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ÉTAPES D'ULTRAFILTRATION ET PROCÉDÉS DE FORMULATION DANS LA PRODUCTION DE BIOPESTICIDES À BASE DE *BACILLUS THURINGIENSIS* EN UTILISANT DES EAUX USÉES ET DES BOUES D'ÉPURATION COMME SUBSTRAT

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DÉDICACE

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RÉSUMÉ

Le but principal de ce projet de recherche est d'évaluer la nécessité d'insérer une étape d'ultrafiltration dans le procédé de production de biopesticides à base du *Bacillus thuringiensis* (Bt) variété *kurstaki* (Btk) en utilisant les milieux résiduels comme substrat de fermentation, et de développer une formulation des biopesticides ainsi obtenus. En effet, il a été prouvé, que l'usage des milieux résiduels réduit de moitié le coût de production des biopesticides Bt par rapport au milieu conventionnel de soja. Les milieux résiduels utilisés comme substrats de fermentation sont: la boue secondaire non-hydrolysée (NH), boue secondaire thermo-hydrolysée (TH) et les eaux usées des industries d'amidon (EUA). Le milieu semi-synthétique de soja est aussi utilisé comme milieu de référence. Les bouillons fermentés, qui comprennent les composants actifs (cellules, spores viables, cristaux de proteines, enzymes, proteines végétatives insecticides, etc.), sont obtenus par fermentation de Btk en bioréacteur de 15 litres. Les composants actifs sont récupérés par centrifugation des bouillons fermentés, sous forme de concentré (''culot'').

Toutefois, des pertes des composants actifs ont été identifiées dans le surnageant. C'est ainsi qu'un procédé d'ultrafiltration (un des principaux objectifs de cette étude), a été optimisé pour récupérer, dans un volume réduit (rétentat), les composants actifs des surnageants des quatre milieux en vue d'augmenter l'entomotoxicité des biopesticides. Des mélanges entre les concentrés de la centrifugation (culot) et ceux d'ultrafiltration (rétentat) ont été réalisés comme de possibles formulations. Cependant, les différents mélanges obtenus, qui ont une rhéologie particulièrement complexe, ne présentaient pas toutes les propriétés requises pour une bonne l'application. D'où la nécessité de développer une formulation appropriée (autre objectif de cette étude) par la détermination et l'optimisation des concentrations des additifs de formulations anti-UV et antimicrobienne par rapport aux différents milieux (EUA, NH, TH, soja) et à travers des tests de formulation (ce sont des tests d'exposition aux radiations UV et de détermination des contaminations d'autres organismes que Bt après une durée de conservation).

Un dernier objectif de cette étude est de déterminer les conditions optimales de production des poudres humides de biopesticides en séchant par pulvérisation des bouillons fermentés. La

procédure consiste à optimiser les paramètres opératoires de séchage par le plan composite centré (PCC) et la méthode de réponse en surface (MRS). Dans cette étude, les cellules totales, les spores viables, le potentiel insecticide (entomotoxicité) et les activités enzymatiques sont les variables de mesure et d'analyse des résultats.

Les résultats des travaux sur l'ultrafiltration ont montré qu'une membrane de 5 kDa (diamètre du pore de 0.015 µm) permet de récolter tous les composés actifs du surnageant, surtout les composés de virulence (les enzymes) qui jouent un rôle important dans le potentiel entomotoxique des biopesticides. L'efficacité d'ultrafiltration en termes de récupération d'entomotoxicité des milieux de soja, EUA, NH et TH est de 100% avec des valeurs d'entomotoxicité en spruce budworn units par litre (SBU/L) de 11.6×10^9, 14.6×10^9, 13.5×10^9 et 9.5×10^9 dans les rétentats respectifs. L'évaluation des effets des composés de virulence (protéases et chitinases) à travers la mesure d'entomotoxicité du meilleur ratio du mélange culot – rétentat, (4 g de culot + 1 mL de rétentat) a donné une augmentation de l'entomotoxicité (en SBU/L) de 4.3×10^9, 1.6×10^9, 1.4×10^9 et 4.8×10^8 respectivement pour les milieux de soja, EUA, NH et TH. Le calcul du bilan de masse des matières en suspension montre une rétention de matière sur la membrane. Cette rétention de matière qui varie avec les milieux se traduit aussi par des pertes des composés actifs par adsorption sur des particules retenues sur la membrane, d'où la nécessité d'une étude de colmatage des membranes.

La rétention de la matière, qui est essentiellement une adsorption physique sur la membrane, est très élevée pour les surnageants de TH (68% des matières en suspension contre 15%, 12%, 7% respectivement pour les milieux de soja, EUA et NH). Compte tenu de ces résultats, l'étude de colmatage est essentiellement portée sur les milieux résiduels EUA et TH. Elle est faite sur une membrane de 0.2 m^2 avec des volumes de 2 L et 4 L de chacun des surnageants EUA et TH. Les résultats ont montré que l'augmentation du colmatage de la membrane évaluée à travers la variation du flux du perméat présente une phase stationnaire dans laquelle la résistance apparente due à la couche de colmatage est la plus élevée. Ces résistances, calculées pour 2 L de filtration, sont de 3.5×10^{12} et 1.8×10^{12} (m^{-1}) respectivement pour EUA et TH; alors qu'avec 4 L, elles deviennent 1.2×10^{12} et 2.1×10^{13} (m^{-1}) pour EUA et TH respectivement.

Pour la formulation anti-UV, les résultats obtenus avec les tests d'exposition aux radiations UV montrent qu'en absence des additifs de protection, les boues d'épuration possèdent une meilleure protection contre les radiations UV-A (400-315 nm) et UV-B (315-280 nm). Les demi-vies d'entomotoxicité sont NH (3.4 j)>TH (3.25 j)>EUA (1.9 j)>soya (1.8 j). Avec les différents additifs de protection, les résultats ont montré que la formulation avec l'acide p-amino benzoïque à 0.20% (p/p) a augmenté la demi-vie d'entomotoxicité de EUA (7.8 j) et de soya (5.9 j) alors que l'acide lignosulfonique à 0.20% (p/p) donne un meilleur résultat dans le cas de TH (7.3 j) et NH (8 j).

Concernant les formulations antimicrobiennes, après trois années de conservation, Parmi les microorganismes visés (salmonelles, streptocoques fécales, coliformes fécales, les staphylocoques, les levures et moisissures), seules les moisissures ont été décelées dans certaines formulations à faible concentration des additifs (0.1% p/p) et dans les contrôles. L'efficacité des additifs évalué à travers les valeurs d'entomotoxicité et des spores viables ont montré que l'acide propionique donne de meilleurs résultats dans les cas de soya, EUA et NH avec des concentrations respectives de 0.5%, 0.5% et 0.3% (p/p). Pour le milieu de TH, c'est le métabisulfite de sodium qui paraît le plus efficace à 0.3% (p/p).

L'optimisation de la production des poudres humides de biopesticides a donné des valeurs de températures de sortie relativement faibles et qui sont loin d'affecter les spores et les cristaux de protéines insecticides. Aussi, les coefficients de détermination sont très faibles avec les valeurs d'entomotoxicité. Alors, seules les valeurs de spores viables ont servi aux analyses statistiques de la MRS (coefficient de détermination : EUA - 92% et TH - 94%) pour déterminer les conditions optimales de production de poudre humide. Le débit d'alimentation de 0.29 g/min, le débit d'aspiration d'air chaud de 0.51 m^3/min, la température d'entrée de 180 ^oC et la pression d'atomisation de 0.10 MPa sont les valeurs optimales dans le cas de EUA. Les valeurs optimales dans le cas de TH sont 0.45 g/min, 0.49 m^3/min, 170^oC et 0.096 MPa. Avec ces conditions optimales on obtient des pertes de spores viables de 18% et 13% respectivement pour les poudres humides d'EUA et TH par rapport à leurs bouillons fermentés initiaux respectifs.

ABSTRACT

The principal objective of this research project is to evaluate the necessity of ultrafiltration step in the production process of *Bacillus thuringiensis* (Bt) var. *kurstaki* (Btk) by using wastes as substrate. It has been established that the use of the residual media and especially the wastewater and wastewater sludge, decreases the production cost of Bt biopesticides by almost half when compared to conventional media. The culture media used for the study were: non-hydrolyzed sludge (NH), hydrolyzed sludge (TH) and starch industry wastewater (SIW). Soya semi-synthetic medium was included as a control. Fermented broths comprising the active components (viable cells, spores, protein crystals, enzymes, etc.) are obtained by fermentation of Btk in a fermentor of 15 liters.

The active components are recovered by centrifugation of the fermented broths, in the form of concentrate ("base"). Mostly, losses of the active components were detected in the supernatant of centrifugation. Thus, a process of ultrafiltration (one of the principal objectives of this study), was optimized to recover, in a decreased volume (retentate), the active components in the supernatants of the four media in order to increase the entomotoxicity of the biopesticides. A mixture of concentrate of centrifugation (base) and that of ultrafiltration (retentate) was evaluated as possible formulate concentrates. Additionally, the various mixtures obtained have a complex rheology, and do not encompass the necessary properties to be used during application. It is thus necessary and essential to develop a suitable formulation in order to circumvent the drawbacks of the fermented broths. The biopesticides thus formulated will possess the properties and characteristics required for their marketing and application. This objective comprises determination and optimization of anti-UV and antimicrobial additives in various media of SIW, NH, TH and soya. The study included variation (screening) and optimization of the concentrations of these additives through various tests of formulation. Tests of exposure to UV radiations and contamination after storage time were performed.

Final objective of this study is to determine the optimum conditions for the production of wet powder formulation of biopesticides of SIW and TH by drying the fermented broth in a spray dryer. The procedure consisted of optimizing the operational parameters by central composite

plan (CCP) and the response surface method (RSM). The spray drying operational parameters (inlet temperature, flow of aspiration of hot air, atomization pressure and feed rate) constitute independent variables, whereas the outlet temperature, moisture content, number of viable spores and entomotoxicity are dependent variables. It should be noted that to accomplish all the objectives of this study, total cells, viable spores, insecticidal potential (entomotoxicity) and enzymatic activity are the variables of measurement and analysis of the results.

For the first objective (ultrafiltration), the results showed that a membrane of 5 kDa (pore diameter of 0.015 µm) with a surface area of 0.1 m^2 was optimal for concentration of all composants present in supernatant, especially the virulence factors (enzymes, vegetative insecticidal proteins, zwittermicin-A) which play a significant role in the entomotoxicity of the biopesticides. Efficiency of ultrafiltration in terms of entomotoxicity recovery for soya, SIW, NH and TH were 100% with entomotoxicity values of 11.6, 14.6, 13.5 and 9.5 (x 10^9 SBU/L) in the respective retentates. The evaluation of the effects of virulence factors (proteases and chitinases) for a ratio of the centrifugate-retentate, (4g of centrifugate + 1mL of retentate) gave an increase in the entomotoxicity of 4.3, 1.6, 1.4 and 4.8 (10^9 SBU/L) for soya, SIW, NH and TH, respectively. Meanwhile, there were losses of active components on the ultrafiltration membrane through the adsorption of matter on the membrane, necessitating further experiments on membrane fouling.

The retention of organic matter, via physical adsorption on the membrane, is very high for the supernatants of hydrolyzed sludges (68% of the suspended matter as compared to 15%, 12%, 7% for soya, SIW and NH, respectively). Taking into account the preceding results and objectives of this work, the membrane fouling study was performed for 0.2 m^2 membrane with a volume V (2 L and 4 L) of supernatants of SIW and TH. The results showed that increase in fouling of the membrane evaluated through the variation of the flow of permeate presented a stationary phase in which apparent resistance of the fouled layer was maximum. This resistance, calculated for 2 L of filtration, was $3.5x10^{12}$ and $1.8x10^{12}$ (m^{-1}) for SIW and TH, respectively. These values were 1.2 x10^{12} for SIW and 2.1x10^{13} (m^{-1}) for TH in the case of 4 L of filtration.

For the anti-UV formulation, the results obtained during UV exposure tests showed that in the absence of protection additives, the wastewater sludge gave better protection against UV-A and UV-B radiations. The half-lives calculated in terms of entomotoxicity were NH (3.4 d)>TH (3.25 d)>SIW (1.9 d)>soya (1.8 d). The study of various additives of UV protection showed that the formulation with p-amino benzoic acid at 0.20% w/w increased the half-life in terms of entomotoxicity for SIW (7.8 d) and soya (5.9 d) whereas lignosulfonic acid at 0.20% w/w gave better results for TH (7.3 d) and NH (8 d).

For tests using antimicrobial formulations, after three years of conservation, Among the microorganisms concerned (Salmonella, Streptocoques fecal, coliforms, Staphylococci, yeasts and moulds), only the moulds were detected in certain formulations with weak concentration of the additives (0.1 w/w) and in controls. The effectiveness of the additives evaluated through the values of entomotoxicity and viable spores showed that the propionic acid gives better results in the cases of soya, SIW and NH with respective concentrations of 0.5%, 0.5% and 0.3% (w/w). For the medium of TH, it is the sodium metabisulfite which appears most effective to 0.3% (w/w).

The optimization of production of wet powder formulations showed that the values of outlet temperatures were relatively low for the two media (SIW ant TH), and are far from affecting the spores and the insecticidal crystal proteins. Also, the model determination coefficients are certainly very low for entomotoxicity. Thus, only the viable spore values were used for statistical analyses by response surface method. With the spores, the model determination corficient of optimal conditions for the production of wet powders gave 92% and 94%, for SIW and TH, respectively. The feed rate of 0.29 g/min, aspiration of hot air of 0.51 m^3/min, the inlet temperature of 180 ^{O}C and the pressure of atomization of 0.10 MPa were the optimal values of independent variables in the case of SIW. The optimal values in the case of TH are 0.45 g/min, 0.49 m^3 / min, 170 ^{O}C and 0.096 MPa. With these optimal conditions, viable spore losses of 18% and 13% respectively, were obtained for the wet powders of SIW and TH compared to their respective initial fermented broths.

LISTE DES ABBRÉVIATIONS

Français

Bt	*Bacillus thuringiensis*
CPI	cristaux de protein insecticide
DDT	dichlorodiphenyltrichloroéthane
EUA	eau uses d'amidon
KDa	Kilodalton
MRS	Méthode de réponse en surface
PVA	Produit à valeur ajoutée
Tx	Entomotoxicité
UF	Ultrafiltration
UV	Ultraviolet
UF	Ultrafiltration

Anglais

ANOVA	Analysis of variance
C	Centrifugate
C_{feed}	Concentration of suspended solids in feed
$C_{permeate}$	Concentration of suspended solids in permeate
$C_{retentate}$	Concentration of suspended solids in retentate
CFU	Colony forming units
J_w	Flux of Permeate
MWCO	Molecular weight cut-off
NH	Non-hydrolyzed sludge
P	Permeat
R	Retentate
S	Surnageant

SBU	Spruce budworm units
SIW	Starch industry wastewater
SS	Suspended solids
TH	Thermo-hydrolyzed sludge
TMP	Transmembrane pressure gradient
TS	Total solids
USEPA	United States Enviornnemental Protection Agency
Vips	Vegetative insecticidal protein
VS	Viables spores

Chapitre 1.
Synthèse

INTRODUCTION

L'agriculture et la forêt sont depuis toujours endommagées par des insectes ravageurs, entraînant ainsi d'importantes pertes économiques. Des récentes études réalisées au Canada ont montré que la tordeuse des bourgeons de l'épinette (*Choristoneura fumiferana: Tortricidae*) est l'insecte le plus ravageur des forêts (Kneeshaw, 2001; Belle-Isle *et al.,* 2007). Presque 30 millions d'hectares de forêts sont partiellement ou gravement ravagés par des insectes (Industrie Canada, 1998). Ainsi, entre 1980 et 1996, on a observé des années successives de défoliation causée par la tordeuse des bourgeons de l'épinette sur plus de 69 millions d'hectares de forêts représentant 17 % des forêts canadiennes (Écozones, 1996). Face à ce fléau, les insecticides chimiques comme le dichlorodiphényltrichloroéthane (DDT) étaient auparavant utilisés, mais malheureusement avec des effets secondaires néfastes sur l'homme et son environnement (Guo *et al.,* 2009). En fait, une fois libérés dans l'environnement, ces pesticides chimiques (non biodégradables et non spécifiques aux insectes cibles) vont toucher directement (par absorption, ingestion, respiration, etc.) ou indirectement (par pollution, contamination, etc.) d'autres organismes et d'autres sites que ceux visés par leurs applications.

Afin de pallier ces effets indésirables et dangereux des pesticides chimiques, et dans le souci de promouvoir les objectifs du développement durable qui visent la protection des ressources environnementales, de la faune et de la flore, les recherches pour une lutte biologique sont de plus en plus recommandées et encouragées par les autorités nationales et internationales (AGCan et CCNUPA, 2001; NBPBCO, 2001a et b). Les biopesticides ont plusieurs avantages et sont sans danger pour l'environnement, et plusieurs recherches ont été portées sur les différents procédés de leur production (Smirnoff, 1972 et 1983; Lisansky *et al.*, 1993; Dale, 1999; NRC, 1999; Elda, 2000). Cependant, il ressort de ces études que le coût de production des pesticides biologiques est très élevé à cause du prix des milieux de culture utilisés. Cela limite leur accessibilité, d'où la nécessité de trouver un milieu de culture à faible coût.

Avec les nouvelles technologies visant à valoriser des résidus encombrants issus des activités industrielles et domestiques, il est opportun que les recherches sur la production à faible coût des pesticides biologiques explorent la piste d'utilisation de milieux résiduels. Ces milieux sont,

par exemple, les eaux usées industrielles et les boues d'épuration municipale, dont la gestion est une véritable problématique pour les pouvoirs publics, les responsables des industries et des usines de traitement des eaux. Cette difficulté de gestion des eaux usées industrielles et des boues d'épuration dont la quantité ne cesse d'augmenter au fil des années constitue une autre problématique qui fait actuellement l'objet d'intenses recherches. À ce propos, les boues d'épuration et les eaux usées des industries agroalimentaires comportent les éléments nutritifs nécessaires, principalement le carbone et l'azote, permettant la croissance d'un grand nombre de microorganismes, en particulier le *Bacillus thuringiensis* (Bt) employé pour l'obtention de bioinsecticides (Yezza *et al.*, 2006a; Vu *et al.*, 2009). C'est dans ce cadre qu'intervient cette étude qui contribuera au développement du procédé en aval permettant de finaliser la production des biopesticides à partir des eaux usées agroalimentaires et des boues d'épuration municipales.

Ce chapitre 1 comporte quatre parties: *La première* aborde la problématique générale du coût élevé des biopesticides, ainsi que celle de la gestion des boues d'épuration. Elle présente également les problèmes spécifiques de cette étude à travers la description des différentes étapes du procédé de production des biopesticides à base de Bt. *La seconde* présente une synthèse bibliographique afin de définir les objectifs spécifiques de cette étude. *La troisième partie* décrit les hypothèses et objectifs de l'étude. Enfin, *la dernière partie* expose très brièvement la démarche méthodologique ainsi que les principaux résultats et discussions qui seront traités de manière plus détaillée dans les articles constituant les autres chapitres de cette thèse.

