ou améliorés à une faible concentration en OD (30%). La variation du OUR a contribué à la détermination de la capacité du fermenteur à fournir de l'oxygène, ainsi que de la croissance de la biomasse. Les profils rhéologiques ont démontré la variabilité des eaux usées durant la fermentation causée par la croissance mycélienne et la conidiation. Afin d'estimer la consommation d'énergie, les besoins en agitation et en aération ont été quantifiées en termes de surface sous les courbes, l'agitation vis-à-vis le temps (rpm), et l'aération vis-à-vis le temps (lpm). Cette simple et nouvelle stratégie pour l'opération de fermenteur apparaît comme très efficace et pourrait être adaptée à d'autres champignons utilisés en lutte biologique.

**Mots-clés:** Agents de lutte biologique, conidies, oxygène dissout, rhéologie, eaux usées de l'industrie de l'amidon, *Trichoderma viride*.

**Abstract**

Dissolved oxygen (DO) concentration was selected as a principal parameter for translating results of shake flask fermentation of *Trichoderma viride* (biocontrol fungi) to a fermenter scale. All fermentations were carried out in a 7.5 l automated fermenter with a working volume of 4 l. Fermentation performance parameters such as volumetric oxygen transfer coefficient ($k_La$), oxygen uptake rate (OUR), rheology, conidia concentration, glucose consumption, soluble chemical oxygen demand, entomotoxicity and inhibition index were measured. The conidia concentration, entomotoxicity and inhibition index were either stable or improved at lower DO concentration (30%). Variation of OUR aided in assessing the oxygen supply capacity of the fermenter and biomass growth. Meanwhile, rheological profiles demonstrated the variability of wastewater during fermentation due to mycelial growth and conidiation. In order to estimate power consumption, the agitation and the aeration requirements were quantified in terms of area under the curves, agitation vs. time (rpm.h), and aeration vs. time (lpm.h). This simple and novel strategy of fermenter operation proved to be highly successful which can be adopted to other biocontrol fungi.

**Keywords** Biocontrol agents, conidia, dissolved oxygen, rheology, starch industry wastewater, *Trichoderma viride*.

## INTRODUCTION

The biocontrol agents (BCAs) market has been growing continuously over the last few decades due to the adverse environmental impacts of chemical pesticides [11]. Additional factors such as production cost, resistance development in pests, and stricter government policy have also inhibited the utilization of chemical pesticides. In particular, fungal BCAs share considerable market due to their broad spectrum of biological activity and environmental safety [29]. Amongst fungal BCAs, conidial formulations of many *Trichoderma* spp. have been shown to be effective in killing and/or preventing growth of several plant pathogens, namely *Rhizoctonia*, *Pythium*, *Fusarium* and *Cylindrocladium* [7, 10]. In addition to the production of antimicrobial compounds, conidia of *Trichoderma* fungi are preferred over mycelia and chlamydospores due to their high rate of production [18]. However, a significant amount of conidia are lost during the formulation step and soil application due to air-drying and mechanical stress during handling. Thus, higher *Trichoderma viride* conidia concentrations would be required at the end of the submerged fermentation process in the formulated product for field application [15]. The biological control properties of *Trichoderma* spp. can be further enhanced by applying them in combination with existing novel crop protection strategies [28]. Moreover, production cost has been a major obstacle to the success of many biopesticides [15], including *Trichoderma* spp. This stimulated the exploration of cheap and waste-based raw materials, e.g. wastewater sludge [31] and food-processing waste [35], to minimize the overall production cost. However, none of the studies using waste as a substrate were carried out in fermenter, a pre-requisite for mass-scale production.

Although conidiation in submerged fermentation is a difficult phenomenon [15, 26], yet it offers several advantages over solid state fermentation. *Trichoderma* spp. fermentation is an aerobic process and oxygen mass transfer therefore is a prominent obstacle. Moreover, conidiation and production of antimicrobial compounds are highly affected by oxygen transfer (aeration and agitation). Furthermore, the oxygen transfer in submerged fermentation is also largely affected by viscosity and morphology (mycelia and pellet formation) [5, 25]. These parameters create heterogeneity in the medium, resulting in compartmentalization of the fermentation broth in terms of dissolved oxygen concentration, pH, and substrate availability [14, 36]. Therefore, scaling up is a function of rheological parameters that needs to be studied explicitly. Further, more complex rheology

of a fermentation medium necessitates study on bioreactors for the optimization of critical parameters such as agitation and aeration (oxygen transfer), which have a direct impact on operating costs [14, 26]. It has also been reported that high shear on fungal cells resulted in sporulation and cell rupture, which hampered the conidia production rate [12]. Therefore, a critical analysis of operating parameters would be a vital step in economizing the mass production of *Trichoderma* sp.-based BCAs.

This study was conducted for process development of *Trichoderma viride* conidia and antagonist metabolites production by using starch industry wastewater (SIW) as raw material. The optima of aeration and agitation intensities and incubation time were determined, so as to decrease the final cost of these BCAs. Bioassay of fermented broth was also conducted on larvae of a forest pest, spruce budworm (*Choristonuera fumiferana*), and on a fungal phytopathogen, *Cylindrocladium floridanum*, to assess the biocontrol efficacy of the fermentation process.

## MATERIALS AND METHODS

### *Chemicals*

The analytical grade chemicals were purchased from Sigma-Aldrich or BDH (Toronto, Canada). Microbiological media and fermentation-related chemicals (e.g., anti-foam, acid and base) were of commercial grade.

### *Raw Material (Starch Industry Wastewater)*

Starch industry wastewater (SIW) used in this study was obtained from a local starch industry (ADM-Ogilvie, Candiac, Quebec). Table 1 represents the physio-chemical characteristics of SIW, which showed metal concentrations in accordance with Québec guidelines for agricultural application [21]. The SIW was stored for a maximum of 3 weeks at 4 ± 1°C to minimize microbial degradation.

*Starter Culture, Pathogens and Inoculum*

The *Trichoderma viride* fungus was a commercial strain [31] isolated from soil. It was found to be active against phytopathogenic fungi, *Fusarium* sp. and *Cylindrocaldium floridanum* and spruce budworm (SB) insect larvae, as verified in our laboratory. For fungal pathogen testing, *Cylindrocladium floridanum*, pathogenic to spruce trees was obtained from the Laurentian Forestry Centre (LFC, Quebec, Canada). The insecticidal bioassay was carried out using third instar larvae of eastern spruce budworm (SB) (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) provided by Natural Resources Canada (Sault Ste. Marie, Ontario).

The *Trichoderma viride* strain was grown on potato dextrose agar (PDA) plates for 4-7 days under dark conditions at 28 ± 1°C and 35 ± 2% relative humidity. It was subsequently maintained at 4 ± 1°C and subcultured monthly. For starter culture, a ≈ ½" x ½" scraped piece of 32-36 h old mycelial mat of subculture was taken. Afterwards, it was aseptically homogenized in 4 ml of sterile tryptic soya broth (TSB, Difco) with a Micro Tissue Grinder® (VWR, Canada) submerged in an ice bath. The starter culture was inoculated into 500 ml Erlenmeyer flasks containing 150 ml of sterile TSB at pH 6.0 ± 0.01. Subsequently, the flasks were incubated in a rotary shaker at 28 ± 1°C and 250 ± 5 rpm for 48 h, and used immediately as inocula for the fermenter.

*Fermentation*

Fermentations were carried out in a bench top glass fermenter (7.5 l capacity) (LABFORS 3, INFORS AG, Switzerland). The fermenter was equipped with a programmable logic controller for pH, agitation, anti-foam, dissolved oxygen and temperature. A data acquisition and process control software (IRIS version 5.01; Infors, Switzerland) was utilized for process automation. The agitator system consisted of two equally spaced Rushton turbine-type impellers and three baffles. Sterilization of 4 l of SIW was carried out for 30 min at 121 ± 1°C. The inoculum comprised 10% v/v starter culture. The pH during fermentation was maintained at 6.0 ± 0.1 by using 2 N $H_2SO_4$ and 2 N NaOH, whereas the temperature was kept constant at 28 ± 1°C. Three dissolved oxygen (DO) levels (≤80, ≥40 and ≥30%) were maintained in separate experiments in duplicate by

varying the agitation speed (PID control) and aeration rate (manually). A maximum of 75-100 ml samples were drawn aseptically at regular intervals and stored at 4 ± 1°C for subsequent analyses. The significance of the fermentation data was based on the average of the duplicate set of fermentations.

*Spore Assessment*

A modified CFU plating method was utilized to measure the *Trichoderma* spore count. In this method, the fungal conidia was separated from mycelia and media agglomerates, as described elsewhere [31].

For CFU plating, appropriately diluted 100 µl samples of the separated conidia of *Trichoderma viride* were plated on tryptic soya agar plates. The agar plates were incubated at 28 ± 1°C and 35 ± 2% relative humidity for 30-36 h in the dark. For statistical significance of data, five replicates for three different dilutions were used for ANOVA enumeration. Standard deviation for CFU count was 8-10%.

*Microscopy*

Smear preparation was carried out with 20-50 µl of fresh samples using a pipette tip cut at the end. Morphological examination of the mycelia and conidia were carried out to assess the effect of agitation during mixing in the fermenter. To this end, a computer-coupled optical microscope (Zeiss Axiolab) equipped with a digital camera (Axiocam HRC Zeiss) was utilized. Also, contamination was checked by qualitative observation of all samples.

*Soluble Chemical Oxygen Demand (SCOD)*

The closed reflux method as described in Standard Methods [2], was used for SCOD determination. Standard deviation was 3-5%, based on triplicate samples of two fermentation runs.

*Volumetric oxygen transfer coefficient ($k_La$), Oxygen uptake rate (OUR), and Power consumption measurements*

The dynamic gassing-out method was used for $k_La$ measurements [1]. The DO control was momentarily stopped during the dynamic gassing-out method to facilitate correct $k_La$ measurements. Oxygen uptake rates were obtained by measuring the slope of DO decrease during air-off of the dynamic gassing-out protocol. For calculation of the cumulative oxygen consumed, the area under OUR curves was manually quantified by approximating the area with rectangles and triangles. The oxygen probe used in this study was based on the Clark polarographic sensor. The probe was equipped with a temperature compensation circuit and a response time ≤ 30s to attain 90% of the final value at 25°C.

For measurement of power consumption in terms of revolutions per minute × hour (rpm.h) and litres per minute × hour (lpm.h), the area under the agitation and aeration profiles, respectively, was measured. To this end, the rectangles between two adjacent Y-ordinates of respective parameters were calculated with the Excel spreadsheet program.

*Rheological Measurements*

Rheological analyses were carried out for fresh samples by using a rotational viscometer (DVII+, Brookfield) equipped with small sample adapter spindle (SC4 34, Brookfield). The calibration and the rheological testing procedure used for the spindle were carried out according to the instrument's manual. The viscosity data were examined by using the software Rheocalc V2.6 (Brookfield Engineering Labs, 1999). The Ostwald-deWaele power law, $\tau = k\,\gamma^n$, Bingham equation, $\tau = \tau_0 + \mu_P \gamma$, and Casson equation, $\sqrt{\tau} = \sqrt{\tau_0} + \sqrt{\mu_P \gamma}$, were investigated to describe the rheological characteristics of all samples.

*Enzyme Activity*

Protease activity (PA) was measured in centrifuged supernatant (7650 g for 20 min at 4 ± 1°C) of fermented broth. The supernatant was appropriately diluted in borate buffer

at pH 8.2 ± 0.01 and used as enzyme aliquot. A modified method [17] was employed for PA measurements in IU/ml.

Cellulase activity was measured as described by Wang et al. [32]. Amylase activity was determined by measuring the appearance of total reducing sugar in the reaction mixture by utilizing the dinitrosalycylic acid reagent method [23]. The enzyme reaction mixture consisted of 40 µl of 0.5 M sodium acetate buffer (pH 6.0), 100 µl of 0.5% soluble starch, 0 to 60 µl of enzyme solution, and ultrapure water to a final volume of 200 µl [8]. The reaction was carried out for 30 min at 50°C and stopped by heating at 96°C for 5 min. One amylase unit was defined as the amount of enzyme which releases 1 mmol of glucose per min (saccharifying activity) from soluble starch.

Standard deviations for all enzyme measurements were up to 9%, based on triplicate samples of two fermentation runs.

*Insecticidal Bioassay*

Entomotoxicity (Tx) of the fermented samples was determined using diet incorporation bioassay [4]. Sample preparation and diet protocol are described elsewhere [6]. Industry standard contained spores and crystals of the bacterium *Bacillus thuringiensis* at a potency of 20.1 x $10^9$ IU/l (International Unit) measured against cabbage looper (*Trichoplusia ni*). By comparison, Tx as spruce budworm unit (SBU) of the *Trichoderma*-fermented sludge reported in this study was 20-25% higher than Tx reported as IU. Tx of sample preparations was expressed in spruce budworm units/µl (SBU/µl) with 8-10% of standard deviation.

*Fungicidal Bioassay*

Two important fungal pathogens of the forestry sector were investigated by slightly modified scored response bioassays as described by Mischke [24]. This study involved submerged fermentation, and therefore no extraction of metabolites was needed as explained by Mischke [24]. In this procedure, serial dilutions of 1/2, 1/4, 1/8 and 1/16 were used in 24-well tissue culture plates. The standard times for scoring and for the ordinal scale of inhibition were similar to those of the original study [24]. The phytopathogen used

in this study was *Cylindrocladium floridanum.* The inhibition effects were quantified as inhibition index *I* in Equation 1:

$$I = \frac{\sum_{i=1}^{n}\left[\left(S_{1,i}.D_i.T_1\right)+\left(S_{2,i}.D_i.T_2\right)\right]}{M} \quad (1)$$

where *S* is the degree of inhibition assigned as numerical score for well "*I*" at time *T* in days. *D* is the dilution factor for well "*I*", e.g., 2, 4, 8, 16, respectively for wells 1, 2, 3, 4 and so on. *M* is the maximum possible *I* for each row "*i*", i.e., *S* is presumed to be equal to 2 for every well in each row "*i*" at every time so that;

$$M = \sum_{i=1}^{n}\left[\left(2.D_i.T_1\right)+\left(2.D_i.T_2\right)\right].$$

## RESULTS AND DISCUSSION

*Fermentation – Conidium Growth Kinetics*

In order to evaluate the feasibility of *Trichoderma viride* conidia production using SIW as substrate, minimum DO concentration was examined. The constant range of DO levels varied between ≥30% and ≥80% in independent experiments (Figures 1–3). The DO outside this limit was extremely deleterious for fermentation (data not shown). In case of DO below 30%, problems like wall growth, very high viscosity due to mycelia, and channeling of air bubbles were observed. However, it was extremely difficult to maintain DO above 80% due to design limitations of the fermenter. In addition, higher agitation to maintain DO > 80% caused damage to the mycelial mass and consequently resulted in lower conidia build up. The detrimental effect of agitation on *Trichoderma* spp. has already been reported by many researchers [12, 33]. Nevertheless, inhibitory effects of oxygen on fungal physiology could not be ruled out. For example, high oxygen concentration resulted in thinning and fragmentation of fungal mycelia, which consequently affected rheological behaviour [20]. Therefore, it was decided to maintain DO levels at ≤80%, ≥40% and ≥30% in three independent experiments.

Conidium growth kinetics for all fermentations were interpreted in terms of aeration and agitation requirements, CFU production, reducing sugar consumption, and SCOD utilization (Figures 1–3). At ≥30% DO, conidia production reached a maximum ($2.84 \times 10^8$ CFU/ml) after approximately 45 h of cultivation. Total reducing sugar (TRS) depleted sharply (3982 to 984 mg/l) for 18 h, followed a by slow decrease towards the end

of fermentation (Figure 1b). The TRS concentration decrease was also evident from the SCOD decrease (17212 to 2236 mg/l), which accounted for all soluble constituents (including TRS) of SIW. Furthermore, the decrease in TRS and SCOD was concurrent with an increase in mycelia growth and aeration requirements (0–24 h; Figure 1a), a well-known fact about *Trichoderma* fungi [26]. Conidiation of *Trichoderma* reached its maximum at ≈ 45 h so that the batch kinetics at ≥30% DO suggested the fermentation batch time to be ≈45 h. However, TRS and SCOD consumption and BCA efficacy (discussed later) in terms of bioassay suggested 96 h as cultivation time. In fact, at around 96 h, TRS and SCOD decreased to a minimum level, and Tx and inhibition index (I) attained their maxima. Meanwhile, micrographs showed that the mycelial mass diminished gradually from 18–96 h, leaving sparse fragments at the end (Figure 4). This observation suggested that conidiation after 96 h was not possible (nearly complete absence of mycelia), thereby rendering it as batch time.

Figure 2 shows the batch kinetics of *Trichoderma* at ≥40% DO. The profiles of conidiation, TRS consumption and SCOD reduction were similar to batch kinetics at ≥30% DO. However, conidiation (≈$2.42 \times 10^8$ CFU/ml at 40 h), the decrease in TRS (4225 to 88 mg/l at 72 h) and SCOD (17453 to 2637 mg/l at 72 h) were faster with respect to batch kinetics at ≥30% DO. Similarly, at ≤80% DO (Figure 3), the incubation time decreased to reach maximum conidiation (≈$2.77 \times 10^8$ CFU/ml at 36 h). In addition, a decrease in TRS (3736 to 0 mg/l, at 48 h) and SCOD (17261 to ≈2800 mg/l at 72 h) was also observed. Thus, conidiation, TRS and SCOD results showed that the batch time of 4 d was sufficient in each case. Nevertheless, it was observed that the increment in final CFU concentration was only marginal at ≥40% and ≤80% DO, in comparison to ≥30% DO (an increase by a factor of 1.22 to 1.25). Furthermore, the bioassay of final fermentation broth suggested superiority of ≥30% DO over ≥40 and ≤80% DO (discussed later).

Thus, DO ≥30% was found to be better for conidia formation in *Trichoderma viride* and it resulted in higher BCA activity in the fermented medium.

*Rheology and Mass Transfer Phenomena*

*Trichoderma* fermentation is a highly aerobic process [12, 18], and therefore, any increase in broth viscosity can hamper oxygen transfer rates. It would consequently be

pertinent to examine the volumetric oxygen transfer coefficient ($k_La$), broth viscosity, aeration and agitation as a function of time [4]. This would facilitate predicting and ensuring a sufficient amount of oxygen for *Trichoderma viride* growth during fermentation. This would also help increase conidia production on the one hand, and develop a strategy for scaling-up of the bioreactor design for mass production on the other hand. Previously, some authors have reported broth rheology and mass transfer limitations in *Trichoderma* fermentation [16, 35]. However, none of them considered conidia production as a main product, which is very important for the production of fungi as biocontrol agent.

Viscosity of SIW was a function of *Trichoderma* fermentation, as observed in Figures 1b, 2b and 3b. Broth viscosity varied between 416 and 1.82 mPa.s for ≥30, 40 and ≤80% DO. The highest viscosity was observed at DO concentrations in the increasing order as 30>40>80%. Irrespective of % DO, a major change in viscosity took place during the first 36 h of cultivation. This was also visualized under microscope and was apparent from the broth appearance inside the glass bioreactor. Visual observation showed an increase in mycelial mass during the first 36 h, followed by a decrease until the end of fermentation (96 h). Moreover, mycelial growth of fungi is well known to impart a viscous behaviour during fermentation [26]. In general, $k_La$ decreases with viscosity; however, in the case of ≥30% DO, the profiles of $k_La$ and the viscosity were similar until 36 h (Figure 1b). This was probably due to an increase in aeration and agitation rates to maintain ≥30% DO (Figure 1a). For ≥40% DO, $k_La$ varied inversely to viscosity until 36 h of fermentation. Subsequently, the decrease in $k_La$ was independent of viscosity and followed aeration and agitation variations. At ≤80% DO, variation in $k_La$ until 36 h was largely dependent on agitation and aeration rates. The $k_La$ decrease between 36–54 h was possibly be due to anti-foam addition.

The OUR profiles of ≥30, ≥40 and ≤80% DO are shown in Figure 5. It shows that the oxygen consumption at ≥30 and 40% DO reached maxima around 12 h and, at ≤80% DO, around 18 h. This was also apparent from mycelial mass formation during this period. The OUR profiles also collectively showed that once significant conidiation had occurred, the system did not need higher oxygen supply. This explains the decrease in OUR values after ≈36 h in all cases. Moreover, it was evident (Figure 5) that the cumulative oxygen

consumption was approximately 158.8 mmol/l at ≥30≈40% DO and 106.1 mmol/l at ≤80% DO. The higher OUR and oxygen consumption at lower DO (≥30 and 40%) with respect to ≤80% DO was probably due to the detrimental effect of agitation on mycelial biomass [12, 33]. This was also apparent in micrographs (Figure 4) and through visual observation of the glass bioreactor. In addition, the viscosity change (in the order of 30>40>>80% DO) was the indirect indicator of mycelial growth. This should explain the higher BCA efficacy of ≥30 and 40% DO fermentation broths (discussed later).

Broth rheology was also analyzed for its non-Newtonian behaviour as shown in Figure 6. The time dependent rheological profiles of *Trichoderma*-fermented SIW at different fermentation times showed a decrease in viscosity with time (thixotropic behaviour). The fermentation samples obeyed the Ostwald-deWaele power law with a confidence of fit of ≥75–93%, which was the highest in comparison with Casson and Bingham laws. The complexity of rheology was evident from the variation in $K$ and $n$, irrespective of % DO. It was noticeable that the respective initial and final values of $K$ and $n$ were almost similar in each case. The range of $K$ varied as 30>40>80% DO, and variation in $n$ closely followed the change in viscosity. The characteristic plots between $\tau$ and $\gamma$ showed all broths to be pseudoplastic in nature, and their viscosity-time profiles confirmed their thixotropic nature [27]. Thus, the rheological profiles suggested that viscosity could be a significant factor during fermentation but would lose its importance during downstream processing.

*Power Consumption*

The aeration and agitation requirements of any large-scale fermentation facility could be correlated to their power consumption [22, 30]. Therefore, it was possible to obtain a preliminary estimate of power consumption for a large-scale *Trichoderma* conidia production, based on this study. In this study, the aeration and agitation requirements were transformed into rpm.h and lpm.h, respectively, in order to correlate them with power requirements. The areas under the curves of the aeration and agitation profiles in Figures 1a, 2a and 3a were calculated and are presented in Table 2. The information from this interpretation is very useful when compared at the three DO levels. For example, an increment of 14 to 80% in agitation and 47 to 350% in aeration power was observed, when

≥30% DO process was compared with ≥40 and ≤80% DO. However, it can be concluded from previous discussions that maintaining a DO level >>30% would be energy and cost intensive. Furthermore, this approximation would be improved if a pilot facility was studied for this purpose.

*BCA Efficacy and Enzyme Activity*

The samples of fermentation broths taken at different times were subjected to insecticidal and fungicidal bioassays. Figure 7 presents the entomotoxicity (Tx), inhibition index (*I*), amylase activity and protease activity profiles at all DO levels. A common feature observed in the two bioassays was that BCA efficacy decreased with increasing level of DO (entomotoxicity: 12467 to 10058 SBU/µl; inhibition index: 0.733 to 0.568). The near maximum Tx and *I* increase could be attained at approximately 66 h in all cases, which was also concurrent with the protease activity. However, the increase in Tx and *I* was not totally dependent on conidia production (Figures 1b, 2b, and 3b). A time lag was observed between conidia formation and Tx and *I* increase. Moreover, physiological characteristics of conidia (function of incubation time) might have influenced Tx and *I* by increasing virulence, i.e., biological efficacy [18]. As the final CFU concentration was almost similar in all cases, the adverse effect on BCA efficacy at higher DO could be due to a decreased concentration of metabolites (lytic enzymes and antibiotics) [7, 8, 34]. The lytic enzymes and antibiotics have been considered as major synergistic factors along with conidia in different bioassays [16, 24]. Moreover, many researchers have suggested that a wide range of microbes contain proteins and polysaccharides (similar to starch, e.g., glycogen) in their cell wall. This enables proteases and amylases produced by *Trichoderma* spp. to antagonize pathogens by degrading their cell wall [32, 34].

Protease activity profiles (Figure 7c) of the fermentation broths showed a decrease in activity after 18, 24 and 31 h, respectively, for ≥30, 40 and ≤80% DO. The decreasing DO profile continued until ≈36 to 48 h, followed by an increase or no net change until the end of fermentation. This could be due to the adverse effects of agitation on mycelial biomass and consecutive lower enzyme production, as also reported in previous studies [19]. It was noteworthy that many enzymes showed activity in the presence of certain co-enzymes and/or co-factors (inducing agents). Therefore, it was also possible that adverse

growth conditions due to agitation (breakage of mycelial mass) might have affected the production of inducing agents. Subsequently, it resulted in a decrease in overall enzyme activity instead of the higher enzyme concentration that could be present due to mycelial lysis. Similarly, amylase and cellulase activity was adversely affected by intense agitation. The decrease in metabolites could be due to lesser mycelial growth at higher agitation conditions, as reported in various studies [9, 13].

The two types of bioassays carried out proved the dual efficacy of *Trichoderma viride*, which would be advantageous from an application point of view. Thus, single application of *Trichoderma* formulation on spruce trees (commercially valuable) would control two spruce pests: (a) an insect pathogen, the spruce budworm, and (b) a fungal pathogen, *Cylindrocladium floridanum*. This would result in pathogen control via *Trichoderma viride* formulation, which would be performant and cost effective.

## CONCLUSIONS

This study investigated the kinetic behaviour of fermentation and rheological parameters. Furthermore, power consumption was estimated in correlation to agitation and aeration requirements. Dissolved oxygen concentration at ≥30% was the most suitable option for *Trichoderma* conidia production (≥ $10^8$ CFU/ml) and biocontrol efficacy (higher entomotoxicity and inhibition index). Higher dissolved oxygen concentrations (≥40 and ≤80%) were ineffective in increasing conidia as well as BCA activity. The amounts of total reducing sugar and soluble COD consumed were similar, irrespective of dissolved oxygen levels, justifying 96 h as optimal batch time. Maximum viscosity increased (54, 188 and 416 mPa.s, respectively, for ≤80% (31 h), ≥40% (12 h) and ≥30% (18 h) owing to less shear on the mycelial mass. Rheological analyses (evaluation of $K$ and $n$) showed the pseudoplastic and thixotropic (time-dependent) nature of *Trichoderma*-fermented wastewater broths. The bioassay proved the dual efficacy (against phytopathogens, *Cylindrocladium floridanum* and spruce budworm larvae) of *Trichoderma viride* fermented starch industry wastewater. Thus, a plausible alternative to costly synthetic medium with simultaneous value-addition of a waste was explored. This batch study will be a critical step for modeling a pilot scale strategy and, finally, large-scale fermentation of BCAs.

## NOMENCLATURE

| | |
|---|---|
| $\tau$ | shear stress (mPa) |
| $\tau_0$ | yield stress (shear stress at 0 rpm of spindle, mPa) |
| $\gamma$ | shear rate ($s^{-1}$) |
| $K$ | consistency index (mPa.$s^n$) |
| $n$ | flow behaviour index (dimensionless) |
| $\mu_P$ | plastic viscosity (mPa.s) |

## ACKNOWLEDGEMENTS

The authors are sincerely thankful to 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 the authors.

## REFERENCES

1. Aiba S, Humphrey AE, Millis NF (1973) Aeration and Agitation. In: Biochemical Engineering. Academic Press, 2nd edition, New York.
2. APHA-AWWA-WPCF (1998) Standard Methods for Examination of Water and Wastewaters. 20th edn. (Eds. Clesceri, L.S., Greenberg, A.E. and Eaton, A.D.), American Public Health Association, Washington, DC, USA.
3. Bailey JE, Ollis DF (1986) In: Biochemical engineering fundamentals, 2nd edition. McGraw-Hill, New York.
4. Beegle, C.C. (1990) Bioassay methods for quantification of *Bacillus thuringiensis* δ-endotoxin, Analytical Chemistry of *Bacillus thuringiensis*, In: Analytical Chemistry of *Bacillus thuringiensis* (Eds. Hickle L.A. and Fitch W.L.), American Chemical Society, pp. 14–21.
5. Bhargava S, Nandakumar MP, Roy A, Wenger KS, Marten MR (2003) Pulsed feeding during fed-batch fungal fermentation leads to reduced viscosity without detrimentally affecting protein expression. Biotechnology and Bioengineering, 81(3), 341–347. doi: 10.1002/bit.10481
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. Butt TM (2000) Fungal biological control agents. In: Pesticide Outlook – October 2000. The Royal Society of Chemistry, pp 186–191.
8. Campos L, Felix CR (1995) Purification and Characterization of a Glucoamylase from *Humicola grisea.* Applied and Environmental Microbiology. 61(1): 2436–2438.
9. Chet I (1987) *Trichoderma* - Applications, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In: Innovative Approaches to Plant Disease Control. I. Chet, ed. John Wiley & Sons, New York. pp 137-160.
10. Cooney JM, Lauren DR (1998) *Trichoderma*/pathogen interactions: measurement of antagonistic chemicals produced at the antagonist/pathogen interface during tubular bioassay. Letters in Applied Microbiol, 27: 283-286.
11. Dinham B (2005) Agrochemical markets soar – pest pressures or corporate design? Pesticides News, 68: 9-11.
12. Felse PA, Panda T (1999) Self-directing optimization of parameters for extracellular chitinase production by *Trichoderma harzianum* in batch mode. Process Biochemistry, 34: 563-566.

13. Harmann GE, Björjmann T (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: *Trichoderma and Gliocladium* Vol. 2. (Harmann, G.E. and Kubicek, C.K., eds), Taylor and Francis Ltd., London, pp 229–265
14. Humphrey A (1998) Shake Flask to Fermentor: What Have We Learned? Biotechnol. Prog, 14: 3–7.
15. Jenkins NE, Heviefo G, Langewald J, Cherry AJ, Lomer CJ (1998) Development of mass production technology for aerial conidia for use as mycopesticides. Biocontrol News and Information, 19(1): 21N-31N.
16. Kanauchi M, Bamforth CW (2001) Growth of *Trichoderma viride* on crude cell wall preparations from barley. J. Agric. Food Chem. 49: 883-887.
17. Kunitz, M. (1947) Crystalline soybean trypsin inhibitor. Journal of General Physiology 30, 291–310.
18. Lewis JA, Papavizas GC (1983) Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. Soil Biol. Biochem. 15(3): 351-357.
19. Lejeune R, Baron GV (1995) Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation. Appl Microbiol Biotechnol, 43: 249–58.
20. McIntyre M, Breum J, Arnau J, Nielsen J (2002) Growth physiology and dimorphism of *Mucor circinelloides* (syn.*racemosus*) during submerged batch cultivation. Appl Microbiol Biotechnol, 58: 495–502.
21. 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.
22. Michell BJ, Miller SA (1962) Power requirements of gas-liquid agitated systems. A.I.Ch.E.J. 8: 262–266.
23. Miller GL (1959) Use of dinitrosalycylic acid reagent for determination of reducing sugars. Anal Chem. 32: 426–8.
24. Mischke S (1997) A quantitative bioassay for extracellular metabolites that antagonize growth of filamentous fungi, and its use with biocontrol fungi. Mycopathologia, 137: 45–52.
25. Olsvik ES, Kristiansen B, (1992) Influence of oxygen tension, biomass concentration, and specific growth rate on the rheological properties of a filamentous fermentation broth. Biotechnol Bioeng, 40: 1293–1299.
26. Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances, 22: 189-259. doi:10.1016/j.biotechadv.2003.09.005
27. Perry RH, Green, DW (1997) Perry's chemical engineers' handbook, 7th ed. New York: McGraw-Hill.
28. Pickett JA, Butt TM, Doughty KJ, Wallsgrove RM, Williams IH (1995) Minimising pesticide input in oilseed rape by exploiting natural regulatory processes. Plenary lecture. Proceedings of the GCIRC 9th International Rapeseed Congress, Cambridge, U. K., 4-7 July 1995. 2, 565–71.
29. Punja ZK, Utkhede RS (2003) Using fungi and yeasts to manage vegetable crop diseases. TRENDS in Biotechnology 21(9): 400–407. doi:10.1016/S0167-7799(03)00193-8
30. Rushton JH, Costich EW, Everett HJ (1950) Power characteristics of mixing impellers. Chemical Engineering Progress. 46: 395–404.
31. Verma M, Brar SK, Tyagi RD, Valéro JR, Surampalli RY (2005) Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. Water Research, 39(15), 3587-3596. doi:10.1016/j.watres.2005.07.001
32. Wang SL, Yen YH, Tsiao WJ, Chang WT, Wang CL (2002) Production of antimicrobial compounds by *Monascus purpureus* CCRC31499 using shrimp and crab shell powder as a carbon source. Enzyme and Microbial Technology 31: 337–344.
33. Weber J, Agblevor FA (2005) Microbubble fermentation of *Trichoderma reesei* for cellulase production. Process Biochemistry, 40: 669–676. doi:10.1016/j.procbio.2004.01.047
34. Zaldívar M, Velásquez JC, Contreras I., Pérez LM. (2001) *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. Electronic Journal of Biotechnology, 4(3): 160-168.
35. Zheng Z, Shetty K (1998) Cranberry processing waste for solid state fungal inoculant production. Process Biochemistry, 33(3): 323-329.
36. Zlokarnik M (1998) Problems in the application of dimensional analysis and scale-up of mixing operations. Chemical Engineering Science, 53(17): 3023–3030.