1. PROBLEMATIQUES GENERALES

1.1. Coût élevé de la production des biopesticides Bt

Les insecticides microbiens sont des préparations à base de bactéries, de virus, de protozoaires ou de champignons. Dans le cas du Bt, ce sont des suspensions formulées à partir de mélanges de spores et de cristaux de protéines insecticides obtenus après une mise en culture, croissance et sporulation de la souche en bioréacteur (fermenteur). En plus des nombreux avantages des bioinsecticides (biodégradables, spécifiques aux insectes cibles, et sans effets secondaires sur l'environnement), leur homologation est simple, rapide et peu coûteuse (MDDEP, 2006) par rapport aux pesticides chimiques. Cependant, ces avantages ne favorisent pas pour autant leur développement et leur commercialisation, car leur marché est loin de concurrencer celui des insecticides chimiques. En effet, les biopesticides s'implantent difficilement sur le marché, et demeurent en grande partie des solutions de rechange souhaitées, plutôt que des applications reconnues. Selon Caron *et al.* (2006) il y a une disparité persistant entre le désaveu des pesticides chimiques et l'accessibilité à des produits plus respectueux de l'environnement au Canada. Cela se justifie par la production industrielle et l'efficacité des biopesticides formulés ainsi que les exigences de leur homologation.

Parmi toutes ces raisons, le coût de production des biopesticides est un facteur déterminant pour leur commercialisation. En effet, ils sont encore dispendieux à cause du coût des matières premières qui représente 35 à 59% dans un procédé conventionnel de production (Lisansky *et al.*, 1993; Stambury *et al.*, 1993). Cela limite donc la taille du marché des biopesticides. Actuellement, plus de 98% des pesticides utilisés à travers le monde sont d'origine chimique pour un marché global de 32 milliards en dollar US (Marrone, 1999; Jarvis, 2001). Estimé à 105 millions de dollar US en 1991, le marché global des biopesticides a atteint plus de 400 millions de dollars en 2000, soit 1,5% du total des pesticides (Gaugler, 1997; Navon, 2000). Ce marché pourrait atteindre entre 2 à 4 milliards de dollars au cours des 10 ou 15 prochaines années.

Malgré cette prévision prometteuse des chiffres d'affaires des biopesticides, force est de constater que beaucoup reste à faire dans le cadre de la lutte biologique face aux pesticides chimiques dont la force, contrairement aux biopesticides, réside dans leur rapidité d'action insecticide et surtout dans leur faible coût de production. Ainsi, afin de réduire le coût d'obtention des insecticides microbiens, il est important de trouver un milieu de culture alternatif, disponible partout et à moindre coût pour produire des biopesticides efficaces et économiquement accessibles. C'est l'une des problématiques générales de cette étude.

1.2. Gestion des boues d'épuration

La croissance démographique et le développement des activités économiques entraînent non seulement des besoins en eau de plus en plus croissants mais aussi engendrent d'importantes pollutions de cette ressource. Par exemple, au Canada, selon des estimations faites en 1999, les effluents urbains représentaient à eux seuls 14,4 millions de mètres cubes d'eaux usées traitées par jour par 1 118 municipalités (Environnement Canada, 1999). Ces eaux usées sont traitées dans des stations d'épuration avant d'être rejetées dans l'environnement (rivières, cours d'eau, fleuves). Ces traitements qui sont de plus en plus sophistiqués génèrent d'importantes quantités de boues d'épuration municipale dont la gestion et le traitement représentent des coûts exorbitants. Par exemple, au Québec, en 2002, sur les 218 000 tonnes de boues produites, seules 11% ont été valorisées en agriculture alors que 40% ont été enfouies et 45% incinérées (Recyc-Québec, 2003). D'après MDDEP (2003) cette proportion de boues municipales valorisées (11%) est même en régression, étant donné que la quantité de boues valorisées plafonne depuis 1994, alors que celles générées par les stations d'épuration ne cessent d'augmenter à raison de 20 % en 8 ans (MDDEP, 2003). L'enfouissement est un moyen simple et peu coûteux, mais la réduction des sites d'enfouissement, les règlementations et les taxes de plus en plus sévères limitent cette pratique. Aussi, dans la plupart des décharges, une partie des éléments fertilisants et des produits toxiques sont parfois entraînés par les eaux superficielles et rejoignent ainsi les nappes souterraines. Pour ce qui est de l'incinération, elle est onéreuse due à une déshydratation préalable, à la disposition des cendres et à la dépollution des gaz de combustion. Face à ces problématiques d'élimination des boues d'épuration, il est important de trouver d'autres moyens de gestion des boues. C'est ainsi qu'avec les innovations technologiques de valorisation des

déchets, l'idée de mise en valeur des boues d'épuration devient de plus en plus intéressante pour beaucoup de chercheurs (Ndegwa *et al.*, 2001; Mannan *et al.*, 2007; Lazcano *et al.*, 2008). Transformer les boues d'épuration en produit à valeur ajoutée (PVA), constituerait la solution idéale à la problématique de la gestion des boues.

1.3. Procédé de production des biopesticides à base de Bt à l'INRS

C'est dans la perspective d'apporter une contribution à la recherche de solutions aux problèmes des coûts élevés des biopesticides-Bt et de la gestion boues d'épuration et eaux usées qu'intervient cette recherche. Elle s'inscrit dans la continuité d'un projet de bioconversion de milieux résiduels en PVA. Vu le jour à l'Institut National de la Recherche Scientifique (INRS), il est dénommé ''Projet Bt-INRS''. Il utilise le potentiel insecticide d'une bactérie entomopathogène le *Bacillus thuringiensis* (Bt) et le pouvoir nutritif des matières résiduelles putrescibles comme les boues d'épuration et les eaux usées agroalimentaires pour produire des biopesticides. Ainsi, ce projet se donne pour défi de faire de la problématique des boues d'épuration, la solution à celle du coût élevé des biopesticides. Ce procédé de biotransformation, consiste en une fermentation biologique, qui selon Demain et Davies (1999) fait intervenir des réactions organiques catalysées par des microorganismes pour obtenir des PVA. Ainsi, par ce procédé de biotransformation, le projet Bt-INRS donne non seulement un espoir et un nouvel élan à la lutte biologique pour la protection de la forêt et de l'agriculture contre des insectes nuisibles, mais aussi apporte un début de solution au difficile et complexe problème de gestion des boues. Toutefois, la résolution de ces problématiques générales du projet Bt passe par une série de problèmes spécifiques dont certains font l'objet de ce projet de recherche.

1.4. Problématiques spécifiques de cette étude

L'une des étapes principales dans la production des biopesticides est le procédé de bioréaction. Dans le cadre du procédé Bt-INRS qui a commencé depuis plus d'une décennie, plusieurs travaux ont été consacrés aux différentes situations spécifiques relatives à la fermentation. Ainsi, des études sur la composition du substrat, la concentration des solides totaux, les procédés de

prétraitement des boues, ainsi que les possibilités de mise en échelle des procédés de fermentation ont été effectuées (Vydiarthi *et al.*, 2000; Lacchab *et al.*, 2001; et 2002; Barnabé *et al.*, 2004; Yezza *et al.*, 2004 et 2006; Vu *et al.*, 2009). Ces recherches avaient essentiellement pour objectifs d'augmenter la concentration des δ-endotoxines (principaux composants actifs de Bt), des spores, des enzymes, etc., qui déterminent le potentiel insecticide du bouillon fermenté de cet entomopathogène.

Cependant, le bouillon de Bt obtenu après l'étape de la fermentation des boues d'épuration ou des eaux usées des industries d'amidon ne présente pas toutes les propriétés et les caractéristiques nécessaires pour donner de bons résultats lors de son application. En effet, d'après Burges (1998), pour un bon rendement, les biopesticides doivent avoir les caractéristiques suivantes **(i)** une stabilité et une bonne conservation contre les contaminations, **(ii)** une bonne suspension/dispersion pour être compatible avec les équipements d'application, **(iii)** une bonne résistance face aux facteurs environnementaux (radiations UV, pluie, température, etc.), **(iv)** un potentiel insecticide élevé. C'est le manque ou l'insuffisance de ces propriétés au niveau des bouillons fermentés à base de Bt des eaux usées et boues d'épuration qui constituent les problématiques spécifiques de cette étude. En plus, il faut noter que les bouillons fermentés des boues d'épuration ont la particularité d'avoir une rhéologie complexe de type non-newtonien (Brar *et al.*, 2004), c'est – à – dire que leur viscosité varie avec le mouvement de cisaillement (écoulement) appliqué. Face à ces problèmes spécifiques dans les procédés de production des biopesticides à partir des milieux résiduels, il est important de développer après l'étape de la fermentation, un procédé en aval efficace avec une formulation appropriée afin que les biopesticides obtenus puissent avoir des propriétés et des caractéristiques requises pour leur commercialisation et leur application sur le terrain. Ce sont ces travaux de procédé en aval et de formulation appropriée des bouillons fermentées à base de Bt des boues d'épuration et des eaux usées des industries agroalimentaires qui constituent l'objet de cette étude que nous présentons dans les paragraphes suivants une synthèse bibliographique.

2. REVUE DE LITTERATURE

2.1. *Bacillus thuringiensis*

2.1.1. Découverte et importance de Bt

Bacillus thuringiensis (Bt) a été isolé en 1901 à partir des vers à soie par le bactériologiste japonais S. Ishiwata, (Milner, 1994). Bt est une bactérie ubiquiste de l'environnement et elle est entre autres présente dans les boues activées des stations d'épuration municipale (Mizukiet *et al.*, 2001 ; Mohammedi *et al.,* 2006). Les connaissances sur Bt ont connu une évolution rapide. Déjà en 1915, les études ont fait remarquer la présence des toxines dans les cultures sporulées de Bt mais pas dans les jeunes cellules (Beegle et Yamamoto, 1992). Existant sous plusieurs variétés, on distingue aussi plusieurs souches de Bt (Schallmey *et al.,* 2004) parmi lesquelles certaines sont entomopathogènes et sont commercialisées sous forme de biopesticides pour la lutte biologique contre les insectes. Plus de 200 variétés de biopesticides à base de Bt sont commercialisées à travers le monde (Schnepf *et al.,* 1998). Les premières formulations commerciales furent réalisées en France en 1938 sous le nom de ''Sporéine'' et étaient utilisées comme bioinsecticides agricoles (Burges, 2001). En 1990, les produits Bt représentaient 90 à 95% du marché des biopesticides (Feitelson *et al.*, 1992).

2.1.2. Caractéristiques et variétés de Bt

Mobile grâce à des flagelles, Bt est une bactérie aérobie, gram-positive et sporulante (Noris, 1971; Benoît *et al.,* 1990; Avignone-Rossa et Mignone, 1995). C'est une bactérie en forme de bâtonnet droit (0,5-2,5 x 1,2-10µm) et les cellules végétatives se présentent souvent en paire ou en chaîne sous des conditions normales de croissance (Sneath, 1984). Les cellules contiennent une seule spore ovale qui réfracte la lumière au microscope à contraste de phase **(Figure 1).**

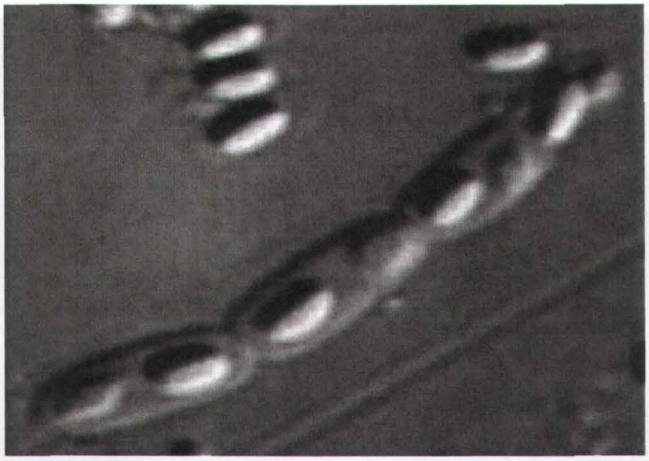

Figure 1. Cellules, spores et cristaux protéiques insecticides de *Bacillus thuringiensis* var. *kurstaki*. Microscopie à contraste d'interférence différentielle (X1600). Image modifiée avec le logiciel Adobe Photoshop de Adode Systems Inc. (É.-U.).

Bt est surtout caractérisé par la production d'inclusions cristallines protéiques parasporales qui le distinguent des autres bacilles. Ces inclusions contiennent des delta-endotoxines ou des cristaux de protéines insecticides qui constituent le principal ingrédient actif contre une grande variété d'insectes (Schnepf *et al.,* 1998; Bravo *et al.,* 2007; Pardo-Lopez *et al.,* 2009). La composition, la forme et le nombre des delta-endotoxines dépendent des variétés du microorganisme. Les cristaux de protéines de Bt ont une structure pyramidale, ovoide, plate ou cubique. Ce sont des agrégats appelés protoxines ou les δ-endotoxines ou des protéines Cry de tailles comprises entre 130 et 140 kDa. Une fois dans l'intestin de l'insecte (pH alcalin), les protoxines sont fragmentées et produisent des toxines dont les tailles sont comprises entre 60 et 80 kDa (Whiteley et Schepf, 1986). Les études ont aussi montré qu'en plus des spores et cristaux de protéines, Bt secrète d'autres composés comme des protéines insecticides végétatives, des antibiotiques et des enzymes (protéases, chitinases, phospholipases, etc.) dont la présence augmente son potentiel insecticide (Schnepf *et al.,* 1998; Liu *et al.*, 2002; Yezza *et al.*, 2006b).

On distingue plusieurs variétés Bt (au moins 34 variétés) ainsi que plusieurs souches. Les chiffres varient suivant les documents, toutefois, on peut estimer environ à 35 000 le nombre de souches de Bt (Lecadet *et al.*, 1999), dont environ 800 ont été isolées (Schallmey *et al.*, 2004). Parmi les variétés de Bt, on peut citer celles qui sont utilisées pour la protection de l'homme et

de son environnement; Il s'agit de Bt *israelensis* (contre diptères comme les moustiques et les mouches noires); Bt *tenebrionis* (contre les coléoptères); Bt *aizawai* et Bt *kurstaki* (contre les lépidoptères). Parmi les variétés les plus hautement entomotoxiques, il y a Btk (variété *kurstaki*) qui est utilisée dans le cadre de cette étude. Reconnu comme un insecticide biologique sans danger pour l'environnement, la santé humaine, les oiseaux et les mammifères (Sopfim, 2001), les formulations de Bt var. *kurstaki* sont commercialisées sous plusieurs dénominations comme Biobit®, Dipel®, Javelin® etc. Au Québec, dans le domaine forestier, les seuls insecticides biologiques utilisés sont à base de Btk, et ils occupent 3% de tous les insecticides utilisés (Sopfim, 2001). Malgré ce grand nombre de variétés de Bt, son mode d'action est en général très semblable pour toutes.

2.1.3. Les différentes phases d'action de Bt

Contrairement aux pesticides chimiques pour lesquels le mode d'action est rapide au moindre contact avec l'insecte, le mode d'action de Bt est lent. Il commence après que les insectes aient ingéré le produit contenant les cristaux de protéines, les spores, et autres composés (enzymes, protéines insecticides végétatives, etc.) encore appelés composés de virulence.

Cinq phases résument le mode d'action de Bt (Schnepf *et al.,* 1998; Cooper, 1994; Aronson et Shai, 2001): Les inclusions cristallines (les protoxines), les spores et les composés de virulences sont ingérés par les larves d'insecte *(phase 1)*. Elle est suivie de celle où les protoxines sont solubilisées dans l'intestin de la larve en raison du pH alcalin et sont donc fragmenter pour donner des toxines actives *(phase 2)*. Au fait, au cours de cette phase 2, les extrémités COOH- et/ou NH2- terminales des protéines Cry sont hydrolysées par les protéases de l'insecte afin de libérer les toxines. Ensuite, les toxines entrent réversiblement en contact avec des récepteurs spécifiques des cellules épithéliales de l'intestin *(phase 3)*. Les toxines forment sur ces récepteurs des pores transmembranaires conduisant à un influx d'électrolytes et d'eau aboutissant à la lyse des cellules épithéliales *(phase 4)*. Cette destruction progressive des structures du tube digestif permet la germination des spores (ingérées avec le cristal) et la multiplication végétative des cellules bactériennes. Ainsi, les tissus de l'insecte sont peu à peu envahis, si bien que la larve

cesse de s'alimenter et finalement meurt *(phase 5)*. Le schéma de la **Figure 2** résume brièvement le mode d'action des delta-endotoxines de Bt.

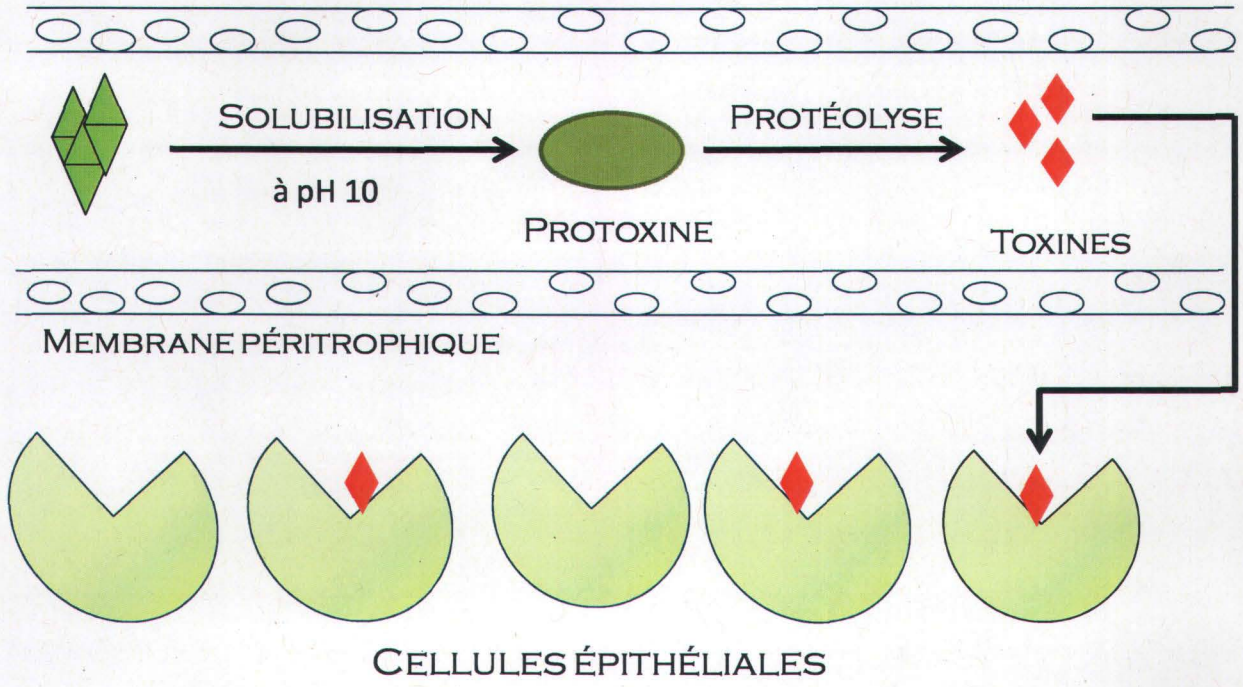

Figure 2. Résumé du mode d'action des cristaux de protéines de Bt

Pour que Bt soit efficace, il faut que les principales caractéristiques et propriétés des cristaux de protéines soient fonctionnelles ; à savoir 1°) une bonne solubilité dans le milieu intestinal de l'insecte, 2°) une conversion efficace des protoxines en toxines, 3°) une adéquation de la formation des liaisons entre les toxines actives et les récepteurs spécifiques de la membrane épithéliale et 4°) la formation de pores membranaires. Ce sont ces conditions qui déterminent la spécificité d'un cristal de protéine insecticide (Kwang-Bo et Coté, 2000), ainsi que le potentiel entomotoxique des biopesticides.

2.1.4. Entomotoxicité

L'entomotoxicité est le paramètre de mesure du potentiel insecticide des biopesticides-Bt. Elle est essentiellement fonction des delta-endotoxines qui sont les agents les plus importants de cet insecticide bactérien. Les cristaux de protéines insecticides de Bt sont toxiques essentiellement pour les insectes nuisibles des ordres de Diptères, Lépidoptères et Coléoptères (Krieg et Langenbruck, 1981). Ils sont aussi toxiques pour les insectes d'autres ordres comme Hymenoptera, Homoptera, Orthoptera et Mallophaga ainsi que les nématodes et les protozoaires (Schnepf *et al.*, 1998). Cette spécificité d'actions de Bt est due aux gènes codant encore appelés les gènes cry. Il existe jusqu'à ce jour au total 25 gènes qui se classent en cinq groupes (Copping et Menn, 2000): cry I et cry II (contre les lépidoptères), cry III (contre les coléoptères), cry IV (contre les diptères) et cry V (contre les coléoptères et lépidoptères). Ainsi, la variété de Bt utilisée dans cette étude, c'est à dire Bt var. *kurstaki* ou BT HD-1 possède les cinq gènes à savoir *cryIAa*, *cryIAb*, *cryIAc*, *cryIIA* et *cryIIB* qui sont distribués sur 12 plasmides (Aronson, 1993; Baum et Malvar, 1995).

Pendant la fermentation, les cristaux de protéines insecticides (CPI) sont produits après la phase exponentielle de croissance dans des inclusions protéiques intracellulaires. Leur accumulation coïncide avec la phase de sporulation de Bt. La production des CPI est favorisée par un pH compris entre 6,5 et 7,5 (Sikdar *et al.*, 1991). Aussi, une oxygénation trop élevée ou trop faible réduit la production de toxines (Avignone-Rossa et Mignone, 1992). Il est démontré qu'une longue limitation d'oxygène pendant la production peut entraîner l'arrêt de la formation de ces cristaux (Yang et Wang, 1998). Un supplément de NaCl et de Tween 60 peut augmenter l'entomotoxicité en favorisant le passage des composés dans la cellule bactérienne et rendant plus accessibles les protéines solubles (Morris *et al.*, 1996). En effet, selon la force ionique et le pH du milieu, le NaCl influence la solubilité des protéines nécessaires à la formation des CPI.

En plus des CPI qui jouent le rôle important dans le potentiel entomotoxique de Bt, il faut aussi noter que ce sont les spores qui achèvent son action en provoquant la septicémie chez les larves d'insectes, en se multipliant d'une manière végétative dans le sang de la larve provocant ainsi sa mort (Mohd-Salleh et Lewis, 1982; Porcar et Juarez-Pérez, 2003). Plusieurs études ont cherché à

quantifier l'effet des spores dans la mesure d'entomotoxicité. En effet, Li *et al.* (1987), Miyasono *et al.* (1994), Liu *et al.* (1998), Donavan *et al.* (2001) ont montré l'effet positif des spores augmentant l'entomotoxicité de Bt, alors que Farrera *et al.* (1998), contestent cette conclusion.

Tout comme dans le cas des spores, des études ont démontré que certains enzymes de Bt, particulièrement (les protéases et les chitinases), ont un effet positif sur son entomotoxicicité. En effet, Yezza *et al.* (2006b) ont montré qu'une activité protéolytique élevée peut augmenter le potentiel des biopesticides-Bt. Toutefois, même si la cellule de Bt secrète des protéases intracellulaires et des protéases extracellulaires (Andrews *et al.*, 1985), compte tenu de l'effet des protéases intestinales des larves des insectes, l'influence des protéases de Bt est parfois confuse et même controversée. En ce qui concerne les chitinases, Liu *et al.* (2002) ont conclu qu'une activité élevée de ces enzymes augmente le potentiel insecticide par le phénomène de synergie facilitant la pénétration à travers les chitines dans l'intestin des larves.

2.2. Obtention de Bt par bioréaction

L'obtention de Bt se fait par bioréaction dans un milieu liquide riche en composants nutritifs à base de carbone et d'azote. Cette fermentation se fait dans un bioréacteur où le pH, la température et l'oxygène sont contrôlés. Les valeurs optimales sont données par un pH 7 et une température de 30°C (Dulmage *et al.*, 1990, et Braun, 2000). Le pourcentage de saturation d'oxygène dans le bioréacteur, contrôlé par l'aération et la vitesse d'agitation, doit être supérieur à 20% (v/v). Plusieurs études ont été réalisées sur la composition et la concentration des différents milieux de culture ainsi que sur les différentes phases de croissance de Bt pendant la fermentation (Luthy *et al.*, 1982; Yudina *et al.*, 1993; Faloci *et al.*, 1993; Alves *et al.*, 1997; Khuzamshukurov *et al.*, 2001; Saskinchai *et al.*, 2001 et Ozkan *et al.*, 2003).

Étant donné que le coût des substrats (matière première) constitue un élément important dans la production et la commercialisation des produits Bt, plusieurs chercheurs dont Alves *et al.* (1997), Tirado-Montiel *et al.* (1998), Rojas *et al.* (1999), Sachveda *et al.* (1999a), Yang et Wang (1998) et Zouari et Jaoua (1999) ont travaillé sur plusieurs produits ou sous-produits comme

milieu de culture alternatif pour l'obtention de Bt. Dans tous les cas, mises à part quelques difficultés sur le plan nutritif ou rhéologique, il faut noter que tous les substrats alternatifs ayant fait l'objet d'étude jusqu'à maintenant, sont peu coûteux, et certains permettent d'obtenir Bt par fermentation. Cependant, ces milieux sont restreints à l'échelle locale (disponible dans certaines localités seulement) et leurs frais de transport dissuadent leurs utilisations (Tirado-Montiel, 1997; Sachevda *et al.*, 1999b ; Saksinchai *et al.*, 2001 ; Vidyarthi *et al.*, 2002). C'est pourquoi l'initiative du projet Bt-INRS, qui cherche à utiliser le plein potentiel nutritif des eaux usées agroalimentaires et des boues d'épuration (disponible en quantité et partout), apparaît comme une meilleure solution.

2.3. Boues d'épuration municipale

2.3.1. Différents types de boues

Les boues sont les principaux résidus produits par une station d'épuration. Ce sont des matières résiduaires qui sont surtout constituées de bactéries mortes ainsi que de matières organiques et minérales (Inoue *et al.*, 1996; Jardé, 2002). Selon le type de station d'épuration et de traitement appliqué, on peut classer les boues en trois grandes catégories: (1) les boues primaires obtenues après une simple décantation des eaux usées. Elles présentent des concentrations très élevées en matières minérales (sable, terre, etc) et en matières organiques en pleine évolution; (2) les boues physico-chimiques, elles sont obtenues par décantation améliorée par des floculants (sel de fer, d'aluminium, etc.) pour agglomérer les particules fines qui se décantent difficilement; (3) les boues secondaires ou biologiques obtenues après un traitement biologique (boues activées, disques biologiques, lits bactériens, etc.). Ces boues essentiellement organiques sont principalement composées de bactéries et de leurs sécrétions. Dans cette catégorie de boues, on distingue: (i) les boues mixtes (mélange de boues primaires et secondaires) obtenues après un traitement complet (décantation primaire – boues activées – décantation secondaire); (ii) les boues d'aération prolongée qui sont obtenues sans décantation primaire mais avec des matières polluantes suffisamment aérées. Ce sont des boues peu concentrées et moins organiques.

2.3.2. Composition des boues

Actuellement désignées sous le terme de ''biosolides'' à cause des effets bénéfiques de leur utilisation en agriculture (Walker, 1998), les boues d'épuration municipale sont constituées de 40 à 80% de matières organiques. Ces composés proviennent des excréments humains, des microorganismes morts ou vivants. C'est un mélange complexe de polysaccharides (sucres, cellulose, lignine), d'azote organique (protéines et acides aminés) et inorganique ($N\text{-}NH_4^+$ et $N\text{-}NO_2^-\text{-}NO_3^-$), de phosphore organique et inorganique ($P\text{-}PO_4^{3-}$), de graisses (huiles, lipides et acides gras). Il y a également des métaux, des matières humiques et fulviques, ainsi que des composés récalcitrants comme les hydrocarbures aromatiques. Les concentrations de ces composés dépendent de la composition des eaux usées et du type de traitement appliqué. Certains composés peuvent être facilement biodégradables alors que d'autres nécessitent des procédés de dégradation par hydrolyse, ozonation, ultrasonication, etc. (Barnabé *et al.*, 2004; Pham *et al.*, 2009)

2.3.3. Rhéologie des boues

Outre son origine et sa concentration, une boue est aussi caractérisée par d'autres données numériques. Il s'agit de la siccité (pourcentage en matières sèches), des matières en suspension dissoutes et volatiles, de la consistance ou rhéologie. En effet, d'après (OIE, 2000), il y a des boues liquides (siccité de 0 à 10 %), des boues pâteuses (siccité de 10 à 25 %), des boues solides (siccité de 25 à 85 %) et des boues sèches (siccité supérieure à 85 %). La rhéologie, mesurée à travers la viscosité et la taille des particules donne une caractéristique importante des boues. En biotechnologie, la connaissance de la rhéologie du milieu de culture est importante pour toute manipulation de ce bouillon (Aiba *et al.*, 1973; Kõnig *et al.*, 1981; Walling *et al.*, 2001; Pollard *et al.*, 2002; O'Cleirigh *et al.*, 2005). Dans le cas particulier des boues d'épuration, Brar (2007) étudié la rhéologie des boues fermentées par Bt ainsi que celle de la formulation aqueuse du bouillon fermenté. Elle aboutit à la conclusion que la rhéologie intervient et influence toutes les étapes du traitement depuis la composition des milieux de culture et les prétraitements jusqu'à l'étape de la formulation en passant par la fermentation et les techniques de récupération des composants actifs. La rhéologie est aussi nécessaire pour le choix des additifs ou adjuvants de

formulation afin d'avoir un bon synergisme entre ceux-ci et le milieu de culture. En effet, il existe entre les diverses particules et composants actifs, des interactions dues aux différentes forces de liaisons (Liaisons hydrogène, forces électrostatiques, hydrophobes, Van der Waals, etc.) (Israelachvili, 1992), et le choix des additifs doit en tenir compte pour la mise en formulation.

2.4. Formulation

C'est l'étape de production qui suit celle de la fermentation. Elle comprend les procédés de récupération des ingrédients actifs du bouillon fermenté (procédé en aval ou pré-formulation), et la formulation proprement dite qui consiste à la sélection des additifs et leurs concentrations en vue de l'obtention d'un produit fini répondant aux attentes du marché.

2.4.1. Procédés en aval (pré-formulation)

À la fin de la fermentation, les composants actifs du bouillon fermenté de Bt sont les spores viables, les cristaux protéiques, les enzymes (protéases, chitinases, phospholipases), les protéines végétatives insecticides et autres (Rowe et Margaritis, 2004). Avant la formulation, il est important de récupérer avec des procédés de séparation efficaces et économiques ces composants actifs du bouillon fermenté (Rowe et Margaritis, 2004). Le choix du procédé utilisé pour la récupération de ces composants actifs est très important car ces techniques peuvent augmenter ou diminuer l'activité insecticide du produit final à cause parfois de la faible concentration des composants actifs dans le bouillon fermenté ou de leur faible résistance thermique et physique (par exemple cellules de Bt). Plusieurs techniques de séparation solide-liquide ont fait l'objet de recherches approfondies, Parmi elles, on peut citer:

2.4.1.1. Centrifugation du bouillon fermenté

Parmi les techniques de récupération des composants actifs du bouillon fermenté, la centrifugation est le plus souvent utilisée (Zamola *et al.*, 1993 ; Maybury *et al.*, 2000). Aussi, dans le cadre du projet Bt-INRS, Brar *et al.* (2006) ont appliqué ce procédé de centrifugation au

bouillon fermenté à base de Bt obtenu à partir des boues d'épuration et des eaux usées des industries d'amidon. Cependant, malgré l'efficacité et la performance des résultats obtenus, la centrifugation ne permet pas de récupérer tous les composants actifs. Ainsi, des pertes des composants actifs sont identifiées dans le surnageant obtenu après la centrifugation. Il s'agit surtout de composants solubles comme les enzymes (protéases, chitinases, phospholipases, etc.) qui ne peuvent pas être récupérés par la centrifugation, mais aussi des spores et des cristaux protéiques. Pour cela, il est important de les récupérer du surnageant par un autre procédé.

2.4.1.2. Filtration membranaire du surnageant de la centrifugation

Les anciennes techniques de récupération des composants actifs du surnageant, comme les procédés d'adsorption, d'évaporation et de précipitation, donnent des rendements faibles (de Barjac *et al.*, 1966; Benz *et al.*, 1966; Kim *et al.*, 1970). Aussi, les méthodes peu récentes de séparation par extraction utilisant une association de PEG/sel (K_2HPO_4) (Tzeng et Hsu, 1994), sont longues et leurs mises à l'échelle difficiles. Les techniques de récupération des solutés d'un surnageant les plus efficaces, consistent à utiliser un procédé de filtration membranaire. C'est le cas, par exemple, des procédés de récupération des composés organiques par microfiltration, ultrafiltration, ou nanofiltration utilisant différentes types de membranes (Cabassud *et al.*, 1991; Russoti *et al.*, 1995; Cui *et al.*, 1997; Adikane *et al.*, 1999; Tzeng *et al.*, 1999; Christy *et al.*, 2002; Darnon *et al.*, 2003). Ces auteurs ont appliqué la filtration membranaire à plusieurs milieux synthétiques, ainsi que dans les procédés de production d'eau potable, mais pas sur les surnageants des milieux résiduels.

La différence fondamentale entre les diverses techniques de filtration réside d'une part dans le principe (filtration normale ou frontale et filtration tangentielle) et, d'autre part, dans le choix de la taille des pores de la membrane. En effet, la taille des pores des membranes d'ultrafiltration est supérieure à celle des membranes de nanofiltration et inférieure à celle des pores pour microfiltration. Les seuils de coupure des membranes d'ultrafiltration varient entre 1 et 100 kDa avec une pression opératoire de 50 à 500 kPa et un flux du perméat (filtrat) moins élevé qu'en microfiltration (Anselme et Jacob, 1996). Étant donné la taille des pores des membranes d'ultrafiltration, elles sont capables de récupérer les colloïdes, les particules, les macromolécules

mais aussi les bactéries et les virus (Bouchard *et al.*, 2003c). **Le Tableau 1** présente les caractéristiques des différents procédés membranaires.

Tableau 1. Caractéristiques des filtrations membranaires à gradient de pression

Procédé	Origine de la sélectivité	Force motrice	Diamètre des pores	Seuil de coupure	Consommation émergétique	Procédés concurrents
Microfiltration (MF)	Différence de taille entre les particules ou molécules à séparer	Pression (0.1 à 3 bar)	0.1 à 10µm	-	1 - 10 KWh/m^3	Filtration Centrifugation
Ultrafiltration (UF)	Différence de taille et de charge entre les particules ou molécules à séparer	Pression (0.1 à 10 bar)	0.01 à 0.1µm	1 - 300 kDa	1 - 10 KWh/m^3	Évaporation Distillation Échange d'ion
Nanofiltration (NF)	Différence de taille et de charge entre les particules ou molécules à séparer	Pression (10 à 50 bar)	~ 1 nm	0.2 - 1 kDa	5 - 50 KWh/m^3	Évaporation Osmose inverse Échange d'ion
Osmose Inverse (OI)	Différence de solubilité et de diffusion dans la membrane des molécules à séparer	Pression (30 à 100 bar)	Membrane dense	< 0.2 kDa	10 - 200 KWh/m^3	Évaporation Distillation Échange d'ion

La filtration membranaire donne des résultats intéressants et promet un avenir encourageant (Mallevialle *et al.*, 1996). Cependant, le problème le plus souvent rencontré est le colmatage des membranes (Bacchin *et al.*, 2005; Thekkedath *et al.*, 2007). En effet, malgré les nombreuses études portant sur ce handicap, la prédiction de l'évolution du colmatage de la membrane au cours de la filtration de différents types de milieux demeure une problématique complexe qui nécessite plus d'approfondissement. Le colmatage d'une membrane peut être un phénomène physique ou chimique dû à un dépôt des particules du milieu sur la membrane qui est un matériau comportant des pores et caractérisé par une résistance donnée. Ce dépôt se fait d'une part par obstruction mécanique des pores dans la profondeur de la membrane, et d'autre part, par adsorption des particules à la surface de la membrane due à des phénomènes physico-chimiques liés à la composition du milieu et aux propriétés de la membrane. L'épaisseur de cette adsorption est appelée couche de polarisation dans laquelle la concentration des particules augmente du liquide vers la surface de la membrane. Cette couche de polarisation peut être assimilée à un milieu poreux caractérisé par une résistance hydraulique

Vu les conclusions des travaux de Brar *et al.* (2006) sur la centrifugation (notamment les pertes observées dans les surnageants) et compte tenu de ce qui précède sur les différents procédés de récupération membranaire, il apparaît que l'ultrafiltration est une alternative valable. Elle peut donc être associée à la centrifugation dans les procédés en aval de la fermentation de Bt des boues d'épuration et eaux usées des industries d'amidon afin de récupérer les composants actifs solubles (surtout les enzymes) pour augmenter le potentiel entomotoxique des biopesticides.

2.4.2. Formulation des biopesticides

2.4.2.1. Généralités et Définitions

En général, une formulation est un ensemble de savoir-faire nécessaire pour développer ou fabriquer un produit commercial caractérisé par certaines propriétés, afin de répondre à un besoin spécifique. Le produit formulé est le résultat d'une combinaison ou mélange de diverses matières d'origine synthétique ou naturelle. Ces matières se classent généralement en deux groupes à savoir, les composants actifs qui assurent la fonction principale recherchée au niveau du produit, et les adjuvants/additifs de formulation qui assurent les fonctions secondaires facilitant la préparation et la mise en œuvre du produit. En principe, la formulation touche toutes les étapes de transformation du produit depuis les procédés en amont produisant les composants actifs (matières premières) jusqu'aux procédés en aval, directement en contact avec l'utilisateur final (industriel ou grand public) (Burges, 2001). Elle recherche un meilleur compromis possible entre la performance, la facilité d'application du produit, la sécurité des utilisateurs et un coût minimal. Ce compromis évolue constamment avec les modes et le niveau de vie de la population qui constitue le champ de compétition des entreprises.