**Table 1.** Characteristics of starch industry wastewater

| Parameter | Concentration ± σ* (mg/kg, unless stated otherwise) |
|---|---|
| Total solids (g/l) | 16.7 ± 0.872 |
| Total volatile solids (g/l) | 13.1 ± 0.601 |
| Suspended solids (g/l) | 2.1 ± 0.060 |
| Suspended volatile solids (g/l) | 1.9 ± 0.104 |
| pH | 4.0 ± 0.01 |
| Total carbon | 401000 ± 25000 |
| Total nitrogen | 59200 ± 2700 |
| Total phosphorus | 12015 ± 808 |
| $N\text{-}NH_3$ | 579 ± 47 |
| $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ | 42.8 ± 2.7 |
| $P\text{-}PO_4^{3-}$ | 782 ± 56 |
| Al | 195.8 ± 8.8 |
| Ca | 26000 ± 2000 |
| Cd | 0.9 ± 0.1 |
| Cr | 9.6 ± 0.6 |
| Cu | 100.5 ± 6.56 |
| Ni | 12.6 ± 1 |
| Fe | 5623 ± 306 |
| K | 1785 ± 141 |
| Pb | 15.3 ± 0.9 |
| Mn | 54.2 ± 2.8 |
| S | 684 ± 51 |
| Zn | 612 ± 35 |
| Na | 6810 ± 501 |

* σ Standard deviation

**Table 2** Power requirements in terms of rpm.h and lpm.h for fermentation

| *% DO (≥)* | *rpm.h* | *lpm.h* |
|---|---|---|
| 80 | 63303 | 348.13 |
| 40 | 40139 | 133.26 |
| 30 | 35585 | 90.48 |

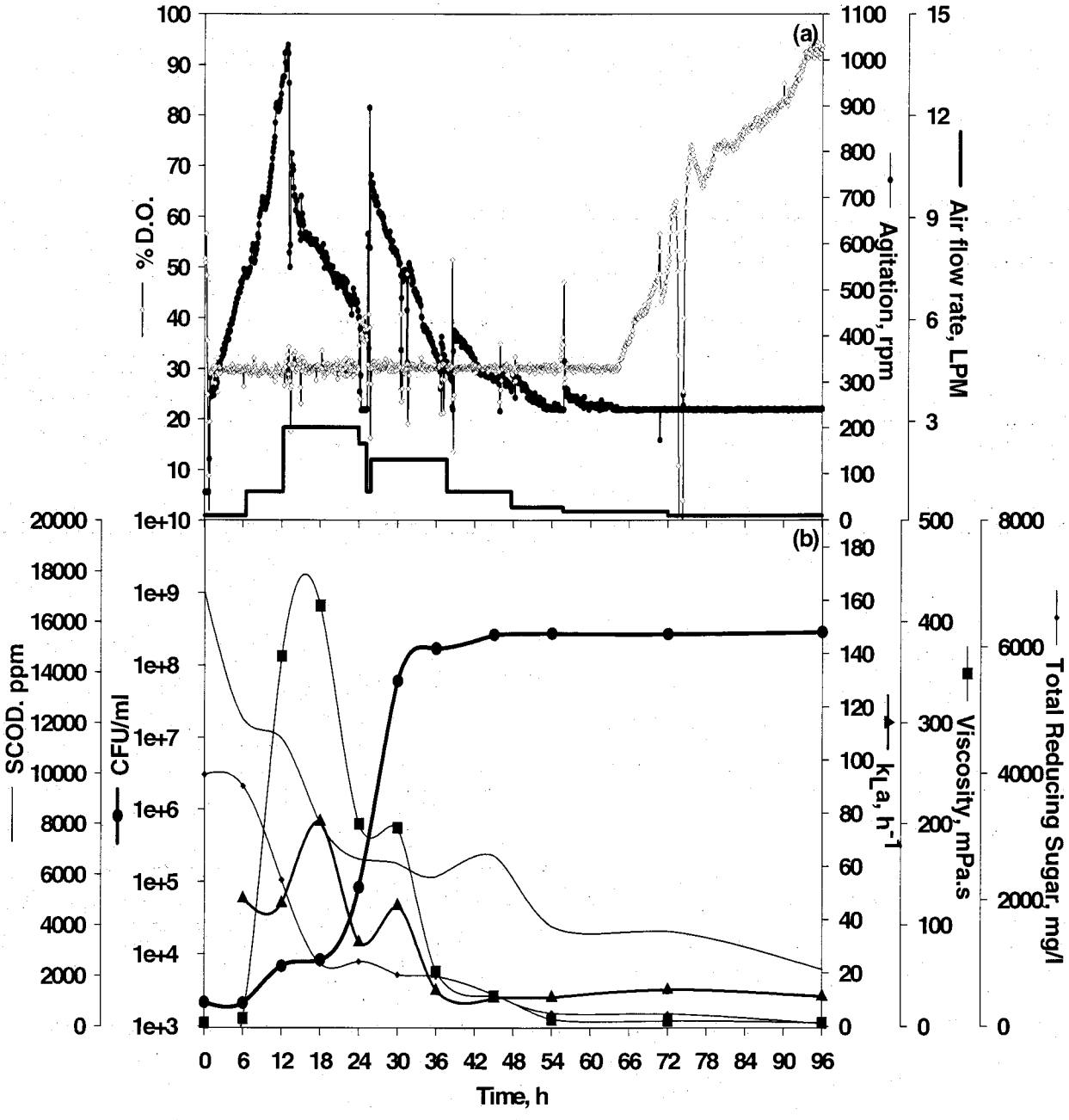



**Figure 1.** Fermentation profile of *T. viride* in SIW at ≥30% DO; (a) operational parameters, (b) growth parameters.

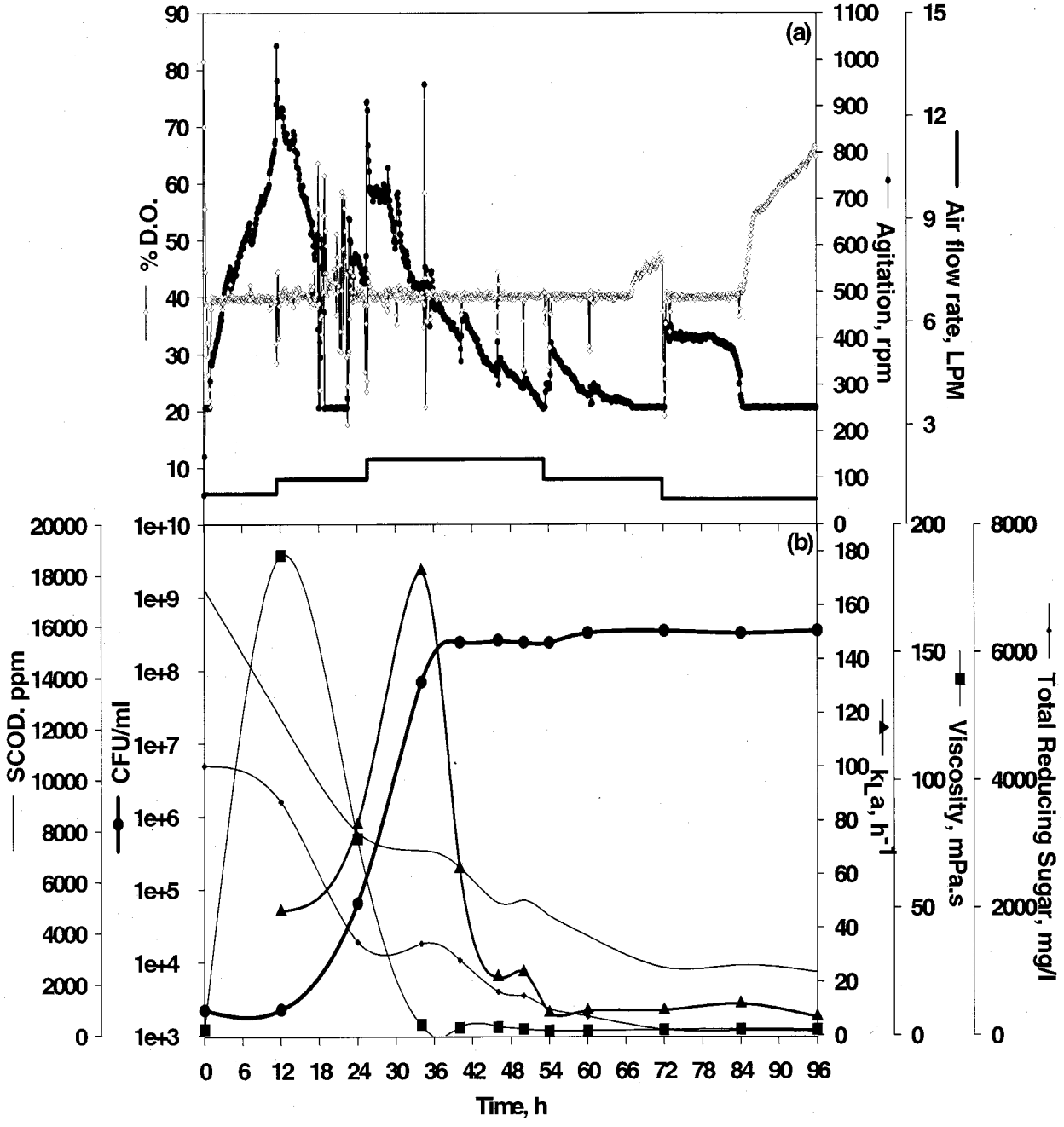



**Figure 2.** Fermentation profile of *T. viride* in SIW at ≥40% DO; (a) operational parameters, (b) growth parameters.

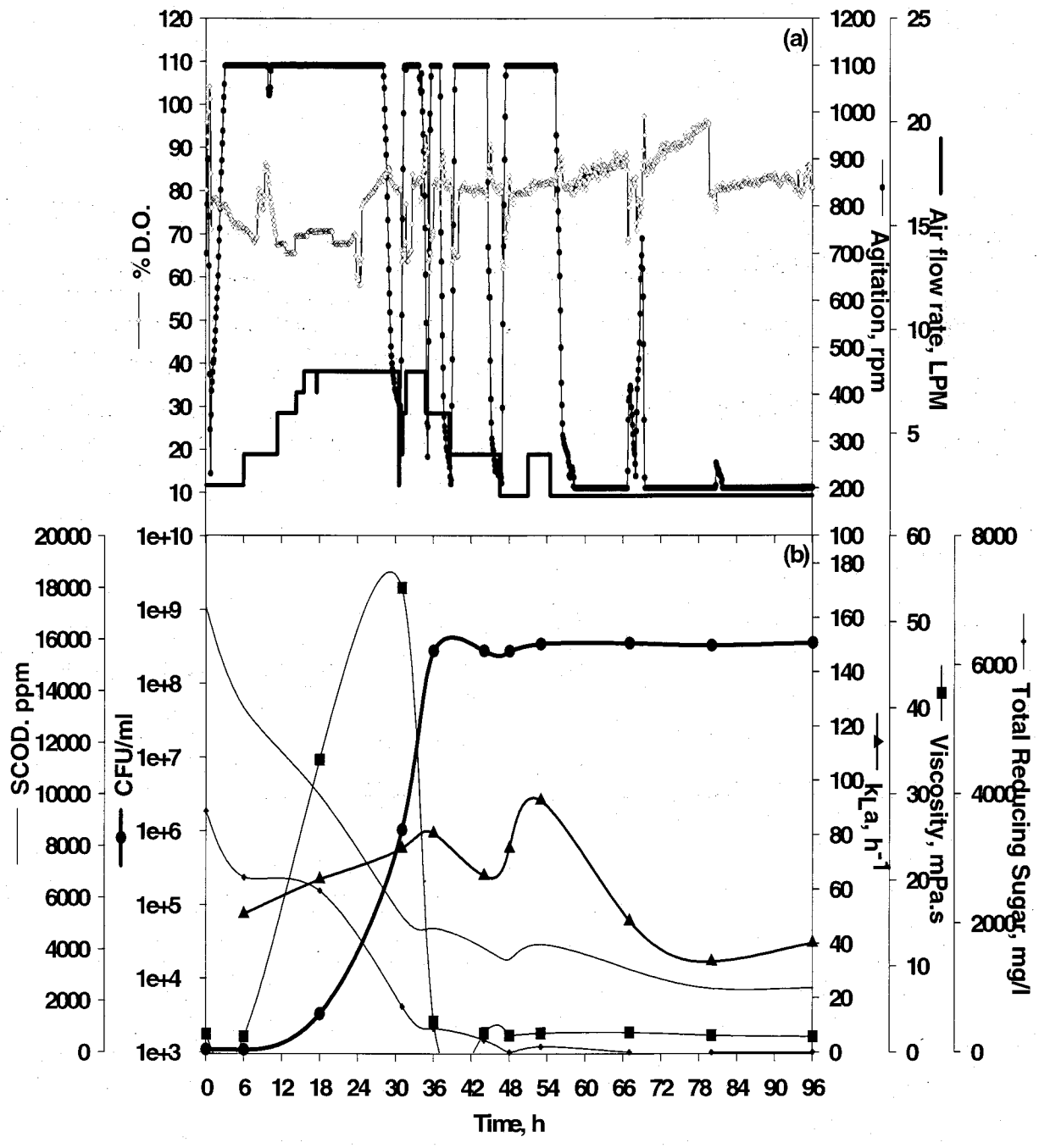



**Figure 3.** Fermentation profile of *T. viride* in SIW at ≤80% DO; (a) operational parameters, (b) growth parameters.

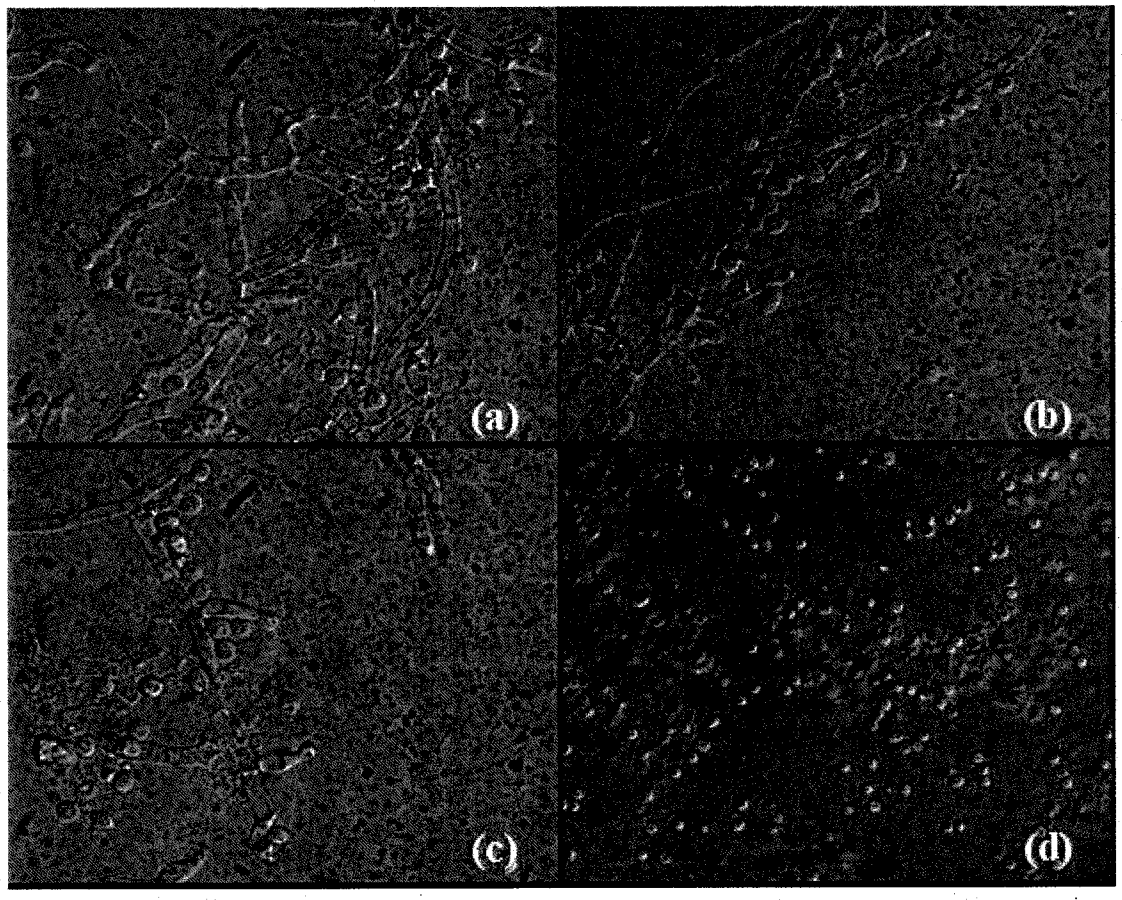



**Figure 4.** SIW ≥30% DO; (a) vegetative growth as mycelia at 18 h, (b) partial conidiation at 36 h and (c) 45 h, and (d) almost complete conidiation at 96 h.

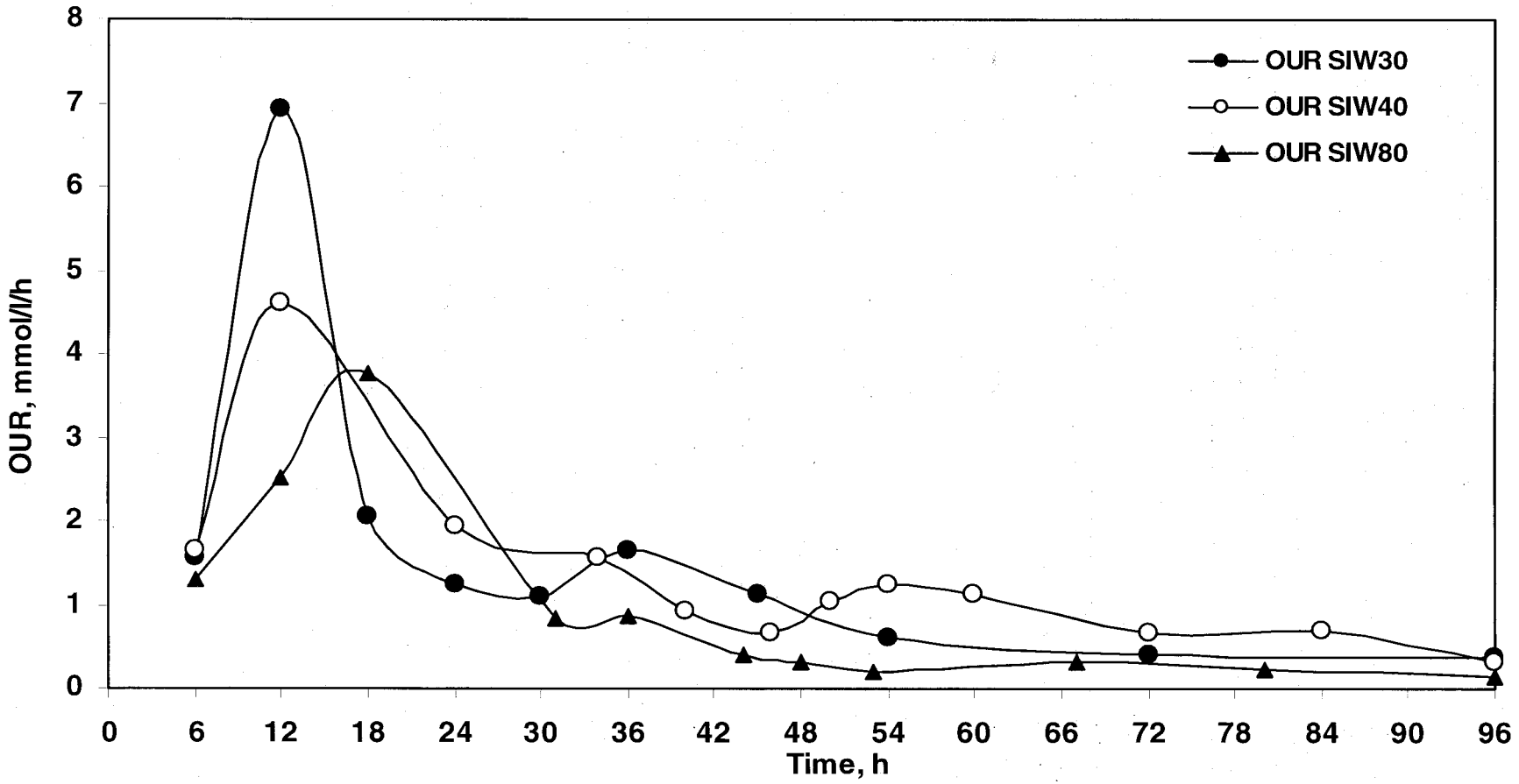



**Figure 5.** OUR profile of *T. viride* in SIW at ≥30, 40 and ≤80% DO

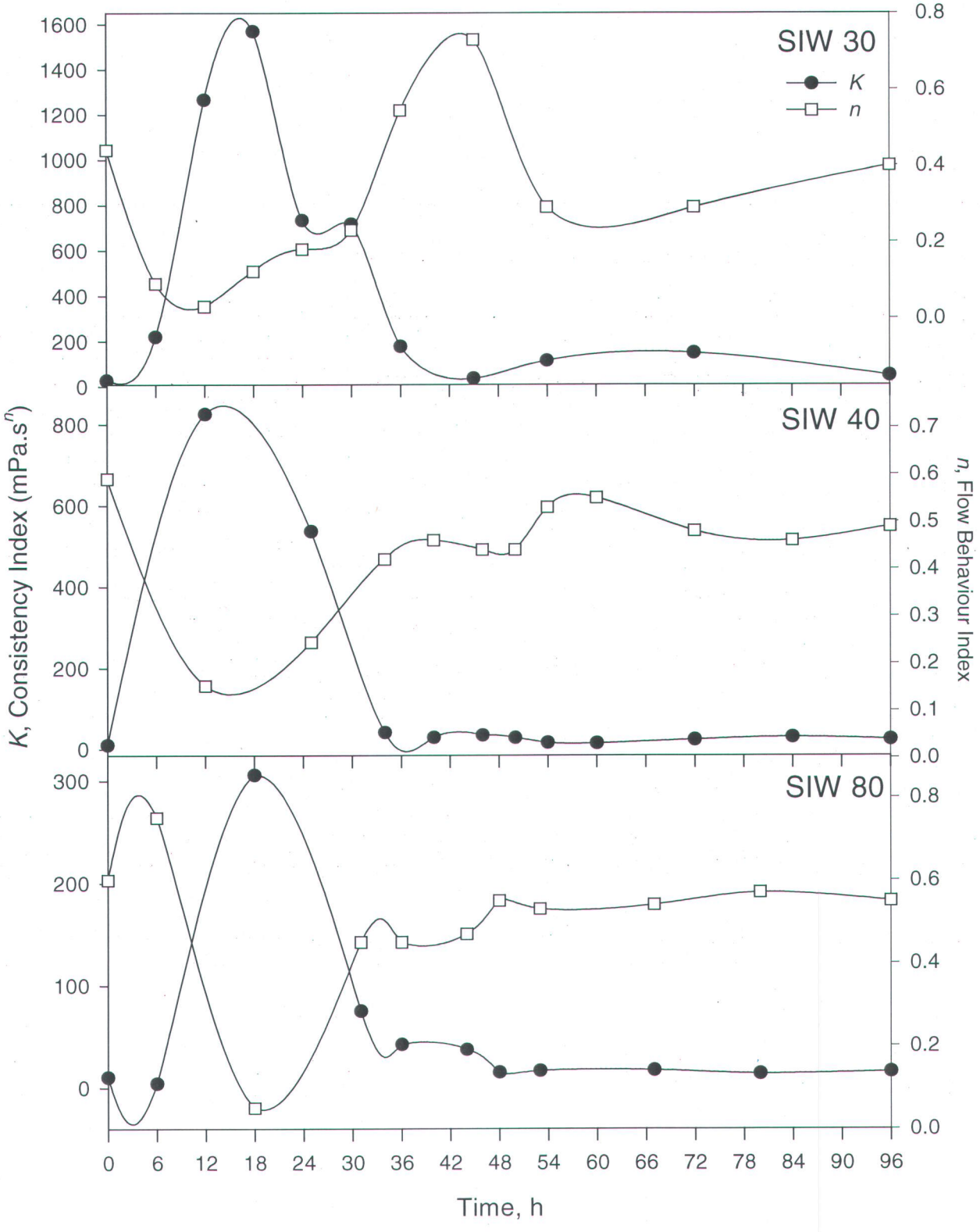



**Figure 6.** Rheological profile of *T. viride* in SIW at ≥30, 40 and ≤80% DO

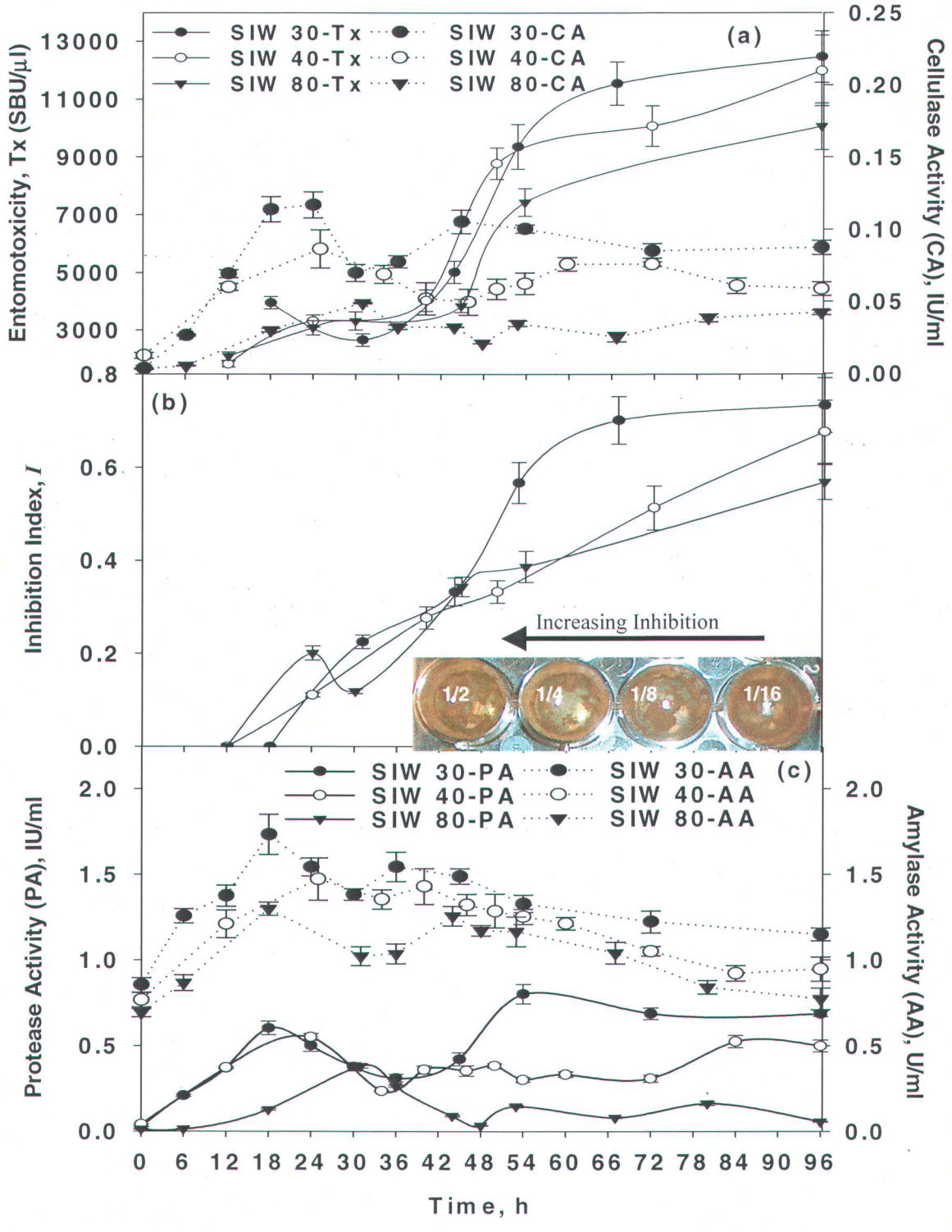



**Figure 7.** BCA activity of *Trichoderma viride*; (a) entomotoxicity, cellulase activity and (b) inhibition index profile (Inset – appearance of fungal bioassay wells), and (c) amylase and protease activities with incubation time.

## Part IV

# Fed batch fermentation strategy for antagonist *Trichoderma viride* production using unstructured mathematical modeling in starch industry wastewater

Mausam Verma[1], Satinder K. Brar[1], R. D. Tyagi[1,*], V. Sahai[2], D. Prévost[3], J. R. Valéro[1] and R. Y. Surampalli[4]

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

[2]Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Delhi, 110 016, India

[3]CRDSGC, Agriculture et Agro-alimentaire Canada, 2560 boul. Hochelaga, Québec, Canada G1V 2J3

[4]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117

(Communication: R.D. Tyagi, E-mail: tyagi@ete.inrs.ca; Phone No. : 1(418) 654 2617; Fax No. : 1(418) 654 2600)

**(Submitted: Biochemical Engineering Journal)**

**Modèle mathématique pour l'établissement d'une stratégie de production d'un antagoniste à base de *Trichoderma viride* par fermentation en mode «fed batch» en utilisant des eaux usées de l'industrie d'amidon**

**Résumé**

Un modèle mathématique a été établi pour la production en mode de bio-réaction «fed batch» fermentation, du champignon antagoniste basé sur *Trichoderma viride*. Des eaux usées d'amidon ont été employées comme matières premières de fermentation et ce, en utilisant un volume variable du procédé «fed batch». Les données de fermentation ont été employées comme des paramètres cinétiques de l'obtention des conidies, de l'accroissement de l'activité de lutte biologique et de la consommation du substrat. La concentration des conidies, l'activité entomotoxique et le taux de sucre résiduel ont été pris en compte pour développer le modèle mathématique. De plus, huit (8) paramètres cinétiques ont aussi été considérés par rapport aux fermentations et cela, afin de simuler les valeurs des variables de fermentation, soit les conidies, le sucre résiduel total et l'entomotoxicité. La production d'amylase, de protéase et de cellulase atteint des valeurs maximales durant les premières 18–72 h de fermentation, puis diminue à la fin de l'opération. L'entomotoxicité et l'activité anti-fongique augmentent au cours des premières 96 h puis commence à diminuer après 108 h. La concentration de conidies se stabilise après 30 h, puis suit le même pattern que l'entomotoxicité et l'action fongicide. La durée de fermentation en mode «fed batch» a été établie à 108 h. Le modèle mathématique pourra permettre d'une part de prédire de façon avantageuse la phase de production des conidies et d'autre part de contribuer à planifier la stratégie d'addition des substrats au cours de la fermentation en mode «fed batch».

**Mots clés:** Lutte biologique, entomotoxicity, fed-batch, fermentation, activité anti-fongique, modèle mathématique, *Trichoderma viride*.

**Abstract**

A mathematical model was developed for fed-batch fermentation of an antagonistic fungus, *Trichoderma viride* based on batch cultivation. Starch industry wastewater was used as fermentation medium using variable volume fed-batch process. The batch cultivation data were used to guesstimate kinetic parameters for conidia production, biocontrol activity increase and substrate consumption. Conidia concentration, entomotoxicity concentration and total residual sugar concentration were considered for the unstructured mathematical model development. In addition, eight kinetic parameters were also taken into account on the basis of batch cultivations in order to simulate model values of fermentation variables, namely, conidia, total residual sugar and entomotoxicity. The production of amylase, protease and cellulase enzymes showed maximum values during 18–72 h, which decreased at the end of the fermentation. Entomotoxicity and fungicidal activity increased until around 96 h and started declining after 108 h. The conidia concentration initially stabilized around 30 h, however, it followed similar growth profile as entomotoxicity and fungicidal activity. Therefore, fed-batch fermentation time was determined to be 108 h. The mathematical model developed in this study can aid in precise prediction of conidia production and also in determination of nutrient feeding strategy in fed-batch fermentation.

Keywords: Biocontrol, entomotoxicity, fed-batch, fermentation, fungicidal activity, mathematical model, *Trichoderma viride*.

**Introduction**

*Trichoderma* spp. based biocontrol agents (BCAs) have been found to be very effective due to its bidentate approach, namely, the plant growth promotion as well as its action against a wide range of plant pathogenic fungi including *Pythium* spp., *Rhizoctonia solani* , *Fusarium* spp., among others (Papagianni, 2004). Despite the enormous potential of *Trichoderma* spp. based BCAs, they have not yet reached the desired level in order to compete profitably in the world market. The largest technological impediment to the use of *Trichoderma* spp. as BCAs is the lack of both technology and facilities for biologically- and economically-effective production (Silva et al., 2005). Thus, in the pursuit of reducing the production costs, various researchers have carried out extensive studies by using alternative raw materials like agricultural and industrial residues but most of these studies

pertained to enzymes production (Doppelbauer et al., 1987; Maheshwari et al., 1994). Meanwhile, our earlier research was concentrated on the use of wastewater and wastewater sludge as potential raw materials for the growth of *Trichoderma* spp. yielding encouraging results (Verma et al., 2005, 2006 a,b). The studies proved starch industry wastewater to be an excellent proponent for *Trichoderma* spp. production with high spore counts and improved entomotoxicity (antagonistic potential) and enzyme production (Verma et al., 2006a).

As discussed earlier, mass scale production of *Trichoderma* spp. is limited. However, there are many fermentation techniques available which can improve the mass scale production of BCA. Fed-batch strategy is a widely studied technique which is subject to many permutations and combinations (Stanbury et al., 1995). Typical fed-batch fermentation could comprise many sets of data of feed input and feed rates which are conventionally dealt by trial and error approach. However, adoption of a mathematical modeling approach would reduce the number of experiments to achieve a set of fed-batch fermentation operation conditions and is a more rational and rapid approach to develop a scale-up strategy. Earlier fed-batch strategies in the case of *Trichoderma* spp. were mainly related to on-line control strategies and suffered disadvantages of lack of reliability and robustness (Riva et al., 2000) due to reasons like limitations of reliable online analysis of fermentation products (parameters). On the other hand, off-line determination (feed-forward fed-batch strategy, in this case) of fermentation parameters (spore concentrations, substrate and product) could be reliably used to interrelate with possible mathematical models. Thus, model simulations can be used to determine the best set of fermentation parameters based on minimal number of experiments, thereby, saving time and resources. In particular, Verma et al., (2006a) have reported that conidia production by *T. viride* was enhanced by about 1–2 log cycles by increasing total residual concentration (in terms of soluble starch up to 20 g $l^{-1}$). Therefore, it was expected that conidia production can be enhanced by SIW, if fresh SIW (source of starch) would be introduced at suitable feed rate and fermentation time. Meanwhile, to the best of our knowledge, no study has been reported on model based feed forward fed-batch strategy for *Trichoderma* spp. based BCA production on waste.

While incorporating the input parameters to the model, various factors need to be taken into consideration, namely, biomass production, substrate consumption and BCA activity (translated through entomotoxicity) which will dictate the overall process. In addition, varying morphology of *Trichoderma* spp. based BCAs are known to present special challenge in optimization and scale-up (Wang et al., 2005). Thus, the model presented herein, based on our laboratory fed-batch studies is unique as it will establish the strategy for mass scale production of *Trichoderma* spp. from wastes enhancing their commercial potential.

In this perspective, the performance of a feed forward fed-batch model for the production of *Trichoderma* spp. conidia and entomotoxicity (Tx) in starch industry wastewater was studied. The model consisted of key variables, namely, concentrations of conidia, total residual sugar and Tx coupled to the mass balance equations. Principal objectives of this study were: Development of a mathematical model to estimate fermentation parameters for the production of *T. viride* conidia and Tx followed by validation of the best set of fed-batch operation parameters obtained by several simulation runs of the model. In addition, assessment of BCA activity of the *T. viride* conidia in soil should also be carried out to prove its commercial potential.

## Materials and Methods

*Chemicals*

All reagents and other chemicals used for enzyme activity measurements were of analytical grade and were obtained from EM Sciences (Ontario, Canada).

*Starch industry wastewater*

The raw material for fermentation was procured as wastewater from a local wheat starch processing industry (ADM Ogilvie, Candiac, Quebec). The metal content of SIW were similar to the values reported by us earlier with a maximum variation of about 5–8%. The solids content of the SIW were 17.3 g/l and 14.9 g/l, respectively, for total solids (TS) and dissolved solids (DS), measured as per APHA (1998). The pH of SIW was 3.8 ± 0.01 at the time of sampling. The concentration of metals in the SIW was also determined as per APHA (1998) and was within the limits of Québec Govt. guidelines for agricultural

application (MENV, 2004). The SIW was stored for a maximum of 2-3 weeks at 4 ± 1°C to minimize microbial growth.

*Microorganisms for antagonism and phytopathogenesis*

*Trichoderma viride* was utilized in this study for antagonism assessment (Verma et al., 2006). The inoculum preparation and maintenance methods for *T. viride* for shake flask starter culture are described in Verma et al., (2006). In brief, 500 ml shake flasks, containing 150 ml sterile (121 °C for 15 minutes in autoclave) tryptic soya broth (TSB, Difco) at pH 6.0 were inoculated with 4-7 day old ½" × ½" size mycelial mat of *T. viride*. The shake flaks were incubated in a rotary shaker at 28 °C and 250 rpm for 48 h. An inoculum prepared in TSB medium of 10% (v $v^{-1}$) was employed for SIW fermentation in shake flasks and fermenter. The preparation method for starter culture inoculum of phytopathogenic fungus (*Fusarium* sp., Verma et al., 2006) in shake flask was similar to *T. viride*, except that it was cultured at pH 7.0, 25 °C and incubated for 24 h. Further, an inoculum of 5% (v $v^{-1}$) of *Fusarium* sp. was utilized for the production in bench scale fermenter (15 l capacity).

The *T. viride* inoculum for 7.5 l (5 l, working volume) bench scale fermenter was prepared in two to three 500 ml shake flasks using TSB as fermentation medium. The operating pH, temperature and dissolved oxygen (DO) were maintained, respectively, at 6.0, 28 °C and ≥ 30% as already established for optimal batch scale production of *T. viride* conidia (Verma et al., 2006).

*Bench scale fermenter operation*

The bench scale fermentation study of *T. viride* on SIW was carried out in a 7.5 l (5 l, working volume) fermenter (LABFORS 3, INFORS AG, Switzerland). The fermenter unit comprised automated control of pH, agitation, anti-foam addition, dissolved oxygen, temperature and air flow rate. The data acquisition was linked to a process control software (IRIS version 5.01; Infors, Switzerland) and was utilized for fermentation process automation. The aeration system was equipped with a tubular sparger with perforated end for fine bubbles, two equally spaced Rushton turbine-type impellers and three baffles. The

SIW for *T. viride* were sterilized inside the fermenter for 30 min at 121 °C. The DO level was maintained at ≥ 30% for *T. viride* to avoid oxygen limitation on growth by varying the agitation speed via PID control and manual control of aeration rate. Fermentation broth samples (50-75 ml) were aseptically drawn at regular intervals and stored at 4 ± 1°C for subsequent analyses. The duplicate set of fermentations was carried out for significance tests of the data.