Dans le cas des biopesticides, la formulation a pour but d'apporter aux bouillons fermentés, des propriétés et caractéristiques nécessaires contre certains effets néfastes ou problématiques spécifiques rencontrées lors de l'utilisation des biopesticides (Burges, 2001). Il s'agit des effets : (1) des facteurs environnementaux (actions des radiations UV, lessivage par la pluie, impacts des feuillages, température); (2) liés à l'applicabilité des biopesticides sur le terrain

(contaminations pendant le stockage, suspension et dispersion, adhérence aux feuilles, potentiel entomotoxique).

2.4.2.2. Effets des facteurs environnementaux

2.4.2.2.1. Lumière solaire et radiations UV

L'action insecticide de Btk sur les larves de lépidoptère est très connue (Knowles *et al.*, 1987), de même que son effet bénéfique pour l'environnement. Cependant, un des problèmes rencontrés dans l'application de biopesticides Btk sur le terrain est sa courte durée d'action insecticide due en partie aux radiations solaires. En effet, l'efficacité des actions des protoxines de Btk se trouve affectée par son inactivation par la lumière solaire, plus particulièrement par les radiations UV (Griego *et al.*, 1978; Ignoffo *et al.*, 1978; Pozsgay *et al.*, 1987; Becker *et al.*, 1992).

Plusieurs études portant sur les différents moyens ou techniques de protections contre l'inactivation de Bt par les UV ont été réalisées. Parmi elles, il y a des formulations sous forme de microcapsules (Ignoffo *et al.*, 1991), des formulations granulaires (Ahmed *et al.*, 1973), ou des formulations par ajout d'additifs de protection contre les UV (Dunkle *et al.*, 1989). Toutefois, certains additifs par leur nature et leurs concentrations, peuvent améliorer ou dégrader la stabilité de la formulation (Dunkle *et al.*, 1989), d'où la nécessité d'une étude de synergisme des divers additifs par rapport au milieu de culture et une optimisation de leurs concentrations. Ainsi, étant donné la particularité des milieux de culture utilisés (boue d'épuration et eaux usées des industries agroalimentaires) utilisés dans cette étude, une recherche des additifs qui tiennent compte des caractéristiques de ces milieux est indispensable.

2.4.2.2.2. Pluie

Pour que les biopesticides puissent agir longtemps après leur pulvérisation, il faut qu'ils adhérent aux feuilles et aux plantes le plus longtemps possible. Cependant, il arrive que la pluie entraîne par lessivage une partie des biopesticides déposés sur les feuilles, diminuant ainsi leurs

potentiels insecticides. Il est montré qu'une pluie de 3 cm peut réduire de 20% l'efficacité des biopesticides (Behle *et al.*, 1977). Navon (1993) a aussi montré que la rosée peut diminuer l'efficacité de Bt sur les feuilles. Pour pallier à cet effet néfaste de la pluie, plusieurs études ont été réalisées pour augmenter l'adhérence des biopesticides sur les feuilles. Ainsi, McGuire *et al.* (1994) ont montré que les formulations à base d'amidon peuvent augmenter l'activité insecticide de Bt à cause du fait que ce produit a une propriété adhésive. Aussi, certains composés comme les polysaccharides, les protéines et les glycoprotéines présents dans le corps de certaines cellules comme le *Pseudomonas* peuvent favoriser la propriété adhésive sur les feuilles (Burges, 1998). Cependant les cellules de Bt ne contiennent pas ces éléments, donc ne possèdent pas cette propriété. D'où l'importance de développer une formulation qui donne cette propriété adhésive aux biopesticides Bt. Ceci est possible soit, par une formulation avec ajout d'additifs comme mélasse, gomme xanthane, methyl carboxycellulose, soit par une formulation de type microcapsule à base d'amidon comme support.

2.4.2.2.3. Température

La température optimale de croissance de Bt est 30°C. Cependant, l'application des produits Bt sur le terrain peut être influencée par la température. En effet, Ignoffo (1992) a montré que seules les valeurs de températures comprises entre 10 et 30°C permettent une bonne activité des bactéries entomopathogènes. Ainsi, les températures extrêmes peuvent causer une inactivation des produits Bt (Cohen, 1991). Cet impact de la température sur ces bioinsecticides dépend des régions et des périodes d'application. Contrairement aux régions tropicales où la chaleur élevée peut entraîner la dégradation des formulations (Morris, 1983), au Canada par exemple l'activité des produits Bt est encore possible jusqu'à 0°C (Molloy, 1990). L'étude de la stabilité à la température des formulations Bt obtenues à partir des boues d'épuration et des eaux usées des industries d'amidon a été largement abordée par Brar *et al.* (2007).

2.4.2.2.4. Impacts des feuillages

L'activité biologique de Bt est aussi influencée par certains effets négatifs des feuilles. En effet, les études ont montré que l'activité de Bt sur les feuilles ombragées est plus élevée que celle sur

des feuilles non ombragées (Beckwith et Stelzer, 1987). En effet, l'ombre limite les effets de la lumière et des radiations UV qui réduisent l'activité des cristaux des protéines de Bt. Aussi, la concurrence entre l'action insecticide de Bt et la croissance des plantes (donc augmentation de la surface des feuilles) est une autre problématique dont on doit tenir compte dans la fréquence d'application de Bt aussi bien en agriculture qu'en forêt. De même, Diverses études ont montré que certains composés volatils comme des aldéhydes, des cétones, des acides carboxyliques, etc. présents sur les feuilles ont des effets inhibiteurs sur les composants actifs de Bt (Smirnoff, 1972; Ferry *et al.*, 2004). Étant donné que toutes ces études ont été réalisées avec les milieux synthétiques, il serait intéressant de reprendre ces études avec les milieux des eaux usées et les boues fermentées, vu leurs particularités (rhéologie et composition).

2.4.2.3. Effets liés à l'applicabilité des biopesticides sur le terrain

Outre les facteurs environnementaux, la formulation de biopesticides Bt tient également compte des problèmes liés à sa commercialisation et à son application sur le terrain (suspension/dispersion, contamination par d'autres microorganismes, potentiel entomotoxique, etc.).

2.4.2.3.1 Suspension et dispersion

Une bonne pulvérisation des biopesticides exige que le produit formulé présente une structure permettant une bonne suspension et dispersion du mélange. Le nombre de spores et de cristaux de protéines insecticides par gouttelette est un paramètre très important pendant la dispersion. En effet, si l'insecte ingère une gouttelette de dose non létale, il peut arrêter de manger pendant plusieurs jours mais survivra et pourra continuer à faire des dégâts (Smirnoff et Valéro, 1983). Donc, il est important que les tailles des particules permettent une bonne suspension/dispersion (que le mélange ne décante pas) afin que la calibration des gouttelettes ne varie pas pendant la pulvérisation. Aussi, le contrôle ou la mise en suspension peut se faire parfois par certains additifs ou adjuvants de suspension ou de dispersion. Dans la production industrielle des biopesticides, la stabilité de la dispersion à long terme a une grande importance pour la qualité du produit final. En effet, la stabilité de la dispersion d'une particule dépendra de l'équilibre

entre les forces répulsives et attractives qui existent entre elles lorsqu'elles s'approchent les unes des autres. Il est donc important de prédire l'évolution de la stabilité de la dispersion dans le temps. Ainsi, étant donné que l'interrelation entre les particules peut être déterminée par la mesure du potentiel zêta et de la dispersion, il est nécessaire de faire une étude de prédiction de la dispersion des particules par le potentiel zêta.

2.4.2.3.2. Préservation contre les contaminations

Pendant leur transport ou l'entreposage, les préparations Bt peuvent être sujettes à des contaminations par des microorganismes. Pour cela, en général les formulations des préparations Bt sont préservées à un pH compris entre 5 et 7 (milieu acide) afin d'empêcher ces contaminations. De même, à ce pH, les cristaux protéiques insecticides sont à l'abri de toute solubilisation protéolytique, car ces cristaux se solubilisent en milieu basique (Dubois *et al.* 1993). En effet, un pH très acide ou très basique peut inactiver les ingrédients actifs du produit Bt (Griffiths, 1982; Salama and Moris, 1993). Il est aussi démontré qu'un pH optimal de conservation peut augmenter la durée de vie du produit formulé (Date, 1970). Cette formulation (par exemple granules, briquettes ou microcapsules) peut avoir un certain lien structural pour garder son intégrité pendant un bon moment et prévenir ainsi toutes contaminations (Burges, 1998). Aussi, l'usage des additifs antimicrobiens comme l'acide sorbique et l'acide propionique, le métabisulfite de sodium permet de réaliser un milieu tampon à pH acide (4-6) afin de préserver les biopesticides Bt. Couch et Ignoffo (1981) estiment qu'une durée de vie de 18 mois est le minimum pratique pour les pesticides microbiens. Cependant, actuellement certains pesticides chimiques commerciaux peuvent avoir une durée de vie minimale de deux ans. Rhodes (1993) indique donc qu'une durée de vie de quatre ans serait souhaitable pour les biopesticides afin de favoriser leur commercialisation par rapport aux pesticides chimiques.

2.4.2.3.3. Phagostimulation

Il s'agit d'ajouter aux produits formulés des additifs dont le but est de stimuler ou encourager les insectes à absorber rapidement les préparations formulées. Au laboratoire, il n'est pas facile de mettre les insectes dans les conditions naturelles de compétition dans un petit espace sur lequel

le produit formulé est appliqué. En effet, la dose des cristaux ou de spores mangée sur une feuille uniformément traitée doit être proportionnelle à la quantité de feuilles mangées, mais les résultats de mortalité ne sont pas proportionnels (Burges, 1998).

2.4.2.4. Les additifs de formulation

D'après ce qui précède, il est nécessaire de compléter l'activité principale des biopesticides (pouvoirs insecticides des composants actifs) par des propriétés secondaires apportées par des additifs (ou adjuvants) de formulation. Il s'agit d'additifs (1) de conservation des agents anti-microbiens permettant d'accroître la stabilité du produit afin de conserver son intégrité biologique pour une longue durée; (2) améliorant l'adhérence du produit sur les feuilles; (3) de protection contre les radiations UV et la photodégradation; (4) pour la suspension et la dispersion du produit; (5) émulsifiants et anti-moussants facilitant la manipulation du produit; (6) phagostimulants permettant de donner un goût plus attractif au produit formulé et augmentant ainsi le potentiel entomotoxique. Selon Santé Canada (1992), les adjuvants entrant dans la formulation du produit doivent être évalués et réputés non toxiques aux doses utilisées. Ces adjuvants doivent aussi respecter les reglementations des listes III-A et III-B de USEPA. Dans tous les cas, les adjuvants de formulation doivent être inertes sans interactions avec les composants actifs. Aussi, le choix ou la sélection des adjuvants doit tenir compte de types de formulation souhaitée, de la rhéologie du milieu formulé et des conditions d'application. Le **Tableau 2** donne les fonctions, les effets et des exemples de quelques adjuvants/additifs de formulation.

Tableau 2. Fonctions et effets de quelques additifs (ou adjuvants) de formulation

Fonctions	Effets	Exemples des adjuvants	Référence
Agents de protection contre UV	Protège la formulation contre l'inactivation des composants actifs de Bt par les radiations solaires	Mélasse, acide lignosulfonique de sodium, rouge congo, acide p-amino benzoique, acide folique, etc.	(Bell et Kanavel, 1978; Shapiro *et al.*, 1983; Ignoffo *et al.*, 1991)
Agents de suspension	Maintient la formulation en suspension	Sorbitol, sucrose, monophosphate de sodium, métabisulfite de sodium, silicate de sodium, vergum, gomme arabique, polymethacrylate de sodium, etc.	(En-Xian *et al.*, 2003; Styliane *et al.*, 2005)
Phagostimulants	Attire et stimule les insectes à manger suffisamment une bonne quantité du produit formulé avant la fin de l'action des toxines sur les feuilles	Glucose, sucrose, mélasse, fleur de soya, farine de mais, extrait de levure, pheast^{TM} etc.	(Montaya *et al.*, 1966; McGuire *et al.*, 1994; Farrar *et al.*, 1995). (Grochulski *et al.*, 1995; Guillon 1995)
Agents anti-microbiens	Protège la formulation contre les contaminations d'autres micro-organismes	Acide propionique, acide scorbique, acide citrique, acide lactique, métabisulfite de sodium, etc.	
Adhésifs	Augmente l'adhérence de la formulation aux feuilles, et donc permet à la formulation de résister aux effets de lessivage par la pluie	Mélasse, gomme ghatti, carboxy methyl cellulose, gomme xanthane, sorbitol, etc	(Jones, 1988a; Angus, 1959; shasha *et al.*, 1995; Roome, 1975)

2.4.2.5. Différents types de formulation

Les biopesticides sont disponibles en différentes formulations qui se présentent sous plusieurs aspects et en deux catégories: Les formulations liquides (suspensions concentrées et émulsions) et les formulations solides (poudres humides, granules ou briquettes). Le choix du type de formulation dépend de plusieurs facteurs dont l'insecte destructeur visé, la durée d'action du potentiel insecticide, la nature de l'environnement à traiter ainsi que son accessibilité.

2.4.2.5.1. Les formulations solides

Ce sont souvent des formulations sèches sous forme de grains de poussières, de granules, de briquettes, de capsules et de poudres humides. Le choix du support de base dépend de la dureté, la densité et le taux de dilution du produit souhaité (Polon, 1973). Les grains de poussières de tailles comprises entre 5 et 20 mm sont à base d'argile ou de silice de proportion variable en fonction de la densité désirée (Matthews, 1992) et la concentration en microorganismes est d'environ 10%. Il faut noter dans le cas d'une application directe sous forme de poudre, que les grains de poussières sont souvent emportés par le courant d'air à tel point que seuls 10% seulement atteignent la surface cible (Burges, 1998).

Les granules, capsules et briquettes sont à base de minerais d'argile, de polymère d'amidon, de fertilisants solides etc (Ross et Lembi, 1985). La concentration en Bt varie entre 5 à 20% (Burges, 1998). Les granules pénètrent facilement le couvert végétal pour se rendre dans l'eau du gîte. Pour cela elles sont utilisées lorsque la végétation interfère avec l'application aérienne du produit. Pour leur application, les granules de volume compris entre 5-10 mm^3 exigent un équipement d'une calibration précise avec une pulvérisation uniforme sans endommager le produit (Walker, 1976). Les formulations solides particulièrement les granules et les briquettes ont aussi l'avantage d'être distribuées ou pulvérisées facilement à la main (Burges, 1998). Ainsi, les équipements d'application vont d'une simple poignée de main à la pulvérisation par avion en passant par des tracteurs d'application. En ce qui concerne les formulations en poudres humides, elles commencent à prédominer parmi les produits commerciaux des biopesticides. C'est une formulation en poudre avec des additifs souvent près à être mélangés à l'eau peu de temps avant

la pulvérisation. La formation de petites boules pâteuses (cake) pendant le mélange est à surveiller car elles peuvent boucher les pulvérisateurs. En effet, l'usage des silices en petite quantité aide à prévenir contre cette situation (Burges, 1998).

2.4.2.5.2. Les formulations liquides

Pour ce type de formulation, c'est le liquide qui joue le rôle de support. Les additifs de suspension, de dispersion et les surfactants permettent d'obtenir une bonne suspension du milieu liquide, d'où l'importance de leur choix. Il s'agit de trouver un équilibre entre la viscosité du milieu et la vitesse de décantation des particules. Les formulations liquides de Bt sont les plus largement utilisées (Burges, 1998) en raison de leurs facilités de manipulation et de leurs propriétés dispersantes en eaux courantes. Dans les suspensions, les dispersants diminuent le taux de la sédimentation de ces particules qui s'agglomèrent de façon réversible par floculation. Dans les formulations liquides, les surfactants sont identifiés par leurs caractères hydrophiles et lipophiles. Par contre, dans les émulsions, leur sédimentation est réduite à cause des surfactants qui ont une propriété stabilisatrice.

En conclusion, la formulation des biopesticides permet d'obtenir, à partir des ingrédients actifs du bouillon fermenté et des différents agents/additifs de formulation, un produit final : (1) **stable** (pendant la production, la distribution et le stockage); (2) **facile à appliquer** sur le terrain (compatibilité avec les équipements, bonne performance et efficacité des différents types de formulation); (3) **sans danger** pour l'environnement et avec (4) avec **un potentiel insecticide élevé** par rapport aux insectes ou organismes cibles. Les caractéristiques et la rhéologie du milieu de culture jouent un rôle très important. En effet, contrairement aux biopesticides Bt produits à partir de milieux semi-synthétiques (comportement approximativement newtonien), ceux obtenus à partir de boues d'épuration et des eaux usées d'industrie d'amidon ont une rhéologie complexe avec un comportement non-newtonien dont il faut tenir compte, d'où le développement d'une formulation propre et adaptée à ces milieux résiduels. Ainsi, en se basant sur la littérature et les travaux déjà réalisés sur le projet Bt, les hypothèses et objectifs ci-dessous sont formulés dans le cadre de ce projet de recherche.

3. HYPOTHESES - OBJECTIFS - ORIGINALITE

3.1. Hypothèses de recherche

Par rapport aux problématiques spécifiques de cette étude et tenant compte de la revue de littérature, les hypothèses de recherche suivantes ont été formulées:

1° Les études de Brar *et al.* (2006) sur la centrifugation du bouillon fermenté obtenu à l'aide des boues d'épuration et eaux usées d'industrie d'amidon ont permis de déterminer les conditions optimales de récupération (récolte) des composés actifs sous forme d'un concentré appelé **"culot"**. Mais, ce procédé ne permet pas de récolter tous les composés actifs surtout les enzymes produites par Bt qui selon Yezza *et al.* (2006b) et Liu *et al.* (2002) peuvent augmenter le potentiel entomotoxique des biopesticides. D'où:

***Hypothèse 1**: La récupération (récolte) du reste des composés actifs (spécialement les enzymes) contenu dans le surnageant des bouillons fermentés des milieux résiduels devrait permettre d'augmenter l'entomotoxicité des biopesticides.*

2° D'après Burges, (1998), l'efficacité d'un additif dans une formulation dépend de sa concentration et de son synergisme avec la rhéologie du milieu de culture. D'où :

***Hypothèse 2 :** L'optimisation qualitative et quantitative des différents additifs de formulation en rapport avec la rhéologie des milieux devrait permettre d'obtenir des biopesticides stables et efficaces pour un meilleur rendement des applications sur le terrain*

3° Brar (2007) a développé des formulations de suspensions aqueuses des bouillons fermentés des milieux résiduels. D'autres études dont celles par Burges (1998) ont montré les avantages des formulations en poudres humides avec les milieux synthétiques. Alors :

***Hypothèse 3**: il est possible d'obtenir, à partir des bouillons fermentés des eaux usées et des boues d'épuration, des biopesticides en poudres humides pouvant présenter les mêmes avantages que ceux obtenus avec des milieux synthétiques*

3.2. Objectifs de recherche

L'objectif principal de cette recherche est de déterminer des paramètres scientifiques essentiels et minimaux pour la mise au point d'une formulation des biopesticides Bt en utilisant des eaux usées et des boues fermentées comme substrats de fermentation. Il s'agit d'obtenir un produit formulé avec des fonctions, propriétés et caractéristiques nécessaires pour sa commercialisation et son application sur le terrain. Ainsi, *en se basant sur les hypothèses et la synthèse bibliographique d'une part, la faisabilité et le temps alloué à cette étude d'autre part, les objectifs spécifiques sont définis:*

■ Pour la pré-formulation (procédé en aval)

1. Optimiser les paramètres d'ultrafiltration pour récupérer les composants actifs (spores, cristaux de protéines, enzymes, etc.) des surnageants de la centrifugation des bouillons fermentés obtenus à partir des boues d'épuration et les eaux usées des industries d'amidon.