*Conidia measurement*

The conidia concentration was measured in terms of CFU on tryptic soya agar (TSA) plates as described in Verma et al., (2005). In this method, the conidia were separated from fermentation broth by using a small autoclavable filtration unit. Further, the CFU plating was conducted automatically on TSA medium using Whitley Automatic Spiral Plater 2, Fisher Scientific, Quebec, Canada. For statistical significance of data, five replicates of three different dilutions of two fermentation runs were employed for ANOVA enumeration.

*Total reducing sugar*

Substrate concentration of SIW was assessed in terms of total reducing sugar concentration. For this, the fermentation broth supernatant was assayed by the dinitrosalicylic acid (DNS) method as explained for cellulase enzyme activity.

*Biocontrol Assays*

Entomotoxicity

The insect control potential of samples was determined by using eastern spruce budworm (SB) larvae (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) as per method described by Brar et al. (2005). Entomotoxicity (Tx) of *T. viride* fermented samples was measured in SB units $\mu l^{-1}$ (SBU $\mu l^{-1}$). In our earlier studies, one SBU has been reported to be about 20% lesser than one international unit (IU) of entomotoxicity. The statistical significance of the Tx data was assured by using twenty replicates at three different dilutions of two fermentation runs of the fermentation samples. The data were treated by ANOVA enumeration.

Fungicidal activity

Modified scored response bioassay of Mischke (1997) was employed. The phytopathogen insect utilized for fungicidal activity was also a spruce tree pathogen *Fusarium* sp. and it was procured from the Laurentian Forestry Centre (LFC, Quebec, Canada). In order to remove conidia from fermentation broth, the broth was centrifuged at 12000 g for 15 min at 4 °C followed by filtration through ϕ 0.45 µm pore-size glass fiber filter (Whatman paper 934-AH). Afterwards, serial dilutions of 1/2, 1/4, 1/8 and 1/16 in ultrapure water were used in 24-well tissue culture plates for evaluation of antifungal activity of the extracellular metabolites of *T. viride*. The scoring time and the inhibition scale were same as that in the original study (Mischke, 1997).

Plant biocontrol

Plant growth promotion and disease prevention assessments of *T. viride* fermented sludge (96 h) in soil were carried out on three commercially important plants, namely, green pepper (*Capsicum annuum grossum*), tomato (*Lycopersicon esculentum*) and soya (*Glycine max*) under greenhouse conditions (30 °C, 60% relative humidity and 16 h photoperiod). The soil for the bioassay was utilized in four combinations; (1) without any treatment, (2) mixed with *T. viride* conidia at $10^6$ CFU (g soil) $^{-1}$ (commonly used conidia concentrations, Yedidia et al., 1999), (3) mixed with spores of *Fusarium* sp. at $10^6$ CFU (g soil) $^{-1}$ soil, and (4) mixed with *T. viride* conidia and *Fusarium* sp., each at $10^6$ CFU (g soil) $^{-1}$. The statistical significance of data was assured by carrying out bioassay under randomized blocks design containing a total of 20 plastic pots for each plant. Each plant was sown in separate pots (about 7–9 seeds) containing 1.2 kg soil at 0 d and after 2 weeks period, only 3 germinated plants per pot were retained for bioassay purpose. Analysis of variance (ANOVA) test was also carried out at the level of $p < 0.05$ to determine the significant difference within as well as between treatments.

*Microscopy*

Microscope samples were obtained by preparing smear of approximately 50 µl of fresh fermentation broth. The smears were examined under microscope for detection of mycelia and conidia, and contamination, if any. A computer coupled optical microscope

(Zeiss Axiolab) equipped with a digital camera (Axiocam HRC Zeiss) was utilized for the image of microbial cultures.

*Enzyme activities*

All enzyme activity assays were carried out utilizing soluble phase of the fermentation medium. For this, *T. viride* fermented sludge samples were centrifuged at 7650 g for 20 min at 4 ± 1°C and the supernatant was used as an enzyme aliquot for subsequent assays. The specific methods for different enzymes are described as follows.

Amylase

Amylase activity was determined by a slightly modified method of Bergmeyer and Bernt (1974). According to this method, the reaction mixture consisted of 40 µl of 0.5 M sodium acetate buffer at pH 6.0, 60 µl of enzyme solution, 100 µl of 0.5% (w $v^{-1}$) soluble starch, and ultrapure water to make up the volume to 200 µl. The enzymatic reaction was allowed to be carried out at 50 °C for 30 min and was stopped by heating the reaction tubes in a water bath at 96 °C for 5 min as described in Campos and Felix (1995). The glucose production from the reaction mixture was analyzed using glucose assay kit from Sigma-Aldrich Chemicals (GAGO20-1KT). The unit of amylase enzyme activity was defined in terms of the amount of enzyme that could release 1 µmol of glucose per min from soluble starch.

Protease

Protease activity was measured at 275 nm in a spectrophotometer by estimating the concentration of tyrosine ontained from casein as enzyme substrate. The enzyme activity was determined in IU $ml^{-1}$ as per modified method of Kunitz (1947). The supernatant was appropriately diluted in borate buffer at pH 8.2 and used for enzymatic assay.

Cellulase

The cellulase enzyme activity was measured as per method described by Wang et al. (2002). In this method, 100 µl of enzyme aliquot was mixed with 400 µl of substrate solution (1.25% (w $v^{-1}$) carboxy methyl cellulose (CMC) in an acetate buffer solution (125

mM, pH 5)). Later, the enzymatic reaction mixture was retained at 37 ± 1 °C for 10 min. The enzyme mixture was then centrifuged at 7650 g for 10 min, and the amount of reducing sugar generated in the supernatant was assayed by the dinitrosalicylic acid (DNS) method (Miller, 1959). The unit of cellulase activity was defined as the generation of 1 μmol of reducing sugar per minute at 37 °C and a pH of 5.

In all enzyme activity measurements, the standard deviation was established to be ≤ 8%, based on triplicate samples of two fermentation runs.

*Oxygen mass transfer coefficient ($k_La$)*

The dynamic gassing-out method was used for $k_La$ measurements (Aiba et al., 1973). The DO control was momentarily stopped during the dynamic gassing-out method to facilitate correct $k_La$ measurements. The calibration methods of pH and DO probes and the measurements of $k_La$ are explained in (Brar et al., 2005). The oxygen sensor probe used in this study was based on Clark polarographic sensor. The probe was equipped with a temperature compensation circuit and a response time ≤ 30s to attain 90% of the final value at 25°C.

*Viscosity measurements*

The fresh fermentation media were examined for rheological behaviour by measuring viscosity and shear stress vs. shear rate profiles. The measurements were carried out using a rotational viscometer (DVII+, Brookfield) equipped with a small sample adapter spindle (SC4 34, Brookfield). The details of the viscometer are explained in earlier studies (Verma et al., 2006). The Ostwald-deWaele power law, $\tau = k\,\gamma^n$ (widely studied) was investigated to describe the rheological characteristics of all samples.

*Mathematical model development*

*T. viride* fermentation on substrate like SIW poses challenges like entangled growth of mycelium on fibrous residues present in SIW as suspended matter. Therefore, growth of mycelial biomass was very difficult to measure by using centrifugation, filtration or

washing of the mycelial pellets in the fermentation broth. At this juncture, in order to overcome this problem, an unstructured model was preferred to depict *T. viride* batch fermentation process. In the past, several researchers have also followed unstructured models, which in many cases provided satisfactory agreement with experimental data by adopting a simple mathematical approach (Phisalaphong et al., 2006; Dalsenter et al., 2005; Koutinas et al., 2003). Based on prior studies of batch fermentation of *T. viride* on SIW, exponential growth rate was found to be suitable to describe kinetic of conidia production (Verma et al., 2006a). Hence, for the simplicity of the mathematical model, the specific growth rate ($\mu$) of the conidia was represented by Monod expression. In our prior studies it was established that the dissolved oxygen (DO) at $\geq$ 30% would be most favourable for conidia production as well as biological activity of *T. viride* grown in SIW (Verma et al., 2006b), therefore, DO was maintained at $\geq$ 30% throughout the fermentation. Furthermore, it was also observed in our earlier studies that conidia production in SIW was dependent on total reducing sugar concentration (Verma et al., 2006b). In fact, the increase in conidia concentration ceased when total reducing sugar concentration decreased to minimum (around 36-42 h). Thus, for fermenter operation, the model was based on following assumptions:

1. The total reducing sugar in SIW was considered as the limiting nutrient.
2. Temperature, pH and DO were constant during the fermentation.

The conidia production, substrate consumption and entomotoxicity increase with fermentation time were correlated to conidia concentration (X) and/or total residual sugar concentration (R) as follows:

conidia: $$\frac{dX}{dt} = \mu X \quad (1)$$

entomotoxicity: $$q_p = \frac{1}{X}\frac{dTx}{dt} \quad (2)$$

SIW (substrate): $$-\frac{dR}{dt} = \frac{1}{Y_{X/R}}\left(\frac{dX}{dt}\right) + \frac{1}{Y_{Tx/R}}\left(\frac{dTx}{dt}\right) + K_M X \quad (3)$$

Furthermore, the Monod's kinetics resulted in following Equations:

$$\mu = \frac{\mu_m R}{K_S + R} \quad (4)$$

$$q_p = \frac{q_{pm} R}{K_p + R} \tag{5}$$

The optimization of kinetic parameters set depended significantly on the prior experience of the *T. viride* batch fermentation process. The parameter values for $\mu_m$, $q_{pm}$, $K_S$ and $K_P$, were extrapolated from Monod rate equation of the data of batch fermentation by linearization of the respective Equations. Both of the yield coefficients, namely, $Y_{X/R}$ and $Y_{Tx/R}$ were obtained from earlier batch fermentation experiments. The remaining kinetic parameters were estimated by trial and error method until the best visual fit was obtained between experimental and simulated data. The best fit values of all kinetic parameters at optimal environmental conditions (pH, 6; 28 °C and ≥ 30% DO – Verma et al., 2006b) are presented in Table 1. Least-squares method was used to minimize the sum of squared errors between the predicted and experimental data. The ordinary differential equations of the model considered, were solved by using the routine ODE 15s available in MATLAB (version 7.0.1). The ordinary differential equations were integrated by using a program *odesolve*, developed by Polking (2005). The program provides a Graphical User Interface (GUI) for use of MATLAB's differential equation solvers.

*Feed forward fermentation strategy*

The fed-batch fermentation was based on the extension of batch mathematical model. For this, the feed rate to the fermenter was estimated on the basis of following Equations:

$$\frac{dV}{dt} = F \tag{6}$$

$$\frac{dX}{dt} = \mu X - \left(\frac{F}{V} X\right) \tag{7}$$

$$q_{pm} = \frac{1}{X}\frac{dTx}{dt} - \frac{1}{X}\left(\frac{F}{V} Tx\right) \tag{8}$$

$$-\frac{dR}{dt} = \frac{1}{Y_{X/R}}\left(\frac{dX}{dt}\right) + \frac{1}{Y_{Tx/R}}\left(\frac{dTx}{dt}\right) + K_M X - \left(\frac{F}{V} R\right) \tag{9}$$

The model Equations expressed above {Eqs. (1)–(9)} were simulated using the best-fit parameters values.

## Results and Discussion

*Simulations studies using proposed model*

Different feed rates (from 0.5 to 3.0 l $d^{-1}$, in stepwise increments of 0.5 l $d^{-1}$) at different fermentation times (from 0 h to 72 h at each 12 h intervals) were examined by running several offline fed-batch model simulations on computer. The different model simulations suggested that a feed rate of 2.0 l $d^{-1}$ started at 48 h resulted the maximum possible increment in conidia concentration and Tx value at the end of fermentation. Meanwhile, it was also observed from batch fermentation experiments that around 48 h, total residual sugar concentration of the fermentation broth decreased to about 96.2% (≈163.2 mg $l^{-1}$) of initial total reducing sugar concentration. Therefore, introduction of fresh SIW medium at this stage was reasonable. Before 48 h, the simulations of lower feed rates (0.5 to 1.0 l $d^{-1}$) predicted no significant increase in the final conidia production and higher feed rates from (2.5 and 3.0 l $d^{-1}$) predicted only nominal increase in conidia production as well as the fermentation time (in comparison to batch fermentation data at 96 h). On the other hand, after 48 h, the simulations of lower feed rates (from 0.5 to 1.0 l $d^{-1}$) showed nominal increase in the final conidia production, whereas, the higher feed rates (from 2.5 and 3.0 l $d^{-1}$) resulted in nominal increase in final conidia production (in comparison to batch fermentation data at 96 h). No significant increase in conidia concentration and Tx were predicted after 120 h fermentation. The Tx value of the *T. viride* fermented broth in the model was considered as a linear function of conidia (derived from previous experimentd, Figure 1a). In fact, the fed-batch fermentation results of Tx were also linearly related to conidia concentration (Figure 1b). Thus, based on feed forward model simulation for SIW as a fermentation medium under best set of fed-batch conditions (feed started at 48 h of the fermentation time at a rate of 2.0 l $d^{-1}$) the final conidia concentration and Tx predicted were approximately, $1.12 \times 10^9$ CFU $ml^{-1}$ and 17883.2 SBU $\mu l^{-1}$, respectively around 120 h.

*Model verification with experimental result*

The conidia production of fed-batch fermentation process adopted in this study was actually a batch fermentation process until 48 h of fermentation and was switched to fed-

batch fermentation mode from 48h (as predicted through simulation) and operated in this mode until the end of fermentation (120 h) at a feed rate 2.0 l $d^{-1}$.

The batch fermentation was started with an initial fermentation medium volume of 3 l. The conidia production, Tx increase and total residual sugar consumption under the aforestated fed-batch operating conditions are shown in Figure 2. The model simulated profiles of conidia concentration, Tx value and total residual sugar concentration are also plotted by dotted line. It can be seen that the model simulated values are in high concordance with the experimental values between 24 to 108 h of the fermentation as is evident from Figure 2b.

The fed-batch fermentation model showed its limitation in terms of disagreement during 0–24 h and > 108 h where a slight decrease in experimental value of conidia concentration was observed in both the cases. The model also failed to predict the Tx value at t > 108h. This was due to the fact that during 0-24 h there was mycelium growth and therefore no conidia production during this time. The conidia production resulted after the vegetative growth phase (mycelial biomass production), hence, an obvious lag phase for conidia production was observed. The earlier reaserchers (Phisalaphong et al., 2006; Dalsenter et al., 2005) did not observe this lag as they used overall biomass concentration growth parameter rather than conidia. Moreover, production of inhibitory compounds during extended fermentation period could have adverse effect on conidia at t > 108h, as explained later in the text.

Furthermore, the conidia concentration around 48 h was $2.22 \times 10^8$ CFU $ml^{-1}$, which started decreasing from 48 to 66 h (down to $5.29 \times 10^7$ CFU $ml^{-1}$) due to combined effects of dilution of the fermentation medium with fresh SIW as well as germination of existing conidia to form mycelial biomass. The vegetative growth was also observed under microscope as well and was also evident from the glass vessel of the fermenter. The model predicted profile during 48–72 h resulted in constant decrease as only dilution of the fermentation broth with fresh SIW medium was taken into account to decrease conidia and not the germination of the conidia. After about 72 h, the increase in conidia was consistent over an extended period of fermentation time (> 120 h).

The experimental data could show an increase in conidia only up to about 108 h ($6.56 \times 10^8$ CFU $ml^{-1}$) followed by consistent decrease until 120 h ($2.87 \times 10^8$ CFU $ml^{-1}$)

due to reasons explained earlier. Therefore, the suitable fermentation time for conidia and Tx harvest was determined to be 108 h for furture experiments, e.g., the fermentation broth for plant growth promotion bioassay (explained later) was harvested at around 108 h. Thus, the unstructured mathematical model for the fed-batch strategy in the case of *T. viride* grown on SIW was validated as an increase in conidia concentration of about 61.43% was observed in contrast to batch fermentation process.

Nevertheless, a more complex model could be postulated and simulated in future for better predictability of the fermentation kinetic parameters, specifically, for initial and extended phases of the fermentation process. For this, spore germination or loss and inhibitory effects of extended fermentation periods should be incorporated in the model in terms of kinetic parameters.

*Model limitations*

As said before, the limitations of the simple model adopted in this study is that it could not account for any decrease in conidia concentration and Tx value at the end of the fermentation. As a matter of fact, the kinetic constants for conidia germination or loss and Tx decrease due to production of inhibitory compounds during extended fermentation periods which could not be incorporated in the model. Therefore, it was logical to expect that the actual conidia concentration and Tx value might not follow the model simulation values under extended fermentation time conditions.

*Entomotoxicity and fungicidal bioassay*

The Tx and fungicidal activity values of *T. viride* fermented SIW medium are shown, respectively, in Figure 2b and Figure 3a. As mentioned earlier, Tx was incorporated into the fed-batch model which showed good fit ($R^2$ = 0.89) between experimental and model simulated Tx values until about 108 h. Similar profile for fungicidal activity (modified scored response bioassay) was also observed, except, between 36 to 72 h, an increase was observed in contrast to Tx. The fungicidal activity was measured for extracellular metabolites of *T. viride* which were mostly produced during vegetative growth phase. Therefore, it could be possible that the feed input at 48 h helped in formation of vegetative biomass (mycelia), thereby, increasing the extracellular metabolites. The value of Tx and fungicidal activity (inhibition index, *I*) obtained around

108 h of fermentation were 15823.7 SBU $\mu l^{-1}$ and 0.816, respectively, which were higher than the batch fermentation (26.91 and 11.32%, respectively, for Tx and *I*) reported by Verma et al. (2006b). Thus, the fed-batch fermentation strategy also helped in increasing the antagonistic potential of *T. viride* with respect to batch fermentation.

*Enzyme activity*

*Trichoderma* spp. are well known for production of lytic enzymes on simple as well as complex substrates (Whipps and Lumsden, 2001). Moreover, the lytic enzymes produced by these fungi have been reported to be useful in their biocontrol action (Ahmad and Baker, 1987). Therefore, the fermentation broth in this study was also subjected to assays for lytic enzymes, namely, amylase, protease and cellulase. The enzyme activity profiles of these enzymes are shown in Figure 3b. The enzyme activity profiles until 48 h were similar to the batch fermentation as reported earlier (Verma et al., 2006b). However, after 48 h, the change in enzyme activity profiles was apparently affected by dilution effect of fed-batch fermentation mode. Furthermore, the effect of agitation and aeration on enzyme activities also possibly played an important role in changing the enzyme activities during the course of fermentation.

Figure 4a shows the aeration, agitation, DO and $k_La$ profiles which suggested that the change in agitation and aeration were abrupt at many times and could affect the enzyme activities, especially, in the case of mycelia forming microorganisms like *T. viride* (Lejeune and Baron et al., 1995). The enzyme activities for amylase, protease and cellulase enzymes were, respectively, 0.96 U $ml^{-1}$, 0.34 IU $ml^{-1}$ and 0.81 IU $ml^{-1}$ at around 108 h of fermentation. In general, it was observed that the enzyme production did not increase with respect to batch fermentation, possibly due to the dilution of the fermentation broth caused by concurrent feed input. Therefore, in future, fixed volume fed-batch strategy should also be examined for higher enzyme activities.

*Viscosity behaviour of fermentation broth*

Viscosity plays an important role in determining power requirements in case of submerged fermentation as well as media pumping and centrifugation. Therefore, viscosity was also measured during fermentation (Figure 4b). Although, the batch fermentation data were already available from our earlier experiments, it was expected that introduction of

fresh feed during fermentation might increase viscosity due to resultant mycelial growth (in contrast to Bhargava et al. 2003). However, the experimental results showed the decrease in viscosity after 18 h was consistent until the end of the fermentation and no apparent effect of fed-batch mode could be observed. The maximum viscosity obtained was 393.92 m Pa.s (at 18 h). The pseudoplastic behaviour of the fermentation broth was also assessed by measuring consistency index ($K$) and flow behaviour index ($n$) as shown in (Figure 4b). The variation in $K$ and $n$ showed that the rheology of SIW was highly variable during fermentation and was more pseudoplastic at the end of fermentation as $n$ was < 1.0. Therefore, it was concluded that the fermentation broth would impose higher power requirements to the agitation unit only during the initial fermentation stage (0–42 h).

*Plant growth bioassay*

The plant growth promotion bioassay results are also shown in Figure 5.The results showed that *T. viride* was active in soil and could be utilized for disease control as well as growth promotion. It was inferred that irrespective of the type of the plants (tomato, pepper and soya), *Fusarium* sp. adversely affected plant growth. In addition, *T. viride* acted in disease prevention to a greater extent in case of pepper and soya in comparison to tomato. Meanwhile, *T. viride* was not effective as a fertilizer in this case, as application of minimal fertilization was required after 4-5 weeks due to poor soil nutrient conditions. However, it is expected that repeated application of *T. viride* would certainly enhance soil nutrients as also mentioned by many researchers (Whipps and Lumsden, 2001; Ahmad and Baker, 1987) as it is a slower process (3–6 months) compared to 1–2 months span of plant bioassay experiment.

**Conclusions**

Batch fermentation of *T. viride* was extended to feed forward fed-batch mode using a simple unstructured mathematical model in starch industry wastewater as raw material. The kinetic parameters of fermentation were estimated based on extrapolation of data and initial guesses were based on prior studies. The experimental and model simulated values fitted very well during the course of fermentation, except, during initial and extended fermentation period due to nature of growth of conidia and production of inhibitory compounds or loss of conidia. However, future studies involving more kinetic parameters

like conidia germination or loss rate constant and inhibition constants might provide better fit for extended fermentation mode. Nevertheless, the model helped in minimizing several fermentation runs that would normally be required to achieve the best set of operating parameters for fed-batch mode. The increase in conidia concentration, entomotoxicity and inhibition index were, respectively, 61.43, 26.91 and 11.32% for fed-batch mode in comparison to batch fermentation. Besides, the viscosity measurements also confirmed that fed-batch mode would not impose any increase in viscosity at the end of fermentation. The increase in lytic enzymes production was normalized due to dilution of fermentation medium. Meanwhile, the plant growth bioassay proved that the fungi *T. viride* was active in soil and therefore, possesses immense commercial potential.

**Acknowledgements**

The authors are thankful to 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.

**Nomenclature**

| | |
|---|---|
| $\mu$ | specific growth rate ($h^{-1}$) |
| $\mu_m$ | maximum specific growth rate ($h^{-1}$) |
| BCA | Biocontrol agent |
| DO | Dissolved oxygen (%) |
| $F$ | feed flow rate ($l\ d^{-1}$) |
| $K$ | Consistency index (m Pa. $S^n$) |
| $K_M$ | maintenance constant ($h^{-1}$) |
| $K_P$ | saturation production constant ($g\ l^{-1}$) |
| $K_S$ | saturation growth constant ($g\ l^{-1}$) |
| $n$ | Flow behavior index |
| $q_p$ | specific production rate ($h^{-1}$) |
| $q_{pm}$ | maximum specific production rate ($h^{-1}$) |
| R | Total reducing sugar |
| SIW | Starch industry wastewater |
| TSB | Tryptic soya broth |
| Tx | Entomotoxicity (SBU $\mu l^{-1}$) |
| $V$ | volume (l) |
| $Y_{Tx/R}$ | yield coefficient for entomotoxicity increase with respect to substrate used ($10^6$ SBU $g^{-1}$) |
| $Y_{X/R}$ | yield coefficient for conidia production with respect to substrate used ($10^3$ CFU $g^{-1}$) |

## References


Ahmad JS and Baker R. Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutants of *Trichoderma harzianum*. Phytopathology 1987; **77**: 358-362.

Aiba S, Humphrey AE, Millis NF (1973) Aeration and Agitation. In: Biochemical Engineering. Academic Press, 2nd edition, New York.

APHA–AWWA–WPCF 1998. Standard Methods for Examination of Water and Wastewaters. 20th edn. (Eds. Clesceri, L.S., Greenberg, A.E. and Eaton, A.D.), American Public Health Association, Washington, DC, USA.

Bergmeyer, H. U., and E. Bernt. 1974. Determination off glucose with oxidase and peroxidase, p. 1205–1215. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 3. Verlag-Chimie-Academic Press, New York.

Bhargava S, Nandakumar MP, Roy A, Wenger KS, Marten MR (2003) Pulsed feeding during fed-batch fungal fermentation leads to reduced viscosity without detrimentally affecting protein expression. Biotechnol Bioeng 81(3):341–347. DOI: 10.1002/bit.10481

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.

Campos L, Felix CR (1995) Purification and Characterization of a Glucoamylase from *Humicola grisea*. Applied and Environmental Microbiology. 61(1): 2436–2438.

Dalsenter, F.D.H. Viccini, G., Barga, M.C., Mitchell, D.A., Krieger, N.A. A mathematical model describing the effect of temperature variations on the kinetics of microbial growth in solid-state culture, Process Biochem. 40 (2005) 801–807.

Doppelbauer, R., Esterbauer, H., Steiner, W., Lafferty, R. & Steinmuller, H. 1987. The use of cellulosic wastes for production of cellulases by *Trichoderma reesei. Appl. Microbiol. Biotechnol.* **26**: 485-494.

Koutinas, A.A., Wang, R., Kookos, I.K., Webb, C. Kinetic parameters of *Aspergillus awamori* in submerged cultivations on whole wheat flour under oxygen limiting condition, Biochem. Eng. J. 16 (2003) 23–34.

Kunitz, M. (1947) Crystalline soybean trypsin inhibitor. Journal of General Physiology 30, 291–310.

Lejeune R, Baron GV (1995) Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation. Appl Microbiol Biotechnol 43:249–58.

Maheshwari DK, Gohade S, Paul J, Varma A 1994. Paper mill sludge as a potential source for cellulase production by *Trichoderma reesei* QM 9123 and *Aspergillus niger* using mixed cultivation. Carbohydrate Polymers **23**: 161-163.

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.

Miller GL (1959) Use of dinitrosalycylic acid reagent for determination of reducing sugars. Anal Chem. 32: 426–8.

Mischke S (1997) A quantitative bioassay for extracellular metabolites that antagonize growth of filamentous fungi, and its use with biocontrol fungi. Mycopathologia, 137: 45–52.

Papagianni, M. 2004. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances, 22: 189-259.

Phisalaphong, M., Srirattana, N., Tanthapanichakoon, W. Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation. Biochemical Engineering Journal, 28 (2006) 36–43.

Polking, J.C. 2005. Ordinary differential equations using MATLAB. http://math.rice.edu/~polking/index.html.

Silva, R.G., Cruz, A.J.G., Hokka, C.O., Giordano, R.L.C. and Giordano, R.C. 2000. A hybrid feedforward neural network model for the cephalosporin c production process. Brazilian Journal of Chemical Engineering, 17, 4-7.

Stanbury, P.F., Whitaker, A., Hall, S.J. 1995. Microbial growth kinetics. In: Principles of fermentation technology, 2 ed. pp. 13–31. Elsevier Science Inc. New York, U.S.A.

Verma M, Brar SK, Tyagi RD, Valéro JR, Surampalli RY (2005) Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. Water Research, 39(15), 3587-3596. doi:10.1016/j.watres.2005.07.001

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006a. Starch industry wastewater as a substrate for antagonist, *Trichoderma viride* production. *Bioresource Technology*, DOI:10.1016/j.biortech.2006.08.032 (in press).

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006b. Dissolved oxygen as principal parameter for conidia production of biocontrol fungi *Trichoderma viride* in a non-Newtonian wastewater. *Journal of Industrial Microbiology & Biotechnology*, 33, 941–952. DOI 10.1007/s10295-006-0164-6.

Wang SL, Yen YH, Tsiao WJ, Chang WT, Wang CL (2002) Production of antimicrobial compounds by *Monascus purpureus* CCRC31499 using shrimp and crab shell powder as a carbon source. Enzyme and Microbial Technology 31: 337–344.

Wang, L., Ridgway, D., Gu, T., Moo-Young, M. 2005. Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. Biotechnology Advances 23, 115–129.

Whipps JM and Lumsden RD. 2001. Commercial use of fungi as plant disease biological control agents: status and prospects. In: *Fungal Biocontrol Agents: Progress;, Problems and Potential,* (eds T. Butt, C. Jackson & N. Magan), Wallingford: CABI Publishing, pp 9-22.

Yedidia, I., Benhamou, N., Chet, I. 1999. Induction of defense responses in cucumber by the biocontrol agent *Trichoderma harzianum. Appl. Environ. Microbiol.,* 65: 1061-1070.

**Table 1.** Best-fit kinetic parameters based on batch fermentation

| Kinetic parameters | Units | Value |
|---|---|---|
| $\mu_m$ | $h^{-1}$ | 0.046 |
| $q_{pm}$ | $h^{-1}$ | $1.67 \times 10^{-4}$ |
| $Y_{X/R}$ | $10^3$ CFU $g^{-1}$ | 12.48 |
| $Y_{Tx/R}$ | $10^6$ SBU $g^{-1}$ | $3.97 \times 10^{-5}$ |
| $K_M$ | $h^{-1}$ | 0.0017 |
| $K_S$ | g $l^{-1}$ | 10.63 |
| $K_P$ | g $l^{-1}$ | $1.2 \times 10^{-4}$ |

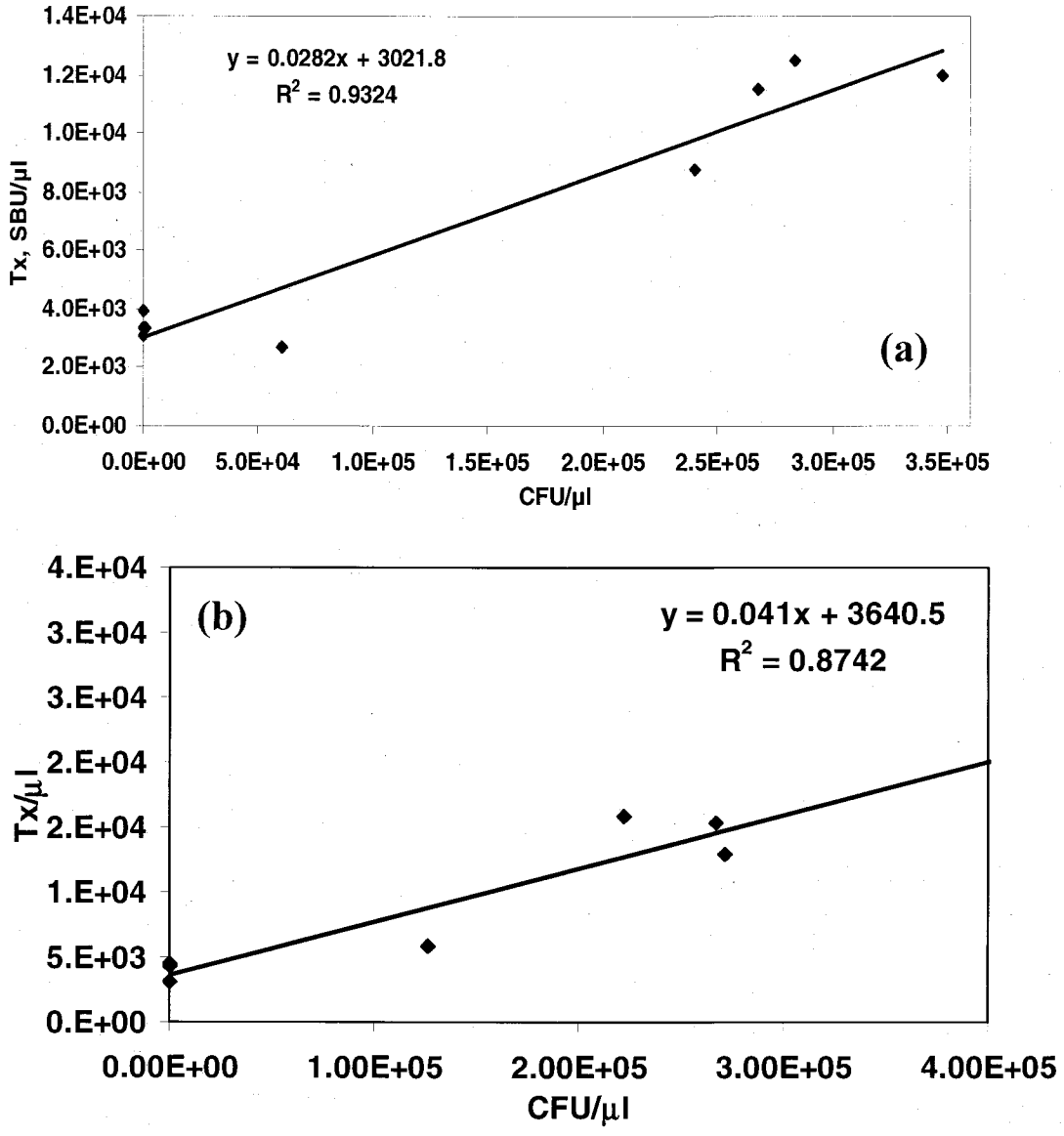



**Figure 1.** Linear correlation between Tx and conidia concentration of *T. viride* during: (a) batch fermentation (Verma et al., 2006b), (b) fed-batch fermentation (present study).

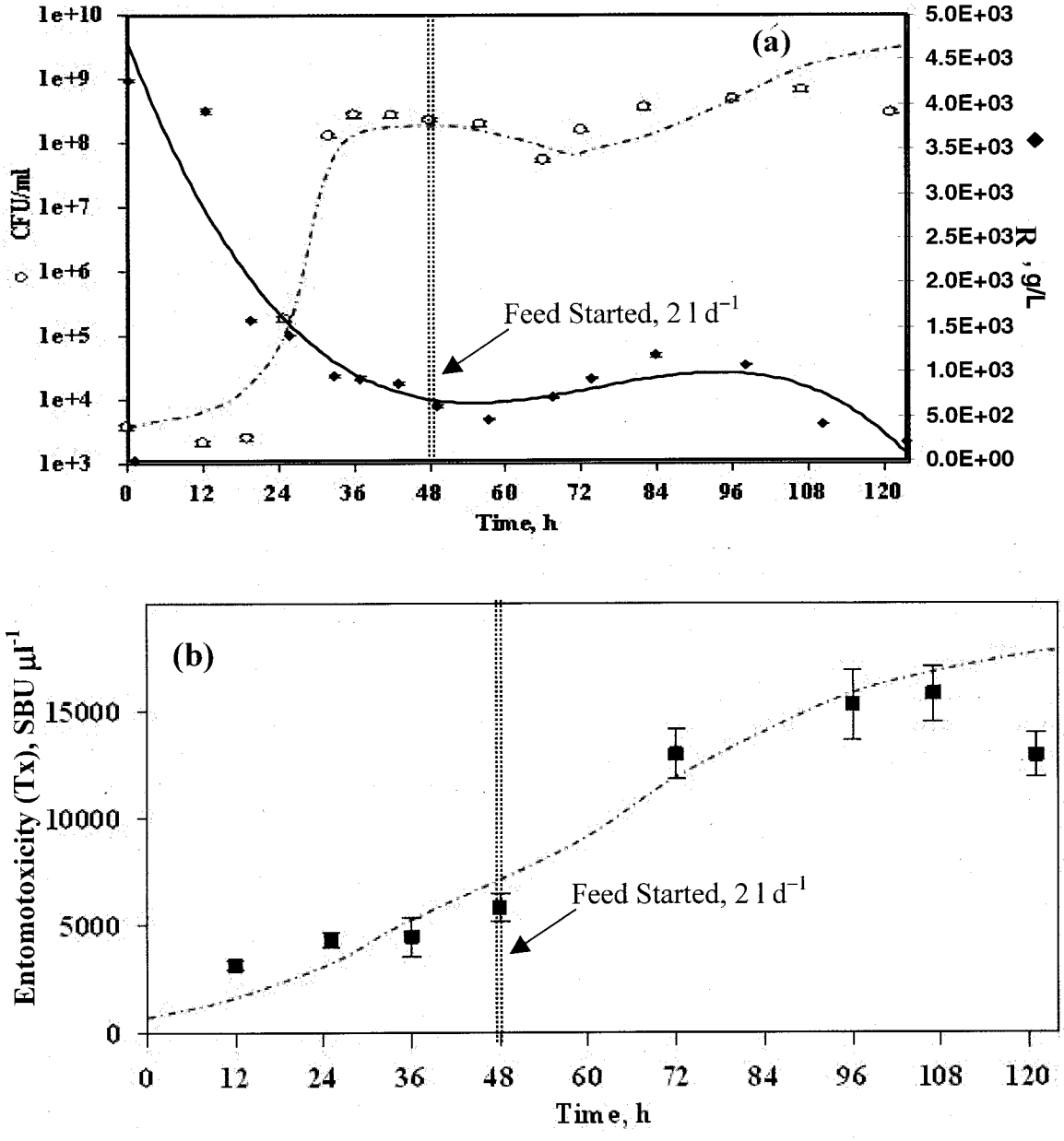



**Figure 2.** Fed-batch mode profiles (legends – experimental data; dotted-line – simulation data) of (a) conidia production and total reducing sugar, R and (b) entomotoxicity concentration.

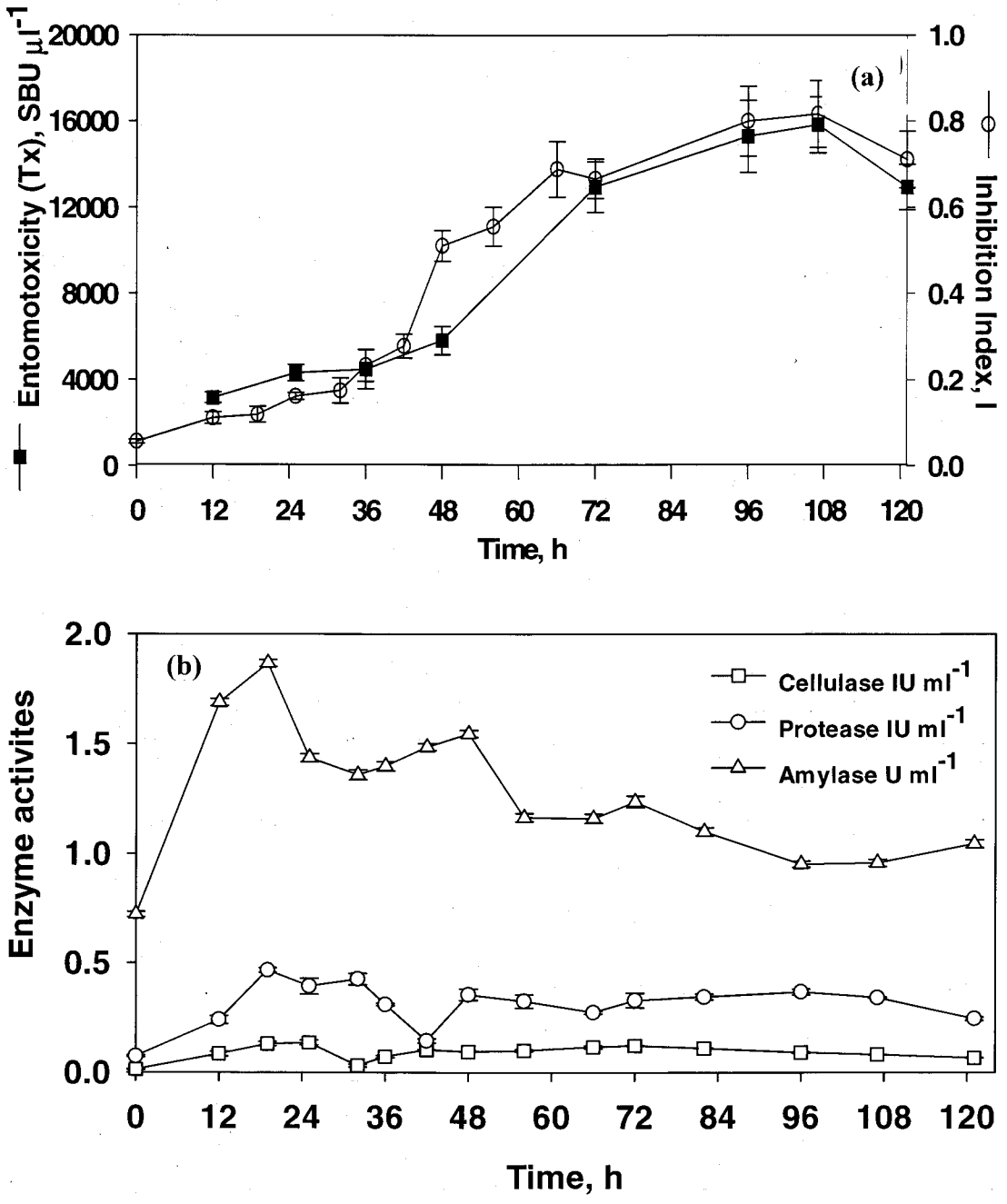



**Figure 3.** Profiles of (a) entomotoxicity and fungicidal bioassays and (b) enzyme production during fed-batch mode.

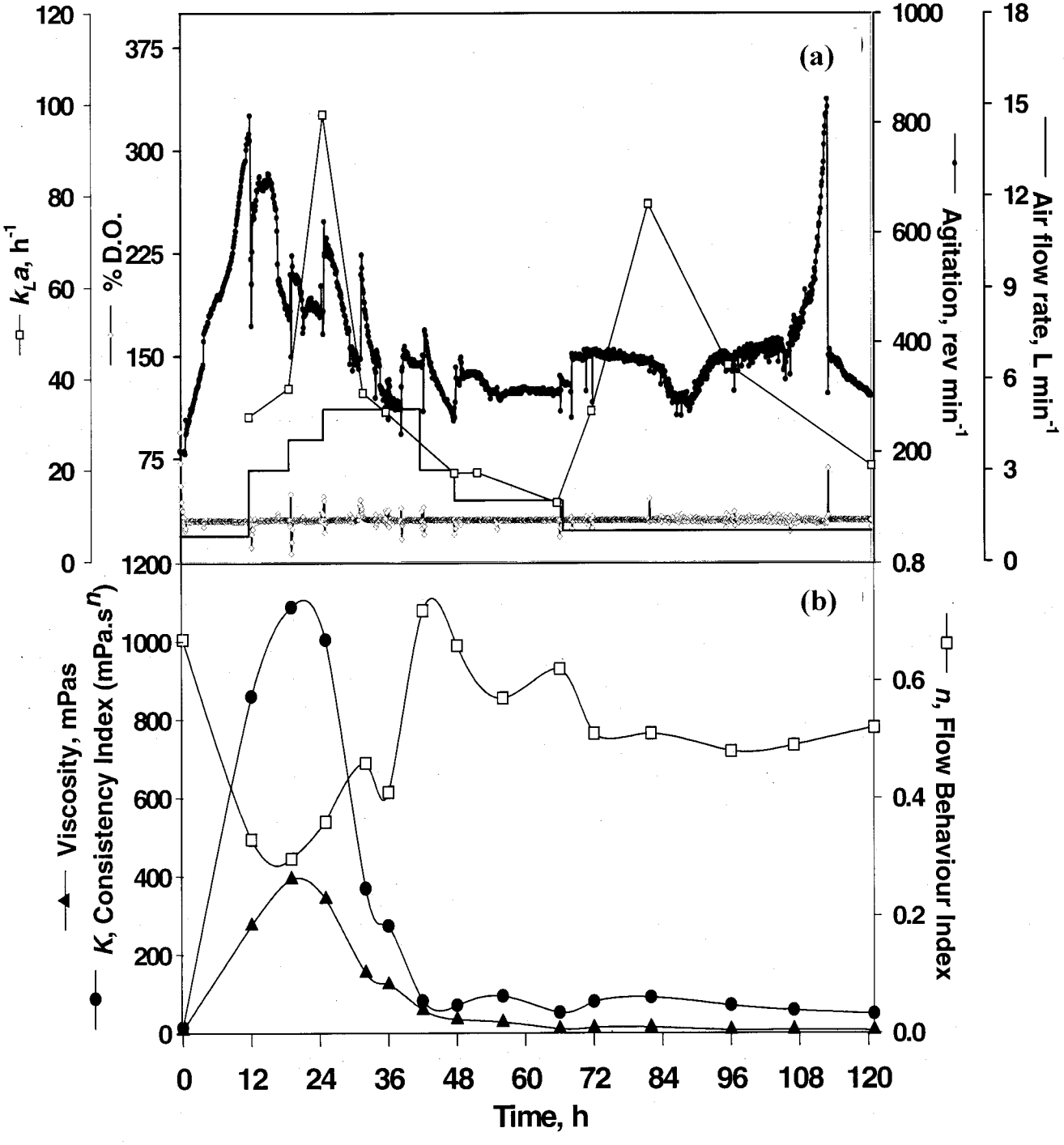



**Figure 4.** Fed-batch fermentation operation parameters, (a) $k_La$, D.O., agitation and air flow rate, (b) viscosity, $K$ and $n$.

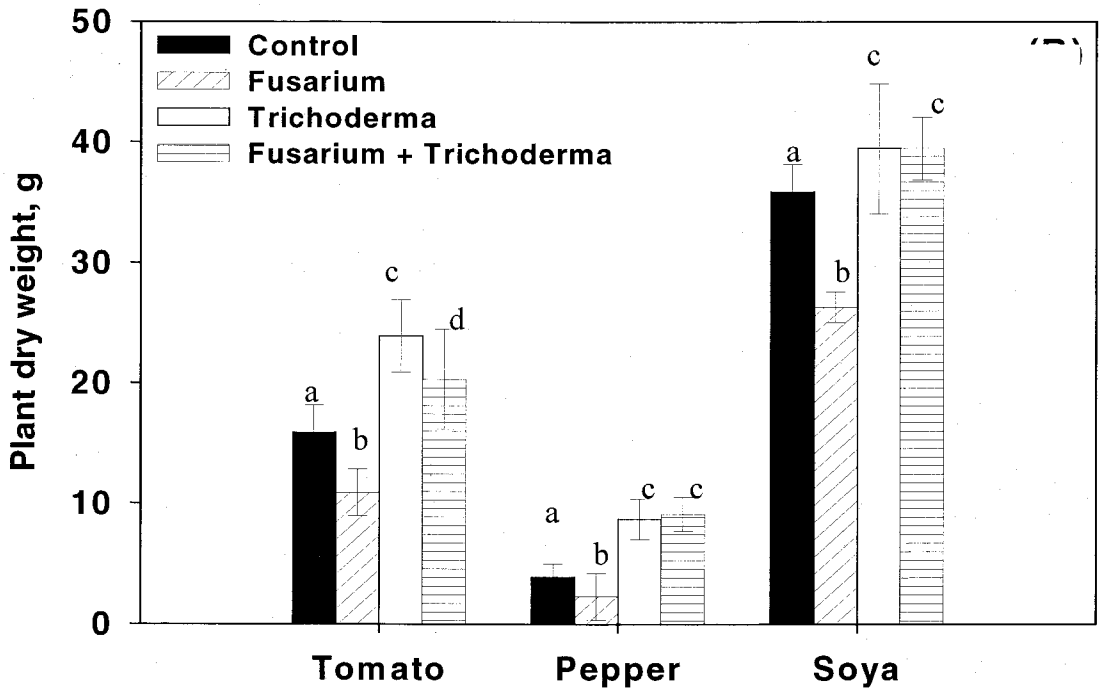



**Figure 5.** Plant growth promotion results (plant grown from seeds).

# CHAPITRE 5.
# ÉTUDE TECHNICO-ÉCONOMIQUE DE LA PRODUCTION DE *TRICHODERMA* SPP.

# Techno-economic study of production of conidial propagules of biocontrol fungus *Trichoderma* from alternative raw materials

**M. Verma[1], Satinder K. Brar[1], R. D. Tyagi[1,*], R. Y. Surampalli[2], and J. R. Valéro[1,3]**

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

[2]US EPA, P.O. Box-17-2141, Kansas City, KS 66117, U.S.A.

[3]NRCan Canadian Forestry Services, Laurentian Forestery Centre des 1055 du P.E.P.S., P.O. Box 3800, Sainte-Foy, Québec Canada, G1V 4C7

**(To be submitted)**

## Étude technico-économique de la production par fermentation de conidies de *Trichoderma* spp. en utilisant des matières premières alternatives

**Résumé**

Actuellement les préparations de *Trichoderma* spp. dominent le marché des biopesticides à base de champignons. Toutefois, le coût élevé et la méconnaissance de ces produits nuisent à leur emploi pour la lutte contre les ravageurs. Dans cette optique, plusieurs chercheurs ont exploré auparavant les possibilités d'appliquer *Trichoderma* spp. pour le contrôle biologique. En fait, la plupart de leurs travaux ont démontré que *Trichoderma* spp. a une activité fortement antagoniste vis-à-vis divers microorganismes et insectes, *in-vitro* aussi bien que *in-situ*. Par contre, les processus d'obtention de masse par fermentation *Trichoderma* spp. ainsi que les coûts n'ont pas fait l'objet d'études détaillées. Dans ce but, la production avec succès en termes de technico-économie de *Trichoderma* spp., en utilisant des matières premières alternatives bon marché a fait l'objet ici d'études exhaustives. Les données du processus d'obtention de *Trichoderma* spp. en employant des boues d'épuration municipales et des eaux usées de divers industries ont été analysées du point de vue technico-économique. Ceci a été comparé à l'obtention conventionnelle conduisant à des préparations de *Trichoderma* spp. présentement sur le marché ayant une concentration de $10^6$–$10^7$ conidies par g de produit final. L'échelle de production a été estimée en fonction de la matière première et du coût minimal du produit (4.69 - 6.67 $ CDN/kg) égal ou inférieur aux préparations commerciales actuelles (25 $ CDN/kg) de ce biopesticides. Ces comparaisons ont permis de déterminer que les eaux usées industrielles et un milieu synthétique spécifique peuvent être utilisés pour une production de masse de *Trichoderma* spp, alors que l'emploi des boues d'épuration municipales requiert des recherches supplémentaires pour permettre et valider leur utilisation comme substrats pour l'obtention de cet agent biologique. Par ailleurs, les résidus déshydratés peuvent être des substituts valables pour la production de suspensions d'agents biologiques à base de *Trichoderma* spp. Cette étude sera aussi fort utile dans le futur pour évaluer les possibilités d'employer différentes matières premières pour l'obtention de *Trichoderma* spp aussi bien que pour valider les processus de production par bio-réaction.

**Mots clés:** Agent de lutte biologique, conidia, coût, technico-économique, *Trichoderma* spp., boues d'épuration.

**Abstract**

Currently, *Trichoderma* spp. based products dominate the fungal biocontrol agents market worldwide. However, these products are still far from application in field crops due to cost implications and unawareness. In the past, several researchers have explored many *Trichoderma* spp. for biocontrol. In fact, almost all of them have reported *Trichoderma* spp. to be highly antagonistic against several phytopathogenic microbes and insects, *in-vitro* as well as *in-situ*. However, the fine details of production cost issue of *Trichoderma* spp. at mass scale has never been assessed so far. Therefore, in this study, techno-economy of successful *Trichoderma* spp. production from cheaper alternative raw materials has been investigated in detail. The *Trichoderma* spp. production process data used for the techno-economics study were taken for municipal wastewater sludge and several industrial wastewaters. The basis of production was compared to commercial products of *Trichoderma* spp. present in market and was taken as $10^6$–$10^7$ conidia per g of final product depending upon the antagonistic potential of conidia. The scale of production was varied according to raw material at which the product cost was minimal or < that of the available related commercial product. Industrial wastewaters and a particular synthetic medium based processes utilized could be directly subjected to mass scale production, whereas, municipal wastewater sludge would require further research to facilitate its feasibility. On the other hand, dewatered sludge would be a great substitute of talc as a bulking agent (carrier) in *Trichoderma* spp. biocontrol agent formulations. Hence, the present study will be very helpful in screening of raw materials as well as production processes for *Trichoderma* spp. based biocontrol agents in future.

**Keywords:** Biocontrol agent, conidia, cost, techno-economics, *Trichoderma* spp., wastewater sludge.

## Introduction

Biocontrol agents (BCAs) are becoming increasingly popular amongst organic food producers as well as conventional consumers due to its beneficial long term effects and environmental friendliness in contrast to harmful chemical pesticides. *Trichoderma* spp. based BCAs have gained considerable fraction of BCAs market due to their wide spectrum application (Whips and Lumsden, 2001; Pliar et al, 2006; Rabeendran et al., 2006; Dubey, 2007). There are several studies reported by various researchers in which antagonism of *Trichoderma* spp. was examined. However, due to lack of cost effectiveness of *Trichoderma* spp. based BCAs, the mass application (in field crops and forest sector) has still not been achieved. In fact, other fungal based BCAs also suffer problems of cost effectiveness that restrain the mass application (Jenkins et al., 1998; Brewer and Larkin, 2005). The important factors behind the commercial incompetence of fungal BCAs with respect to chemical pesticides were costly raw materials and lower spore production (Vega et al., 2003). In addition, unawareness of consumers regarding sustainability and long term benefits of BCAs has been a major set back to the commercialization (Dinham, 2005). As a matter of fact, presently, only a few major manufacturers are involved in the production of BCAs, whilst the existing BCA producers are of small to moderate scale (Evans, 2004). Moreover, existing manufacturing and application facilities were mainly designed for chemical pesticides, therefore, fungal BCAs face challenges on all grounds (Jackson et al., 1991). For example, submerged fermentation based processes could be a commercial success if the pre-existing fermentation facilities could be utilized. Unfortunately, the lower product value in market in comparison to contemporary fermentation products e.g., pharmaceuticals, enzymes and others limit BCAs from mass scale success. In general, the major manufacturers are reluctant to take unknown risk and are required to be convinced exhaustively before endorsing any novel technique for the production of BCAs. In this context, it would be imperative to mention that several researchers have explored many fungal BCAs and few products have been commercialized as well (Batta, 2004a,b; Krauss et al., 2006; Rojo et al., 2006; Singh et al., 2007). However, techno-economic evaluation of the production processes of fungal BCAs could be rarely cited in the literature which possibly deterred the fungal BCAs processes from successful commercialization. Therefore, it was evident that for successful implementation of *Trichoderma* fungi based

BCAs in field crops and forest, laboratory scale production processes should be subjected to techno-economic evaluation.

It is possible that realistic estimate of the cost and the risk involvement of fungal BCAs processes obtained through these studies would encourage industries to invest in BCA sector. Earlier, we have successfully demonstrated the utilization of alternative raw materials for the production of conidia of *T. viride* that could apparently lower the cost of *Trichoderma* spp. based BCAs (Verma et al., 2005, 2006 a,b,c). Meanwhile, utilization of wastewater and wastewater sludges would also aid in sustainable waste management. Nevertheless, in order to assess the commercial feasibility of the waste based fungal BCAs, technical cost evaluation of these processes would be highly legitimate. The present techno-economic study of *T. viride* based BCA production processes incorporates all potential raw materials explored earlier in our research and the final cost has been compared with existing *Trichoderma* spp. based BCAs. Furthermore, market presence of *Trichoderma* spp. based BCAs has been reported by several researchers, however, information regarding mass scale production and cost analysis was absent (Batta, 2004a,b; Krauss et al., 2006). Therefore, this study will be helpful in encouraging *Trichoderma* spp. based BCAs by providing facts about mass scale production and consecutive beneficial impacts on agriculture and environment.

**Methods**

Production processes

Different fermentation processes for the production of *T. viride* conidia from industrial wastewaters and wastewater sludges have been described earlier (Verma et al., 2005; 2006a,b,c,d). In this study, waste amendments conditions (solids concentration adjustments, pre-treatments and sterilization), the fermentation time to reach maximum conidia production and maximum conidia concentration were considered based on earlier studies. The possible scenarios for different *T. viride* based BCA are listed in Table 1. Thus, for the industrial wastewaters and wastewater sludges considered in this study, a total of 15 production process scenarios were possible.

Basis of techno-economic analysis

The different *T. viride* production processes were analyzed by developing a general process flow sheet comprising of all unit operations and by modification of process units as per specific requirements (Figure 1). The costs of equipments were referred from Peters and Timmerhaus (1980) and Ulrich (1984) and the present cost estimate was determined using conversion factors obtained from Marshall and Swift (2005) and chemical engineering plant cost index (2005). The cost shown for all equipments, utilities and services are in Canadian dollars. Total annual working days was assumed to be 300 d as also suggested in literature for realistic approach (Peters and Timmerhaus, 1980). The annual plant production capacity was varied from $10^{13}$ to $10^{17}$ CFU/year for different raw materials depending upon the optimal values at which the product cost was minimum possible. These production rates were adequate for application requirements of up to $10^{5}$ – $10^{9}$ ha land at $10^{8}$ CFU/ha as also recommended by many *Trichoderma* spp. based BCAs manufactures.

The approach adopted for techno-economic evaluation of the *T. viride* production processes have been discussed in the following sections:

*General plant design considerations*

The *T. viride* based BCA production plant could be considered as a fluid processing plant, except the optional drying unit (D) towards the end for powder formulation (Figure 1). A typical process operation will include, supply of raw material and pumping in storage tank (T). Afterwards, the raw material might pass through centrifuge unit (DVC), if solids adjustments are required, otherwise, the raw material will be transferred to main fermenter for sterilization or, thermal hydrolysis. Meanwhile, inoculum should be prepared up to pre-fermenter step. The fermenter will be inoculated after the completion of sterilization or thermal hydrolysis step and the lowering of the fermenter temperature by passing cold water through the fermenter limpet coils. In the meantime, preparation for the inoculation for next batch would be started. After the completion of fermentation, the fermented broth would be aseptically transferred to mixing tank using compressed air supply. The fermented broth could then be utilized either for liquid suspension preparation or for dry powder formulation. In general, the cost estimate of piping of overall plant was assumed

for that of a fluid processing plant. For all equipments, the purchase cost was assumed and installation cost was predicted as described in Peters and Timmerhaus (1980).

*Utility section*

The major utility section for the *T. viride* conidia production plant considered were gas fuelled steam generator, process air compressor unit, cooling tower and demineralized water plant. Table 2 lists specifications and cost of all major process equipments and related accessories. Operating costs of all utilities were referred from Peters and Timmerhaus (1980).

*Raw material procurement, sterilization and pre-treatments*

In this study, the raw materials considered were, wastewater sludge, dewatered sludge and starch industry, pulp and paper industry and slaughter house wastewaters. These raw materials were compared with a conventional semi-synthetic medium as well as a synthetic medium, previously explored by the authors (Verma et al., 2006c). The transportation cost of all wastewaters and wastewater sludges was estimated for a 25 km distance from the source, which was ≈0.006 $ per kg as per model formulated by Boileau & Associates (1989) by incorporating present day cost of fuel. For solids amendments, a disc type continuous centrifuge was considered, in addition, all peripheral pumps for wastewater transfer lines were also assumed for the centrifuge unit as shown in Figure 1. All raw materials excluding wastewater were to be sterilized before fermentation, whereas, the wastewater sludge was to be subjected to thermal alkaline hydrolysis which also served the purpose of sterilization (dual advantages). Sterilization and thermal hydrolysis were assumed to be carried out directly in the fermenter by using superheated steam, which could be generated by gas fuelled steam generator as shown in process flow diagram (Figure 1).

*Fermentation stage*

All raw materials were assumed to be fermented under optimal operating conditions as described elsewhere (Verma et al., 2005; 2006a,b,c,d). In short, inoculum for pre-pre fermenter would be prepared in shake flasks for up to ≤ 20 l, afterwards, bench top fermenter(s) should be employed. For transfer of inoculum, sterile compressed air would

be used by using inoculum transfer lines connected to the fermenters. Moreover, the valves used for fermentation transfer lines would be strictly of diaphragm type. The total fermentation batch time was taken as, medium pumping (1 h) + safety checks (1 h) + sterilization and cooling (8 h) + inoculum transfer (1 h) + fermentation time (72 h) + cleaning and wait time (10 h) = 93 h. Thus, a maximum of 77 fermentation batches per year were possible.

*Harvesting and formulation*

Liquid formulations of *T. viride* will be prepared by mixing antimicrobial agents and fermented broth in mixing tank after fermentation unit as shown in process flow sheet (Figure 1). The composition for liquid formulation is mentioned in Table 1. The liquid formulation will pass through a homogenizer unit (ball mill) in order to achieve a uniform and recommended size of conidia suspension. In case of dry powder formulation, fermented broth will be mixed with inert material (dewatered sludge or, talc powder) in a rotary dryer as shown in Figure 1. The *T. viride* based formulations (liquid suspension and dry powder) could then be packaged and marketed.

Techno-economic analysis

Based on the plant cost estimates including, fixed and working capital investments and manufacturing cost of *T. viride* based BCA formulation, techno-economic evaluation of the process was carried out. In particular, Ulrich (1984) and Peters and Timmerhaus (1980) were consulted for techno-economic analysis of the feasibility of the overall process. The fixed capital investment was obtained by summation of cost of all equipments, installation, land and services. The working capital was assumed to be 15% of total capital investment as suggested in Ulrich (1984) and Peters and Timmerhaus (1980). The manufacturing cost was obtained by estimating raw materials and utilities cost, labour and administrative cost, depreciation, taxes, insurance, marketing and interests as comprehensively presented in Table 3. The life-time of project as well as all equipments was assumed to be 10 years. Further, the profitability of the process was determined in terms of discounted cash flow rate of return (DCFRR) as per the iterative procedure described in Ulrich (1984). According to Peters and Timmerhaus (1980), many industrial concerns demand a predicted pretax rate of return to be $\geq 35\%$ preposition so that it could be regarded as safe. Therefore,

in order to justify the investments, DCFRR were calculated using a program in Microsoft EXCEL for all processes. The payback period (PBP) for the investment was also calculated for all processes on the basis of net profit, depreciation and tax. Mathematically, PBP could be defined as,

$$\text{PBP} = \frac{\text{depreciable fixed capital investment}}{(\text{avg profit/yr} + \text{avg depreciation/yr})} \quad (1)$$

**Results and Discussion**

Total capital cost

Total capital costs of the *T. viride* based BCA production plant for all scenarios for liquid suspension processes are presented in Table 2. For all other scenarios (Table 1), the capital investment required for liquid suspension (Table 2) was appended with suitable rotary dryer cost and were utilized for economic analysis. Meanwhile, the total capital investments for all other scenarios are not presented. The total capital investments for the processes assessed were in the order, PPN > SSM > SIW > TH > TSB. It was observed that the investment for medium handling and storage was the highest in the case of TH due to requirement of centrifugation. The capital investment in fermentation stage seemed to be most significant that had consequential effect on overall capital investment. As explained earlier, the total capital investment could be broadly divided into direct and indirect costs as per Peters and Timmerhaus (1980), which also followed the similar trend for obvious reasons. Furthermore, the production scale for different scenarios was determined by maintaining the product cost for most of the scenarios $\leq$ about 25 $ CDN per kg (commercial *Trichoderma* based formulation containing $\geq 10^7$ CFU/g), except for TH and TSB (discussed later). Thus, it was estimated that for lower conidia producing media (TH and TSB, $\approx 10^7$ CFU/ml fermented broth), even at $10^{13}$ CFU/year and lower conidia concentration in the *T. viride* formulation ($10^6$ CFU/g, suggested by Yedidia et al, 1999) would be challenging and should be analyzed for techno-economy. On the other hand, for higher conidia producing media (SSM, SIW and PPN, > $10^8 - 10^{10}$ CFU/ml fermented broth), about $10^{15} - 10^{17}$ CFU/year scale of production was found to be competitive to existing commercial formulations of *Trichoderma* spp. For example, size of fermentation unit required for TH and TSB media was excessively higher (of the order of 1-2 $\times$ $10^3$ cu m) than the one required for SSM, SIW and PPN media. Therefore, the total capital

investment suggested that despite lower capital requirements in TH and TSB, the scenarios were less attractive due to lower conidia concentration of the processes.

Manufacturing cost

The product cost was estimated based on the annual investment required for product manufacture. For this, all direct production costs including raw materials, labor, utilities, fixed charges and general expenses were evaluated on the basis of fixed capital investment and are presented in Table 3 for all possible scenarios. Meanwhile, a total of 12 operators were assumed to work in 3 shifts (4 operators per shift) through out the year for all possible scenarios. It was observed that annual production cost was in the order, TSB ≥ TH > PPN > SSM > SIW. The categories that principally affected overall annual production costs in all scenarios were operating labor, depreciation, plant-overhead costs and annual interest. The production cost distribution for different liquid suspension based scenarios has been presented in Figure 2. Meanwhile, raw material was also an important parameter, in case of SSM, SIW-T, PPN-LS and PPN-T. In contrast, patent and royalties, wastewater treatment and rent were assumed to be negligible with respect to overall annual production cost. The compositions and bulk price of media used for inoculum preparation, fermentation and formulation were presented in Table 4. It was also evident from that soya flour, starch, potassium hydrogen phosphate, di-potassium hydrogen phosphate, potassium sorbate and talc powder were the major factors for costs of TSB and SSM media. Furthermore, on comparison of dry powder formulation based on talc and dewatered sludge, it was evident that dewatered sludge was economical and hence could easily replace talc for mass scale production. Therefore, the cheaper alternatives as raw materials as mentioned here would be helpful in reducing the overall product cost.

Economic analysis and profitability

The economic analyses of all possible scenarios were carried out by calculating DCFRR and payback period of total capital investment as DCFRR reflects the interests on investment (Horwitz, 1980). The results of DCFRR and payback period are presented in Table 3. For calculation of DCFRR, it was assumed that in the first year of the project, total capital investment would be utilized for the erection of plant as well as for initiation of plant operation. In fact, even during the second year, the sales income was assumed to be only 50% of the actual value. Subsequently, the sales income was assumed to be actual

until the completion of the project (i.e., 10 years). Furthermore, at the end of 10 years, the depreciation cost and working capital were added to the net cash income of the project. These strategies were also recommended by previous authors so that a realistic approach could be achieved. In fact, during the calculation of DCFRR, several simulations were also plotted for different rates of return and was concluded that all possible scenarios were capable of having PBP $\leq$ 4 years for up to 40%. It was observed that for the given rate of returns from 40 - 65%, the PBP increased sharply towards $\geq$ 10 years. The profitability of each scenario was judged on the basis of risk involved as well as production scale. Thus, according to Ulrich (1984) and Peters and Timmerhaus (1980) all the scenarios seemed to be risk free as the DCFRR varied between (55.2 to 67.62%). However, DCFRR could not be considered as a sole evaluation parameter for profitability due to its dependence on predicted future interest rates. For example, it was evident that despite having higher DCFRRs in all scenarios of TSB and TH media, the product cost would be not feasible for marketability. Meanwhile, for all scenarios for SSM, SIW and PPN, the product costs were well below the existing *Trichoderma* spp. based BCA formulations, thereby, suggesting negligible risk and possible market success.

**Conclusions and Recommendations**

In this study, realistic cost estimation was made with a possible variation of about 20-30%, which would be plausible approach to evaluate *T. viride* based BCA processes which are still at a natal stage of development. This approach will help in eliminating wastage of costly research resources on non-feasible processes. Moreover, based on the problem(s) depicted in this study, it will be possible to modify the process(es) in future. Meanwhile, wastewater sludges and several industrial wastewaters rich in biodegradable organic matter pose threat to the environment. Therefore, the efforts to utilize these wastes as safe biocontrol agents by the production of *Trichoderma viride* should be evaluated for process feasibility. In this view, the results of starch industry wastewater, pulp and paper industry wastewater and soluble starch medium were encouraging enough to recommend these media for mass scale production without further increase in conidia concentration. On the other hand, it was deduced that wastewater sludge should be further investigated for increase in conidia concentration so that the process would be feasible at mass scale. Dewatered sludge proved to be an excellent bulking agent for dry powder formulations. As

a matter of fact, dewatered sludge would eliminate the burden of mixing talc in soil as it constitutes biological matter. Thus, huge amounts of dewatered sludge could also be managed in a sustainable manner (98.85% w/w per kg of *Trichoderma viride* formulation). The cost of *T. viride* based liquid suspensions and dry powder formulations for best scenarios (starch industry wastewater, pulp and paper industry wastewater and soluble starch medium) were in the range of 4.69 - 6.67 $ CDN/kg of product, which was much lower than existing *Trichoderma* spp. based BCA products. The economic analysis also confirmed the profitability of the processes and acceptable payback period. Thus, it could also be interpreted that conidia concentrations > $10^8$ CFU/ml of fermented broth would provide feasible mass scale production of *T. viride* in the case of waste based raw materials. However, in the case of synthetic or semi- synthetic raw materials, > $10^{10}$ CFU/ml would be essential to render the process economical. Importantly, in this study, the basis of production was taken to be conidia concentration in order to compare with existing *Trichoderma* spp. based products. In addition, no studies could be cited by the authors that deal with techno-economic evaluation of fermentation process of *Trichoderma* fungi. On the other hand, the existing techno-economic studies describe mostly solid fermentation based processes for other biocontrol fungi, thereby, justifying the need for the present cost evaluation.

**Acknowledgements**

The authors are thankful to 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.

**List of Abbreviations**

| | |
|---|---|
| *BCA* | *Biocontrol agents* |
| D | Drying unit |
| DCFRR | Discounted cash flow rate of return |
| DVC | Centrifuge unit |
| GP | Gear pump |
| IM | Impeller motor |
| PBP | Payback period |
| PPN | Pulp and paper non-treated wastewater |
| PPN - DS | Pulp and paper non-treated - dewatered sludge amended (formulation stage) |
| PPN -LS | Pulp and paper non-treated - liquid suspension (formulation stage) |
| PPN -T | Pulp and paper non-treated - talc amended (formulation stage) |

| | |
|---|---|
| SIW | Starch industry wastewater |
| SIW - DS | Starch industry wastewater - dewatered sludge amended (formulation stage) |
| SIW -LS | Starch industry wastewater - liquid suspension (formulation stage) |
| SIW -T | Starch industry wastewater - talc amended (formulation stage) |
| SSM | Soluble starch medium |
| SSM- DS | Soluble starch medium - dewatered sludge amended (formulation stage) |
| SSM-LS | Soluble starch medium - liquid suspension (formulation stage) |
| SSM-T | Soluble starch medium - talc amended (formulation stage) |
| T | Storage tank |
| TH | Thermal alkaline hydrolyzed sludge |
| TH - DS | Thermal alkaline hydrolyzed sludge - dewatered sludge amended (formulation stage) |
| TH -LS | Thermal alkaline hydrolyzed sludge - liquid suspension (formulation stage) |
| TH -T | Thermal alkaline hydrolyzed sludge - talc amended (formulation stage) |
| TSB | Tryptic soya broth |
| TSB- DS | Tryptic soya broth – dewatered sludge amended (formulation stage) |
| TSB-LS | Tryptic soya broth - liquid suspension (formulation stage) |
| TSB-T | Tryptic soya broth - talc amended (formulation stage) |
| V | Valves |

## References


Bae, Y.S., Knudsen, G.R. 2007. Effect of sclerotial distribution pattern of *Sclerotinia sclerotiorum* on biocontrol efficacy of *Trichoderma harzianum*. *Applied Soil Ecology*, 35: 21-24.

Batta, YA. 2004a. Effect of treatment with *Trichoderma harzianum Rifai* formulated in invert emulsion on post harvest decay of apple blue mold. *International Journal of Food Microbiology*, 96: 281– 288.

Batta, YA. 2004b. Postharvest biological control of apple gray mold by T*richoderma harzianum Rifai* formulated in invert emulsion. *Crop Protection*, 23: 19– 26.

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.

Brewer, M.T., and Larkin, R.P. 2005. Efficacy of several potential biocontrol organisms against *Rhizoctonia solani* on potato. *Crop Protection* 24:939-950.

Dinham, B. 2005. Agrochemical markets soar—pest pressures or corporate design? Pestic News, 68: 9–11.

Dubey, S.C., M. Suresh, and B. Singh. 2007. Evaluation of *Trichoderma* species against *Fusarium oxysporum f.* sp. *ciceris* for integrated management of chickpea wilt. Biological Control 40:118-127.

Evans, J. 2004. *Interview*: Shifting perceptions on biopesticides. *Agrow* **455:** pp18–21. PJB Publications Ltd.

Horwitz, B.A., 1980. The mathematics of discounted cash flow analysis. *Chemical Engineering*, pp 169-174.

Jackson, A.M., Whipps, J.M., Lynch, J.M. 1991. Production, delivery systems, and survival in soil of four fungi with disease biocontrol potential. *Enzyme and Microbial Technology*, 13: 636-642.

Jenkins, NE, Heviefo, G, Langewald, J, Cherry, AJ, Lomer, CJ. 1998. Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information*, 19(1), 21N-31N.