2. Quantifier l'effet des composants actifs solubles (protéases et chitinases) sur le potentiel entomotoxique (entomotoxicité) des biopesticides.

3. Évaluer les possibilités de mise à l'échelle du procédé d'ultrafiltration des surnageants des bouillons fermentés des eaux usées et des boues d'épuration.

■ Pour la formulation

4. Étudier les effets des radiations UV sur les composants actifs des biopesticides avant et après la formulation, tout en déterminant les demi-vies d'entomotoxicité et les spores viables. Il

s'agit d'une étude qualitative et quantitative des différents additifs de formulation pour la protection contre les radiations UV.

5. Sélectionner et étudier l'efficacité de différents additifs antimicrobiens de conservation et de protection du produit formulé contre les contaminations extérieures par d'autres microorganismes que Bt.

6. Concevoir et optimiser les procédés de production de biopesticides en poudre humide et comparer le potentiel (en termes de spores et d'entomotoxicité) avec les suspensions aqueuses.

3.3. Originalité de la recherche

La production des biopesticides avec des eaux usées et des boues d'épuration permet d'atteindre des résultats encourageants aussi bien dans l'étape de prétraitement que celle de la fermentation. Concernant la formulation, les travaux de Brar sur la rhéologie des bouillons fermentés, la centrifugation et la réalisation de la formulation en suspension aqueuse ont permis une avancée considérable de cette étape. Cependant, il est important et souhaitable d'une part, d'augmenter le potentiel insecticide des biopesticides obtenus par ces milieux et, d'autre part, d'obtenir un produit formulé stable, répondant aux exigences du marché. Ce type de recherche n'a pas été répertorié dans la littérature. Ainsi, *l'originalité de cette étude est, d'une part, de récuperer efficacement les composants actifs de Bt des bouillons fermentés des eaux usées et les boues d'épuration et, d'autre part, d'optimiser la formulation des biopesticides afin d'obtenir un produit stable de potentiel élevé.*

4. DEMARCHE METHODOLOGIQUE

4.1. Plan expérimental

Le plan expérimental de cette étude peut être représenté par la figure suivante :

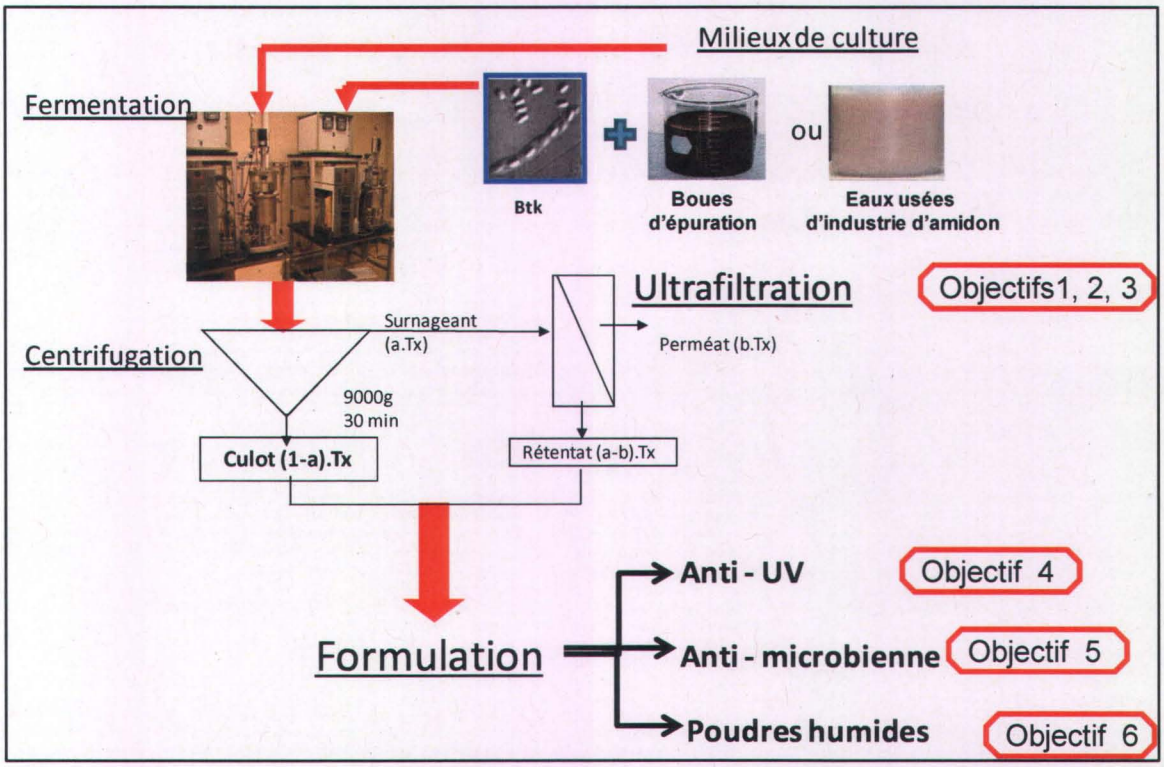

Figure 3. Schéma récapitulatif du plan expérimental

4.2. Démarche méthodologique

Les démarches méthodologiques détaillées des objectifs spécifiques sont présentées dans les différents chapitres relatifs à ces objectifs. En effet, les parties I et II du chapitre 2 portent sur les travaux d'ultrafiltration (Objectifs 1, 2, 3). Les Parties I, II et III du chapitre 3 concernent respectivement les objectifs 4, 5 et 6 sur la formulation.

5. RESULTATS

5.1. Fermentation et Centrifugation

Le Tableau 3 donne les valeurs de solides totaux, des spores viables et de l'entomotoxicité des bouillons fermentés (après 48 heures de fermentation) et des culots de centrifugation des quatre milieux. Dans cette partie présentant la synthèse des résultats, les différents milieux de culture utilisés dans cette étude à savoir les eaux usées d'industrie d'amidon, les boues secondaires hydrolysées et non-hydrolysées et le milieu synthétique de soja seront désignés respectivement par SIW, TH, NH et soya en conformité avec les articles présentés des chapitres suivants.

Tableau 3. Résumé des résultats de la fermentation et de la centrifugation

	Bouillon fermenté				Culot de centrifugation			
Milieux	Soja (soya)	Eaux usées d'amidon (SIW)	Boues non hydrolysées (NH)	Boues hydrolysées (TH)	Culot de soya	Culot de SIW	Culot de NH	Culot de TH
Solides totaux (g/L)	21	15 - 17	25	25	102.8	57	92	98.8
Spores viables (10^8CFU/mL)	2.3	4.1	2.5	3.4	25	15	7.8	160
Entomotoxicité (10^9SBU/L)	10.5	14.9	11.7	18.7	22.9	21.2	16.2	22.2

À la lumière de ces résultats, il ressort que les bouillons fermentés des milieux résiduels SIW, TH et NH ont un nombre de spores viables et d'entomotoxicité supérieurs aux valeurs du milieu de référence de soya. Cela est dû au potentiel nutritif élevé de ces milieux résiduels pour la culture du Bt. Aussi, ces valeurs sont toutes élevées dans les différents culots de centrifugation. Cependant, étant donné qu'après la formulation du culot, le nombre de spores viables et l'entomotoxicité diminuent à cause de l'ajout des additifs de formulation et évidemment de l'eau (pour la bonne dissolution des additifs), il est important de chercher à augmenter le potentiel entomotoxique des différents culots en récupérant les composants actifs (surtout les composants actifs solubles) perdus dans les surnageants.

5.2. Ultrafiltration des surnageants de centrifugation

5.2.1. Taille de la membrane – Volume du rétentat – taux de récupération

L'étude de sélection de la membrane d'ultrafiltration a permis le choix de la membrane de 5 kDa. Avec une telle membrane, la récupération des composants actifs (spores viables, entomotoxicité, protéases et chitinase) a donné un meilleur résultat dans un volume de rétentat variant entre 10 et 20% du volume du surnageant. En effet, l'augmentation d'entomotoxicité dans les rétentats de soya, SIW, NH et TH sont respectivement 7.9%, 10.5%, 9.0% et 5.7% des entomotoxicités des surnageants respectifs. La faible augmentation de TH est due aux pertes des composants actifs sur la membrane d'ultrafiltration. Ainsi, une étude de bilan de masse effectuée avec la filtration de 1 L de surnageant, a montré que la rétention des matières en suspension au niveau de la membrane varie suivant les milieux. Cette rétention, qui est une adsorption physique sur la membrane, est très élevée pour les surnageants des boues hydrolysées (68% des matières en suspension contre 15%, 12%, 7% respectivement pour Soya, SIW et NH). En ce qui concerne la quantification des effets des composées de virulence (protéases et chitinases) à travers la mesure d'entomotoxicité du mélange culot – rétentat, le meilleur ratio (4 g de culot + 1 mL de rétentat) a donné une augmentation de l'entomotoxicité de 4345, 1552 1386 et 475 (10^6 SBU/L) respectivement pour soya, SIW, NH et TH. On remarque encore ici des pertes considérables des composés de virulence sur la membrane. À la lumière de ces résultats, une optimisation des paramètres opératoires pour limiter les pertes sur la membrane et une étude du colmatage pour évaluer les possibilités de mise en échelle du procédé d'ultrafiltration s'avèrent nécessaires.

5.2.2. Optimisation du flux d'alimentation et de la pression transmembranaire

Les valeurs optimales de la pression transmembranaire et du flux d'alimentation sont respectivement 90 kPa et 550 $L.h^{-1}.m^{-2}$ pour le milieu semi-synthétique de soya, SIW et NH. Ces valeurs sont 110 kPa et 720 $L.h^{-1}.m^{-2}$ pour TH. Avec ces conditions optimales, les valeurs des concentrations des spores viables de soya, SIW, NH et TH sont respectivement de $4.3x10^6$, $6.1x10^6$, $6.0x10^5$ et $7.1x10^6$ (CFU/mL). Le fait que les milieux de soya, SIW et NH aient une

même condition optimale peut être justifié par leur viscosité plus ou moins identiques (± 1.3 mPa.s) contre 1.8 mPa.s pour celle de TH. Cette valeur élevée de la viscosité de TH justifie la facilité de colmatage par rapport aux autres milieux, d'où les valeurs de pression transmembranaire et de flux d'alimemtation élevés, afin de réduire le colmatage et maintenir le flux du perméat plus ou moins constant. Étant donné que les milieux de soya, SIW et NH ont une même condition optimale et une même viscosité, l'étude de mise en échelle est effectuée avec un de ces milieux en l'occurrence SIW et le milieu de TH.

5.2.3. Étude de colmatage – Approche de mise en échelle

L'approche de mise en échelle du procédé d'ultrafiltration est basée sur l'étude de colmatage. Elle est faite avec une membrane de 0.2 m^2 avec 2 L et 4 L des surnageants de SIW et TH. L'augmentation du colmatage de la membrane évaluée à travers la variation du flux du perméat peut être subdivisée en trois phases dont une stationnaire et plus longue (1 h 18 min 52 sec pour SIW et 1 h 57 min 20 s pour TH). Ainsi, à l'opposé des flux des perméats qui diminuent, les pertes en solides totaux sur la membrane augmentent et sont plus élevées dans le cas de TH que celui de SIW. La phase stationnaire, dans laquelle la résistance due au colmatage est plus élevée, semble être plus constante dans le cas de SIW que TH.

Cette résistante de la membrane dont la valeur apparente calculée dans le cas de la filtration de 2 L est $3.5x10^{12}$ et $1.8x10^{12}$ (m^{-1}) respectivement pour SIW et TH. Avec une filtration de 4 L de surnageant, ces valeurs sont $1.2\ x10^{12}$ (m^{-1}) pour SIW et $2.1x10^{13}$ (m^{-1}) pour TH. La valeur élevée de la résistance apparente dans le cas de TH s'explique par la difficulté de filtration de surnageant de ce milieu à cause de la petite taille de ses particules et de sa viscosité élevée. Ces résultats montrent qu'avec une membrane de 0.2 m^2, une filtration de 2 L et 4 L de surnageant de SIW donne un même ordre de grandeur de la résistance de la couche de colmatage (10^{12}). Par contre, pour la même surface de membrane et dans le cas du surnageant de TH, l'ordre de grandeur de la résistance de la couche de colmatage est multiplié par 10 quand on passe de 2 L à 4 L de surnageant. Étant donné que les paramètres opératoires sont optimisés avec un nombre élevé de spores viables, une augmentation de la pression transmembranaire ou du flux du feed dans le cas

de TH, dans le but de réduire la résistance de la couche de colmatage, peut entraîner une destruction de la membrane ou affecter la viabilité des spores.

5.3. Formulation anti - UV

L'étude des effets des radiations UV sur les composants actifs des quatre milieux montre qu'en absence des additifs de protection, par rapport aux milieux synthétiques, les milieux issus des boues secondaires (hydrolysées ou non) présentent plus de protection naturelle contre les radiations UV. Les demi-vies d'entomotoxicité sont de 3.4 et 3.25 jours respectivement pour NH et TH, contre 1.9 et 1.8 jours pour les milieux de soya et des eaux usées d'industrie d'amidon respectivement. Cette différence de demi-vies d'entomotoxicité entre les boues secondaires et les milieux de soya et SIW peut être essentiellement expliquée par le fait qu'à une longueur donnée, l'absorbance en fonction du temps des radiations par un milieu dépend de sa composition et de ses caractéristiques. Il apparait donc que les boues secondaires absorbent plus longtemps dans les UV que les milieux de soya et des eaux usées d'industries d'amidon. Cela est dû au fait que les boues contiennent des composés chromophores et auxochromes comme la mélanine, des acides fulviques et humiques qui absorbent dans les UV (Manka *et al.*, 1974), et par conséquent, jouent un rôle plus protecteur contre ces radiations que les milieux de soya et SIW.

En présence des additifs de protection, les études démontrent que: (1) le choix des additifs dépend du milieu de culture, et pour un milieu donné, le niveau de protection des composants actifs contre les radiations UV est fonction de la concentration de l'additif ainsi que de leurs intervalles d'absorption dans l'UV: (2) l'acide p-amino benzoique à 0.20% p/p donne une meilleure protection avec les milieux de SIW et soya avec des demi-vies respectives de 7.8 et 5.9 jours avec des pertes d'entomotoxicité au bout de 6 jours de 44 et 53% respectivement contre 90% en absence des additifs; (3) l'acide lignosulfonique à 0.20% w/w est efficace pour les boues hydrolysées et non-hydrolysées avec respectivement 7.25 et 8 jours de demi-vies d'entomotoxicité avec une perte d'entomotoxicité au bout de 6 jours de 45% contre 67% en absence des additifs.

5.4. Formulations antimicrobiennes

Après trois ans de conservation, aucune contamination par les microorganismes visés n'a été décelée dans presque tous les échantillons sauf dans les contrôles de TH et NH ainsi que les mélanges de SIW et de Soya contenant respectivement 0.1% p/p de métabisulfite de sodium et 0.1% p/p d'acide ascorbique. Cela peut être justifié par le fait que, d'après les résultats des pH, l'ajout de 0.1% p/p de concentration de chacun des trois additifs n'a pas fait varier le pH des mélanges par rapport à ceux des contrôles. Les pH des formulations avec 0.3% p/p et 0.5% p/p de concentrations d'additifs varient entre 5.5 et 6. Les résultats d'entomotoxicité au bout de trois années, ont donné une perte de potentiel dans toutes les formulations des quatre milieux ainsi que dans les contrôles. Ces pertes sont parfois plus significatives pour les concentrations de 0.3% w/w que celles de 0.5% p/p. En ce qui a trait à l'efficacité des additifs évalué à travers les résultats des spores viables et d'entomotoxicité, l'acide propionique donne de meilleurs résultats dans les cas de soya, SIW et NH avec des concentrations respectives de 0.5%, 0.5% et 0.3% (p/p). Pour le milieu de TH, c'est le métabisulfite de sodium qui paraît le plus efficace à 0.3% p/p.

5.5. Optimisation du procédé de séchage par pulvérisation pour la production des poudres humides de biopesticides

Les concentrations des bouillons fermentés des eaux usées d'amidon (SIW) et des boues hydrolysées (TH) ayant servi à alimenter le spray dryer sont de 15 g/L et 25 g/L respectivement. Pour toutes les expériences du plan composite centré, les valeurs des températures de sortie sont relativement faibles pour les deux milieux, et sont loin d'affecter les spores et les cristaux de protéines insecticides. De même, les valeurs d'humidité sont presque toutes inférieures à 10%. Ainsi donc, seules les valeurs de spores viables et de l'entomotoxicité sont utilisées pour les analyses de l'ANOVA de la méthode de réponse en surface. En prenant les spores comme réponses, la détermination des conditions optimales de production des poudres humides a donné des résultats avec de bons coefficients de corrélations 92% et 94% respectivement pour SIW et TH. Ces coefficients sont certes faibles avec l'entomotoxicité comme réponses (57% pour SIW et 58% pour TH). Ces bas coefficients obtenus avec l'entomotoxicité peuvent s'expliquer par le

fait que la mesure de l'entomotoxicité fait intervenir d'autres paramètres tels que les insectes tests, la diète et les conditions environnementales (température et humidité extérieure) qui peuvent influencer les valeurs obtenues. Étant donné que les faibles coefficients de détermination obtenus avec les résultats d'entomotoxicité ne pouvent qu'expliquer partiellement les variabilités d'un modèle, seules les réponses des spores viables sont utilisées pour déterminer les conditions optimales de séchage des bouillons fermentés.