Krauss, U., Martijn ten Hoopen, G., Hidalgo, E., Martinez, A., Stirrup, T., Arroyo, C., Garcia, J., Palacios, M. 2006. The effect of cane molasses amendment on biocontrol of frosty pod rot (*Moniliophthora roreri*) and black pod (*Phytophthora* spp.) of cocoa (*Theobroma cacao*) in Panama. *Biological Control* 39: 232-239.

Pilar Santamarina, M., Rosello, J. 2006. Influence of temperature and water activity on the antagonism of *Trichoderma harzianum* to *Verticillium* and *Rhizoctonia*. *Crop Protection*, 25: 1130-1134.

Rabeendran, N., Jones, E.E., Moot, D.J., Stewart, A. 2006. Biocontrol of *Sclerotinia* lettuce drop by *Coniothyrium minitans* and *Trichoderma hamatum*. *Biological Control*, 39: 352-362.

Rojo, F.G., Reynoso, M.M., Ferez, M., Chulze, S.N., Torres, A.M. 2006. Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. *Crop Protection*. In Press, Corrected Proof.

Singh, A., Srivastava, S., Singh, H.B. 2007. Effect of substrates on growth and shelf life of *Trichoderma harzianum* and its use in biocontrol of diseases. *Bioresource Technology*, 98: 470-473.

Vega, F.E., Jackson, M.A., Mercadier, G., Poprawski, T.J. 2003. The impact of nutrition on spore yields for various fungal entomopathogens in liquid culture. *World Journal of Microbiology & Biotechnology,* 19: 363-368.

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006a. Industrial wastewaters and dewatered sludge: rich nutrient source for production and formulation of biocontrol agent, *Trichoderma viride. World Journal of Microbiology and Biotechnology* (submitted).

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006b. Starch industry wastewater as a substrate for antagonist, *Trichoderma viride* production. *Bioresource Technology*, DOI:10.1016/j.biortech.2006.08.032 (in press).

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006c. Dissolved oxygen as principal parameter for conidia production of biocontrol fungi *Trichoderma viride* in a non-Newtonian wastewater. *Journal of Industrial Microbiology & Biotechnology*, 33, 941–952. DOI 10.1007/s10295-006-0164-6.

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y. and Valéro, J.R. 2006d. Bench-scale fermentation of *Trichoderma viride* on wastewater sludge: rheology, lytic enzymes and biocontrol activity. *Enzyme and Microbial Technology,* (to be submitted).

Verma, M., Brar, S.K., Tyagi, R.D., Valéro, J.R. and Surampalli, R.Y. 2005. Wastewater Sludge as a Potential Raw Material for Antagonistic Fungus (*Trichoderma* sp.): Role of Pre-treatment and Solids Concentration. *Water Research,* 39(15), 3587-3596. DOI:10.1016/j.watres.2005.07.001.

Yedidia, I, Benhamou, N, Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum. Applied and Environmental Microbiology*, 65(3): 1061–1070.

**Table 1.** Different possible scenarios for *T. viride* based BCA process

| Formulation | CODE (suffix) | Description |
|---|---|---|
| Liquid suspension | LS | Liquid suspension will comprise *T. viride* fermented broth, Tween 80 (0.15% w/v) and potassium sorbate (0.5% w/v). |
| Dry powder–I | T | Talc powder (98.85% w/v) will be mixed with *T. viride* fermented broth to achieve dry powder (≤ 5% w/v moisture content) of desired conidia concentration. The excess moisture will be removed during drying process. |
| Dry powder–II | DS | Dewatered sludge (98.85% w/v) will be mixed with *T. viride* fermented broth to achieve dry powder (≤ 5% w/v moisture content) of desired conidia concentration. The excess moisture will be removed during drying process. |

**Table 2.** Equipment details and capital investments of all scenarios[#]

| Equipment details and fixed capital | TSB | | | SSM | | | TH | | | SIW | | | PPN | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Unit-100: Medium handling, storage and concentration** | **Specification** | **Quantity** | **Value $ 000 US** | **Specification** | **Quantity** | **Value $ 000 US** | **Specification** | **Quantity** | **Value $ 000 US** | **Specification** | **Quantity** | **Value $ 000 US** | **Specification** | **Quantity** | **Value $ 000 US** |
| 101: Centrifugal pump (cu m, psi) | 10, 100 | 2 | 6.4 | 40, 100 | 2 | 8.7 | 10, 100 | 2 | 6.4 | 40, 100 | 2 | 8.7 | 40, 100 | 2 | 9.2 |
| 102: Medium storage tank (cu m) | 20 | 1 | 15.8 | 80 | 1 | 30.4 | 20 | 1 | 15.8 | 80 | 1 | 30.4 | 100 | 1 | 50.6 |
| 103: Disc centrifuge (cu m per h) | | 0 | 0.0 | | 0 | 0.0 | | 1 | 229.7 | | 0 | 0.0 | | 0 | 0.0 |
| **Subtotal** | | | 22.2 | | | 39.1 | | | 251.9 | | | 39.1 | | | 59.8 |
| **Unit-200: Medium Preparation Utilities** | | | | | | | | | | | | | | | |
| 201: De-mineralization plant (cu m per s) | 0.0005 | 1 | 90.1 | 0.001 | 1 | 120.1 | 0.0005 | 1 | 90.1 | 0.001 | 1 | 120.1 | 0.001 | 1 | 120.1 |
| 202: Cooling tower (cu m per s) | 0.01 | 1 | 30.0 | 0.05 | 1 | 120.1 | 0.01 | 1 | 30.0 | 0.05 | 1 | 120.1 | 0.05 | 1 | 120.1 |
| 203: Steam generator ($10^3$ kg steam per h, °C) | 1.1, 121 | 1 | 42.7 | 4.87, 121 | 1 | 108.4 | 1.1, 121 | 1 | 42.7 | 4.28, 121 | 1 | 94.9 | 6.34, 121 | 1 | 122.0 |
| **Subtotal** | | | 162.8 | | | | 348.6 | | | | 162.8 | | | | 335.1 |
| **Section-300: Fermentation** | | | | | | | | | | | | | | | |
| 301: Air compressor (scfm, kW) | 900, 75 | 1 | 137.8 | 1000, 75 | 4 | 597.3 | 900, 75 | 1 | 137.8 | 1000, 75 | 3 | 448.0 | 1000, 75 | 4 | 597.3 |
| 302: Fermenter (cu m, kW) | 16.5, 2 | 1 | 697.5 | 74, 28 | 1 | 1060.3 | 16.5, 2 | 1 | 694.7 | 65, 28 | 1 | 1015.1 | 96, 28 | 1 | 1257.8 |
| 303: Pre-fermenter (cu m, kW) | 1.65, 0.2 | 1 | 208.1 | 7.4, 5 | 1 | 297.8 | 1.65, 0.2 | 1 | 207.4 | 6.5, 5 | 1 | 286.7 | 9.6, 5 | 1 | 323.3 |
| 304: Pre pre-fermenter (cu m, kW) | 0.16, 0.1 | 1 | 65.6 | 0.74, 1 | 1 | 89.8 | 0.16, 0.1 | 1 | 65.4 | 0.65, 1 | 1 | 86.9 | 0.96, 1 | 1 | 96.5 |
| 305: Alkali storage tank (cu m) | 1.5 | 1 | 14.4 | 6 | 1 | 36.0 | 1.5 | 1 | 14.4 | 6 | 1 | 36.0 | 6 | 1 | 36.0 |
| 306: Alkali pump (cu m per h) | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 |

| Equipment details and fixed capital | TSB | | | SSM | | | TH | | | SIW | | | PPN | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 307: Acid storage tank (cu m) | 1.5 | 1 | 14.4 | 6 | 1 | 36.0 | 1.5 | 1 | 14.4 | 6 | 1 | 36.0 | 6 | 1 | 36.0 |
| 308: Acid pump (cu m per h) | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 |
| 309: Anti-foam tank (cu m) | 1.5 | 1 | 14.4 | 6 | 1 | 36.0 | 1.5 | 1 | 14.4 | 6 | 1 | 36.0 | 6 | 1 | 36.0 |
| 310: Anti-foam pump (cu m per h) | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 | 6.8 | 1 | 6.9 |
| **Subtotal** | | | 1173.0 | | | 2173.9 | | | 1169.3 | | | 1965.4 | | | 2403.7 |
| **Section-400: Harvesting and product recovery** | | | | | | | | | | | | | | | |
| 403: Gear pump (cu m per h) | 10 | 2 | 9.2 | 10 | 2 | 15.2 | 10 | 2 | 9.2 | 10 | 2 | 15.2 | 10 | 2 | 18.4 |
| **Subtotal** | | | 9.2 | | | 15.2 | | | 9.2 | | | 15.2 | | | 18.4 |
| **Section-500: Formulation and packaging** | | | | | | | | | | | | | | | |
| 501: Gear pump (cu m per h) | 10 | 2 | 9.2 | 10 | 2 | 15.2 | 10 | 2 | 9.2 | 10 | 2 | 15.2 | 10 | 2 | 18.4 |
| 502: Mixing tank (cu m, kW) | 16.5, 2 | 1 | 193.5 | 74, 20 | 1 | 414.0 | 16.5, 2 | 1 | 191.8 | 65, 20 | 1 | 386.3 | 74, 20 | 1 | 478.6 |
| 503: Ball mill (ton per h) | 10 | 1 | 137.8 | 10 | 1 | 137.8 | 10 | 1 | 137.8 | 10 | 1 | 137.8 | 10 | 1 | 137.8 |
| **Subtotal** | | | 340.5 | | | 567 | | | 338.8 | | | 539.3 | | | 634.8 |
| **Equipment subtotal** | | | 1708 | | | 3144 | | | 1932 | | | 2894 | | | 3479 |
| **Total continuous power, kW** | 102.3 | | | 377 | | | 105.4 | | | 296 | | | 381 | | |
| **Direct costs (recommended % range*)** | | | | | | | | | | | | | | | |
| Purchased equipment-delivered (100) | | | 1707.7 | | | 3143.9 | | | 1932.1 | | | 2894.1 | | | 3479.0 |
| Purchased equipment-installation (47) | | | 802.6 | | | 1477.6 | | | 908.1 | | | 1360.2 | | | 1635.1 |
| Instrumentation and controls (18) | | | 307.4 | | | 565.9 | | | 347.8 | | | 520.9 | | | 626.2 |
| Piping (installed) (66) | | | 1127.1 | | | 2075.0 | | | 1275.2 | | | 1910.1 | | | 2296.1 |
| Electrical (installed) (11) | | | 187.8 | | | 345.8 | | | 212.5 | | | 318.4 | | | 382.7 |
| Buildings (including services) (18) | | | 307.4 | | | 565.9 | | | 347.8 | | | 520.9 | | | 626.2 |
| Yard improvements (10) | | | 170.8 | | | 314.4 | | | 193.2 | | | 289.4 | | | 347.9 |

| Equipment details and fixed capital | TSB | SSM | TH | SIW | PPN |
|---|---|---|---|---|---|
| Service facilities (installed) (70) | 1195.4 | 2200.7 | 1352.5 | 2025.9 | 2435.3 |
| Land (purchase required) (6) | 102.5 | 188.6 | 115.9 | 173.6 | 208.7 |
| **Total direct costs** | **4542.4** | **8362.7** | **5139.4** | **7698.4** | **9254.0** |
| **Indirect costs (recommended % range)** | | | | | |
| Engineering and supervision (33) | 563.5 | 1037.5 | 637.6 | 955.1 | 1148.1 |
| Construction expenses (41) | 700.1 | 1289.0 | 792.2 | 1186.6 | 1426.4 |
| Contractor's fee (21) | 358.6 | 660.2 | 405.7 | 607.8 | 730.6 |
| Contingency (42) | 717.2 | 1320.4 | 811.5 | 1215.5 | 1461.2 |
| **Total indirect costs** | **2339.5** | **4307.1** | **2647.0** | **3964.9** | **4766.2** |
| **Total fixed-capital investment, $C_{FC}$** | **6881.9** | **12669.8** | **7786.4** | **11663.3** | **14020.2** |
| **Working capital, $C_{WC}$** | **1214.5** | **2235.8** | **1374.1** | **2058.2** | **2474.2** |
| **Total capital investment, $C_{TC}$** | **8096.4** | **14905.6** | **9160.5** | **13721.5** | **16494.4** |

\# Equipments for only liquid suspension are listed; for dry powder formulation, rotary dryer was added to all scenarios.

* Recommended % range for a fluid processing plant

**Table 3.** Manufacturing cost details of all possible scenarios[#]

| Category | Cost, 000 $US/year, unless stated, otherwise | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Direct production costs** | **TSB-LS** | **TSB-T** | **TSB-DS** | **SSM-LS** | **SSM-T** | **SSM-DS** | **TH-LS** | **TH-T** | **TH-DS** | **SIW-LS** | **SIW-T** | **SIW-DS** | **PPN-LS** | **PPN-T** | **PPN-DS** |
| Raw materials | 158 | 337 | 163 | 701 | 1211 | 443 | 82 | 196 | 25 | 313 | 761 | 86 | 462 | 1126 | 127 |
| Operating labor | | | | | | | | 490 | | | | | | | |
| Direct supervisory and clerical labor (10% $C_{OL}$) | | | | | | | | 49 | | | | | | | |
| Utilities | | | | | | | | | | | | | | | |
| *Steam* (2.044$ US/1000 lb) | 15 | 15 | 15 | 64 | 64 | 64 | 14 | 14 | 14 | 57 | 57 | 57 | 84 | 84 | 84 |
| *Electricity* (0.045$US/kWh) | 33 | 33 | 33 | 122 | 122 | 122 | 34 | 34 | 36 | 96 | 96 | 96 | 123 | 124 | 124 |
| *Process water* (0.4856 $US/1000 liter) | 2 | 2 | 2 | 7 | 7 | 7 | 2 | 2 | 2 | 6 | 6 | 6 | 9 | 9 | 9 |
| *Wastewater treatment* (Negligible (≤ 0.0003 cu m/s)) | | | | | | | | 0 | | | | | | | |
| Maintenance and repairs (2% $C_{FC}$) | 138 | 150 | 150 | 253 | 265 | 265 | 156 | 168 | 156 | 233 | 240 | 240 | 280 | 293 | 293 |
| Operating supplies (0.2% $C_{FC}$) | 14 | 15 | 15 | 25 | 27 | 27 | 16 | 17 | 16 | 23 | 24 | 24 | 28 | 29 | 29 |
| Laboratory charges (10% $C_{OL}$) | | | | | | | | 49 | | | | | | | |
| Patents and royalties (0%) | | | | | | | | 0 | | | | | | | |
| Fixed charges | | | | | | | | | | | | | | | |
| Depreciation[†] (10% $C_{FC}$) | 454 | 494 | 494 | 836 | 876 | 876 | 514 | 554 | 514 | 770 | 793 | 793 | 925 | 965 | 965 |
| Local taxes (0.6% $C_{FC}$) | 41 | 45 | 45 | 76 | 80 | 80 | 47 | 50 | 47 | 70 | 72 | 72 | 84 | 88 | 88 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Insurance (1% $C_{FC}$) | 182 | 198 | 198 | 335 | 350 | 350 | 206 | 222 | 206 | 308 | 317 | 317 | 370 | 386 | 386 |
| Rent | | | | | | | | 0 | | | | | | | |
| Plant-Overhead costs 70% ($C_{OL}$ + 10%$C_{OL}$ + 2%$C_{FC}$) | 474 | 482 | 482 | 555 | 563 | 563 | 486 | 495 | 486 | 541 | 545 | 545 | 574 | 582 | 582 |
| **General Expenses** | | | | | | | | | | | | | | | |
| Administration 15% ($C_{OL}$ + 10%$C_{OL}$ + 2%$C_{FC}$) | 10 | 10 | 10 | 17 | 17 | 17 | 10 | 10 | 10 | 16 | 16 | 16 | 20 | 20 | 20 |
| Distribution and selling (1.8% $C_{TE}$) | 66 | 73 | 69 | 114 | 129 | 112 | 69 | 70 | 63 | 99 | 111 | 96 | 117 | 135 | 113 |
| Research and development (3% $C_{TE}$) | 98 | 110 | 104 | 171 | 193 | 167 | 103 | 105 | 95 | 148 | 166 | 143 | 175 | 203 | 169 |
| Financing (interest) (10% $C_{TC}$) | 810 | 881 | 881 | 1491 | 1562 | 1562 | 916 | 987 | 916 | 1372 | 1413 | 1413 | 1649 | 1721 | 1721 |
| **Annual total product cost ($ CDN), $C_{TE} = (C_{ME} + C_{GE})$** | 3277 | 3650 | 3454 | 5696 | 6441 | 5579 | 3449 | 3503 | 3164 | 4935 | 5537 | 4778 | 5840 | 6757 | 5635 |
| **DCFRR (%) – 10 years** | 65.97 | 67.34 | 64.2 | 62.78 | 67.0 | 59.22 | 61.2 | 58.87 | 57.52 | 59.56 | 64.17 | 56.5 | 58.76 | 64.28 | 54.93 |
| **Pay back period (years)** | | | | | | | | 3.345 | | | | | | | |
| **Total product cost, $ CDN† per kg formulation ($10^6$-$10^8$ CFU/g)** | 327.7 | 365.0 | 345.4 | 5.70 | 6.44 | 5.58 | 344.9 | 350.3 | 316.4 | 4.93 | 5.54 | 4.78 | 5.84 | 6.76 | 5.63 |

**Table 4.** Composition of media used for inoculum preparation, fermentation and formulation of all media

| Raw Material | Consumption (ml/L or, g/L) | Bulk price, $ CDN/kg |
|---|---|---|
| **Inoculum/Fermentation** | | |
| Soyameal flour | **15 (0)** | 0.32 |
| Starch | **5 (30)** | 0.70 |
| Glucose (dextrose) | **5 (0)** | 2.20 |
| Calcium carbonate | **1** | 0.93 |
| Potassium hydrogen phosphate | **1** | 35.00 |
| Di-potassium hydrogen phosphate | **1** | 35.00 |
| Magnesium sulphate | **0.3** | 0.89 |
| Ferrous sulphate | **0.02** | 0.65 |
| Zinc sulphate | **0.02** | 0.70 |
| Sulfuric acid | **6.55** | 0.09 |
| Caustic soda/sodium hydroxide | **0.4** | 0.28 |
| Poly (propylene) glycol | **10** | 0.19 |
| **Formulation** | | |
| Potassium sorbate | **5** | **13** |
| Tween-80 | **1.5** | **3.5** |
| Talc powder | **98.85** | **0.2** |
| Dewatered Sludge | **98.85** | **0.006** |

Values in parenthesis represent specific values for SSM medium

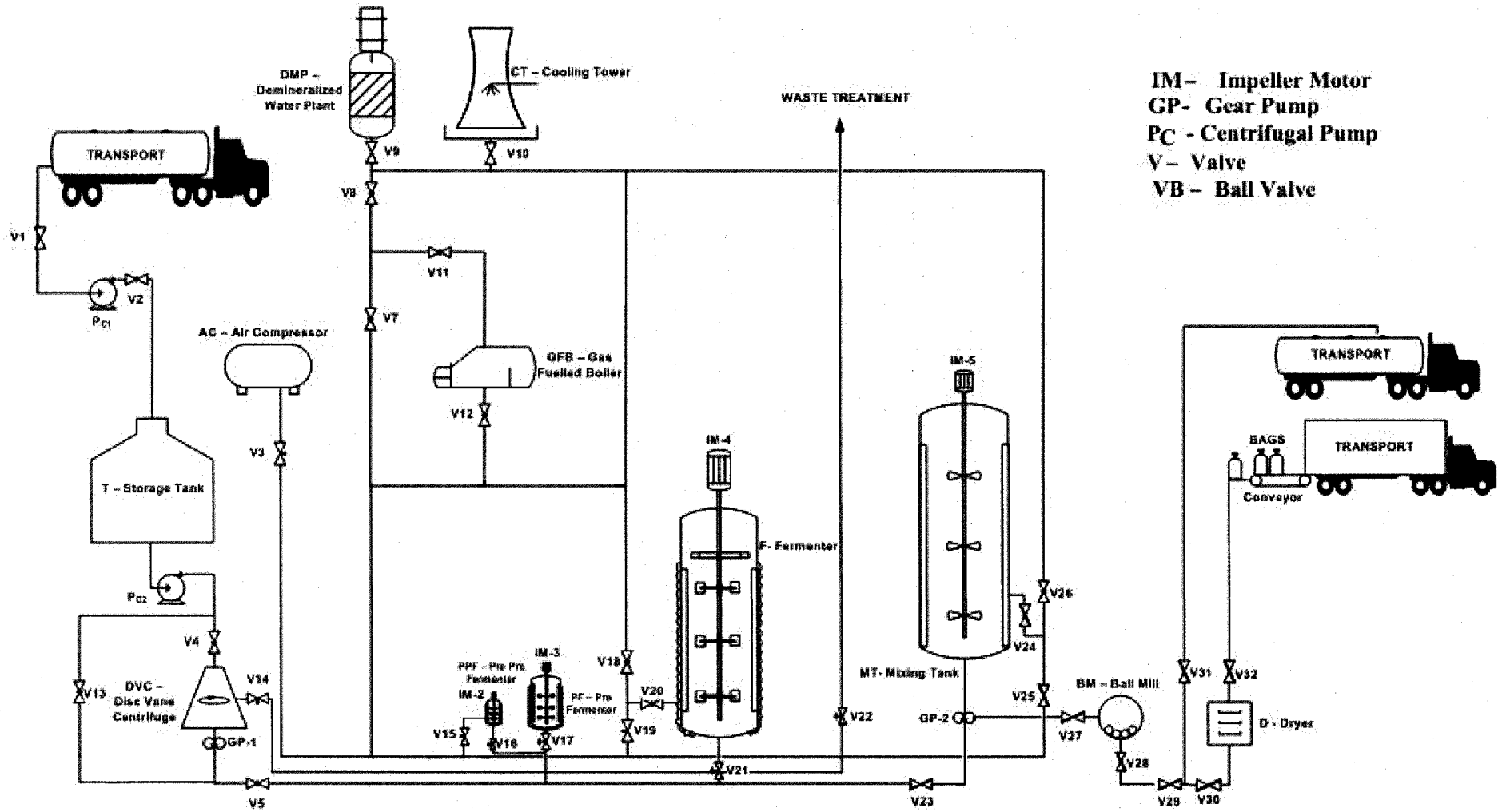



**Figure 1.** Qualitative process flow sheet of *T. viride* based BCA production plant.

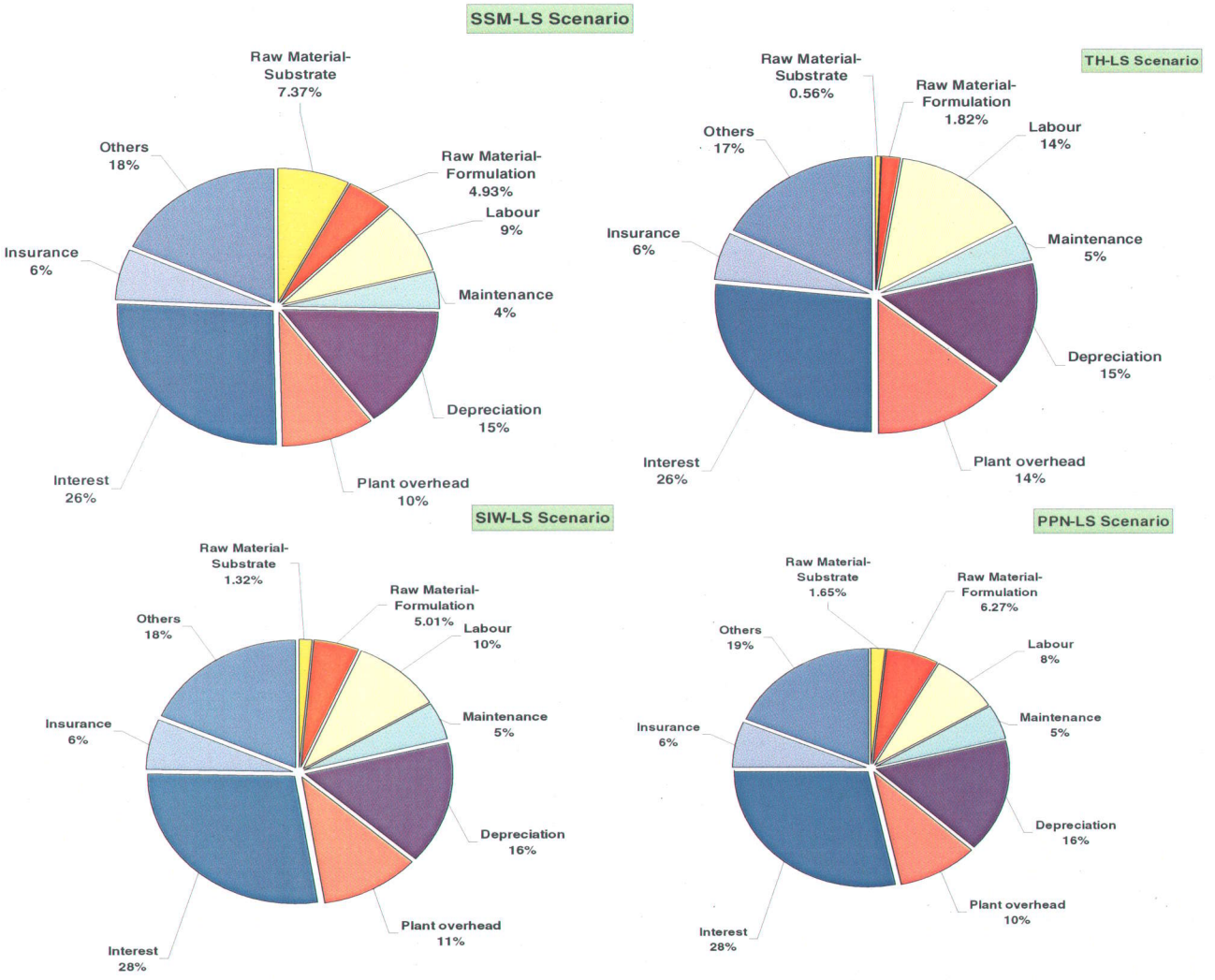



**Figure 2.** Production cost distribution (%) of different liquid suspension based scenarios.

# CHAPITRE 6.
# CONCLUSIONS ET RECOMMANDATIONS

## 6.1. Conclusions

Cette étude démontre que la production d'ALBs basés sur *Trichoderma* sp. serait une option viable pour l'utilisation écologique et durable de diverses eaux usées ou boues d'épuration avec ou sans prétraitement. L'interprétation des résultats de cette recherche et les conclusions émises sont compilées dans les prochaines sous-sections.

### 6.1.1. Chapitre 2: Champignons dans la bioremédiation et la lutte biologique

Les champignons démontrent une excellente capacité à réaliser la bioremédiation des sols. Les champignons causant la craie blanche ont besoin de sources nutritives autres que les polluants. Ainsi, une fois que l'agent épaississant supportant la croissance ou le substrat est épuisé (copeaux de bois, sphaigne, résidus de céréales), le sol doit être ré-inoculé avec un inoculum frais de champignons causant la craie blanche, ce qui peut avoir des répercussions sur son utilisation durable à grande échelle. L'établissement d'un consortium de champignons autosuffisants dans le sol est possible avec les symbioses entre les champignons mycorhiziens et les plantes. Les champignons antagonistes du genre *Trichoderma* sont connus pour utiliser plusieurs produits chimiques persistants dans le sol comme seule source de carbone en raison de leur capacité à produire diverses enzymes lytiques. De plus, l'application de *Trichoderma* spp. comme ALBs dans le sol offre un avantage additionnel en améliorant le contenu en nutriments des sols. Présentement, les modes d'action de ces champignons sont partiellement connus envers certains pathogènes et ont besoin d'être explorés davantage. Le succès dans la commercialisation de ces champignons comme ALBs nécessite le développement de procédés de production faisables. Pour cela, des substrats bons marchés et l'optimisation des paramètres d'opération doivent être considérés.

### 6.1.2. Chapitre 3: Biodégradation et rhéologie des boues d'épuration et production d'ALBs à base de *T. viride*

L'étude de la biodégradabilité des boues d'épuration a été estimée en termes de consommation de solides par les microorganismes. Indépendamment du type de boues, la concentration en solides dissout augmente de façon linéaire avec la concentration en solides totaux et l'ordre de biodégradabilité est le suivant : brute <

stérilisée < hydrolysée. Les résultats indiquent aussi que la concentration en solides est un paramètre significatif pour la biodégradation alors que le prétraitement aide à diminuer la viscosité et le comportement pseudo plastique.

Avec ou sans prétraitement, les boues d'épuration sont adéquates comme matière première pour la production d'ALBs basés sur *Trichoderma* sp.. La méthode modifiée d'étalement pour le compte des unités formatices de colonies, développée dans le cadre de cette recherche pour l'énumération de la masse conidienne des champignons dans des milieux complexes comme les boues, s'est avérée adéquate. Les boues ayant subi une hydrolyse thermo-alcaline (rendement en spores de $1.19 \times 10^7$ CFU/ml à 30 g/L de solides en suspension; environ 604 fois plus élevé que les boues non hydrolysées à 30 g/L de solides en suspension) ont un très grand potentiel pour la croissance de *Trichoderma* sp.. Les bioessais réalisés avec les boues fermentées par *T. viride* ont montré une forte entomotoxicité (≈15000 SBU/µl) et, pour cette raison, sont des ALBs potentiels. La fermentation de boues d'épuration par *T. viride* en fermenteur de laboratoire a aussi été étudiée pour la production simultanée de diverses enzymes lytiques, la prévention de maladies chez les plantes et la promotion de la croissance végétale. Il a été observé que les boues fermentées par *T. viride* étaient actives contre le phytopathogène fongique *Fusarium* sp. dans le sol sous des conditions équivalentes à celles de la pelouse.

Ainsi, le procédé de production de *Trichoderma* sp. en utilisant les boues d'épuration comme matière première a été étudié à différentes étapes (de la production à l'application sur le terrain) et les résultats obtenus pourraient être éventuellement utilisés pour la production de masse d'ALBs à base de *T. viride*.

### 6.1.3. Chapitre 4: Eaux usées industrielles et boues déshydratées comme matières premières potentielles pour la production de *T. viride* et boues déshydratées comme agent épaississant pour la formulation

À l'exception des boues d'épuration, plusieurs eaux usées industrielles et boues déshydratées (un polluant secondaire des stations d'épuration des eaux usées) possèdent aussi des nutriments essentiels pour la croissance de *Trichoderma* sp. Globalement, l'étude suggérait que la fermentation de rejets riches en protéines serait efficace pour la lutte biologique en raison de la production simultanée de plusieurs enzymes lytiques alors que ceux riches en carbone seraient plus adéquats pour la production de conidies. De plus, les

eaux usées de l'industrie de l'amidon étaient beaucoup plus efficaces pour la production de conidies et l'antagonisme. Il a été observé que les plus faibles agitations et aérations pour la production de *Trichoderma* sp. dans les eaux usées de l'industrie de l'amidon permettaient d'accroître le potentiel comme ALB. La stratégie de fermentation en mode « fed-batch » était aussi utile pour accroître la productivité ainsi que le potentiel comme ALB. Le modèle mathématique développé et validé pour déterminer les conditions optimales pour le "fed-batch" sera aussi un outil très utile pour des études ultérieures en rapport avec la production de *Trichoderma* sp. dans des rejets similaires.

6.1.4. Chapitre 5: Analyse technico-économique du procédé de production d'ALBs basés sur *T. viride*

L'estimation des coûts de la production d'ALBs à base de *T. viride* en utilisant les rejets tels que mentionnés précédemment indique clairement que le procédé pourrait être faisable. L'état du marché des ALBs basés sur *Trichoderma* sp. n'est pas disponible dans la littérature en comparaison avec d'autres ALBs comme *Bacillus thuringiensis*. Cependant, la demande actuelle pour des ALBs et le coût-efficacité des ALBs basés sur *Trichoderma* sp. et des rejets pourraient assurer le succès commercial de ces ALBs dans le futur.

## 6.2. Recommandations

Le présent travail de recherche a été exécuté pour développer un procédé économiquement faisable de production des conidies antagonistes de *T. viride*. Le procédé développé pourrait être ultérieurement employé pour la production de masse en considérant les recommandations suivantes :

- La fermentation des boues d'épuration par *T. viride* doit être réalisée en mode « fed-batch » qui pourrait être couplé à l'introduction de composés capables d'induire la production de conidies.
- Les eaux usées d'abattoir et de papetière doivent être étudiées plus en détails car elles se sont montrées efficaces pour la production de conidies et la lutte biologique.
- Le milieu de fermentation doit être étudié quant à son rôle dans l'induction de la production de conidies.

- La biomasse de *T. viride* dans des milieux aussi complexes que les rejets pourrait être indirectement mesurée en estimant le contenu en chitine et en utilisant le contenu initial en chitine des rejets comme contrôle.
- L'étude de la stabilité des formulations de *T. viride* à base de boues déshydratées doit être réalisée. Entre-temps, la suspension liquide pourrait être testée pour des applications en forêt.
- Une étude à l'échelle pilote en fermenteur est requise pour avoir une estimation précise du coût des ALBs à base de *T. viride*.

# ANNEXES

# Annexe – I

## Données

## Biorémediation par les champignons

**Figure 2.4** Comparison chart for bench scale treatability as % destruction and removal efficiency of various processes for TNT contaminated soil bioremediation (Craig et al., 1995).

| **Treatment** | **% Destruction** | **Time, d** |
|---|---|---|
| Composting | 99.5 | 60 |
| Anaerobic Bioslurry | 94.5 | 80 |
| Aerobic Bioslurry | 99.9 | 77 |
| White Rot Fungus Treatment | 41.1 | 120 |

# Annexe – II

# Données

**Pré-traitement des boues d'épuration – Études de biodégradabilité et de rhéologie**

**Figure 1.** Profiles of (a) dissolved solids concentration at 0 d, (b) % reductions in TS, TCOD and DS concentration at 20 d and (c) Rheological behaviour (K and *n*) at 0 and 20 d samples of NH, ST, and TH sludges at 10–50 g $l^{-1}$ TS.

(a) dissolved solids concentration (g/l) at 0 d

| TS, g/l | NH0 | error | ST0 | error | TH0 | error |
|---|---|---|---|---|---|---|
| 10 | 0.7 | 0.1 | 3.3 | 0.4 | 3.7 | 0.5 |
| 20 | 1.9 | 0.3 | 7.4 | 1.1 | 8.9 | 1.4 |
| 30 | 3.6 | 0.5 | 9.8 | 1.3 | 13.4 | 1.7 |
| 40 | 4.8 | 0.5 | 13.7 | 1.4 | 16.6 | 1.7 |
| 50 | 6.5 | 0.6 | 18.5 | 1.8 | 20.3 | 2.0 |

(b) % reductions in TS, TCOD and DS concentration at 20 d

| | % Change Total Solids | | | | | |
|---|---|---|---|---|---|---|
| TS, g/l | NH20 | error | ST20 | error | TH20 | error |
| 10 | 26 | 1.3 | 32 | 1.6 | 40.3 | 2.1 |
| 20 | 26.5 | 1.8 | 36 | 2.4 | 44.5 | 3.0 |
| 30 | 25 | 1.8 | 31 | 2.2 | 40.8 | 2.9 |
| 40 | 13.5 | 1.2 | 28.5 | 2.4 | 36.7 | 3.1 |
| 50 | 16.8 | 0.7 | 22.2 | 1.0 | 29.7 | 1.3 |

| | % Change TCOD | | | | | |
|---|---|---|---|---|---|---|
| TS, g/l | NH20 | Error | ST20 | error | TH20 | error |
| 10 | 16.3 | 0.8 | 29.5 | 1.5 | 53.6 | 2.7 |
| 20 | 25.8 | 1.7 | 41.3 | 2.8 | 56.5 | 3.8 |
| 30 | 23.8 | 1.7 | 32.0 | 2.3 | 42.3 | 3.0 |
| 40 | 15.3 | 1.3 | 27.6 | 2.3 | 43.7 | 3.7 |
| 50 | 11.5 | 0.5 | 22.5 | 1.0 | 31.1 | 1.3 |

| | % Change Dissolved Solids | | | | | |
|---|---|---|---|---|---|---|
| TS, g/l | NH20 | error | ST20 | error | TH20 | error |
| 10 | 20.3 | 1.0 | 57.8 | 4.0 | 61.0 | 3.1 |
| 20 | 41.9 | 2.8 | 66.8 | 5.7 | 66.9 | 4.5 |
| 30 | 39.7 | 1.9 | 56.4 | 6.3 | 61.3 | 4.4 |
| 40 | 38.8 | 4.2 | 51.6 | 7.2 | 54.6 | 4.6 |
| 50 | 31.6 | 1.4 | 41.7 | 3.8 | 52.9 | 2.3 |

(c) Rheological behaviour (K and *n*) at 0 and 20 d samples of NH, ST, and TH sludges at 10–50 g $l^{-1}$ TS.

| Rheological Indices | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TH0 | TH0 | TH20 | TH20 | ST0 | ST0 | ST20 | ST20 | NH0 | NH0 | NH20 | NH20 |
| TS, g/l | K | n | K | n | K | n | K | n | K | n | K | n |
| 10 | 18.4 | 0.59 | 3.2 | 0.88 | 24.2 | 0.50 | 4.2 | 0.63 | 18.1 | 0.52 | 5.2 | 0.62 |
| 20 | 66.7 | 0.53 | 5.6 | 0.83 | 110.0 | 0.49 | 6.6 | 0.63 | 61.2 | 0.48 | 10.1 | 0.55 |
| 30 | 172.3 | 0.45 | 57.9 | 0.59 | 231.0 | 0.50 | 28.0 | 0.56 | 282.0 | 0.48 | 35.5 | 0.52 |
| 40 | 432.7 | 0.46 | 162.4 | 0.53 | 539.7 | 0.44 | 244.0 | 0.52 | 1407.0 | 0.33 | 456.0 | 0.39 |
| 50 | 895.1 | 0.45 | 346.0 | 0.46 | 850.3 | 0.41 | 437.6 | 0.45 | 3139.0 | 0.28 | 1186.3 | 0.33 |

**Figure 2.** (c) biological activity in NH, ST, and TH sludges after 20 d incubation (initial CFU concentration = $4.07 \times 10^7$ for NH and $1.08 \times 10^6$CFU $ml^{-1}$ for ST and TH).

| **CFU / ml** | | | | | |
|---|---|---|---|---|---|
| **TS (g/l)** | **10** | **20** | **30** | **40** | **50** |
| NH0 | 1.1E+06 | 1.1E+06 | 1.1E+06 | 1.1E+06 | 1.1E+06 |
| ST0 | 1.1E+06 | 1.1E+06 | 1.1E+06 | 1.1E+06 | 1.1E+06 |
| TH0 | 1.1E+06 | 1.1E+06 | 1.1E+06 | 1.1E+06 | 1.1E+06 |
| $NH_{20}$ | 3.5E+05 | 2.6E+05 | 5.3E+05 | 2.8E+05 | 2.1E+05 |
| $ST_{20}$ | 1.0E+06 | 2.8E+06 | 4.1E+06 | 9.6E+05 | 4.1E+05 |
| $TH_{20}$ | 1.1E+06 | 1.1E+07 | 2.3E+07 | 1.7E+06 | 4.6E+05 |

**Figure 3.** Viscosity patterns of NH, ST, and TH sludges at 10–50 g $l^{-1}$ TS; a) before incubation (0 d), b) after incubation (20 d). Equations in box represents exponential relationship of viscosity (μ, mPas) versus TS concentration (g $l^{-1}$).

| (a) | Viscosity (mPa.s) | | | | |
|---|---|---|---|---|---|
| **TS (g/l)** | **10** | **20** | **30** | **40** | **50** |
| NH0 | 3.2 | 8.5 | 62.0 | 121.0 | 219.0 |
| ST0 | 3.0 | 7.4 | 43.0 | 90.0 | 170.0 |
| TH0 | 2.8 | 7.0 | 40.0 | 66.0 | 126.0 |
| (b) | | | | | |
| NH20 | 2.9 | 4.5 | 49.7 | 80.3 | 158.0 |
| ST20 | 2.4 | 3.7 | 17.0 | 29.0 | 70.7 |
| TH20 | 2.2 | 3.3 | 11.0 | 20.0 | 40.0 |

**Figure 4.** Shear stress vs. shear rate profiles of NH, ST and TH sludges at 10 - 50 g $l^{-1}$ TS at 0 and 20 d.

| NH – 0 d | | | | | |
|---|---|---|---|---|---|
| | Shear Stress | | | | |
| **Shear Rate** | **10 g/l** | **20 g/l** | **30 g/l** | **40 g/l** | **50 g/l** |
| 0.4 | 0.3 | 0.6 | 1.4 | 9.8 | 24.2 |
| 0.7 | 0.3 | 0.7 | 1.4 | 13.0 | 31.8 |
| 1.8 | 0.4 | 0.9 | 3.3 | 18.8 | 38.7 |
| 3.7 | 0.2 | 0.9 | 6.1 | 22.8 | 45.2 |
| 7.3 | 0.3 | 1.2 | 11.2 | 27.8 | 52.8 |
| 11.0 | 0.3 | 1.4 | 15.2 | 31.1 | 58.2 |
| 14.7 | 0.3 | 1.6 | 17.0 | 33.6 | 62.5 |
| 18.3 | 0.4 | 1.8 | 18.4 | 35.1 | 66.9 |
| 24.5 | 0.6 | 2.1 | 19.9 | 39.8 | 73.0 |
| 30.6 | 0.7 | 2.4 | 22.0 | 41.9 | 77.0 |
| 36.7 | 0.8 | 2.7 | 23.1 | 44.1 | 80.9 |
| 42.8 | 0.9 | 3.0 | 24.6 | 46.6 | 84.2 |
| 48.9 | 1.0 | 3.2 | 26.4 | 48.4 | 87.1 |
| 55.0 | 1.1 | 3.5 | 27.8 | 50.6 | 90.3 |
| 61.2 | 1.2 | 3.8 | 29.6 | 52.0 | 92.9 |
| 67.3 | 1.4 | 4.0 | 30.7 | 53.5 | 95.8 |
| 73.4 | 1.5 | 4.3 | 31.4 | 56.7 | 98.3 |
| 79.5 | 1.6 | 4.5 | 32.2 | 58.5 | 101.2 |
| 85.6 | 1.7 | 4.8 | 33.2 | 61.8 | 104.1 |
| 91.7 | 1.8 | 5.0 | 34.0 | 63.6 | 107.0 |
| 97.8 | 1.9 | 5.2 | 34.7 | 64.7 | 109.5 |
| 104.0 | 2.0 | 5.5 | 35.8 | 66.1 | 112.0 |
| 110.1 | 2.1 | 5.7 | 36.9 | 66.5 | 114.2 |
| 116.2 | 2.2 | 5.9 | 37.6 | 68.3 | 116.7 |

| | | | | | |
|---|---|---|---|---|---|
| 122.3 | 2.4 | 6.1 | 38.7 | 69.4 | 118.9 |
| 128.4 | 2.5 | 6.3 | 39.8 | 70.1 | 120.7 |
| 134.5 | 2.6 | 6.5 | 40.8 | 71.9 | 122.5 |
| 140.6 | 2.7 | 6.7 | 41.6 | 73.0 | 124.7 |
| 146.8 | 2.8 | 6.9 | 42.6 | 74.1 | 126.1 |
| 152.9 | 2.9 | 7.1 | 43.7 | 75.5 | 128.3 |
| 159.0 | 3.0 | 7.4 | 45.2 | 77.0 | 129.7 |
| 165.1 | 3.2 | 7.6 | 46.3 | 78.1 | 131.9 |
| 171.2 | 3.4 | 7.8 | 47.3 | 79.1 | 133.3 |
| 177.3 | 3.7 | 8.0 | 48.1 | 80.9 | 135.2 |
| 183.5 | 4.0 | 8.2 | 49.5 | 82.0 | 137.0 |
| 189.6 | 4.2 | 8.4 | 51.0 | 83.5 | 138.4 |
| 195.7 | 4.5 | 8.6 | 52.8 | 84.9 | 140.2 |
| 201.8 | 4.8 | 8.8 | 53.1 | 86.4 | 142.0 |

| **NH – 20 d** | | | | | |
|---|---|---|---|---|---|
| | **Shear Stress** | | | | |
| **Shear Rate** | **10 g/l** | **20 g/l** | **30 g/l** | **40 g/l** | **50 g/l** |
| 0.4 | 0.1 | 0.2 | 0.5 | 3.3 | 8.0 |
| 0.7 | 0.1 | 0.2 | 0.5 | 4.3 | 10.4 |
| 1.8 | 0.1 | 0.3 | 1.1 | 6.2 | 12.7 |
| 3.7 | 0.1 | 0.3 | 2.1 | 7.7 | 14.8 |
| 7.3 | 0.1 | 0.4 | 3.7 | 9.4 | 17.6 |
| 11.0 | 0.1 | 0.5 | 5.1 | 10.4 | 19.7 |
| 14.7 | 0.1 | 0.5 | 5.6 | 11.2 | 20.9 |
| 18.3 | 0.1 | 0.6 | 6.2 | 11.8 | 22.7 |
| 24.5 | 0.2 | 0.7 | 6.7 | 13.1 | 24.2 |
| 30.6 | 0.2 | 0.8 | 7.4 | 14.2 | 25.9 |
| 36.7 | 0.3 | 0.9 | 7.8 | 14.8 | 27.1 |
| 42.8 | 0.3 | 1.0 | 8.1 | 15.3 | 28.0 |
| 48.9 | 0.3 | 1.1 | 8.8 | 16.3 | 29.0 |
| 55.0 | 0.4 | 1.2 | 9.3 | 17.0 | 30.4 |
| 61.2 | 0.4 | 1.2 | 10.0 | 17.5 | 31.5 |
| 67.3 | 0.5 | 1.3 | 10.2 | 17.5 | 32.1 |
| 73.4 | 0.5 | 1.4 | 10.7 | 19.0 | 32.7 |
| 79.5 | 0.5 | 1.5 | 10.5 | 19.8 | 33.3 |
| 85.6 | 0.6 | 1.6 | 11.1 | 20.8 | 34.1 |
| 91.7 | 0.6 | 1.7 | 11.3 | 21.0 | 35.4 |
| 97.8 | 0.6 | 1.7 | 11.3 | 21.7 | 36.5 |
| 104.0 | 0.7 | 1.8 | 11.8 | 22.0 | 37.4 |
| 110.1 | 0.7 | 1.9 | 12.2 | 21.8 | 38.0 |
| 116.2 | 0.8 | 1.9 | 12.4 | 22.4 | 39.2 |
| 122.3 | 0.8 | 2.1 | 13.0 | 23.0 | 38.9 |
| 128.4 | 0.8 | 2.1 | 13.3 | 23.3 | 40.6 |
| 134.5 | 0.9 | 2.2 | 13.5 | 23.9 | 40.2 |

| | | | | | |
|---|---|---|---|---|---|
| 140.6 | 0.9 | 2.3 | 13.9 | 23.9 | 41.2 |
| 146.8 | 0.9 | 2.3 | 14.0 | 25.1 | 42.0 |
| 152.9 | 1.0 | 2.4 | 14.6 | 25.4 | 43.3 |
| 159.0 | 1.0 | 2.5 | 14.8 | 26.0 | 42.8 |
| 165.1 | 1.1 | 2.6 | 15.7 | 25.8 | 44.0 |
| 171.2 | 1.1 | 2.6 | 16.0 | 26.2 | 45.2 |
| 177.3 | 1.2 | 2.7 | 15.9 | 27.3 | 44.2 |
| 183.5 | 1.3 | 2.8 | 16.3 | 27.9 | 44.8 |
| 189.6 | 1.4 | 2.8 | 17.2 | 28.0 | 46.1 |
| 195.7 | 1.5 | 2.9 | 17.6 | 28.5 | 46.8 |
| 201.8 | 1.6 | 3.0 | 17.4 | 29.2 | 47.8 |

| **ST – 0 d** | | | | | |
|---|---|---|---|---|---|
| | **Shear Stress** | | | | |
| **Shear Rate** | **10 g/l** | **20 g/l** | **30 g/l** | **40 g/l** | **50 g/l** |
| 0.4 | 0.2 | 0.3 | 1.0 | 5.2 | 13.5 |
| 0.7 | 0.2 | 0.4 | 1.7 | 7.4 | 18.5 |
| 1.8 | 0.2 | 0.5 | 2.5 | 10.7 | 22.6 |
| 3.7 | 0.2 | 0.6 | 4.2 | 14.4 | 26.9 |
| 7.3 | 0.3 | 0.8 | 6.9 | 17.9 | 31.0 |
| 11.0 | 0.4 | 1.0 | 8.8 | 20.4 | 34.6 |
| 14.7 | 0.4 | 1.2 | 9.7 | 22.4 | 37.4 |
| 18.3 | 0.5 | 1.4 | 9.8 | 23.6 | 41.0 |
| 24.5 | 0.7 | 1.7 | 11.2 | 27.0 | 46.0 |
| 30.6 | 0.9 | 2.0 | 12.2 | 28.4 | 48.7 |
| 36.7 | 1.1 | 2.2 | 13.0 | 30.2 | 54.1 |
| 42.8 | 1.2 | 2.6 | 14.2 | 33.1 | 58.8 |
| 48.9 | 1.4 | 2.9 | 16.0 | 35.1 | 61.5 |
| 55.0 | 1.5 | 3.1 | 17.0 | 36.9 | 63.7 |
| 61.2 | 1.7 | 3.4 | 18.2 | 38.0 | 65.0 |
| 67.3 | 1.9 | 3.7 | 19.0 | 39.2 | 66.5 |
| 73.4 | 2.0 | 3.9 | 19.8 | 41.4 | 69.5 |
| 79.5 | 2.2 | 4.2 | 20.8 | 42.9 | 71.7 |
| 85.6 | 2.3 | 4.4 | 21.4 | 45.2 | 72.6 |
| 91.7 | 2.5 | 4.7 | 21.9 | 47.8 | 76.5 |
| 97.8 | 2.6 | 4.9 | 22.5 | 47.9 | 79.0 |
| 104.0 | 2.8 | 5.1 | 23.5 | 48.4 | 81.9 |
| 110.1 | 2.9 | 5.4 | 24.3 | 49.8 | 83.0 |
| 116.2 | 3.0 | 5.6 | 26.0 | 50.5 | 84.8 |
| 122.3 | 3.3 | 5.8 | 26.4 | 51.2 | 86.8 |
| 128.4 | 3.3 | 6.1 | 27.6 | 52.8 | 89.4 |
| 134.5 | 3.5 | 6.2 | 28.1 | 54.1 | 90.2 |
| 140.6 | 3.6 | 6.5 | 28.6 | 55.0 | 93.2 |
| 146.8 | 3.8 | 6.8 | 29.7 | 56.2 | 93.1 |
| 152.9 | 4.0 | 7.0 | 31.3 | 57.2 | 96.0 |

| | | | | | |
|---|---|---|---|---|---|
| 159.0 | 4.3 | 7.2 | 32.4 | 59.6 | 97.2 |
| 165.1 | 4.7 | 7.4 | 33.1 | 61.1 | 97.3 |
| 171.2 | 5.1 | 7.7 | 34.3 | 62.0 | 99.4 |
| 177.3 | 5.5 | 7.8 | 35.4 | 63.8 | 100.5 |
| 183.5 | 5.9 | 8.2 | 36.5 | 63.6 | 104.3 |
| 189.6 | 6.1 | 8.3 | 37.4 | 65.0 | 106.1 |
| 195.7 | 6.4 | 8.6 | 39.0 | 68.6 | 108.2 |
| 201.8 | 6.7 | 8.8 | 40.4 | 68.8 | 108.8 |

| **ST – 20 d** | | | | | |
|---|---|---|---|---|---|
| | **Shear Stress** | | | | |
| **Shear Rate** | **10 g/l** | **20 g/l** | **30 g/l** | **40 g/l** | **50 g/l** |
| 0.4 | 0.1 | 0.1 | 0.4 | 1.8 | 4.7 |
| 0.7 | 0.1 | 0.1 | 0.7 | 2.6 | 6.4 |
| 1.8 | 0.1 | 0.2 | 1.0 | 3.7 | 7.6 |
| 3.7 | 0.1 | 0.2 | 1.6 | 5.2 | 9.2 |
| 7.3 | 0.1 | 0.3 | 2.5 | 6.6 | 11.6 |
| 11.0 | 0.2 | 0.4 | 3.1 | 7.5 | 12.3 |
| 14.7 | 0.2 | 0.5 | 3.3 | 8.1 | 13.4 |
| 18.3 | 0.2 | 0.5 | 3.4 | 8.7 | 15.0 |
| 24.5 | 0.3 | 0.6 | 3.9 | 9.9 | 16.6 |
| 30.6 | 0.4 | 0.7 | 4.3 | 10.6 | 17.9 |
| 36.7 | 0.4 | 0.9 | 4.5 | 11.3 | 19.6 |
| 42.8 | 0.5 | 1.0 | 4.9 | 11.9 | 21.1 |
| 48.9 | 0.5 | 1.1 | 5.5 | 12.6 | 22.1 |
| 55.0 | 0.6 | 1.2 | 5.9 | 13.2 | 22.8 |
| 61.2 | 0.7 | 1.3 | 6.4 | 13.8 | 23.4 |
| 67.3 | 0.7 | 1.4 | 6.7 | 14.2 | 24.2 |
| 73.4 | 0.8 | 1.5 | 7.0 | 15.1 | 24.7 |
| 79.5 | 0.9 | 1.6 | 7.0 | 15.9 | 25.4 |
| 85.6 | 0.9 | 1.7 | 7.4 | 16.3 | 26.1 |
| 91.7 | 1.0 | 1.8 | 7.7 | 17.1 | 26.9 |
| 97.8 | 1.0 | 1.8 | 7.7 | 17.2 | 28.7 |
| 104.0 | 1.1 | 2.0 | 8.3 | 17.7 | 30.0 |
| 110.1 | 1.2 | 2.1 | 8.6 | 17.9 | 30.8 |
| 116.2 | 1.2 | 2.1 | 9.3 | 18.6 | 31.4 |
| 122.3 | 1.3 | 2.3 | 9.6 | 18.9 | 31.9 |
| 128.4 | 1.3 | 2.3 | 10.0 | 19.2 | 32.8 |
| 134.5 | 1.4 | 2.4 | 10.1 | 20.2 | 32.9 |
| 140.6 | 1.5 | 2.5 | 10.3 | 20.1 | 34.3 |
| 146.8 | 1.5 | 2.6 | 10.8 | 21.0 | 34.5 |
| 152.9 | 1.6 | 2.7 | 11.2 | 21.5 | 35.5 |
| 159.0 | 1.8 | 2.8 | 11.5 | 22.2 | 35.5 |
| 165.1 | 1.9 | 2.9 | 12.2 | 22.7 | 36.0 |
| 171.2 | 2.0 | 3.0 | 12.6 | 22.9 | 37.6 |

| | | | | | |
|---|---|---|---|---|---|
| 177.3 | 2.2 | 3.1 | 12.8 | 23.7 | 36.7 |
| 183.5 | 2.4 | 3.1 | 13.2 | 23.9 | 38.3 |
| 189.6 | 2.5 | 3.2 | 13.8 | 24.3 | 38.6 |
| 195.7 | 2.6 | 3.3 | 14.2 | 25.0 | 39.3 |
| 201.8 | 2.7 | 3.4 | 14.7 | 25.4 | 40.2 |

| **TH – 0 d** | | | | | |
|---|---|---|---|---|---|
| | **Shear Stress** | | | | |
| **Shear Rate** | **10 g/l** | **20 g/l** | **30 g/l** | **40 g/l** | **50 g/l** |
| 0.4 | 0.0 | 0.0 | 0.7 | 1.4 | 5.1 |
| 0.7 | 0.1 | 0.1 | 2.2 | 2.9 | 7.9 |
| 1.8 | 0.1 | 0.2 | 2.2 | 4.4 | 9.6 |
| 3.7 | 0.2 | 0.3 | 2.9 | 8.0 | 12.3 |
| 7.3 | 0.4 | 0.5 | 3.7 | 11.0 | 14.3 |
| 11.0 | 0.5 | 0.7 | 3.6 | 13.3 | 15.8 |
| 14.7 | 0.6 | 1.0 | 3.7 | 14.3 | 18.2 |
| 18.3 | 0.7 | 1.2 | 2.9 | 16.2 | 21.4 |
| 24.5 | 1.0 | 1.5 | 4.4 | 18.0 | 26.3 |
| 30.6 | 1.2 | 1.8 | 4.4 | 19.8 | 28.6 |
| 36.7 | 1.4 | 2.1 | 5.0 | 20.6 | 34.6 |
| 42.8 | 1.7 | 2.7 | 6.3 | 25.1 | 41.0 |
| 48.9 | 2.0 | 3.0 | 8.0 | 26.7 | 44.8 |
| 55.0 | 2.2 | 3.3 | 8.7 | 28.2 | 46.4 |
| 61.2 | 2.5 | 3.6 | 9.6 | 30.3 | 47.5 |
| 67.3 | 2.7 | 4.0 | 10.2 | 31.5 | 48.2 |
| 73.4 | 2.9 | 4.3 | 11.3 | 32.8 | 51.4 |
| 79.5 | 3.2 | 4.5 | 12.1 | 34.4 | 51.8 |
| 85.6 | 3.3 | 4.8 | 12.7 | 35.7 | 53.2 |
| 91.7 | 3.5 | 5.1 | 13.4 | 38.8 | 55.9 |
| 97.8 | 3.8 | 5.4 | 13.6 | 38.3 | 59.6 |
| 104.0 | 3.9 | 5.5 | 14.7 | 38.9 | 62.5 |
| 110.1 | 4.0 | 5.8 | 15.7 | 39.9 | 65.0 |
| 116.2 | 4.3 | 6.1 | 18.2 | 40.7 | 65.8 |
| 122.3 | 4.6 | 6.4 | 18.4 | 41.8 | 68.5 |
| 128.4 | 4.7 | 6.6 | 19.3 | 44,1 | 72.0 |
| 134.5 | 4.9 | 6.9 | 20.0 | 45.4 | 72.7 |
| 140.6 | 5.1 | 7.3 | 20.1 | 46.2 | 75.6 |
| 146.8 | 5.4 | 7.6 | 21.5 | 47.3 | 76.1 |
| 152.9 | 5.6 | 7.9 | 22.9 | 48.8 | 76.7 |
| 159.0 | 6.3 | 8.2 | 24.4 | 51.0 | 78.7 |
| 165.1 | 6.9 | 8.4 | 24.9 | 53.0 | 78.3 |
| 171.2 | 7.5 | 8.7 | 26.3 | 54.5 | 81.3 |
| 177.3 | 8.1 | 8.9 | 28.0 | 55.0 | 81.9 |
| 183.5 | 8.8 | 9.4 | 29.1 | 55.9 | 86.4 |
| 189.6 | 8.9 | 9.6 | 29.7 | 57.3 | 88.4 |

| | | | | | |
|---|---|---|---|---|---|
| 195.7 | 9.2 | 10.0 | 30.9 | 61.5 | 90.2 |
| 201.8 | 9.7 | 10.2 | 33.1 | 61.4 | 91.2 |

| **TH – 20 d** | | | | | |
|---|---|---|---|---|---|
| | **Shear Stress** | | | | |
| **Shear Rate** | **10 g/l** | **20 g/l** | **30 g/l** | **40 g/l** | **50 g/l** |
| 0.4 | 0.0 | 0.0 | 0.4 | 0.7 | 2.5 |
| 0.7 | 0.0 | 0.1 | 1.1 | 1.4 | 4.0 |
| 1.8 | 0.1 | 0.1 | 1.1 | 2.2 | 4.7 |
| 3.7 | 0.1 | 0.2 | 1.4 | 4.0 | 6.1 |
| 7.3 | 0.2 | 0.3 | 1.8 | 5.4 | 8.3 |
| 11.0 | 0.2 | 0.4 | 1.8 | 6.5 | 8.0 |
| 14.7 | 0.3 | 0.5 | 1.8 | 7.2 | 9.0 |
| 18.3 | 0.4 | 0.6 | 1.4 | 8.0 | 10.8 |
| 24.5 | 0.5 | 0.7 | 2.2 | 9.0 | 13.0 |
| 30.6 | 0.6 | 0.9 | 2.2 | 9.8 | 14.5 |
| 36.7 | 0.7 | 1.0 | 2.5 | 10.5 | 17.3 |
| 42.8 | 0.8 | 1.2 | 2.9 | 11.2 | 19.2 |
| 48.9 | 0.9 | 1.3 | 3.6 | 12.3 | 20.6 |
| 55.0 | 1.0 | 1.5 | 4.0 | 13.0 | 21.3 |
| 61.2 | 1.1 | 1.6 | 4.3 | 13.7 | 21.3 |
| 67.3 | 1.2 | 1.8 | 4.7 | 14.5 | 22.0 |
| 73.4 | 1.3 | 1.9 | 5.1 | 15.2 | 23.1 |
| 79.5 | 1.4 | 2.1 | 5.4 | 15.9 | 23.5 |
| 85.6 | 1.5 | 2.2 | 5.8 | 16.3 | 24.2 |
| 91.7 | 1.6 | 2.3 | 6.1 | 17.3 | 25.7 |
| 97.8 | 1.7 | 2.5 | 6.1 | 17.3 | 27.5 |
| 104.0 | 1.8 | 2.6 | 6.9 | 18.1 | 30.0 |
| 110.1 | 1.9 | 2.7 | 7.2 | 18.4 | 30.7 |
| 116.2 | 2.0 | 2.9 | 8.3 | 19.2 | 31.1 |
| 122.3 | 2.1 | 3.0 | 8.7 | 19.5 | 32.2 |
| 128.4 | 2.2 | 3.1 | 9.0 | 20.2 | 32.9 |
| 134.5 | 2.3 | 3.3 | 9.4 | 21.3 | 33.2 |
| 140.6 | 2.4 | 3.4 | 9.4 | 21.7 | 35.4 |
| 146.8 | 2.5 | 3.5 | 10.1 | 22.4 | 35.8 |
| 152.9 | 2.6 | 3.7 | 10.8 | 23.1 | 35.8 |
| 159.0 | 2.9 | 3.8 | 11.2 | 23.9 | 37.2 |
| 165.1 | 3.2 | 3.9 | 11.6 | 25.3 | 36.9 |
| 171.2 | 3.5 | 4.1 | 12.3 | 24.9 | 39.0 |
| 177.3 | 3.7 | 4.2 | 13.0 | 26.0 | 38.3 |
| 183.5 | 4.0 | 4.3 | 13.4 | 26.4 | 40.8 |
| 189.6 | 4.2 | 4.5 | 14.1 | 26.7 | 40.8 |
| 195.7 | 4.4 | 4.6 | 14.5 | 28.2 | 41.6 |
| 201.8 | 4.6 | 4.8 | 15.5 | 28.5 | 42.6 |

**Figure 5.** Change in TS concentration in case of: a) *Trichoderma viride* (Verma et al., 2005 and unpublished data), and b) *Bacillus thuringiensis* fermentation on wastewater sludge (Brar et al., 2005; Barnabe et al., 2005).

(a) *Trichoderma viride*:

| | *Trichoderma* (TS change, g/l) | | |
|---|---|---|---|
| | ST | AH | TH |
| TS (g/l) | Time, h (96) | Time, h (96) | Time, h (96) |
| 10 | 1.18 | 1.58 | 1.93 |
| 20 | 2.73 | 3.37 | 4.79 |
| 30 | 3.95 | 5.60 | 7.40 |
| 40 | 2.78 | 3.89 | 6.33 |
| 50 | 2.38 | 4.43 | 5.73 |
| | | | |
| | *Trichoderma* (Conidia) | | |
| | ST (CFU/ml) | AH (CFU/ml) | TH (CFU/ml) |
| TS (g/l) | Time, h (96) | Time, h (96) | Time, h (96) |
| 10 | 6.E+03 | 6.E+04 | 1.E+06 |
| 20 | 2.E+04 | 3.E+05 | 2.E+06 |
| 30 | 2.E+04 | 5.E+05 | 1.E+07 |
| 40 | 8.E+03 | 2.E+05 | 9.E+05 |
| 50 | 4.E+03 | 4.E+04 | 4.E+05 |
| | | | |
| | *Trichoderma* (Tx, SBU/ µl) | | |
| | ST | AH | TH |
| TS (g/l) | Time, h (96) | Time, h (96) | Time, h (96) |
| 10 | 2.7E+03 | 4.4E+03 | 5.3E+03 |
| 20 | 5.5E+03 | 8.2E+03 | 1.2E+04 |
| 30 | 6.3E+03 | 1.0E+04 | 1.5E+04 |
| 40 | 4.7E+03 | 6.7E+03 | 9.6E+03 |
| 50 | 1.9E+03 | 3.6E+03 | 7.6E+03 |

(b) *Bacillus thuringiensis*:

| TS (g/l) | Bt (TS change, g/l) ST (g/l) Time, h (48) | TH (g/l) Time, h (48) | TS (g/l) | Bt (Total Cell) ST (CFU/ml) Time, h (48) | TH (CFU/ml) Time, h (48) |
|---|---|---|---|---|---|
| 10 | 0.87 | 1.22 | 10 | 1.0E+06 | 2.0E+06 |
| 20 | 2.34 | 3.92 | 20 | 4.8E+06 | 8.3E+07 |
| 30 | 3.92 | 6.46 | 25 | 1.1E+08 | 1.8E+08 |
| 40 | 4.14 | 6.39 | 30 | 2.0E+08 | 2.6E+09 |
| | | | 40 | 2.3E+06 | 1.3E+07 |

| TS (g/l) | Bt (Total Spore) ST (CFU/ml) Time, h (48) | TH (CFU/ml) Time, h (48) | TS (g/l) | Bt (Tx, SBU/µl) ST Time, h (48) | TH Time, h (48) |
|---|---|---|---|---|---|
| 10 | 1.4E+05 | 1.1E+05 | 10 | 4.2E+03 | 6.2E+03 |
| 20 | 8.5E+05 | 8.4E+06 | 20 | 7.5E+03 | 9.0E+03 |
| 25 | 1.0E+08 | 1.7E+08 | 25 | 9.3E+03 | 1.0E+04 |
| 30 | 1.6E+08 | 2.4E+09 | 30 | 8.6E+03 | 1.5E+04 |
| 40 | 2.0E+06 | 1.0E+07 | 40 | 8.6E+03 | |

# Annexe – III

# Données

**Les boues d'épuration comme matières premières potentielles pour les champignons antagonistes (*Trichoderma* sp.): rôle du pré-traitement et de la concentration en solides**

**Fig. 2.** CFU profile of *T. viride* in NH, AH and TAH sludges.

| NH | | | | | |
|---|---|---|---|---|---|
| Time, h | CCFU | | | | |
| SS, g/l | 10 g/l | 20 g/l | 30 g/l | 40 g/l | 50 g/l |
| 0 | 2833 | 3182 | 2973 | 2906 | 3143 |
| 12 | 2500 | 2545 | 2613 | 2735 | 2952 |
| 22 | 2583 | 2636 | 2703 | 2051 | 2095 |
| 28 | 4250 | 4091 | 4324 | 2991 | 3048 |
| 46 | 8833 | 5000 | 7568 | 5128 | 4190 |
| 54 | 4500 | 5545 | 3784 | 3248 | 9143 |
| 71 | 7500 | 16364 | 18018 | 7778 | 3714 |
| 82 | 6667 | 18182 | 19820 | 10256 | 4095 |
| 96 | 6000 | 19091 | 18919 | 7692 | 3810 |
| | | | | | |
| AH | | | | | |
| Time, h | CCFU | | | | |
| SS, g/l | 10 g/l | 20 g/l | 30 g/l | 40 g/l | 50 g/l |
| 0 | 3394 | 2910 | 2793 | 3005 | 3111 |
| 12 | 2515 | 2561 | 2628 | 2752 | 2970 |
| 22 | 2599 | 2652 | 2719 | 2064 | 2108 |
| 28 | 4276 | 4116 | 4350 | 3010 | 3066 |
| 46 | 7958 | 45045 | 68115 | 28180 | 3775 |
| 54 | 40541 | 49915 | 88136 | 29196 | 19302 |
| 71 | 67568 | 147391 | 252414 | 70064 | 33462 |
| 82 | 66794 | 181445 | 398989 | 102766 | 41035 |
| 96 | 60120 | 291484 | 490089 | 169539 | 38170 |
| | | | | | |
| TAH | | | | | |
| Time, h | CCFU | | | | |
| SS, g/l | 10 g/l | 20 g/l | 30 g/l | 40 g/l | 50 g/l |
| 0 | 3400 | 3500 | 3300 | 3400 | 3300 |
| 12 | 3000 | 2800 | 2900 | 3200 | 3100 |
| 22 | 31000 | 29000 | 30000 | 24000 | 22000 |
| 28 | 58000 | 45000 | 48000 | 35000 | 32000 |
| 46 | 106000 | 95000 | 84000 | 60000 | 44000 |
| 54 | 540000 | 410000 | 420000 | 380000 | 96000 |
| 71 | 900000 | 1800000 | 4000000 | 1100000 | 390000 |
| 82 | 1000000 | 2000000 | 12200000 | 1200000 | 430000 |
| 96 | 1200000 | 2100000 | 10100000 | 900000 | 400000 |

**Fig. 3.** SCOD profile of *Trichoderma viride* in NH, AH and TAH sludges.

| NH - SCOD (ppm) | | | | | |
|---|---|---|---|---|---|
| SS, g/l | 10 | 20 | 30 | 40 | 50 |
| Time, h | | | | | |
| 0 | 2.8E+03 | 3.2E+03 | 3.0E+03 | 2.9E+03 | 3.1E+03 |
| 12 | 2.5E+03 | 2.5E+03 | 2.6E+03 | 2.7E+03 | 3.0E+03 |
| 22 | 2.6E+03 | 2.6E+03 | 2.7E+03 | 2.1E+03 | 2.1E+03 |
| 28 | 4.3E+03 | 4.1E+03 | 4.3E+03 | 3.0E+03 | 3.0E+03 |
| 46 | 8.8E+03 | 5.0E+03 | 7.6E+03 | 5.1E+03 | 4.2E+03 |
| 54 | 4.5E+03 | 5.5E+03 | 3.8E+03 | 3.2E+03 | 9.1E+03 |
| 71 | 7.5E+03 | 1.6E+04 | 1.8E+04 | 7.8E+03 | 3.7E+03 |
| 82 | 6.7E+03 | 1.8E+04 | 2.0E+04 | 1.0E+04 | 4.1E+03 |
| 96 | 6.0E+03 | 1.9E+04 | 1.9E+04 | 7.7E+03 | 3.8E+03 |
| | | | | | |
| AH - SCOD (ppm) | | | | | |
| SS, g/l | 10 | 20 | 30 | 40 | 50 |
| Time, h | | | | | |
| 0 | 3.4E+03 | 2.9E+03 | 2.8E+03 | 3.0E+03 | 3.1E+03 |
| 12 | 2.5E+03 | 2.6E+03 | 2.6E+03 | 2.8E+03 | 3.0E+03 |
| 22 | 2.6E+03 | 2.7E+03 | 2.7E+03 | 2.1E+03 | 2.1E+03 |
| 28 | 4.3E+03 | 4.1E+03 | 4.4E+03 | 3.0E+03 | 3.1E+03 |
| 46 | 8.0E+03 | 4.5E+04 | 6.8E+04 | 2.8E+04 | 3.8E+03 |
| 54 | 4.1E+04 | 5.0E+04 | 8.8E+04 | 2.9E+04 | 1.9E+04 |
| 71 | 6.8E+04 | 1.5E+05 | 2.5E+05 | 7.0E+04 | 3.3E+04 |
| 82 | 6.7E+04 | 1.8E+05 | 4.0E+05 | 1.0E+05 | 4.1E+04 |
| 96 | 6.0E+04 | 2.9E+05 | 4.9E+05 | 1.7E+05 | 3.8E+04 |
| | | | | | |
| TAH - SCOD (ppm) | | | | | |
| SS, g/l | 10 | 20 | 30 | 40 | 50 |
| Time, h | | | | | |
| 0 | 3.4E+03 | 3.5E+03 | 3.3E+03 | 3.4E+03 | 3.3E+03 |
| 12 | 3.0E+03 | 2.8E+03 | 2.9E+03 | 3.2E+03 | 3.1E+03 |
| 22 | 3.1E+04 | 2.9E+04 | 3.0E+04 | 2.4E+04 | 2.2E+04 |
| 28 | 5.8E+04 | 4.5E+04 | 4.8E+04 | 3.5E+04 | 3.2E+04 |
| 46 | 1.1E+05 | 9.5E+04 | 8.4E+04 | 6.0E+04 | 4.4E+04 |
| 54 | 5.4E+05 | 4.1E+05 | 4.2E+05 | 3.8E+05 | 9.6E+04 |
| 71 | 9.0E+05 | 1.8E+06 | 4.0E+06 | 1.1E+06 | 3.9E+05 |
| 82 | 1.0E+06 | 2.0E+06 | 1.2E+07 | 1.2E+06 | 4.3E+05 |
| 96 | 1.2E+06 | 2.1E+06 | 1.0E+07 | 9.0E+05 | 4.0E+05 |

**Fig. 4.** Proteolytic activity of *T. viride* for NH, AH, and TAH sludges.

| NH - Protease (U/ml) | | | | | |
|---|---|---|---|---|---|
| SS, g/l | 10 | 20 | 30 | 40 | 50 |
| Time, h | | | | | |
| 0 | 0.48 | 0.44 | 0.22 | 0.17 | 0.13 |
| 12 | 0.11 | 0.23 | 0.13 | 0.19 | 0.25 |
| 22 | 0.39 | 0.44 | 0.46 | 0.77 | 0.79 |
| 28 | 0.65 | 0.69 | 2.37 | 3.12 | 4.02 |
| 46 | 0.73 | 0.80 | 2.41 | 2.91 | 3.99 |
| 54 | 0.58 | 0.45 | 1.62 | 2.35 | 2.11 |
| 71 | 0.41 | 0.56 | 0.53 | 0.75 | 0.66 |
| 82 | 0.32 | 0.23 | 0.25 | 0.91 | 1.14 |
| 96 | 0.10 | 0.16 | 0.19 | 0.25 | 0.06 |
| | | | | | |
| AH - Protease (U/ml) | | | | | |
| SS, g/l | 10 | 20 | 30 | 40 | 50 |
| Time, h | | | | | |
| 0 | 1.18 | 0.66 | 0.33 | 0.21 | 0.28 |
| 12 | 0.16 | 0.33 | 0.17 | 0.33 | 0.25 |
| 22 | 0.59 | 0.68 | 0.70 | 1.10 | 1.07 |
| 28 | 1.15 | 1.55 | 3.29 | 5.59 | 4.34 |
| 46 | 1.38 | 1.21 | 2.96 | 5.00 | 4.44 |
| 54 | 1.06 | 0.62 | 2.90 | 3.98 | 3.41 |
| 71 | 0.30 | 0.77 | 0.73 | 0.99 | 1.12 |
| 82 | 0.43 | 0.30 | 1.34 | 0.78 | 0.72 |
| 96 | 0.13 | 0.22 | 0.25 | 0.08 | 0.32 |
| | | | | | |
| TAH - Protease (U/ml) | | | | | |
| SS, g/l | 10 | 20 | 30 | 40 | 50 |
| Time, h | | | | | |
| 0 | 0.59 | 1.21 | 1.07 | 0.77 | 1.57 |
| 12 | 0.35 | 1.06 | 1.41 | 0.83 | 0.95 |
| 22 | 1.02 | 1.38 | 2.19 | 1.74 | 2.74 |
| 28 | 2.04 | 3.45 | 6.02 | 7.10 | 6.41 |
| 46 | 2.62 | 2.94 | 6.12 | 7.97 | 7.02 |
| 54 | 2.73 | 3.58 | 5.45 | 6.57 | 6.20 |
| 71 | 0.21 | 1.37 | 2.24 | 2.16 | 2.82 |
| 82 | 0.40 | 0.91 | 0.82 | 0.58 | 2.22 |
| 96 | 1.13 | 0.20 | 0.27 | 0.46 | 1.63 |

# Annexe – IV

# Données

**Fermentation de *Trichoderma viride* dans des boues d'épuration à échelle de laboratoire: rhéologie, enzymes lytiques et activités de lutte biologique**

**Figure 1.** Profiles of; (A) fermentation operational parameters, (B) conidia production and medium rheology of *T. viride* inoculated TH30 sludge.