Ainsi, le débit d'alimentation de 0.29 g/min, le débit d'aspiration d'air chaud de 0.51 m^3/min, la température d'entrée de 180°C et la pression d'atomisation de 0.10 MPa sont les valeurs optimales des variables indépendantes. Cette condition optimale donne un nombre de spores viables élevé de $2.2x10^8$ CFU/mg contre $2.7x10^8$ CFU/mg dans le bouillon fermenté initial, ce qui donne une perte de 18%. Les valeurs optimales dans le cas de TH sont 0.45 g/min, 0.49 m^3/min, 170°C et 0.096 MPa avec un nombre de spores viables de $1.3x10^8$ CFU/mg contre $1.5x10^8$ dans le bouillon fermenté initial de TH (soit une perte de 13 %). Avec ces conditions optimales, les valeurs d'entomotoxicité (10^8 SBU/g) sont de 7.20 pour SIW et 5.99 pour TH contre 9.98 et $7.31x10^8$ dans leurs bouillons fermentés respectifs. Ce qui donne une perte de 28% dans le cas de SIW et de 18% dans le cas de TH. Ces pertes peuvent être expliquées par la diminution de viabilité des composants actifs de Bt (spores, cristaux de protéines insecticide, les enzymes) due aux effets combinés de la température d'entrée et la pression d'atomisation air pendant le séchage. Le fait que cette perte est plus élevée dans le cas de SIW peut-être dû à la faible concentration en solides totaux du bouillon fermenté. En effet, cette concentration donne moins de composants actifs et plus d'humidité dans la gouttelette d'eau, ce qui demande plus de temps pour l'évaporation de l'eau. Ainsi, à haute température, les composants actifs d'un tel milieu consomment plus de chaleur et plus longtemps que pour un milieu de concentration plus élevée.

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Chapitre 2.
Ultrafiltration

PARTIE I

(Résultats des objectifs 1 et 2)

ULTRAFILTRATION RECOVERY OF ENTOMOTOXICITY FROM SUPERNATANT OF *BACILLUS THURINGIENSIS* FERMENTED WASTEWATER AND WASTEWATER SLUDGE

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RÉSUMÉ

L'étude porte sur la récupération des composants actifs (cristaux de protéines insecticides, spores viables et d'autres facteurs de la virulence) du *Bacillus thuringiensis* (Bt) par ultrafiltration des surnageants des bouillons fermentés de biopesticides à base de Bt. Il s'agit des bouillons fermentés des eaux usées d'industrie d'amidon, des boues non-hydrolysées, des boues hydrolysées et le milieu semi-synthétique du soja (comme milieu de référence). Les résultats ont montré que l'ultrafiltration, avec une membrane de 5 kDa, a donné le taux le plus élevé de récupération des composants actifs. On a ainsi une augmentation d'entomotoxicité dans les rétentats de 7.9 %, de 10.5 %, de 9.0 %, de 5.7 % respectivement pour le milieu semi-synthétique de soja, les eaux usées d'industrie d'amidon, les boues non-hydrolysée et les boues hydrolysée. Cependant, la rétention des solides en suspension sur la membrane (mesurée à travers le calcul du bilan de masse) varie suivant les milieux des bouillons fermentés, et est très élevée avec le milieu des boues hydrolysées (milieu semi-synthétiques de soja-15%; eaux usées d'industrie d'amidon-12%; boues non-hydrolysées-7% et boues hydrolysées-68%). Ceci reflète le dépôt de la matière sur la membrane. Dans ce contexte, une étude d'approche de mise à l'échelle du procédé d'ultrafiltration donnerait un meilleur résultat pour les milieux des eaux usées d'amidon et des boues non-hydrolysées par rapport aux milieux de soja et des boues hydrolysées.

Mots-clés: *Bacillus thuringiensis*, Entomotoxicité, Enzymes, Ultrafiltration, Eaux usées, Boues d'épuration.

ABSTRACT

The study investigates the recovery of active components (insecticidal crystal proteins, viable spores and other factors of virulence) of *Bacillus thuringiensis* (Bt) based biopesticides from centrifuged supernatant, by ultrafiltration. The centrifuged fermented broths comprised, starch industry wastewater (SIW), non-hydrolyzed (NH) and hydrolyzed (TH) wastewater sludges and semi-synthetic soya medium (as control). The ultrafiltration membrane of 5 kDa gave the highest recovery of the active components and increased the entomotoxicity in the retentates by 7.9 %, 10.5 %, 9.0 %, 5.7 %, for semi-synthetic soya medium, starch industry wastewater, non-hydrolyzed and hydrolyzed wastewater sludges, respectively. However, the retention of suspended solids on the membrane (measured via mass balance) varied with the type of fermented broths and was very high for hydrolyzed sludge (soya-15%; starch industry wastewater -12%; non-hydrolyzed sludge-7% and hydrolyzed sludge-68%). This reflected the deposit on the membrane. In the given context, scale-up of the ultrafiltration process will give better efficacy for non-hydrolyzed sludge- and starch industry wastewater in comparison to soya and hydrolyzed sludge medium.

Keywords: *Bacillus thuringiensis*, Entomotoxicity, Enzymes, Ultrafiltration, Wastewater, Wastewater sludge.

ABRÉVIATIONS

C_{feed}	Concentration of suspended solids in feed (g/l)
$C_{permeate}$	Concentration of suspended solids in permeate (g/l)
$C_{retentate}$	Concentration of suspended solids in retentate(g/l)
J_w	Flux of Permeate (m/s)
kDa	kilo Dalton
L_p	Permeability of pure water (m/Pa/s)
M	Membrane
$M_{membrane}$	Mass of suspended solids on the membrane
MWCO	Molecular weight cut-off
NH	Non-hydrolyzed sludge
n.d.	Not detectable
P	Permeate
TMP	Transmembrane pressure gradient (Pa)
Q_{feed}	Feed flux (l/h/m^2)
R	Retentate
RCF	Relative centrifugal force (g)
R_m	Intrinsic resistance of membrane (/m)
R_{UF} (%)	The efficiency of recovery of UF
S	Supernatant
SS	Suspended solids (g/l or in %)
SIW	Starch industry wastewater
TH	Thermal alkaline hydrolyzed sludge
TS	Total solid
Tx	Entomotoxicity (SBU/µL)
Tx_{feed}	Entomotoxicity in feed or supernatant (SBU/µl)
Tx_R	Entomotoxicity in retentate (SBU/µl)
Tx_p	Entomotoxicity in permeate (SBU/µl)
μ_m	Viscosity of pure water (Pa.h)
V_{feed}	Volume of the feed (l)
$V_{permeate}$	Volume of the permeate (l)
$V_{retentate}$	Volume of the retentate (l)

1. INTRODUCTION

In order to fulfill the objectives of sustainable development, *Bacillus thuringiensis* (Bt) biopesticides is a control agent against insect pests belonging to different Orders, Lepidoptera, Diptera and Coleoptera [1]. Bt is a Gram positive bacterium, characterized by the production of the insecticidal crystal proteins [2]. In addition to various synthetic media used hitherto for Bt production, several studies constituting the residual biomass, in particular, wastewater and wastewater sludge have been carried out with encouraging results: process optimization in shake flasks and bioreactors [3-5]; enhancement of entomotoxicity by pre-treatment [6]; centrifugal recovery of the active components and development of stable liquid formulation [7, 8]. In addition to the principal contributors of entomotoxicity, namely, crystal proteins and viable spores, there are various other factors like proteases, chitinases, phospholipases, vegetative insecticidal proteins (Vips) and other unknown components which contribute to entomotoxicity [9, 10].

Among the various traditional and advanced techniques for recovery of the active components from the fermented broth, the centrifugation technique is most commonly used [8]. However, Brar [8] observed that in spite of good performance of centrifugation process for Tx recovery (SIW 95 %; NH-90 %; TH-98 % and soya-78 %), the soluble active components responsible for virulence cannot be completely recovered. The substantial losses in the supernatants are due to insecticidal crystals proteins, spores, cells and especially, the soluble components (various enzymes and Vips) which have molecular masses of approximately 30 kDa. Thus, there was a substantial loss of virulence factors of insecticidal activity in the supernatant following centrifugation.

Earlier studies conducted on the recovery of active components from the supernatant by the processes of adsorption, evaporation and precipitation reported lower efficacy [11-13]. Other current methods of separation by extraction like association of polyethylene glycol (PEG)/salt (K_2HPO_4) [14] were long, laborious and the scale-up was difficult. In this context, ultrafiltration (UF) was considered as an effective technique, which can be used for the complex biological

mixture yielding high recovery and high purity. In fact, the ultrafiltration process has been actively employed for the recovery of organic compounds from several synthetic media [15-20]. The selection of the ultrafiltration membrane is a function of the molecular weight cut-off of insecticidal crystal proteins (135 kDa), spore coat protein size (25-30 kDa) and Vips (approximately, 80 kDa). In fact, particles not recovered by the centrifugation process depended on the centrifugal force as detailed by Brar *et al.* [8]. This explained the influence of the centrifugation results on ultrafiltration.

The principal objective of this study was to employ UF process to recover the entomotoxicity or entomocidal components lost in the supernatant of centrifuged fermented broths of non-hydrolyzed sludge, hydrolyzed sludge, starch industry wastewater and semi-synthetic soya medium. The study will comprise various specifique objectives: a) screening of different size membranes to achieve maximum recovery; b) to compare and correlate the UF efficiency at the selected membrane size in terms of various physical (turbidity and suspended solids) and biological (spores viable, entomotoxicity and enzymes) parameters; c) to study the effect of the virulence factors on the entomotoxicity and; d) the possibility of scaling up the process of ultrafiltration for the alternative media.

2. MATERIALS AND METHODS

2.1. Bacterial strain

The bacterial strain used in this study was *Bacillus thuringiensis* subspecies *kurstaki* HD-1 (ATCC 33679). The techniques of culture, maintenance, inoculum and fermentation (for the production of biopesticides) were performed as per earlier studies [5].

2.2. Culture media of Bt

2.2.1. Media and characteristics

The culture media used in this study were: (i) semi-synthetic soya medium (used as a control), that comprised (g/l) soybean meal, 15.0; glucose, 5.0; starch, 5.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.3; $FeSO_4$. $7H_2O$, 0.02; $ZnSO_4$. $7H_2O$, 0.02; $CaCO_3$, 1.0; (ii) starch industry wastewater (SIW) from ADM-Ogilvie (Candiac, Québec, Canada); (iii) secondary sludge from wastewater treatment plant of Communauté Urbaine de Québec (Québec, Canada). The characteristics of different culture media are presented in Table 1. All analytical methods used for characterization were adopted from Standard Methods [21]. After sampling, the starch industry wastewater was directly used for fermentation or was stored at 4°C and was used within one week.

2.2.2. Solids amendment and pre-treatment procedure

The starch industry wastewater (SIW) samples containing total solids (TS) concentration of 17 g/l was directly used for fermentation without pre-treatment but just after sterilization at 121±1°C and 15 psig for 30 min. The secondary wastewater sludge was concentrated approximately from 16 g/l to 50 g/l by centrifugation at 7650 g for 15 min at 20±1°C. The sludge supernatant was used to dilute the samples as per requirements. The homogenization of wastewater sludge was

carried out by using the ''Waring blender''. For hydrolysis, 10 liters of wastewater sludge at TS concentration of 50 g/l was transferred to stainless steel hydrolyzer (SS 316l) equipped with superheated injection and controlled agitation system. The conditions for hydrolysis were 140±1°C at a pressure of 275 kPa (40 psig) for 30 min. [6]. Henceforth, raw and hydrolyzed sludge were designated as NH and TH, respectively. As all the samples are used within a period of a week, there was no significant change in their characteristics.

2.3. Fermentation

Fermentation was conducted in a bioreactor of 15 l (Biogénie Inc., Québec, Quebec) with accessories, connected to a computer with the *iFix 3.5, intellution* software, (Massachusetts, USA) for the control of pH, temperature, air flow, agitation and anti-foam. The detailed procedure of fermentation has been already described by Brar [7]. Once the fermentation was complete, the fermented broth was collected and preserved according to the conditions established by Brar [7]. The temperature and pH of the fermented broth were lowered gradually from 30±1°C to 10±1°C and from 7±0.1 to 4.5±0.1, respectively. Subsequently, the fermented broth was aseptically collected in HDPE bottles of 12 liters (VWR Canlab, Canada) sealed with paraffin and preserved at – 20°C until further use.

2.4. Techniques of recovery of entomotoxicity from the fermented broths

2.4.1. Centrifugation

Fermented broths of the four media (soya, SIW, NH and TH) at pH 4.5 were aseptically centrifuged at 9000 g for 30 min according to the procedure detailed by Brar [7]. The various supernatants after the centrifugation process of the fermented broths were aseptically collected and stored at 4° C, until further used for ultrafiltration study.

2.4.2. Ultrafiltration

2.4.2.1. Operating principle and washing of the filter

The equipment used for ultrafiltration was of tangential flow filtration type (PREP/SCALE-TFF, Cartridges Millipore, Bedford, Massachusetts, US) with recirculation as shown in Fig 1a. The fluid was tangentially pumped along the surface of the membrane. Pressure was applied to force a portion of the fluid through the membrane to the permeate side. The membrane was made up of regenerated cellulose and was of the type: Spiral Wound TFF-1 Module PLCC with a surface area of $0.1m^2$. The cross-section of the membrane is presented in Fig 1b. The supernatant from centrifuge was fed into the ultrafiltration equipment by a pump (Casy Load, Master Flex, Millipore, Bedford, Massachusetts, US) The choice of tangential flow type of filtration was justified by the fact that, contrary to the normal flow system of filtration, fouling chances was reduced resulting in ease of washing.

The process consisted of feeding aseptically a volume V (1l) of the supernatant from the centrifugation step referred to as ''feed'' through the membrane in order to concentrate the active components to a concentrated volume referred to as "retentate" which was 20% of the volume of the supernatant [22]. The flow of the supernatant was obtained by means of a pump whose flow varied between 45 to 180 l/h, which gave a flow of feed through the membrane ranging between 450 and 1800 $l/h/m^2$. As for the permeate flow, it generally depended on the transmembrane pressure and the resistance of the membrane. After the ultrafiltration, the permeate and retentate were collected in pre-sterilized flasks. Sampling of the supernatant, retentate and permeate for various supernatants was carried out for measurements of physical and biological parameters. The supernatant was brought to room temperature (20 to 25°C) in order to conduct ultrafiltration study.

After each ultrafiltration operation, liquid in the membrane was completely drained (back-washed). Taking into account the type of medium used in this study (biological environment), it was recommended to use an alkaline solution (0.1 N NaOH). The alkaline solution was passed

through the membrane until the membrane was clean. Later, the membrane was removed and turned the other side to facilitate complete washing. The performance of the membrane depended on "Normalized Permeability Weight (NWP)". In fact, during the use of the membrane, the value of NWP decreased, and when the value lies between 10 and 20 % of its initial value (that of the new membrane), the membrane was changed. The ultrafiltration study was carried out in three stages:

2.4.2.2. Selection of membrane size

Before proceeding to the optimization of different process parameters, screening of membranes was carried out to select the best performance membrane. Thus, the supernatant of different fermented broths, namely, NH, TH sludge, SIW and semi-synthetic soya medium were passed at a fixed flux rate through the different membranes with molecular weight cut-off (MWCO) of 100, 30, 10 and 5 kDa. For the screening experiments, small membranes of Amocon ultra-15 centrifugal filter devices, with a working volume of 15 ml were used in order to minimize the losses. Samples of supernatant, retentate and permeate were drawn and biological parameters, namely, entomotoxicity (Tx) and viable spores (VS) were measured to judge the performance of each membrane.

2.4.2.3. Optimization of parameters of ultrafiltration

Important parameters to be controlled in UF are transmembrane pressure (TMP) and flux of the feed, "Q_{feed}" which gave the pressure of the feed measured at the entry of the membrane. For the optimization, the experiment was carried out for various values of feed fluxes (450 to 1800 l/h/m^2) and TMP was controlled by the gauge retentate pressure (measured at the outlet of the membrane) so as to have the minimal possible flow of the permeate. In a typical UF process, lower permeate flow results in higher solute concentration in the retentate. The semi-synthetic soya medium, used as a reference, was employed for the optimization study. Samples were drawn to determine the total cells, viable spores and turbidity in the retentate and permeate. Thus, the optimal flux of the feed was the one which gave higher concentration of the active components in the retentate, and the one which preserved the quality of the product with lower shear.

2.4.2.4. Comparative study of four culture media

After selecting the membrane and the process optimal conditions for ultrafiltration, a study of four culture media (soya, SIW, NH, TH) was conducted to recover the entomotoxicity from various supernatants of centrifuged fermented broths. For each medium, 1 liter of supernatant was passed through the membrane under the optimal conditions (optimal pressure and flux) to attain 0.2 liter of retentate. Sampling of the supernatant, retentate and permeate of each medium was carried out to determine the physical and biological parameters. The efficiency of UF recovery which was measured in terms of entomotoxicity was given by the Equation:

$$R_{UF}(\%) = \frac{Tx_{feed} - Tx_P}{Tx_{feed}} \times 100 \quad (1)$$

2.5. Effect of virulence factors on entomotoxicity

The virulence factors are active components, which synergize in augmenting the entomotoxicity. In fact, Bt biopesticides are generally used in the form of formulated products which possess higher entomotoxicity (Tx). This necessitates achievement of highest possible Tx at the end of fermentation. Thus, in order to increase the entomotoxicity, the Bt toxin and spores are recovered by centrifugation process. However, some virulence factors are lost in the supernatant. Furthermore, the centrifugate needs to be mixed with supernatant (from centrifugation) or retentate of UF to recover the lost Tx (as discussed earlier). Additionally, it is important to dilute the centrifugate to obtain a suitable dilution to enhance handling and ease of application. Dilution of centrifugate with retentate (containing virulence factors in concentrated form) will not only adjust the required moisture content but will also enhance the Tx value due to the fact it contains virulence factors in higher concentration. Thus, to define the degree of synergism and suitable moisture content, 1ml of retentate (of respective supernatants) was added to different quantities (1 g, 2 g, 4 g) of centrifugate. The moisture (in % w/w) of centrifugate and the mixture of soya, SIW, NH and TH was measured by using HR 83 Halogen Moisture analyzer (Mettler Toledo, Ontario, Canada). Samples (centrifugate-retentate and centrifugate-saline mixtures) were

subjected to Tx determination. To evaluate the efficiency of UF in term of entomotoxicity, gain of Tx was determined by the Equation 2:

$$Tx(gain) = Tx[mixture(centrifugate + retentate)] - Tx[mixture(centrifugate + \sup erna\tan t)] \quad (2)$$

The effect of dilution was determined by measuring the entomotoxicity of the mixture of centrifugate and saline (0.85 % NaCl without virulence factors) prepared in the similar manner.

2.6. Analysis of parameters

2.6.1. Physical parameters (turbidity and suspended solids)

The turbidity permits determination of the physical effectiveness (total solids and in particular, suspended solids) of UF. It may be also a good biological indicator due to the fact that the spores, the crystals proteins and other virulence factors (protease, chitinase, phospholipase) could be adsorbed on the suspended solids. Turbidity was measured by utilizing a Macro 100 Turbidimeter (de Scientific Inc., Florida, US) with interval of measurement varying from 0 to 1000 NTU at 720 nm. The suspended solids were determined by filtration through the 0.45 μm membrane (Glass Microfibre Filter 934-AH de 42.5mm, Whatman) followed by drying at 105±1°C. The standard deviation of measurement was 3 to 5%. The results of the suspended solids are expressed in the form of mass balance according to the relation:

$$C_{feed}V_{feed} = C_{Retentate}V_{Retentate} + C_{Permeate}V_{Permeate} + M_{membrane} = 100\% \quad (3)$$

2.6.2. Biological parameters

2.6.2.1. Viable spores (VS)

The counting of VS was conducted according to the procedure of Vidyarthi [5] with a slight modification made by Brar [8]. It comprised heating the sample at 80 °C for 10 min in an oil bath (Thermo-lift, Buchler instrument, USA). The sample was finally cooled for 5 min before spreading on the tryptic soya agar plate. The standard deviation was 7 to 8%.