(B)

| TH30 | | | | | |
|---|---|---|---|---|---|
| Time, h | CFU/ml | Surface tension, $Nm^{-1}$ | Kla, $h^{-1}$ | Visc., mPa.s | Protein, mg $l^{-1}$ |
| 0 | 8.0E+03 | 40 | | 3.42 | 5473 |
| 14 | 1.2E+04 | 40.5 | 78.8 | 3.56 | 5187 |
| 24 | 1.8E+04 | 42.7 | 38.6 | 9.78 | 4673 |
| 30 | 3.3E+05 | 38.3 | 20.3 | 11.9 | 4387 |
| 36 | 1.7E+07 | 37.6 | 21.2 | 16.22 | 4327 |
| 42 | 1.8E+07 | 38.6 | 41.6 | 7.88 | 4660 |
| 48 | 2.4E+07 | 36.2 | 44.1 | 9.3 | 3768 |
| 54 | 3.0E+07 | 38.6 | 54.1 | 6.62 | 4141 |
| 60 | 5.0E+07 | 40.8 | 81.5 | 7.6 | 3623 |
| 72 | 4.5E+07 | 39.4 | 85.8 | 8.2 | 3979 |
| 84 | 5.7E+07 | 40.7 | 52 | 7.02 | 4046 |
| 96 | 5.9E+07 | 43.2 | 75.4 | 5.68 | 3628 |

**Figure 2.** Profiles of; (A) fermentation operational parameters, (B) conidia production and medium rheology of *T. viride* inoculated TH35 sludge.

(B)

| **TH35** | | | | | |
|---|---|---|---|---|---|
| Time, h | CFU/ml | Surface tension, $Nm^{-1}$ | Kla, $h^{-1}$ | Visc., mPa.s | Protein, mg $l^{-1}$ |
| 0 | 8.0E+03 | 38.7 | | 2.8 | 5690 |
| 14 | 2.4E+04 | 38.8 | 119.7 | 2.68 | 5568 |
| 24 | 2.3E+04 | 38 | 50.3 | 3.32 | 5094 |
| 30 | 1.1E+05 | 38.7 | 27 | 13.62 | 5359 |
| 36 | 1.0E+06 | 39.1 | 29.7 | 65.99 | 4874 |
| 42 | 3.7E+06 | 39.9 | 27.7 | 53.99 | 4988 |
| 48 | 5.9E+06 | 39.9 | 25.5 | 39.99 | 4739 |
| 54 | 6.0E+06 | 39.4 | 54.1 | 22 | 4526 |
| 60 | 6.6E+06 | 39.1 | 48.3 | 43.99 | 4776 |
| 72 | 5.8E+06 | 38.1 | 84.4 | 22 | 4601 |
| 84 | 6.2E+06 | 38.8 | 62.6 | 6 | 4422 |
| 96 | 6.4E+06 | 38.5 | 58.5 | 3.02 | 4557 |

**Figure 3.** Oxygen uptake rate variations in TH30 and TH35 sludges during *T. viride* fermentation.

| | OUR, mmol/l/h | |
|---|---|---|
| **Time (h)** | **TH 30** | **TH 35** |
| 14 | 1.80 | 0.64 |
| 24 | 4.49 | 2.17 |
| 30 | 2.84 | 2.60 |
| 36 | 2.09 | 2.60 |
| 42 | 1.42 | 1.95 |
| 48 | 1.00 | 1.59 |
| 54 | 0.90 | 1.08 |
| 60 | 0.71 | 0.78 |
| 72 | 0.56 | 0.43 |
| 84 | 0.51 | 0.46 |
| 96 | 0.42 | 0.26 |

**Figure 4.** Rheological profiles of: (A) TH30 and (B) TH35 sludges.

| **TH 30** | | | |
|---|---|---|---|
| **Time, h** | ***K*, Consistency index** | **n, flow index** | **Confidence of Fit, %** |
| 0 | 19.4 | 0.57 | 85.4 |
| 14 | 27.2 | 0.51 | 82.5 |
| 24 | 71.7 | 0.47 | 98 |
| 30 | 98.9 | 0.44 | 93.4 |
| 36 | 130.9 | 0.395 | 78.2 |
| 42 | 56.1 | 0.53 | 93.5 |
| 48 | 112.6 | 0.37 | 95.5 |
| 54 | 84.3 | 0.4 | 93.6 |
| 60 | 86.2 | 0.39 | 91.8 |
| 72 | 91.1 | 0.39 | 91.9 |
| 84 | 68 | 0.45 | 92.4 |
| 96 | 65.9 | 0.44 | 90.3 |
| **TH 35** | | | |
| 0 | 11.4 | 0.64 | 81.2 |
| 14 | 10.7 | 0.64 | 79.6 |
| 24 | 28.3 | 0.591 | 80.9 |
| 30 | 68 | 0.54 | 95.8 |
| 36 | 113.3 | 0.52 | 81.4 |
| 42 | 125.7 | 0.52 | 83.2 |
| 48 | 97.6 | 0.55 | 84.4 |
| 54 | 74.8 | 0.61 | 89.3 |
| 60 | 103.4 | 0.55 | 83.7 |
| 72 | 25.1 | 0.88 | 76.9 |
| 84 | 22.9 | 0.8 | 83.9 |
| 96 | 32.6 | 0.52 | 90 |

**Figure 5.** Enzyme production kinetics of TH30 and TH35 sludges; (A) protease, (B) amylase, (C) cellulase, (D) laccase and (E) chitinase.

| | Protease, IU/ml | | Amylase, U/ml | | Cellulase, U/ml | | Laccase, U/L | | Chitinase, U/ml | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Time, h** | **TH30** | **STDEV** | **TH30** | **STDEV** | **TH30** | **STDEV** | **TH30** | **STDEV** | **TH30** | **STDEV** |
| 0 | 0.58 | 0.01 | 0.62 | 0.02 | 21.59 | 0.66 | 0.13 | 0.01 | 0.09 | 0.00 |
| 14 | 0.88 | 0.07 | 0.73 | 0.01 | 26.91 | 2.08 | 0.18 | 0.02 | 4.22 | 0.32 |
| 24 | 1.23 | 0.09 | 1.22 | 0.03 | 61.47 | 4.79 | 0.17 | 0.00 | 20.18 | 1.58 |
| 30 | 1.53 | 0.11 | 1.14 | 0.02 | 77.71 | 0.60 | 0.17 | 0.00 | 21.15 | 1.16 |
| 36 | 1.60 | 0.07 | 1.17 | 0.00 | 154.92 | 7.48 | 0.31 | 0.02 | 19.37 | 1.72 |
| 42 | 2.14 | 0.10 | 1.28 | 0.01 | 105.94 | 2.28 | 0.31 | 0.01 | 20.40 | 0.50 |
| 48 | 2.14 | 0.03 | 1.14 | 0.02 | 145.66 | 8.33 | 0.36 | 0.02 | 18.71 | 0.87 |
| 54 | 2.04 | 0.03 | 1.20 | 0.02 | 133.49 | 4.62 | 0.35 | 0.02 | 19.81 | 1.42 |
| 60 | 1.23 | 0.08 | 1.09 | 0.01 | 97.77 | 2.21 | 0.30 | 0.01 | 17.44 | 0.37 |
| 72 | 1.47 | 0.09 | 1.31 | 0.03 | 114.95 | 11.27 | 0.38 | 0.01 | 16.96 | 0.79 |
| 84 | 1.96 | 0.13 | 1.13 | 0.02 | 116.54 | 5.00 | 0.41 | 0.02 | 15.16 | 0.20 |
| 96 | 1.90 | 0.10 | 1.22 | 0.02 | 132.51 | 3.52 | 0.37 | 0.02 | 15.12 | 0.73 |
| | **Protease, IU/ml** | | **Amylase, U/ml** | | **Cellulase, U/ml** | | **Laccase, U/L** | | **Chitinase, U/ml** | |
| **Time, h** | **TH35** | **STDEV** | **TH35** | **STDEV** | **TH35** | **STDEV** | **TH35** | **STDEV** | **TH35** | **STDEV** |
| 0 | 0.20 | 0.02 | 0.48 | 0.00 | 18.58 | 1.04 | 0.07 | 0.01 | 0.03 | 0.00 |
| 14 | 0.31 | 0.01 | 0.47 | 0.01 | 11.85 | 0.64 | 0.07 | 0.01 | 3.56 | 0.05 |
| 24 | 0.74 | 0.05 | 0.75 | 0.02 | 20.72 | 0.27 | 0.08 | 0.00 | 8.41 | 0.16 |
| 30 | 1.41 | 0.06 | 0.42 | 0.01 | 47.17 | 2.97 | 0.13 | 0.02 | 14.82 | 0.29 |
| 36 | 1.30 | 0.04 | 0.61 | 0.01 | 83.70 | 1.21 | 0.10 | 0.01 | 13.00 | 0.98 |
| 42 | 1.22 | 0.06 | 0.77 | 0.02 | 60.00 | 0.69 | 0.20 | 0.02 | 9.40 | 0.62 |
| 48 | 1.11 | 0.05 | 0.85 | 0.03 | 77.36 | 1.29 | 0.12 | 0.01 | 7.08 | 0.30 |
| 54 | 1.38 | 0.05 | 0.80 | 0.02 | 104.42 | 1.65 | 0.16 | 0.01 | 12.16 | 0.62 |
| 60 | 1.57 | 0.14 | 0.73 | 0.02 | 118.63 | 2.61 | 0.16 | 0.01 | 12.67 | 0.79 |
| 72 | 1.29 | 0.09 | 0.93 | 0.02 | 116.50 | 9.08 | 0.16 | 0.03 | 8.56 | 0.20 |
| 84 | 1.06 | 0.06 | 0.92 | 0.02 | 67.42 | 2.60 | 0.14 | 0.01 | 7.44 | 0.42 |
| 96 | 0.92 | 0.06 | 0.80 | 0.03 | 83.88 | 5.81 | 0.15 | 0.02 | 4.82 | 0.38 |

**Figure 6.** Antagonism of *T. viride* against, (A) *Fusarium* sp. (inhibition index) (B) spruce budworm larvae (entomotoxicity).

**(A)**

| | TH30 | | TH35 | |
|---|---|---|---|---|
| **Time, h** | **Inhibition Index** | **STDEV** | **Inhibition Index** | **STDEV** |
| 0 | 0.08 | 0.00 | 0.04 | 0.00 |
| 14 | 0.20 | 0.01 | 0.09 | 0.01 |
| 24 | 0.23 | 0.02 | 0.16 | 0.01 |
| 30 | 0.17 | 0.01 | 0.12 | 0.01 |
| 36 | 0.33 | 0.03 | 0.22 | 0.02 |
| 42 | 0.37 | 0.04 | 0.37 | 0.04 |
| 48 | 0.37 | 0.03 | 0.39 | 0.03 |
| 54 | 0.60 | 0.04 | 0.51 | 0.03 |
| 60 | 0.64 | 0.05 | 0.52 | 0.04 |
| 72 | 0.80 | 0.06 | 0.58 | 0.05 |
| 84 | 0.91 | 0.05 | 0.60 | 0.03 |
| 96 | 0.90 | 0.08 | 0.59 | 0.05 |

**(B)**

| | TH30 | | TH35 | |
|---|---|---|---|---|
| **Time, h** | **Tx, SBU/ml** | **STDEV** | **Tx, SBU/ml** | **STDEV** |
| 12 | 4156 | 249 | 3824 | 229 |
| 24 | 5486 | 384 | 4156 | 291 |
| 36 | 11305 | 904 | 5985 | 479 |
| 42 | 13300 | 665 | 6650 | 333 |
| 48 | 12968 | 1167 | 7980 | 718 |
| 72 | 16625 | 1663 | 9532 | 953 |
| 96 | 17290 | 1297 | 9310 | 698 |

**Figure 7.** Effect of *T. viride* conidia on, (A) normal growth of *Fusarium* sp. on Petri (B) growth inhibition of *Fusarium* sp. by *T. viride* on Petri (marked by arrows), (C) *Fusarium* sp. infected soya leaves (marked by arrows) and (D) overall growth of plants.

**(D)**

| **Dry weight, g/l** | **Control** | **STDEV** | ***Fusarium*** | **STDEV** | ***Trichodema*** | **STDEV** | ***Trichodema* + *Fusarium*** | **STDEV** |
|---|---|---|---|---|---|---|---|---|
| **Tomato** | 15.89 | 2.28 | 10.89 | 1.92 | 34.29 | 4.60 | 29.29 | 4.27 |
| **Pepper** | 3.89 | 1.10 | 2.29 | 1.92 | 9.89 | 1.79 | 6.89 | 1.92 |
| **Soya** | 35.89 | 2.28 | 26.29 | 1.30 | 35.49 | 4.10 | 33.69 | 2.51 |

# Annexe – V

# Données

**Les eaux usées industrielles et les boues déshydratées : source riche de nutriments pour la production et la formulation de l'agents de lutte biologique *Trichoderma viride***

**Figure 1.** Optimization of TS concentration (soluble starch concentration, in case of SSM) for *Trichoderma viride* conidia production in different raw materials. (* TS concentration of the sample collected)

| | DS (Total solids optimization) | | | | |
|---|---|---|---|---|---|
| | CFU/ ml | | | | |
| **Time, h** | 10 g/l | 20 g/l | 30 g/l | 40 g/l | 50 g/l |
| **0** | 1.8E+03 | 2.8E+03 | 2.0E+03 | 1.0E+03 | 1.1E+03 |
| **12** | 1.0E+03 | 1.0E+03 | 2.0E+03 | 1.0E+03 | 1.0E+03 |
| **30** | 5.1E+03 | 1.2E+04 | 3.2E+04 | 3.8E+03 | 3.0E+03 |
| **41** | 6.0E+03 | 1.1E+04 | 3.5E+04 | 4.8E+03 | 3.9E+03 |
| **46** | 8.9E+03 | 1.3E+04 | 2.1E+04 | 6.0E+03 | 4.9E+03 |
| **53** | 4.0E+04 | 1.1E+05 | 3.3E+05 | 7.2E+04 | 1.2E+04 |
| **60** | 8.9E+04 | 1.7E+05 | 3.4E+05 | 2.0E+05 | 4.0E+04 |
| **69** | 2.4E+05 | 1.0E+06 | 3.3E+06 | 4.8E+05 | 5.9E+04 |
| **96** | 2.9E+05 | 1.6E+06 | 3.5E+06 | 7.1E+05 | 7.9E+04 |

| | CH (Total solids optimization) | | | |
|---|---|---|---|---|
| | CFU/ ml | | | |
| **Time, h** | 3.8* g/l | 10 g/l | 15 g/l | 20 g/l |
| **0** | 1.8E+03 | 2.5E+03 | 1.3E+03 | 1.2E+03 |
| **12** | 1.0E+03 | 1.0E+03 | 1.7E+03 | 1.0E+03 |
| **30** | 1.5E+04 | 1.7E+05 | 1.1E+04 | 6.0E+03 |
| **41** | 6.5E+04 | 3.0E+05 | 5.0E+04 | 2.0E+04 |
| **46** | 1.7E+05 | 6.1E+05 | 1.1E+05 | 2.7E+04 |
| **53** | 1.3E+06 | 4.5E+06 | 6.9E+05 | 1.2E+05 |
| **60** | 4.5E+06 | 1.9E+07 | 7.8E+05 | 1.5E+05 |
| **69** | 3.2E+06 | 1.1E+07 | 1.9E+06 | 2.1E+05 |
| **96** | 3.9E+06 | 1.9E+07 | 1.6E+06 | 2.0E+05 |

| | PPN (Total solids optimization) | | | |
|---|---|---|---|---|
| | CFU/ ml | | | |
| **Time, h** | 1.7* g/l | 5 g/l | 10 g/l | 15 g/l |
| **0** | 1.7E+03 | 1.0E+03 | 1.3E+03 | 1.2E+03 |
| **12** | 1.0E+03 | 1.0E+03 | 9.3E+02 | 1.0E+03 |
| **30** | 2.3E+04 | 5.0E+04 | 1.2E+04 | 3.3E+03 |
| **41** | 3.1E+04 | 8.2E+04 | 2.4E+04 | 1.2E+04 |
| **46** | 4.8E+04 | 1.1E+05 | 5.0E+04 | 1.5E+04 |
| **53** | 2.3E+05 | 2.0E+06 | 1.2E+05 | 2.0E+04 |
| **60** | 8.9E+05 | 3.4E+06 | 2.9E+05 | 3.1E+04 |
| **69** | 7.8E+06 | 1.9E+08 | 1.0E+06 | 5.3E+04 |
| **96** | 5.0E+06 | 4.9E+07 | 8.3E+05 | 6.0E+04 |

| | PPT (Total solids optimization) | | | |
|---|---|---|---|---|
| | CFU/ ml | | | |
| **Time, h** | 1.3* g/l | 5 g/l | 10 g/l | 15 g/l |
| **0** | 2.4E+03 | 1.3E+03 | 2.4E+03 | 1.2E+03 |
| **12** | 1.0E+03 | 9.3E+02 | 1.0E+03 | 1.0E+03 |
| **30** | 1.5E+04 | 8.3E+03 | 1.9E+04 | 6.0E+03 |
| **41** | 4.8E+04 | 2.4E+04 | 2.9E+04 | 2.0E+04 |
| **46** | 4.8E+04 | 5.0E+04 | 7.8E+04 | 2.7E+04 |
| **53** | 3.1E+05 | 1.7E+05 | 5.9E+05 | 2.9E+04 |
| **60** | 3.1E+05 | 1.7E+05 | 1.2E+06 | 4.8E+04 |
| **69** | 5.0E+05 | 3.5E+05 | 1.1E+06 | 2.4E+04 |
| **96** | 3.7E+05 | 1.4E+05 | 1.6E+06 | 2.2E+04 |

| | SSM (Total solids optimization) | | | | |
|---|---|---|---|---|---|
| | CFU/ ml | | | | |
| **Time, h** | 10 g/l | 20 g/l | 30 g/l | 40 g/l | 50 g/l |
| **0** | 2.4E+03 | 1.3E+03 | 1.0E+03 | 2.4E+03 | 1.2E+03 |
| **12** | 1.0E+03 | 9.3E+02 | 1.0E+03 | 1.0E+03 | 1.0E+03 |
| **30** | 8.9E+03 | 7.2E+04 | 3.4E+05 | 2.0E+04 | 3.0E+03 |
| **41** | 3.2E+04 | 2.9E+05 | 1.0E+06 | 5.9E+04 | 8.9E+03 |
| **46** | 3.6E+04 | 1.2E+06 | 5.3E+06 | 1.3E+05 | 1.3E+04 |
| **53** | 1.5E+05 | 6.5E+06 | 9.9E+07 | 5.9E+05 | 1.6E+04 |
| **60** | 2.6E+05 | 3.8E+07 | 1.2E+08 | 1.7E+06 | 3.0E+04 |
| **69** | 5.3E+05 | 1.9E+07 | 2.5E+10 | 2.9E+06 | 1.6E+05 |
| **96** | 7.1E+05 | 5.8E+07 | 1.0E+10 | 2.9E+06 | 2.2E+04 |

| | SHW (Total solids optimization) | | | |
|---|---|---|---|---|
| | CFU/ ml | | | |
| **Time, h** | 3.2* g/l | 10 g/l | 15 g/l | 20 g/l |
| **0** | 1.8E+03 | 1.9E+03 | 1.3E+03 | 1.2E+03 |
| **12** | 1.0E+03 | 1.1E+03 | 1.7E+03 | 1.0E+03 |
| **30** | 2.1E+04 | 6.0E+04 | 5.4E+03 | 3.1E+03 |
| **41** | 3.2E+04 | 9.9E+04 | 2.7E+04 | 1.3E+04 |
| **46** | 9.3E+04 | 1.8E+05 | 6.0E+04 | 1.2E+04 |
| **53** | 2.7E+05 | 1.7E+06 | 9.3E+04 | 6.5E+04 |
| **60** | 2.6E+06 | 5.3E+06 | 2.4E+05 | 6.0E+04 |
| **69** | 5.4E+06 | 1.6E+07 | 4.8E+05 | 3.9E+04 |
| **96** | 4.5E+06 | 1.6E+07 | 5.8E+05 | 6.0E+04 |

**Figure 3.** Protease activity of *Trichoderma viride* in different raw materials. Values in parentheses show TS concentration (soluble starch concentration, in case of SSM).

| DS-SSM (Industrial Wastewater) – Protease activity (IU/µl) | | | | | | |
|---|---|---|---|---|---|---|
| **Time, h** | **DS** | **CH** | **PPN** | **PPT** | **SSM** | **SHW** |
| 0 | 7.6E-02 | 9.2E-02 | 7.3E-02 | 5.7E-02 | 6.2E-02 | 6.9E-02 |
| 12 | 1.6E-01 | 1.0E-01 | 1.2E-01 | 5.9E-02 | 7.1E-02 | 1.3E-01 |
| 30 | 5.0E-01 | 4.2E-01 | 2.3E-01 | 2.0E-01 | 2.4E-01 | 4.2E-01 |
| 41 | 1.9E+00 | 1.5E+00 | 3.7E-01 | 3.5E-01 | 1.3E+00 | 2.1E+00 |
| 46 | 2.0E+00 | 1.6E+00 | 4.4E-01 | 3.9E-01 | 1.3E+00 | 2.2E+00 |
| 53 | 1.3E+00 | 1.3E+00 | 3.9E-01 | 3.1E-01 | 8.9E-01 | 1.2E+00 |
| 60 | 5.3E-01 | 4.0E-01 | 3.0E-01 | 2.2E-01 | 2.9E-01 | 3.5E-01 |
| 69 | 4.3E-01 | 2.6E-01 | 1.2E-01 | 1.8E-01 | 1.4E-01 | 3.2E-01 |
| 96 | 1.4E-01 | 1.4E-01 | 9.0E-02 | 5.6E-02 | 1.0E-01 | 3.2E-02 |

**Figure 4.** Optimal TS concentration (soluble starch concentration, in case of SSM) with respect to inhibition index of 96 h *Trichoderma viride* fermented samples of all raw materials.

| Supernatant, Index d'inhibition (96h) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Total Solids, g/l** | **DS** | **SSM** | **TS, g/l** | **PPN** | **TS, g/l** | **PPT** | **TS, g/l** | **CH** | **TS, g/l** | **SHW** |
| 10 | 0.34 | 0.21 | 1.70 | 0.33 | 1.30 | 0.23 | 3.80 | 0.27 | 3.20 | 0.35 |
| 20 | 0.41 | 0.42 | 5.00 | 0.49 | 5.00 | 0.25 | 10.00 | 0.48 | 10.00 | 0.70 |
| 30 | 0.61 | 0.53 | 10.00 | 0.44 | 10.00 | 0.33 | 15.00 | 0.41 | 15.00 | 0.59 |
| 40 | 0.45 | 0.32 | 15.00 | 0.27 | 15.00 | 0.29 | 20.00 | 0.33 | 20.00 | 0.45 |
| 50 | 0.28 | 0.22 | | | | | | | | |

**Figure 5.** Shelf-life stability of *Trichoderma viride* conidia in the DS sludge formulation of different raw materials.

| | **CH** | | **PPN** | | **SSM** | | **SHW** | |
|---|---|---|---|---|---|---|---|---|
| **Temperature** | **4°C** | **25°C** | **4°C** | **25°C** | **4°C** | **25°C** | **4°C** | **25°C** |
| **Time, d** | **CFU/g** | | | | | | | |
| 0 | 1.0E+07 | 1.0E+07 | 1.1E+07 | 1.1E+07 | 1.1E+07 | 1.0E+07 | 1.1E+07 | 1.0E+07 |
| 30 | 9.3E+06 | 7.1E+06 | 9.1E+06 | 7.3E+06 | 9.5E+06 | 7.1E+06 | 9.9E+06 | 8.7E+06 |
| 60 | 8.5E+06 | 6.2E+06 | 8.7E+06 | 5.8E+06 | 9.0E+06 | 6.2E+06 | 9.4E+06 | 5.6E+06 |
| 90 | 7.6E+06 | 5.0E+06 | 7.8E+06 | 5.3E+06 | 8.3E+06 | 4.6E+06 | 7.5E+06 | 5.1E+06 |
| 120 | 6.9E+06 | 4.5E+06 | 7.1E+06 | 4.2E+06 | 7.1E+06 | 4.4E+06 | 7.5E+06 | 3.7E+06 |
| 150 | 6.2E+06 | 3.6E+06 | 5.7E+06 | 3.4E+06 | 5.8E+06 | 2.8E+06 | 6.5E+06 | 3.2E+06 |
| 180 | 6.0E+06 | 3.2E+06 | 5.3E+06 | 3.0E+06 | 5.8E+06 | 2.9E+06 | 5.0E+06 | 3.1E+06 |

# Annexe –VII

# Données

**Les eaux usées de l'industrie de l'amidon comme un substrat pour la production de l'antagoniste *Trichoderma viride***

**Figure 1.** CFU and residual glucose profile of *T. viride* in SIW with, (a) varying glucose supplement, (b) varying N-source and soluble starch supplements.

| Time, h | SW1 | SW2 | SW3 | SW4 | SW5 | SW6 | SW7 | SW8 | SW2 Glucose | SW3 Glucose | SW4 Glucose |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.3E+01 | 2.7E+01 | 5.8E+01 |
| 12 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 7.6E+00 | 2.3E+01 | 5.3E+01 |
| 28 | 2.4E+05 | 1.6E+05 | 1.2E+05 | 7.0E+04 | 8.0E+04 | 1.8E+05 | 2.1E+05 | 2.0E+05 | 4.6E+00 | 1.4E+01 | 4.8E+01 |
| 40 | 2.2E+06 | 3.6E+06 | 1.7E+06 | 7.0E+05 | 6.4E+06 | 4.9E+06 | 3.1E+06 | 3.0E+05 | 1.1E+00 | 9.6E+00 | 4.7E+01 |
| 46 | 2.2E+07 | 2.1E+07 | 6.3E+06 | 2.5E+06 | 2.0E+07 | 2.5E+07 | 1.2E+07 | 1.5E+06 | 5.6E-01 | 6.3E+00 | 4.4E+01 |
| 52 | 2.5E+07 | 3.4E+07 | 1.0E+07 | 3.0E+06 | 5.5E+07 | 5.7E+07 | 3.3E+07 | 2.8E+07 | 1.2E-01 | 5.4E+00 | 4.4E+01 |
| 60 | 1.5E+08 | 1.1E+08 | 5.2E+07 | 3.8E+07 | 2.8E+08 | 3.0E+08 | 1.7E+08 | 2.6E+07 | 7.0E-02 | 5.1E+00 | 4.3E+01 |
| 72 | 1.7E+08 | 3.2E+08 | 6.4E+07 | 4.0E+07 | 2.6E+08 | 3.4E+08 | 6.4E+08 | 4.7E+07 | 1.0E-02 | 4.9E+00 | 3.9E+01 |
| 96 | 1.6E+08 | 3.6E+08 | 6.2E+07 | 3.0E+07 | 2.3E+08 | 3.7E+08 | 7.5E+08 | 4.8E+07 | 5.0E-03 | 4.1E+00 | 3.6E+01 |

**Figure 4.** Effect of probable conidiation inducers on CFU production of *T. viride* in SIW.

| Time, h | SW17 | SW18 | SW19 | SW20 | SW21 | SW22 | SW23 | SW24 |
|---|---|---|---|---|---|---|---|---|
| 0 | 9.0E+03 | 5.0E+03 | 1.3E+04 | 1.1E+04 | 9.0E+03 | 6.0E+03 | 8.0E+03 | 8.0E+03 |
| 14 | 7.0E+03 | 4.0E+03 | 8.0E+03 | 9.0E+03 | 6.0E+03 | 3.0E+03 | 5.0E+03 | 6.0E+03 |
| 28 | 9.1E+06 | 2.1E+06 | 9.7E+06 | 8.0E+04 | 1.0E+06 | 2.7E+06 | 4.6E+06 | 1.2E+07 |
| 40 | 4.1E+07 | 5.6E+07 | 5.6E+07 | 1.1E+06 | 2.4E+07 | 2.9E+07 | 4.0E+07 | 4.9E+07 |
| 46 | 1.1E+08 | 2.5E+08 | 2.4E+08 | 8.5E+05 | 2.0E+08 | 3.0E+08 | 2.7E+08 | 3.8E+08 |
| 52 | 3.1E+07 | 3.7E+07 | 1.4E+07 | 7.0E+06 | 1.1E+08 | 1.8E+08 | 3.3E+08 | 1.8E+09 |
| 60 | 3.5E+07 | 1.1E+08 | 9.0E+07 | 1.5E+07 | 3.5E+07 | 3.2E+07 | 1.3E+09 | 2.9E+09 |
| 72 | 6.5E+07 | 2.0E+08 | 3.5E+08 | 2.1E+07 | 1.2E+08 | 4.0E+07 | 3.0E+10 | 4.2E+10 |
| 96 | 4.7E+07 | 2.8E+08 | 1.8E+08 | 4.1E+07 | 2.0E+07 | 7.5E+07 | 1.7E+10 | 8.8E+09 |

| CODE | Composition | CODE | Composition | CODE | Composition | CODE | Composition | CODE | Composition |
|---|---|---|---|---|---|---|---|---|---|
| SW1 | Blank (SIW only) | SW5 | 0.1%(w/v),Meat Peptone | SW9 | 0.1 N, Na2CO3 | SW13 | 0.1%(w/v), L-Glutamic acid | SW18 | 0.1%(w/v), Maleic acid |
| SW2 | 0.5%(w/v), Glucose | SW6 | 0.5%(w/v),Meat Peptone | SW10 | 0.1 N, K2CO3 | SW14 | 0.1%(w/v), Oxalic acid | SW19 | 0.1%(w/v), Humic acid |
| SW3 | 2.0%(w/v), Glucose | SW7 | 1.0%(w/v),Meat Peptone | SW11 | 0.2 N, MgCO3 | SW16 | 0.1%(w/v), Benzoic acid | SW20 | 0.1%(w/v), EDTA |
| SW4 | 5.0%(w/v), Glucose | SW8 | 0.2 N, CaCO3 | SW12 | 0.1%(w/v), L-Arginine | SW17 | 0.1%(w/v), Propionic acid | SW21 | 0.1%(w/v), Pyridine |
| SW22 | 0.1%(w/v), Glycerol | SW23 | 1%(w/v), Starch soluble | SW24 | 2%(w/v), Starch soluble | | | | |

**Figure 3.** Soluble COD consumption of *T. viride* in SIW with varying supplements.

| Time, h | SIW | 0.5% Glucose | 2% Glucose | 5% Glucose | 0.1% Meat Peptone | 0.5% Meat Peptone | 1% Meat Peptone | 1% Slouble Starch | 2% Slouble Starch |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 1.8E+04 | 2.3E+04 | 3.7E+04 | 6.7E+04 | 1.9E+04 | 2.3E+04 | 2.5E+04 | 2.4E+04 | 2.5E+04 |
| 12 | 1.8E+04 | 2.3E+04 | 3.6E+04 | 6.5E+04 | 1.9E+04 | 2.3E+04 | 2.5E+04 | 2.1E+04 | 2.5E+04 |
| 28 | 1.6E+04 | 2.1E+04 | 3.7E+04 | 6.2E+04 | 1.8E+04 | 2.1E+04 | 2.4E+04 | 1.6E+04 | 2.4E+04 |
| 40 | 1.4E+04 | 1.7E+04 | 3.6E+04 | 4.6E+04 | 1.6E+04 | 1.8E+04 | 2.3E+04 | 1.4E+04 | 2.3E+04 |
| 46 | 1.5E+04 | 1.9E+04 | 3.7E+04 | 4.9E+04 | 1.5E+04 | 1.6E+04 | 2.2E+04 | 1.4E+04 | 2.1E+04 |
| 52 | 1.5E+04 | 1.7E+04 | 3.7E+04 | 5.2E+04 | 1.5E+04 | 1.8E+04 | 1.9E+04 | 1.3E+04 | 1.9E+04 |
| 60 | 1.3E+04 | 1.6E+04 | 3.6E+04 | 4.8E+04 | 1.5E+04 | 1.6E+04 | 2.1E+04 | 1.2E+04 | 1.8E+04 |
| 72 | 1.3E+04 | 1.4E+04 | 3.2E+04 | 4.4E+04 | 1.2E+04 | 1.4E+04 | 1.8E+04 | 1.2E+04 | 1.7E+04 |
| 96 | 9.3E+03 | 1.0E+04 | 2.9E+04 | 4.8E+04 | 1.1E+04 | 1.3E+04 | 1.8E+04 | 1.1E+04 | 1.6E+04 |