2.6.2.2. Entomotoxicity

Entomotoxicity (Tx) was measured by the bioassay method by using the 2nd instar spruce budworm larvae (*Choristoneura fumiferana*) imported from Natural Resources Canada (Sault Ste-Marie, Ontario). The larvae were reared on artificial diet for 4 days to obtain the third and fourth instar stages. The bioassays were conducted using the methods of Beegle [23]. Five dilutions for each sample were prepared in a saline solution of 0.85 % (w/v). Each 1.5 ml of dilution was mixed with 30 ml of diet according to the composition given by Tirado-Montiel [4]. The mixture thus obtained was distributed in a stack of 20 glass tubes of 15 x 45 mm (VWR, Canlab, Canada). The control contained 60 tubes with 1 ml of diet in each tube. Other controls were made by mixing 1ml of sterilized medium of soya, SIW, NH and TH with 30 ml diet. After solidification and cooling of the diet, a larva of third instar was introduced into each tube. The tubes were closed by perforated stoppers and kept at 25±1°C for 7 days. Later, the mortality in each stack of 20 tubes was evaluated. The mortality was compared to the mortality induced by the commercial forestry formulation of Foray 76B (Abbott Labs, Chicago, Il, USA) and was expressed in terms of relative spruce budworm potency unit (SBU). This product contains a mixture of spores and crystals of Btk at a potency of 20.1 x 10^9 IU/l (international Unit) measured against cabbage looper (*Trichoplusia ni*). The SBU values were 20 % to 25 % higher than IU values [24]. The standard deviation was approximately equal to 7 %.

2.6.2.3. Enzymatic activity (protease and chitinase)

The proteolytic activity was determined according to Kunitz [25] with minor modifications. The standard deviation of measurement was 8 %.

Chitinase activity measurement was based on quantity of N-acetyl glucosamine (NAG) obtained by reduction of colloidal chitin by the enzyme as described by Ueda and Arai [26]. For this purpose, 0.5 ml of the enzymatic solution was added to 1.0 ml of substrate containing 1.0 % of colloidal chitin suspension in an acetate buffer (50 mM, pH 4). The mixture was incubated at 50°C for 30 min. The reaction was concluded by heating the mixture in boiling water for 15 min. Subsequently, 2 ml of potassium ferricyanide (1.5 mmol/l) was added. The mixture was heated for 15 min and cooled at ambient temperature and then filtered. The filtrate was used to measure optical density with spectrophotometer at 420 nm. The chitinase activity was calculated by using the standard curve obtained by plotting the concentrations of NAG (0 – 0.15 g/ml). A unit of chitinase activity was defined as the quantity of chitin which released one μmol of NAG per minute at pH 4 and temperature of 50±1°C. Negative control contained all compounds in the mixture except the substrate and positive control contained all compounds in the mixture except the enzymatic solution. The standard deviation of measurements was 8%.

3. RESULTS AND DISCUSSION

3.1. Selection of membrane

Table 2 shows the results of screening of different membranes for ultrafiltration of Bt supernatant derived from different fermented broths. It was observed that 5 kDa gave best performance in terms of concentration of Tx and VS in the retentate and negligible losses in the permeate. Hence, 5 kDa was used as the molecular weight cut-off (MWCO) for subsequent studies on UF of Bt fermented broths of soya, SIW, NH and TH.

3.2. Selection of operating parameters of ultrafiltration

3.2.1. Transmembrane pressure, TMP

The TMP was given by the relation: $$TMP = \left[\frac{P_{feed} + P_{ret}}{2}\right] - P_{perm} \quad (4)$$

The permeate flux, a very important parameter in ultrafiltration process varied with TMP according to the relation:

$$J_w = L_p * TMP = \frac{TMP}{\mu_m R_m} \quad (5)$$

According to the ultrafiltration principle, minimum flow of permeate will result in minimum loss or no loss of solute (entomotoxic components, in present case) in the permeate and will provide higher concentration in the retentate. However, the minimal flow of the permeate involved a minimal pressure in the tube of the permeate. And due to low diameter (0.5 mm) of the permeate, one can neglect this pressure compared to that of the feed and the retentate. Taking into account the ultrafiltration equipment used in this study, the minimal flow of the permeate was obtained due to an optimal value of the TMP which is a function of the pressure of the retentate (measurement by the pressure gauge) via the retentate valve. Thus, for a given value of feed flow

(with known pressure of the feed), one can find a value of the pressure of the retentate to ultimately obtain an optimal transmembrane pressure. The transmembrane pressure thus obtained was maintained constant at 193 kPa (28 psig) with the help of a manual valve. This pressure corresponded to the minimal and constant flow of permeate as required by the basic principle of tangential flow ultrafiltration. Similar values of TMP have been reported for different studies of ultrafiltration like recovery of thuringiensin (4 % w/v) by micellar-enhanced ultrafiltration process with cellulose acetate membrane, which has 30 kDa as MWCO [17]. Other similar study on ultrafiltration of synthetic biological solution constituted from yeast extract and β-lactoglobulin with molecular weight cut-off (MWCO)of 15 kDa [19].

In this study, soya medium was used as reference and was employed for the determination of the optimal pressure. Moreover, supernatants of four fermented media, namely, soya, SIW, NH, TH used in this study demonstrated low viscosities of 1.7, 1.7, 1.3 and 3.0 mPa.s, respectively. The low viscosity can be explained by low suspended solids which was nearly 0.2 % w/v and total solids were approximately 2.0 % w/v for the four media. The viscosity changes were mainly due to the particle size which was small in the case of TH. Thus, the optimal TMP value obtained with soya could be applied for each medium. This will render a judicious comparison of the results, especially, concerning the retention of suspended solids on the membrane. It should be noted that the substances retained by the membrane (bulk concentration) can gradually block the pores and thus decrease the flux of the permeate. Thus, the maintenance of constant flux requires an increase in the TMP. But in this study, taking into account the volume of the feed (1l) and the flux of the feed, the phenomenon of membrane obstruction (pore block) lightly influenced the flux of the permeate.

3.2.2. Feed Flux

Profiles of turbidity, viable spores and total cell counts in the retentate vs. the feed flux are presented in Fig 2. These values were negligible in the permeate as size of the spore coat protein of Bt vary between 25 and 30 kDa [27] which is greater than the MWCO of the membrane (5 kDa). Soya medium was used as a reference for this study which was carried out by keeping the circulation of fluid at flow rate between 45 $l.h^{-1}$ (lowest speed) and 180 $l.h^{-1}$ (high speed

induced foam formation) through the pump. This range of pump flow rate corresponding to the feed flux between 450 to 1800 $l.h^{-1}m^{-2}$ was recommended by Millipore [21] according to the solute concentration. In fact, the feed flux through the membrane causes a transport of solute towards the surface of membrane.

A part of solute accumulates at the surface of membrane if the solute flow towards the membrane is greater than the solute passing through the membrane. This accumulation forms a concentration which is known as concentration polarization [28]. Contrary to the system of normal flow filtration, one of the main advantages was that tangential flow filtration accelerated the deposition of particles on the membrane. In all, tangential flow will significantly reduce the fouling of the membrane.

So, according to the total solids concentration of the media (2 % w/v) used in this study, this range of feed flux was suitable. Thus, the feed flux of 900 $l.h^{-1}m^{-2}$ gave highest values of total cell count concentration (3.6 E+06), viable spores (2.6 E+6) and turbidity (855 NTU) in the retentate. In fact, the total solids (particularly suspended solids) are good indicators of turbidity which can also be a biological indicator (as explained earlier). Thus, if the cell and viable spore count increase, the turbidity will also increase which can be a complex parameter comprising suspended solids and enzyme proteins. The cell and spore count decreased at the feed fluxes of 1400 $l.h^{-1}m^{-2}$ and 1800 $l.h^{-1}m^{-2}$. This decrease could be due to the fact that a high flux can also degrade the quality of the product due to the generation of turbulence effect [22]. In fact, at a flux of 180 $l.h^{-1}$ corresponding to a flow rate of 255 $m.s^{-1}$ (diameter of the feed pipe being 1 mm), and a density of the feed equalized to 2 % w/v, will lead to Reynolds number value of approximately 3000. This number being higher (> 2000), one can conclude a turbulent flow. However, higher turbulence can create intense foam in the retentate stream which will create a vacuum and further decrease the permeate flux below the optimum value and hence govern the overall performance of the UF system. For all the supernatants of fermented media used in this study, the total solids concentrations were nearly 2.0 % w/v, and that of the suspended solids was approximately 0.2 % w/v. Thus, the optimal flux determined with the supernatant of soya medium was applied to the supernatant of SIW, NH and TH. Moreover, as soya medium was used as a reference, it was preferable to compare the rest of the media under similar optimal

conditions as soya. The optimal value of feed flux (900 $l.h^{-1}m^{-2}$) corresponded to a Reynolds number of 1498 < 2000, therefore one can conclude that this optimal flow involved a laminar flow.

3.3. Study of four culture media

3.3.1. Physical parameters (Turbidity and suspended solids)

The mass balance of suspended solids in the supernatant, retentate and permeate are presented in Fig. 3. The concentration of SS was higher in the retentate; however, the mass balance calculations made it feasible to calculate the mass of suspended solids retained on the membrane. This retention varied according to the medium and was higher for TH at 68 % mass of suspended solids in the supernatant. This significant retention of suspended solids on the membrane could be due to the fact that the supernatant of TH showed higher viscosity (3.0 mPa.s) when compared to other media. In fact, the supernatant of TH contained higher concentration of dissolved solids (which increased the viscosity), and the suspended solids possessing smaller particle size [7]. Hence, TH supernatant possessed higher viscosity, which could increase the probability of concentration polarization on the membrane. The retention of suspended solids on the membrane in the case of supernatant of soya semi-synthetic medium, SIW and NH were respectively 15%, 12% and 7% of the mass of suspended solids in the respective supernatants. These values were relatively low.

Similarly, Table 3 showed that the value of turbidity in the retentate of TH was slightly lower than supernatant of TH. This reduction of turbidity in TH retentate could be due to the significant retention of suspended solids on the membrane. For soya, SIW and, NH, the values of turbidity in retentates were 855, 16 and 750 NTU, respectively in comparison to 51, 10 and 119 NTU for the respective supernatants owing to the increase of cell and VS count in the retentate. It was observed that in comparison to SIW, the turbidity in the retentates of soya and NH were higher than those in the respective supernatants. Moreover, the turbidity of supernatant of SIW was very low and there was also a small loss in the permeate of SIW. However, in order to improve the

ultrafiltration efficacy for supernatant of the fermented hydrolyzed sludge, it would be recommended to precede ultrafiltration by pre-filtration to decrease the viscosity and hence, the concentration of suspended solids in the supernatant.

3.3.2. Biological parameters

3.3.2.1. Viable spores and Entomotoxicity

The viable spores and entomotoxicity at different stages of centrifugation and ultrafiltration for four Bt fermented media are presented in Fig. 5. The viable spores and entomotoxicity in the permeate of supernatants of different fermented broths were not detectable (Fig. 5). The high Tx values in retentate were justified due to the fact that apart from spores (25-30 kDa) and insecticidal proteins (60 and 70 kDa) [29] Vips (about 80 kDa) were also recovered due to their higher molecular weight than MWCO of the membrane (5 kDa).

The viable spores in the retentate of soya, SIW, NH and TH were 1.14, 1.10, 1.07 and 1.03 log units, respectively, as compared to the respective supernatants (Fig. 5). The entomotoxicity in the retentate of soya, SIW, NH and TH were 8, 11, 9, 6 times as compared to the respective supernatants (Fig. 5). It should be noted here that the Tx value in the supernatants was of the same order (1389-1678 SBU/µl, Fig. 5). However, Tx value in TH retentate was very low as compared to others. The fact that Tx in permeate was not detectable, confIrmed that all factors responsible for Tx were recovered in the retentate stream. Therefore, it is evident that some of the entomotoxic factors (spores, toxins, Vips, chitinases, and proteases) were lost as a deposit on the membrane along with the solids. These factors may be adsorbed on the suspended solids, which in turn were deposited on the membrane and hence lost from the retentate. In case of TH, only 32 % of the suspended solids present in original supernatant were present in the retentate as suspended solids. The rest (68 % of SS) was lost or deposited on the membrane. This loss of solids on the membrane for other cases was much lower than TH (15 % - soya, 12 % -SIW, 7 % - NH). Thus, the Tx loss (due to deposition on the membrane) was higher in the case of TH than other cases (Soya, SIW and NH) providing comparatively lower Tx in TH retentate.

3.3.2.2. Protease and chitinase

The results of protease and chitinase analysis for different streams are presented in Table 3. It was observed that the proteolytic activity increased in the retentates of soya, SIW, NH and TH with the respective values of 2.65, 1.81, 2.25 and 2.62 IU/ml in comparison to 0.580, 0.44, 0.53 and 1.24 IU/ml in the respective supernatants. The proteolytic activity was negligible in different permeates. In comparison to soya, SIW and NH, the increase of proteolytic activity in the retentate of TH when compared to that of the respective supernatants was low. This could be explained by the high retention of virulence factors including enzymes on the membrane along with solids (as discussed earlier).

The chitinase activity was very high in retentate of NH (79 U/ml), when compared to the supernatant (25 U/ml). This was due to the fact that the losses of suspended matter on the membrane were very low in the case of NH. On the other hand, for TH, 68 % of the suspended matter in the supernatant was retained on the membrane and only 32 % was recovered in the retentate. This loss for TH justified the lower chitinase activity of the retentate compared to that of the supernatant (55 U/ml in the supernatant to 58 U/ml in the retentate), due to the adsorption of chitinase on the SS which was in turn deposited on the membrane. The chitinase activity was very low in supernatants of soya and SIW media, and consequently did not show an appreciable increase in the retentates. It has been already established that the presence of chitinase can increase the entomotoxicity through synergy as it enhances the penetration of various virulence factors into the midgut epithelial tissue of larvae [30]

Thus, to study the effect of proteases and chitinases in conjugation with other virulence factors on entomotoxicity, mixtures of centrifugate-retentate in different ratios were compared with mixtures of centrifugate-supernatant as described in the next section.

3.3.2.3. Synergy of virulence factors – repercussions

To define the effect of synergism, it was considered better to take into account ratio of 2:1 [2 g of centrifugate paste + 1 ml retentate (or 1ml supernatant)] and 4:1[4 g of centrifugate paste + 1 ml retentate (1 ml supernatant)]. However, the study of 1:1 ratio [1 g of centrifugate paste + 1 ml retentate] was carried out only for retentate samples of the four media, but they did not yield any synergism (data unreported). On the contrary, effects of dilution on the Tx values were prominent, and hence the study was not carried out for supernatant.

The results of entomotoxicity of the mixture of centrifugate and retentate in the ratio 2:1 and 4:1 for all the media are shown in Fig 5. It was observed that the effect of dilution evaluated for the centrifugate-saline mixture was well evident. In fact, the increase of entomotoxicity in the mixtures of centrifugate-supernatant and centrifugate-retentate when compared to the centrifugate-saline mixture demonstrated the effect of factors of virulence. The increase in entomotoxicity was higher in the centrifugate-retentate mixture in comparison to centrifugate-supernatant mixture or centrifugate-saline mixture. The efficacy in term of entomotoxicity of ultrafiltration in the mixtures was determined by the gain of entomotoxicity (Fig 5). Thus, for the three media (soya, SIW and NH sludge), it was noted that the values of the gain of entomotoxicity was higher in the 4:1 (4 g centrifugate paste + 1 g retentate) ratio (4345, 1552 and 1386 x 10^6 SBU/l) than the 2:1 ratio (3348, 1330 and 831 x 10^6 SBU/l). On the contrary, the gain of entomotoxicity (as defined in Equation 2) was lower for TH medium (475 x 10^6 SBU/l for the two ratios). Further, when the values of entomotoxicity of centrifugate-retentate mixture in the 4:1 ratio for the four media (soya, SIW, NH, TH) were compared with those of the respective centrifugates, it was noted that the increase of Tx by virulence factors in the mixture of centrifugate-retentate compensated for the effects of dilution. Thus, the centrifugate-retentate in the 4:1 ratio [4 g centrifugate +1 ml retentate (respective supernatant)] was recommended for the formulation of various fermented broths with enhanced synergism which varied in the following order: soya > SIW > NH > TH.

3.3.3. Applicability-UF Efficacy

In the light of the results presented above, the possibility of scale-up of the process of ultrafiltration for media investigated in this work will depend on two factors: losses of the active components on the membrane and/or value of Tx in the retentate. Concentration of suspended solids on the surface of the membrane for soya, SIW, TH and NH sludge were 0.035 %, 0.014 %, 0.094 % and 0.007% w/v, respectively. The entomotoxicity being a biological parameter, its losses on the membrane cannot be determined by difference in supernatant and retentate values as in case of suspended solids. Hence, Tx cannot be taken as a precise parameter for eventual scale-up as other parameters could intervene in the measurement of Tx, such as, viability and stress of the larvae.

Nevertheless, the VS deposit can be evaluated as a potential measure of Tx, through mass balance. In fact, by knowing the concentrations of VS in the supernatant and retentate, the VS deposited on the membrane can be evaluated by difference. Moreover, the volume of supernatant utilized was 1L and the retentate was 20 % as seen in Fig. 5. The number or quantity of VS in 1L of the supernatants of soya, SIW, NH and TH were 4.3×10^8, 1.5×10^8, 4.5×10^8 and 1.6×10^8 (CFU), respectively. Further, the number or quantity of VS in respective retentates (0.2 l) were 5.2×10^8, 8.8×10^7, 2.2×10^8 and 4.7×10^7 (CFU) (Fig. 5). Meanwhile, the VS count declined in the retentates. The decrease was due to the deposit of spores on the membrane along with other solids. Furthermore, Yezza *et al.* [31] have demonstrated an exponential relationship between Tx and VS. Thus, the deposit (loss) of spores on the membrane could be considered as an indirect parameter to measure loss of Tx.

The losses of VS on the membrane can throw light on the efficacy of UF system. Moreover, for scale-up of the process, it is necessary to maintain the flux of permeate constant by controlling the transmembrane pressure and by reducing the concentration in the bulk by regulating the feed flux (as discussed earlier). Within the framework of this study, one can evaluate the effectiveness of ultrafiltration system by mass balance according to the concentration of VS in the bulk/supernatant. In fact, the VS deposit on the membrane for the four different media varied in

the following order: NH > TH > SIW > Soya. Hence, the UF performance would follow the same order.