**Figure 5.** Total ATP vs. CFU correlation of *T. viride* in – y1, SIW; y2 SIW + 0.5% glucose; y3 SIW + 2% glucose; y4 SIW + 5% glucose; y5 SIW + 1% soluble starch; y6 SIW + 2% soluble starch.

| Time, h | SIW-DATP | DATP + 0.5% (w/v) Dextrose | DATP + 2% (w/v) Dextrose | DATP + 5% (w/v) Dextrose | SIW-TATP | TATP + 0.5% (w/v) Dextrose | TATP + 2% (w/v) Dextrose | TATP + 5% (w/v) Dextrose | DATP + 1% (w/v) Soluble starch | DATP + 2% (w/v) Soluble starch | TATP + 1% (w/v) Soluble starch | TATP + 2% (w/v) Soluble starch |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.09 | 0.48 | 0.48 | 0.89 | 1.49 | 7.45 | 22.17 | 0.71 | 1.78 | 1.57 | 497.99 | 403.13 |
| 12 | 0.48 | 0.38 | 0.37 | 0.71 | 113.71 | 14.36 | 21.84 | 1.70 | 2.07 | 0.37 | 574.81 | 52.12 |
| 28 | 1.32 | 1.11 | 0.51 | 0.76 | 314.79 | 407.12 | 41.75 | 2.76 | 1.30 | 0.53 | 496.02 | 50.54 |
| 40 | 5.45 | 6.73 | 0.06 | 0.05 | 485.20 | 581.89 | 0.30 | 1.90 | 9.37 | 1.81 | 904.95 | 994.99 |
| 46 | 16.20 | 5.74 | 2.32 | 0.30 | 791.68 | 465.57 | 105.22 | 4.47 | 7.17 | 3.92 | 909.89 | 552.49 |
| 52 | 12.13 | 14.21 | 1.07 | 0.87 | 720.21 | 611.88 | 142.29 | 18.51 | 4.07 | 3.84 | 1823.0 | 1567.1 |
| 60 | 6.21 | 16.71 | 3.30 | 0.55 | 835.85 | 1048.30 | 186.46 | 25.51 | 6.81 | 6.93 | 5447.7 | 3507.7 |
| 72 | 2.29 | 2.42 | 2.08 | 1.63 | 1196.3 | 1604.79 | 544.69 | 39.25 | 7.35 | 11.10 | 5943.3 | 7630.2 |
| 96 | 2.75 | 4.31 | 5.34 | 1.21 | 1126.3 | 1616.86 | 838.14 | 55.28 | 10.63 | 30.35 | 6552.4 | 8608.4 |

| Time, h | DATP + 0.1%(w/v) Meat peptone | DATP + 0.5%(w/v) Meat peptone | DATP + 1%(w/v) Meat peptone | TATP + 0.1%(w/v) Meat peptone | TATP + 0.5%(w/v) Meat peptone | TATP + 1%(w/v) Meat peptone |
|---|---|---|---|---|---|---|
| 0 | 0.1 | 0.5 | 0.5 | 0.9 | 1.5 | 7.5 |
| 12 | 0.5 | 0.4 | 0.4 | 0.7 | 113.7 | 14.4 |
| 28 | 1.3 | 1.1 | 0.5 | 0.8 | 314.8 | 407.1 |
| 40 | 5.5 | 6.7 | 0.1 | 0.1 | 485.2 | 581.9 |
| 46 | 16.2 | 5.7 | 2.3 | 0.3 | 791.7 | 465.6 |
| 52 | 12.1 | 14.2 | 1.1 | 0.9 | 720.2 | 611.9 |
| 60 | 6.2 | 16.7 | 3.3 | 0.6 | 835.9 | 1048.3 |
| 72 | 2.3 | 2.4 | 2.1 | 1.6 | 1196.3 | 1604.8 |
| 96 | 2.8 | 4.3 | 5.3 | 1.2 | 1126.3 | 1616.9 |

| Time, h | SIW-TATP | TATP + 0.5% (w/v) Dextrose | TATP + 2% (w/v) Dextrose | TATP + 5% (w/v) Dextrose | TATP + 0.1%(w/v) Meat peptone | TATP + 0.5%(w/v) Meat peptone | TATP + 1%(w/v) Meat peptone | TATP + 1% (w/v) Soluble starch | TATP + 2% (w/v) Soluble starch |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 8.0E+03 | 8.0E+03 |
| 12 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 1.0E+04 | 5.0E+03 | 6.0E+03 |
| 28 | 2.4E+05 | 1.6E+05 | 1.2E+05 | 7.0E+04 | 8.0E+04 | 1.8E+05 | 2.1E+05 | 4.6E+06 | 1.2E+07 |
| 40 | 2.2E+06 | 3.6E+06 | 1.7E+06 | 7.0E+05 | 6.4E+06 | 4.9E+06 | 3.1E+06 | 4.0E+07 | 4.9E+07 |
| 46 | 2.2E+07 | 2.1E+07 | 6.3E+06 | 2.5E+06 | 2.0E+07 | 2.5E+07 | 1.2E+07 | 2.7E+08 | 3.8E+08 |
| 52 | 2.5E+07 | 3.4E+07 | 1.0E+07 | 3.0E+06 | 5.5E+07 | 5.7E+07 | 3.3E+07 | 3.3E+08 | 1.8E+09 |
| 60 | 1.5E+08 | 1.1E+08 | 5.2E+07 | 3.8E+07 | 2.8E+08 | 3.0E+08 | 1.7E+08 | 1.3E+09 | 2.9E+09 |
| 72 | 1.7E+08 | 3.2E+08 | 6.4E+07 | 4.0E+07 | 2.6E+08 | 3.4E+08 | 6.4E+08 | 3.0E+10 | 4.2E+10 |
| 96 | 1.6E+08 | 3.6E+08 | 6.2E+07 | 3.0E+07 | 2.3E+08 | 3.7E+08 | 7.5E+08 | 1.7E+10 | 8.8E+09 |

**Figure 6.** Protease activity of *T. viride* in SIW with, (a) varying glucose supplement, (b) varying N-source supplement, and (c) varying soluble starch supplement.

| Time, h | SW1 | SW2 | SW3 | SW4 | SW5 | SW6 | SW7 | SW23 | SW24 |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0.02 | 0.03 | 0.03 | 0.06 | 0.10 | 0.03 | 0.04 | 0.03 | 0.04 |
| 12 | 0.09 | 0.03 | 0.05 | 0.10 | 0.28 | 0.39 | 0.29 | 0.10 | 0.07 |
| 28 | 0.11 | 0.33 | 0.32 | 0.09 | 0.56 | 1.58 | 1.75 | 0.70 | 0.09 |
| 40 | 0.50 | 0.66 | 1.56 | 1.24 | 0.92 | 2.19 | 2.22 | 0.97 | 0.84 |
| 46 | 0.19 | 0.41 | 1.36 | 1.14 | 0.85 | 2.12 | 2.24 | 0.72 | 0.43 |
| 52 | 0.10 | 0.32 | 0.68 | 0.18 | 0.37 | 0.65 | 0.65 | 0.12 | 0.19 |
| 60 | 0.20 | 0.22 | 0.34 | 0.22 | 0.21 | 1.10 | 0.93 | 0.11 | 0.18 |
| 72 | 0.11 | 0.21 | 0.53 | 0.23 | 0.43 | 0.60 | 0.57 | 0.09 | 0.07 |
| 96 | 0.10 | 0.13 | 0.26 | 0.29 | 0.26 | 0.80 | 0.46 | 0.08 | 0.11 |

| Time, h | 1% Meat Pepttone + SIW - Frementer |
|---|---|
| 0 | 0.07 |
| 6 | 0.56 |
| 12 | 0.82 |
| 21 | 0.85 |
| 25 | 0.80 |
| 30 | 1.09 |
| 34 | 0.99 |
| 48 | 1.08 |
| 58 | 1.04 |
| 72 | 1.06 |
| 96 | 0.98 |

**Figure 7.** Effect of nutritional supplements on entomotoxicity of *T. viride* and fermenter scale feasibility.

| Samples | Tx (BSBU/l) | CFU | PA, U/ml (per 10 min) | CFUxPA | Tx × PA |
|---|---|---|---|---|---|
| TSB | 8.0 | 1.0E+04 | | | |
| SIW | 10.4 | 1.6E+08 | 1.0 | 1.5E+08 | 10.1 |
| SIW (1% soluble starch) | 9.9 | 1.7E+10 | 0.8 | 1.4E+10 | 8.1 |
| SIW (2% soluble starch) | 9.2 | 8.8E+09 | 1.1 | 9.8E+09 | 10.2 |
| SIW (2% glucose) | 8.9 | 6.2E+07 | 2.6 | 1.6E+08 | 23.3 |
| SIW (5% glucose) | 8.8 | 3.0E+07 | 2.9 | 8.8E+07 | 26.0 |
| SIW (0.5% meat peptone) | 10.9 | 2.6E+08 | 8.0 | 2.1E+09 | 86.5 |
| SIW (1% meat peptone) | 12.3 | 7.5E+08 | 4.6 | 3.5E+09 | 57.0 |
| SIW (1% meat peptone -Fermenter) | 13.7 | 4.2E+09 | 9.8 | 4.1E+10 | 133.4 |

# Annexe – VIII

# Données

**L'oxygène dissout comme paramètre principale pour la production des conidies du champignon de lutte biologique *Trichoderma viride* dans des eaux usées non Newtoniennes**

**Figure 1.** Fermentation profile of *T. viride* in SIW at ≥30% DO; (a) operational parameters, (b) growth parameters.

| Time, h | CFU/ml | Total Reducing Sugar, mg/l | Kla, h-1 | Visc., mPa.s | SCOD, ppm | Protein, mg/l |
|---|---|---|---|---|---|---|
| 0 | 2.1E+03 | 4.0E+03 | | 3.4 | 1.7E+04 | 4.9E+03 |
| 6 | 2.1E+03 | 3.8E+03 | 47.9 | 7.9 | 1.2E+04 | 3.9E+03 |
| 12 | 6.8E+03 | 2.3E+03 | 46.2 | 365.9 | 1.1E+04 | 3.6E+03 |
| 18 | 8.2E+03 | 9.8E+02 | 76.8 | 416 | 8.0E+03 | 3.1E+03 |
| 24 | 8.4E+04 | 1.0E+03 | 31.5 | 200 | 6.6E+03 | 3.3E+03 |
| 30 | 6.0E+07 | 8.2E+02 | 45.3 | 195.9 | 6.4E+03 | 3.3E+03 |
| 36 | 1.7E+08 | 7.9E+02 | 13.3 | 53.9 | 5.9E+03 | 3.6E+03 |
| 45 | 2.6E+08 | 5.0E+02 | 10.5 | 30 | 6.7E+03 | 3.3E+03 |
| 54 | 2.7E+08 | 2.1E+02 | 10.8 | 6.7 | 3.9E+03 | 4.3E+03 |
| 72 | 2.7E+08 | 2.0E+02 | 13.7 | 5.5 | 3.8E+03 | 4.0E+03 |
| 96 | 2.8E+08 | 4.0E+01 | 11.1 | 3.4 | 2.2E+03 | 3.6E+03 |

**Figure 2.** Fermentation profile of *T. viride* in SIW at ≥40% DO; (a) operational parameters, (b) growth parameters.

| Time, h | CFU/ml | Total Reducing Sugar, mg/l | Kla, h-1 | Visc., mPa.s | SCOD, ppm | Protein, mg/l |
|---|---|---|---|---|---|---|
| 0 | 2.2E+03 | 4.2E+03 | | 2.4 | 1.7E+04 | 4.5E+03 |
| 12 | 2.3E+03 | 3.7E+03 | 46.2 | 188 | 1.2E+04 | 5.6E+03 |
| 24 | 6.4E+04 | 1.5E+03 | 78.4 | 77 | 7.9E+03 | 5.2E+03 |
| 34 | 7.0E+07 | 1.4E+03 | 172.8 | 4.3 | 7.2E+03 | 4.7E+03 |
| 40 | 2.4E+08 | 1.2E+03 | 62 | 3.1 | 6.6E+03 | 4.8E+03 |
| 46 | 2.6E+08 | 6.9E+02 | 21.8 | 3.3 | 5.2E+03 | 5.1E+03 |
| 50 | 2.4E+08 | 6.2E+02 | 23.4 | 2.5 | 5.3E+03 | 5.2E+03 |
| 54 | 2.4E+08 | 4.2E+02 | 8.4 | 1.9 | 4.7E+03 | 4.8E+03 |
| 60 | 3.2E+08 | 3.0E+02 | 9.2 | 1.9 | 3.9E+03 | 5.0E+03 |
| 72 | 3.4E+08 | 8.8E+01 | 9.3 | 2.2 | 2.6E+03 | 4.6E+03 |
| 84 | 3.2E+08 | 7.4E+01 | 11.8 | 2.5 | 2.7E+03 | 6.4E+03 |

**Figure 3.** Fermentation profile of *T. viride* in SIW at ≤80% DO; (a) operational parameters, (b) growth parameters.

| Time, h | CFU/ml | Total Reducing Sugar, mg/l | Kla, h-1 | Visc., mPa.s | SCOD, ppm | Protein, mg/l |
|---|---|---|---|---|---|---|
| 0 | 1.1E+03 | 3.7E+03 | | 2.1 | 1.7E+04 | 5.8E+03 |
| 6 | 1.1E+03 | 2.7E+03 | 50.9 | 1.8 | 1.3E+04 | 5.5E+03 |
| 18 | 3.3E+03 | 2.5E+03 | 63.7 | 34 | 1.0E+04 | 5.3E+03 |
| 31 | 1.0E+06 | 7.1E+02 | 74.9 | 54 | 5.3E+03 | 5.0E+03 |
| 36 | 2.8E+08 | 3.7E+02 | 80.5 | 3.5 | 4.8E+03 | 4.7E+03 |
| 44 | 2.8E+08 | 1.9E+02 | 65 | 2.2 | 4.0E+03 | 4.5E+03 |
| 48 | 2.8E+08 | 0.0E+00 | 75.1 | 1.9 | 3.6E+03 | 4.0E+03 |
| 53 | 3.4E+08 | 8.8E+01 | 92.6 | 2.2 | 4.2E+03 | 4.6E+03 |
| 67 | 3.5E+08 | 1.0E+00 | 48.3 | 2.3 | 3.2E+03 | 5.1E+03 |
| 80 | 3.3E+08 | 0.0E+00 | 33.4 | 2 | 2.5E+03 | 4.4E+03 |
| 96 | 3.6E+08 | 0.0E+00 | 40.2 | 1.8 | 2.5E+03 | 3.7E+03 |

**Figure 5.** OUR profile of *T. viride* in SIW at ≥30, 40 and ≤80% DO.

| Time, h | mmol/l/h | Time, h | mmol/l/h | Time, h | mmol/l/h |
|---|---|---|---|---|---|
| 6 | 1.56 | 6 | 1.65 | 6 | 1.32 |
| 12 | 6.93 | 12 | 4.62 | 12 | 2.51 |
| 18 | 2.06 | 24 | 1.95 | 18 | 3.76 |
| 24 | 1.26 | 34 | 1.56 | 31 | 0.83 |
| 30 | 1.11 | 40 | 0.92 | 36 | 0.87 |
| 36 | 1.65 | 46 | 0.67 | 44 | 0.40 |
| 45 | 1.13 | 50 | 1.04 | 48 | 0.32 |
| 54 | 0.61 | 54 | 1.24 | 53 | 0.19 |
| 72 | 0.40 | 60 | 1.13 | 67 | 0.33 |
| 96 | 0.39 | 72 | 0.66 | 80 | 0.22 |
| | | 84 | 0.69 | 96 | 0.15 |
| | | 96 | 0.33 | | |

**Figure 6.** Rheological profile of *T. viride* in SIW at ≥30, 40 and ≤80% DO.

| SIW 30 | | | |
|---|---|---|---|
| Time, h | *K*,Consistency index | n, flow index | Confidence of Fit, % |
| 0 | 26.3 | 0.44 | 72.5 |
| 6 | 220.3 | 0.09 | 81.9 |
| 12 | 1266 | 0.03 | 76.1 |
| 18 | 1569 | 0.122 | 72.2 |
| 24 | 733.1 | 0.18 | 79.1 |
| 30 | 717.1 | 0.23 | 93.2 |
| 36 | 177.2 | 0.544 | 91.6 |
| 45 | 36.1 | 0.73 | 88 |
| 54 | 114 | 0.29 | 88.2 |

| | | | |
|---|---|---|---|
| 72 | 149.1 | 0.29 | 82.5 |
| 96 | 49.6 | 0.4 | 77 |
| | | | |
| **SIW 40** | | | |
| **Time, h** | ***K*,Consistency index** | **n, flow index** | **Confidence of Fit, %** |
| 0 | 10.4 | 0.59 | 68.8 |
| 12 | 826 | 0.152 | 81.4 |
| 25 | 537 | 0.244 | 71.6 |
| 34 | 41.5 | 0.42 | 81.2 |
| 40 | 28.8 | 0.46 | 81.2 |
| 46 | 34.5 | 0.44 | 83.5 |
| 50 | 28.3 | 0.44 | 76.2 |
| 54 | 16.1 | 0.53 | 74.7 |
| 60 | 14.6 | 0.55 | 73.5 |
| 72 | 23.7 | 0.48 | 78.1 |
| 84 | 30.1 | 0.46 | 81.9 |
| 96 | 24.9 | 0.49 | 81.8 |
| | | | |
| **SIW 80** | | | |
| **Time, h** | ***K*,Consistency index** | **n, flow index** | **Confidence of Fit, %** |
| 0 | 10.3 | 0.6 | 74.8 |
| 6 | 4.35 | 0.75 | 65.9 |
| 18 | 305.8 | 0.05 | 74.4 |
| 31 | 75.2 | 0.45 | 87.9 |
| 36 | 42.4 | 0.45 | 88.9 |
| 44 | 37.3 | 0.47 | 87 |
| 48 | 15.1 | 0.55 | 75.4 |
| 53 | 16.5 | 0.53 | 73.2 |
| 67 | 17.1 | 0.54 | 79.3 |
| 80 | 13.8 | 0.57 | 75.7 |
| 96 | 15.8 | 0.55 | 76.1 |

**Figure 7.** BCA activity of *Trichoderma viride*; (a) entomotoxicity, cellulase activity and (b) inhibition index profile (Inset – appearance of fungal bioassay wells), and (c) amylase and protease activities with incubation time.

| SIW30 | | SIW40 | | SIW80 | | STDEV | | |
|---|---|---|---|---|---|---|---|---|
| Time (h) | Tx(IU/micro l) | Time (h) | Tx(IU/micro l) | Time (h) | Tx(IU/micro l) | SIW 30 | SIW 40 | SIW 80 |
| 18 | 4.0E+03 | 12 | 1.8E+03 | 12 | 2.1E+03 | 2.1E+02 | 1.0E+02 | 1.5E+02 |
| 31 | 2.7E+03 | 24 | 3.3E+03 | 24 | 3.1E+03 | 2.2E+02 | 2.1E+02 | 2.5E+02 |
| 44 | 5.0E+03 | 40 | 4.0E+03 | 30 | 3.3E+03 | 3.9E+02 | 3.7E+02 | 3.2E+02 |
| 53 | 9.4E+03 | 50 | 8.8E+03 | 45 | 3.8E+03 | 7.8E+02 | 5.4E+02 | 2.3E+02 |
| 67 | 1.2E+04 | 72 | 1.0E+04 | 54 | 7.4E+03 | 7.5E+02 | 7.0E+02 | 4.8E+02 |
| 96 | 1.2E+04 | 96 | 1.2E+04 | 96 | 1.0E+04 | 8.9E+02 | 1.2E+03 | 8.0E+02 |

| SIW30 | | | SIW40 | | | SIW80 | | |
|---|---|---|---|---|---|---|---|---|
| Time, h | CA, IU/ml | SD | Time, h | CA, IU/ml | SD | Time, h | CA, IU/ml | SD |
| 0 | 3.4E-03 | 1.0E-04 | 0 | 1.2E-02 | 2.1E-03 | 0 | 2.9E-03 | 2.0E-04 |
| 6 | 2.6E-02 | 6.0E-04 | 12 | 6.0E-02 | 2.4E-03 | 6 | 5.2E-03 | 2.0E-04 |
| 12 | 6.9E-02 | 2.7E-03 | 25 | 8.6E-02 | 1.3E-02 | 18 | 3.0E-02 | 1.2E-03 |
| 18 | 1.1E-01 | 8.8E-03 | 34 | 6.9E-02 | 6.3E-03 | 31 | 4.9E-02 | 4.0E-04 |
| 24 | 1.2E-01 | 9.0E-03 | 40 | 5.2E-02 | 1.1E-02 | 36 | 3.2E-02 | 1.5E-03 |
| 30 | 7.0E-02 | 5.9E-03 | 46 | 5.0E-02 | 9.0E-03 | 44 | 3.2E-02 | 1.0E-04 |
| 36 | 7.8E-02 | 4.2E-03 | 50 | 5.9E-02 | 7.1E-03 | 48 | 2.1E-02 | 0.0E+00 |
| 45 | 1.1E-01 | 8.2E-03 | 54 | 6.2E-02 | 7.5E-03 | 53 | 3.5E-02 | 1.9E-03 |
| 54 | 1.0E-01 | 2.8E-03 | 60 | 7.6E-02 | 4.8E-03 | 67 | 2.5E-02 | 3.3E-03 |
| 72 | 8.5E-02 | 5.0E-03 | 72 | 7.6E-02 | 1.7E-03 | 80 | 3.9E-02 | 2.7E-03 |
| 96 | 8.7E-02 | 4.9E-03 | 84 | 6.1E-02 | 5.4E-03 | 96 | 4.2E-02 | 1.6E-03 |
| | | | 96 | 5.9E-02 | 4.7E-03 | | | |

| SIW30 | | | SIW40 | | | SIW80 | | |
|---|---|---|---|---|---|---|---|---|
| **Time (h)** | **I Index** | **SD** | **Time (h)** | **I Index** | **SD** | **Time (h)** | **I Index** | **SD** |
| 18 | 0.00 | 0.00 | 12 | 0.00 | 0.00 | 12 | 0.00 | 0.00 |
| 31 | 0.23 | 0.02 | 24 | 0.11 | 0.00 | 24 | 0.20 | 0.02 |
| 44 | 0.33 | 0.03 | 40 | 0.28 | 0.02 | 30 | 0.12 | 0.01 |
| 53 | 0.57 | 0.04 | 50 | 0.33 | 0.02 | 45 | 0.34 | 0.02 |
| 67 | 0.70 | 0.05 | 72 | 0.51 | 0.05 | 54 | 0.39 | 0.03 |
| 96 | 0.73 | 0.06 | 96 | 0.68 | 0.07 | 96 | 0.57 | 0.04 |

| SIW30 | | | SIW40 | | | SIW80 | | |
|---|---|---|---|---|---|---|---|---|
| **Time, h** | **AA, IU/ml** | **SD** | **Time, h** | **AA, IU/ml** | **SD** | **Time, h** | **AA, IU/ml** | **SD** |
| 0 | 0.85 | 0.04 | 0 | 0.77 | 0.04 | 0 | 0.69 | 0.02 |
| 6 | 1.26 | 0.04 | 12 | 1.21 | 0.08 | 6 | 0.87 | 0.05 |
| 12 | 1.38 | 0.06 | 25 | 1.47 | 0.12 | 18 | 1.30 | 0.04 |
| 18 | 1.73 | 0.12 | 34 | 1.36 | 0.06 | 31 | 1.02 | 0.06 |
| 24 | 1.54 | 0.05 | 40 | 1.43 | 0.10 | 36 | 1.04 | 0.06 |
| 30 | 1.38 | 0.03 | 46 | 1.32 | 0.06 | 44 | 1.26 | 0.06 |
| 36 | 1.55 | 0.09 | 50 | 1.29 | 0.10 | 48 | 1.17 | 0.03 |
| 45 | 1.49 | 0.05 | 54 | 1.25 | 0.05 | 53 | 1.17 | 0.09 |
| 54 | 1.33 | 0.05 | 60 | 1.22 | 0.04 | 67 | 1.04 | 0.06 |
| 72 | 1.22 | 0.07 | 72 | 1.05 | 0.03 | 80 | 0.84 | 0.04 |
| 96 | 1.15 | 0.04 | 84 | 0.92 | 0.04 | 96 | 0.77 | 0.06 |
| | | | 96 | 0.95 | 0.07 | | | |

| SIW30 | | | SIW40 | | | SIW80 | | |
|---|---|---|---|---|---|---|---|---|
| **Time, h** | **PA, IU/ml** | **SD** | **Time, h** | **PA, IU/ml** | **SD** | **Time, h** | **PA, IU/ml** | **SD** |
| 0 | 0.03 | 0.00 | 0 | 0.04 | 0.00 | 0 | 0.01 | 0.00 |
| 6 | 0.21 | 0.01 | 12 | 0.37 | 0.01 | 6 | 0.01 | 0.00 |
| 12 | 0.37 | 0.01 | 24 | 0.55 | 0.02 | 18 | 0.13 | 0.01 |
| 18 | 0.60 | 0.04 | 34 | 0.24 | 0.00 | 31 | 0.38 | 0.01 |
| 24 | 0.50 | 0.04 | 40 | 0.36 | 0.02 | 36 | 0.26 | 0.01 |
| 30 | 0.38 | 0.03 | 46 | 0.35 | 0.03 | 44 | 0.09 | 0.01 |
| 36 | 0.31 | 0.02 | 50 | 0.38 | 0.01 | 48 | 0.03 | 0.00 |
| 45 | 0.42 | 0.04 | 54 | 0.30 | 0.01 | 53 | 0.15 | 0.01 |
| 54 | 0.80 | 0.06 | 60 | 0.33 | 0.02 | 67 | 0.08 | 0.00 |
| 72 | 0.69 | 0.03 | 72 | 0.31 | 0.02 | 80 | 0.16 | 0.01 |
| 96 | 0.69 | 0.02 | 84 | 0.53 | 0.04 | 96 | 0.06 | 0.00 |
| | | | 96 | 0.50 | 0.04 | | | |

# Annexe – IX

# Données

**Modèle mathématique pour l'établissement d'une stratégie de production d'un antagoniste à base de *Trichoderma viride* par fermentation en mode «fed batch» en utilisant des eaux usées de l'industrie d'amidon**

**Figure 1.** Linear correlation between Tx and conidia concentration of *T. viride* during: (a) batch fermentation (Verma et al., 2006b), (b) fed-batch fermentation (present study).

(a)

| CFU/µl | Tx, SBU/µl |
|---|---|
| 2.1E+00 | 3.2E+03 |
| 1.8E+05 | 4.3E+03 |
| 2.7E+05 | 4.5E+03 |
| 2.2E+05 | 5.8E+03 |
| 4.6E+05 | 1.5E+04 |
| 6.6E+05 | 1.6E+04 |
| 2.9E+05 | 1.3E+04 |

(b)

| CFU/µl | Tx, SBU/µl |
|---|---|
| 8.2E+00 | 4.0E+03 |
| 6.0E+04 | 2.7E+03 |
| 2.6E+05 | 5.0E+03 |
| 2.7E+05 | 9.4E+03 |
| 2.7E+05 | 1.2E+04 |
| 2.8E+05 | 1.2E+04 |
| 8.2E+00 | 4.0E+03 |

**Figure 2.** Fed-batch mode profiles (legends – experimental data; dotted-line – simulation data) of (a) conidia production and total reducing sugar, R and (b) entomotoxicity concentration.

(a)

| Time, h | CFU/ml | Stndev, sd | R, mg/l | Stndev, sd |
|---|---|---|---|---|
| 0 | 3.7E+03 | 3.1E+02 | 4274 | 17 |
| 12 | 2.1E+03 | 2.4E+02 | 3929 | 36 |
| 19 | 2.5E+03 | 2.3E+02 | 1582 | 12 |
| 25 | 1.8E+05 | 2.1E+04 | 1409 | 2 |
| 32 | 1.3E+08 | 1.0E+07 | 947 | 20 |
| 36 | 2.7E+08 | 3.1E+07 | 926 | 19 |
| 42 | 2.7E+08 | 2.1E+07 | 868 | 15 |
| 48 | 2.2E+08 | 1.3E+07 | 609 | 14 |
| 56 | 1.9E+08 | 1.7E+07 | 468 | 4 |
| 66 | 5.3E+07 | 3.7E+06 | 707 | 13 |
| 72 | 1.5E+08 | 1.3E+07 | 923 | 8 |
| 82 | 3.5E+08 | 2.7E+07 | 1182 | 20 |
| 96 | 4.6E+08 | 3.8E+07 | 1078 | 12 |
| 107 | 6.6E+08 | 6.6E+07 | 405 | 3 |
| 121 | 2.9E+08 | 1.9E+07 | 198 | 1 |

(b)

| SIW FB | | |
|---|---|---|
| Time, h | Tx, SBU/ml | STDEV |
| 12 | 3.2E+03 | 2.5E+02 |
| 25 | 4.3E+03 | 3.8E+02 |
| 36 | 4.5E+03 | 9.0E+02 |
| 48 | 5.8E+03 | 6.7E+02 |
| 72 | 1.3E+04 | 1.2E+03 |
| 96 | 1.5E+04 | 1.7E+03 |
| 107 | 1.6E+04 | 1.3E+03 |
| 121 | 1.3E+04 | 1.1E+03 |

**Figure 3.** Profiles of (a) entomotoxicity and fungicidal bioassays and (b) enzyme production during fed-batch mode.

(a)

| SIW FB | | |
|---|---|---|
| Time, h | Tx, SBU/ml | STDEV |
| 12 | 3.2E+03 | 2.5E+02 |
| 25 | 4.3E+03 | 3.8E+02 |
| 36 | 4.5E+03 | 9.0E+02 |
| 48 | 5.8E+03 | 6.7E+02 |
| 72 | 1.3E+04 | 1.2E+03 |
| 96 | 1.5E+04 | 1.7E+03 |
| 107 | 1.6E+04 | 1.3E+03 |
| 121 | 1.3E+04 | 1.1E+03 |

| SIW FB | | |
|---|---|---|
| Time, h | Inhibition Index | STDEV |
| 0 | 0.06 | 0.01 |
| 12 | 0.11 | 0.01 |
| 19 | 0.12 | 0.02 |
| 25 | 0.16 | 0.01 |
| 32 | 0.18 | 0.03 |
| 36 | 0.23 | 0.04 |
| 42 | 0.28 | 0.03 |
| 48 | 0.51 | 0.04 |
| 56 | 0.56 | 0.05 |
| 66 | 0.69 | 0.06 |
| 72 | 0.67 | 0.05 |
| 96 | 0.80 | 0.08 |
| 107 | 0.82 | 0.08 |
| 121 | 0.71 | 0.07 |

(b)

| | **Protease, IU/ml** | | **Amylase, U/ml** | | **Cellulase, IU/ml** | |
|---|---|---|---|---|---|---|
| **Time, h** | **SIW-FB** | **STDEV** | **SIW-FB** | **STDEV** | **SIW-FB** | **STDEV** |
| 0 | 0.07 | 0.00 | 0.72 | 0.01 | 0.01 | 0.00 |
| 12 | 0.24 | 0.02 | 1.69 | 0.02 | 0.09 | 0.01 |
| 19 | 0.47 | 0.01 | 1.87 | 0.02 | 0.13 | 0.01 |
| 25 | 0.39 | 0.04 | 1.44 | 0.02 | 0.14 | 0.01 |
| 32 | 0.43 | 0.03 | 1.36 | 0.02 | 0.03 | 0.01 |
| 36 | 0.31 | 0.01 | 1.40 | 0.02 | 0.07 | 0.01 |
| 42 | 0.14 | 0.01 | 1.49 | 0.02 | 0.10 | 0.00 |
| 48 | 0.36 | 0.03 | 1.55 | 0.02 | 0.09 | 0.00 |
| 56 | 0.33 | 0.03 | 1.16 | 0.02 | 0.10 | 0.01 |
| 66 | 0.27 | 0.01 | 1.16 | 0.02 | 0.12 | 0.01 |
| 72 | 0.33 | 0.03 | 1.24 | 0.03 | 0.12 | 0.00 |
| 82 | 0.34 | 0.01 | 1.10 | 0.02 | 0.11 | 0.00 |
| 96 | 0.37 | 0.01 | 0.95 | 0.01 | 0.09 | 0.00 |
| 107 | 0.34 | 0.00 | 0.96 | 0.02 | 0.08 | 0.00 |
| 121 | 0.25 | 0.01 | 1.04 | 0.02 | 0.07 | 0.00 |

**Figure 4.** Fed-batch fermentation operation parameters, (a) $k_La$, D.O., agitation and air flow rate, (b) viscosity, $K$ and $n$.

| SIW FB | | | | | | | |
|---|---|---|---|---|---|---|---|
| Time, h | Air flow rate, LPM | Time (h) | Kla (h) | Time, h | K, Consistency index | n, flow index | Viscosity |
| 0 | 0.83 | 12 | 31.6 | 0 | 13.5 | 0.67 | 7.0 |
| 12 | 0.83 | 19 | 37.8 | 12 | 858.9 | 0.33 | 275.9 |
| 12 | 3 | 25 | 97.8 | 19 | 1088.0 | 0.30 | 393.9 |
| 19 | 3 | 32 | 36.8 | 25 | 1004.0 | 0.36 | 343.9 |
| 19 | 4 | 36 | 32.7 | 32 | 369.5 | 0.46 | 154.0 |
| 25 | 4 | 48 | 19.3 | 36 | 273.6 | 0.41 | 124.0 |
| 25 | 5 | 52 | 19.4 | 42 | 81.1 | 0.72 | 58.0 |
| 42 | 5 | 66 | 12.8 | 48 | 69.6 | 0.66 | 34.0 |
| 42 | 3 | 72 | 32.9 | 56 | 93.2 | 0.57 | 27.0 |
| 48 | 3 | 82 | 78.2 | 66 | 51.4 | 0.62 | 10.0 |
| 48 | 2 | 96 | 43.0 | 72 | 78.8 | 0.51 | 13.0 |
| 67 | 2 | 121 | 20.9 | 82 | 90.5 | 0.51 | 14.0 |
| 67 | 1 | | | 96 | 70.1 | 0.48 | 7.2 |
| 121 | 1 | | | 107 | 57.4 | 0.49 | 7.8 |
| | | | | 121 | 48.0 | 0.52 | 6.1 |

**Figure 5.** Entomotoxicity and fungicidal activity (a), and (b) plant growth promotion results (plant grown from seeds).

(a)

| | C | | F | | T2 | | T2F | |
|---|---|---|---|---|---|---|---|---|
| Tomato | 15.89 | 2.28 | 10.89 | 1.92 | 23.89 | 3.03 | 20.29 | 4.15 |
| Pepper | 3.89 | 1.10 | 2.29 | 1.92 | 8.69 | 1.67 | 9.09 | 1.41 |
| Soya | 35.89 | 2.28 | 26.29 | 1.30 | 39.49 | 5.37 | 39.49 | 2.61 |

# Annexe – X

# Données

**Étude technico-économique de la production par fermentation de conidies de *Trichoderma* spp. en utilisant des matières premières alternatives**

Figure 2. Production cost distribution (%) of different liquid suspension based scenarios.

| Category | SSM-LS scenario | TH-LS scenario | SIW-LS scenario | PPN-LS scenario |
|---|---|---|---|---|
| Raw Material-Substrate | 7.37 | 0.56 | 1.32 | 1.65 |
| Raw Material-Formulation | 4.93 | 1.82 | 5.01 | 6.27 |
| Labour | 8.6 | 14.2 | 9.9 | 8.4 |
| Maintenance | 4.4 | 4.5 | 4.7 | 4.8 |
| Depreciation | 14.7 | 14.9 | 15.6 | 15.8 |
| Plant overhead | 9.7 | 14.1 | 11.0 | 9.8 |
| Interest | 26.2 | 26.6 | 27.8 | 28.2 |
| Insurance | 5.9 | 6.0 | 6.2 | 6.3 |
| Others | 18.2 | 17.4 | 18.4 | 18.6 |