Despite the gain in entomotoxicity by ultrafiltration, a possibility of scale-up must also be taken into account for detailed techno-economic analyses of the process. However, UF studies yielded significant entomotoxicity recovery from supernatant of all Bt fermented broths. In fact, UF could be considered as a secondary step of centrifugation to recover the entomotoxicity from the fermented broth. This will subsequently lead to development of high potency formulation, which will have higher field efficacy. Further studies are in progress in our laboratory on the development of plausible method for determination of precise Tx loss on the membrane surface along with permeate flux variations, which will serve as a database for eventual scale-up. In the given context, UF can serve as a proponent entomotoxicity enhancement technology reaping benefits for Bt formulation development.

CONCLUSIONS

The following conclusions can be drawn from the preceding study on ultrafiltration of supernatant of Bt fermented broths of wastewater and wastewater sludge:

1) The membrane of 5 kDa was suitable to retain all the virulence factors in the retentate and hence, non-detectable spore count and entomotoxicity in the permeate.

2) The optimum flux to achieve maximum total cell and viable spore count in the retentate was found to be 900 L.h-1.m2.

3) There was a loss of suspended solids through deposition on the membrane. The loss was highest for thermal alkaline hydrolyzed sludge (68 %).

4) There was adsorption of virulence factors on the suspended solids and some of the virulence factors were concomitantly lost with the solids deposited on the membrane resulting in Tx loss.

5) The proteolytic activity was higher in the retentates of all fermented broths from different media whereas chitinase was higher only in the case of non-hydrolyzed sludge.

6) The efficacy of ultrafiltration when translated to formulation stage produced enhanced entomotoxicity with the mixture of 4 g centrifugate (with soya-78; SIW-89; NH-90; TH-90 calculated as moisture content) and 1 ml retentate. This formula will serve as a recipe for future formulations.

7) The ultrafiltration efficacy in terms of viable spores deposited on the membrane surface was in the order: non-hydrolyzed > hydrolyzed > starch industry wastewater > soya.

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Table 1. Characteristics of secondary wastewater sludge and starch industry wastewater

Parameter (s)	Secondary sludge		Starch industry wastewater	
TS (g/l)	18	±1.5	17	±1.1
TVS (g/l)	14	±1.1	14	±1.0
SS (g/l)	15	±1.0	2.2	±0.8
VSS (g/l)	13	±2	2.2	±0.7
pH	5.5	±0.1	3.3	±0.1
Concentration (mg/kg TS)				
C	301097	±5987	700345	±6986
N_t	42307	±500	37089	±1578
P_t	7987	±203	340176	±3001
$N\text{-}NH_3$	889	±198.4	109.8	±59.8
$N\text{-}NO_2^-$,$N\text{-}NO_3^-$	14.7	±1.1	4.8	±1.2
$P\text{-}PO_4^{3-}$	4988	±402	14987	±2801
Al	4999	±437	56987	±3798
Ca	14011	±511	11567	±402
Cd	3.01	±0.9	0.54	±0.1
Cr	27.9	±1.1	1.3	±0.04
Cu	401	±157	338	±171.2
Fe	11987	±603	7986.4	±803.4
K	998	±371	23056	±3124
Pb	27	±4.3	27.4	±4.7
S	4369	±538	2301.4	±62.5
Zn	325	±197	250	±77
Na	1456	±408	2189.4	±231
Ni	10.7	±3.9	-	-

± refers to the standard error

Table 2. Screening of different molecular weight cut-off membranes for ultrafiltration of Bt supernatant derived from different fermented broths

	Tx_R (x 10^9SBU/l)			
MWCO (kDa)	**100**	**30**	**10**	**5**
NH	5	9	11.5	13.5
TH	4	7.7	8.9	9.7
SIW	7	10.8	15.8	15
Soya	4.5	9.5	10.8	12
	Tx_P (x 10^9SBU/l)			
NH	4.2	3.5	2.5	n.d.
TH	3	2.6	1.7	n.d.
SIW	4.2	3.4	2.2	n.d.
Soya	3.8	2.5	1.5	n.d.
	VS_R (x 10^6CFU/ml)			
NH	1.5	2	5	20
TH	0.5	0.8	4.3	10
SIW	0.8	1.1	6.4	11
Soya	4.2	5	7.2	10
	VS_P (x 10^7CFU/ml)			
NH	11	1.5	0.5	n.d.
TH	11	0.5	0.1	n.d.
SIW	9	0.8	0.1	n.d.
Soya	7.3	4.2	0.35	n.d.

Table 3. Results of turbidity, protease and chitinase in supernatant, retentate and permeate of different media

	Turbidity (NTU)			Protease (IU/ml)		Chitinase (U/ml)	
	Supernatant	Retentate	Permeate	Supernatant	Retentate	Supernatant	Retentate
Soya	51	855	0.12	0.5800	2.646	nd	nd
SIW	10	16	0.24	0.4360	1.813	nd	nd
NH	119	750	0.20	0.5310	2.253	24.9500	78.77
TH	806	610	0.29	1.2440	2.617	54.7600	57.72

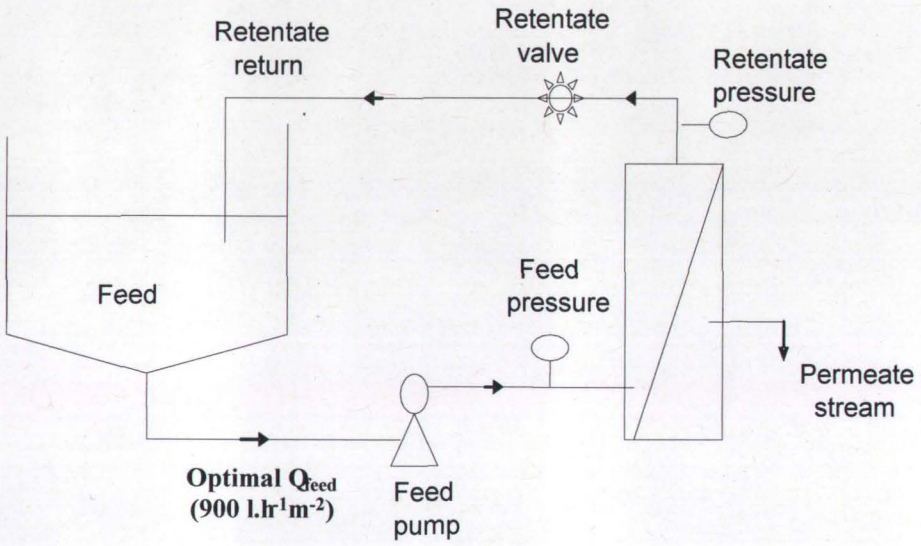

Fig 1a: Schematic representation of UF process

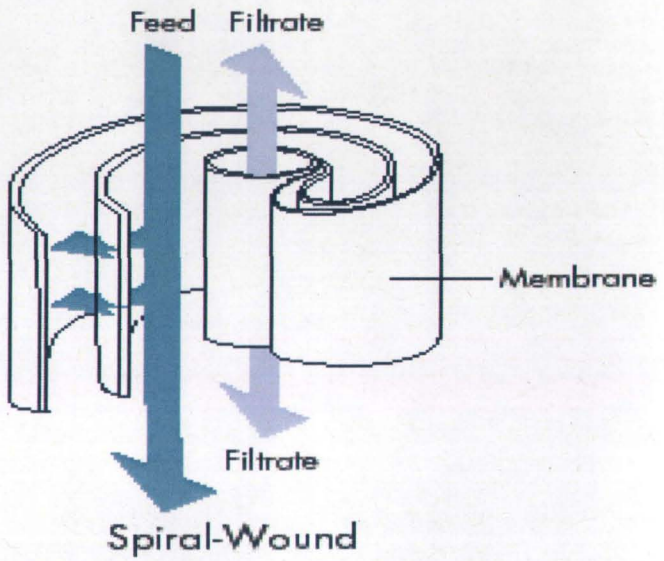

Fig 1b: Cross section of the membrane

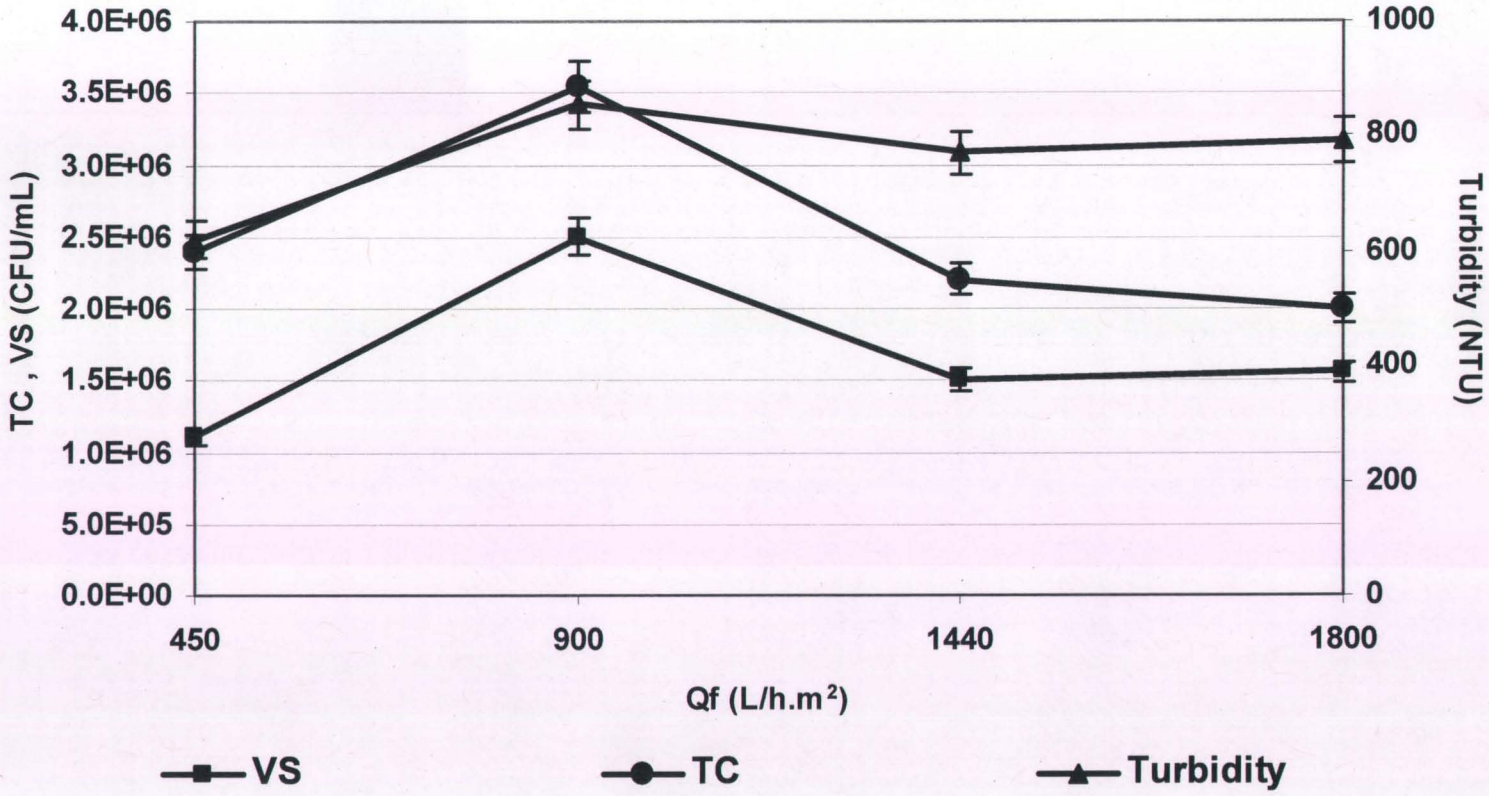

Fig 2. Variation of turbidity, cells and viable spores in the retentate of soya semi-synthetic medium at an optimal value of transmembrane pressure (193 kPa)

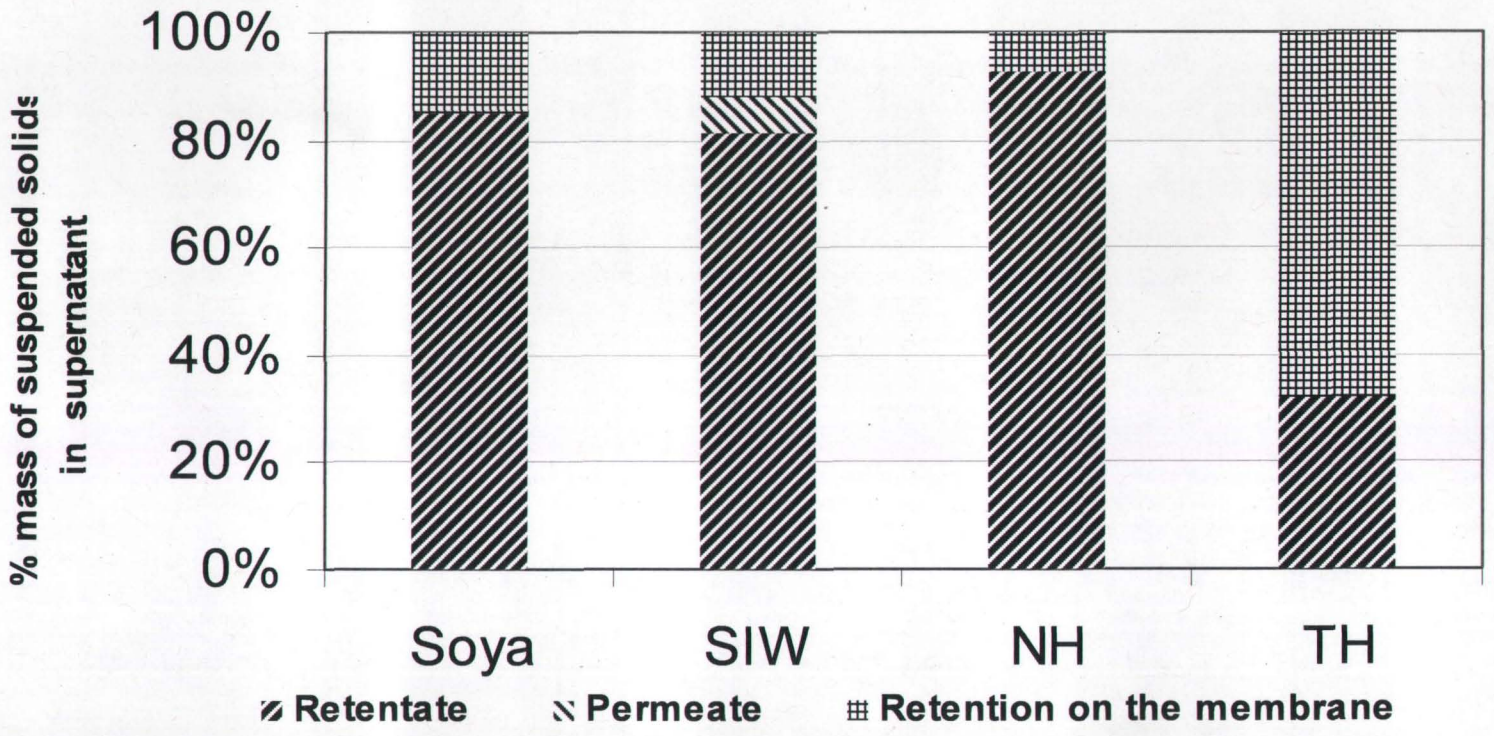

Fig 3. Mass balance of suspended solids for different supernatants of Bt fermented centrifuged media after UF (Percentage of mass of the suspended matter in the retentate and permeate were compared to the mass of suspended matter in the supernatants of various media).

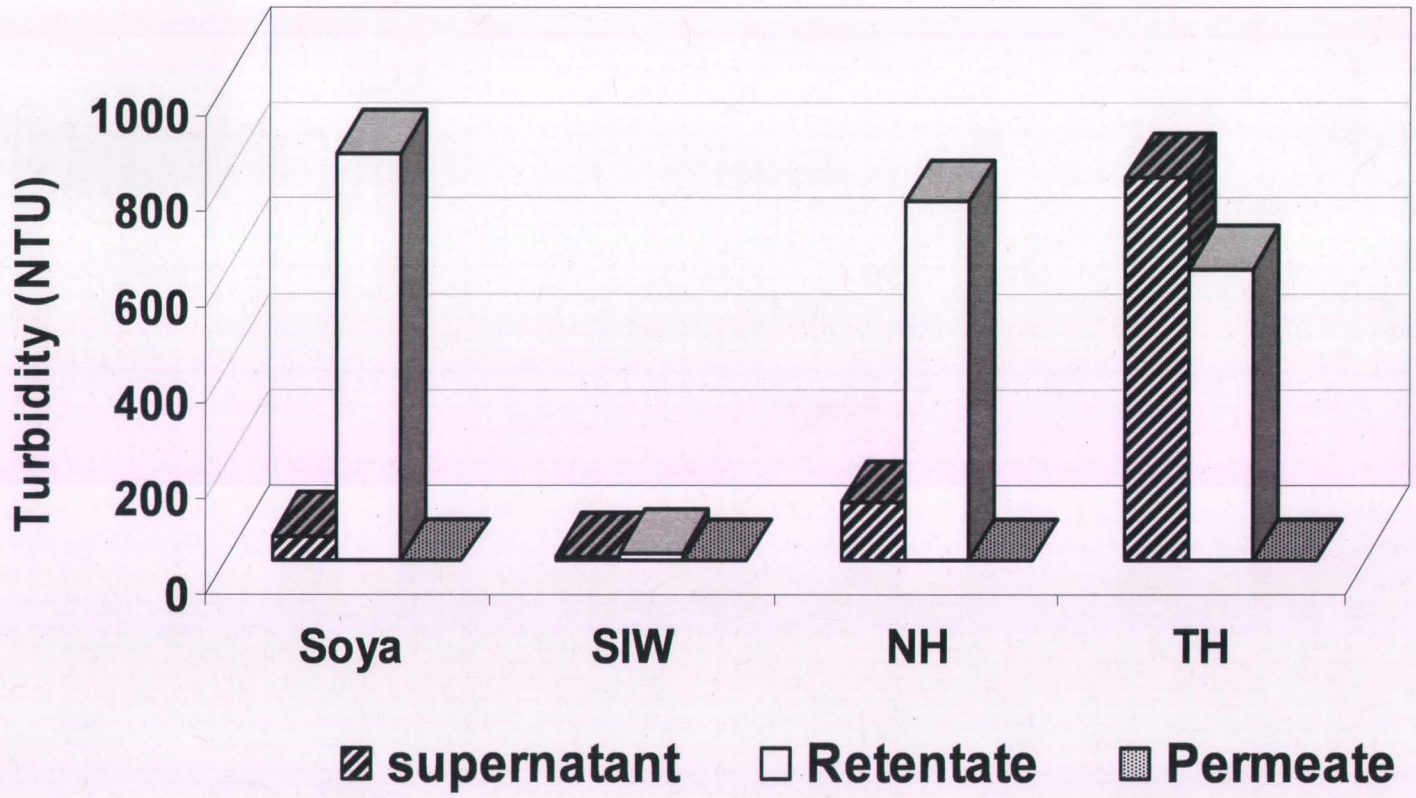

Fig 4. Profiles of turbidity in supernatant, retentate and permeate of different media

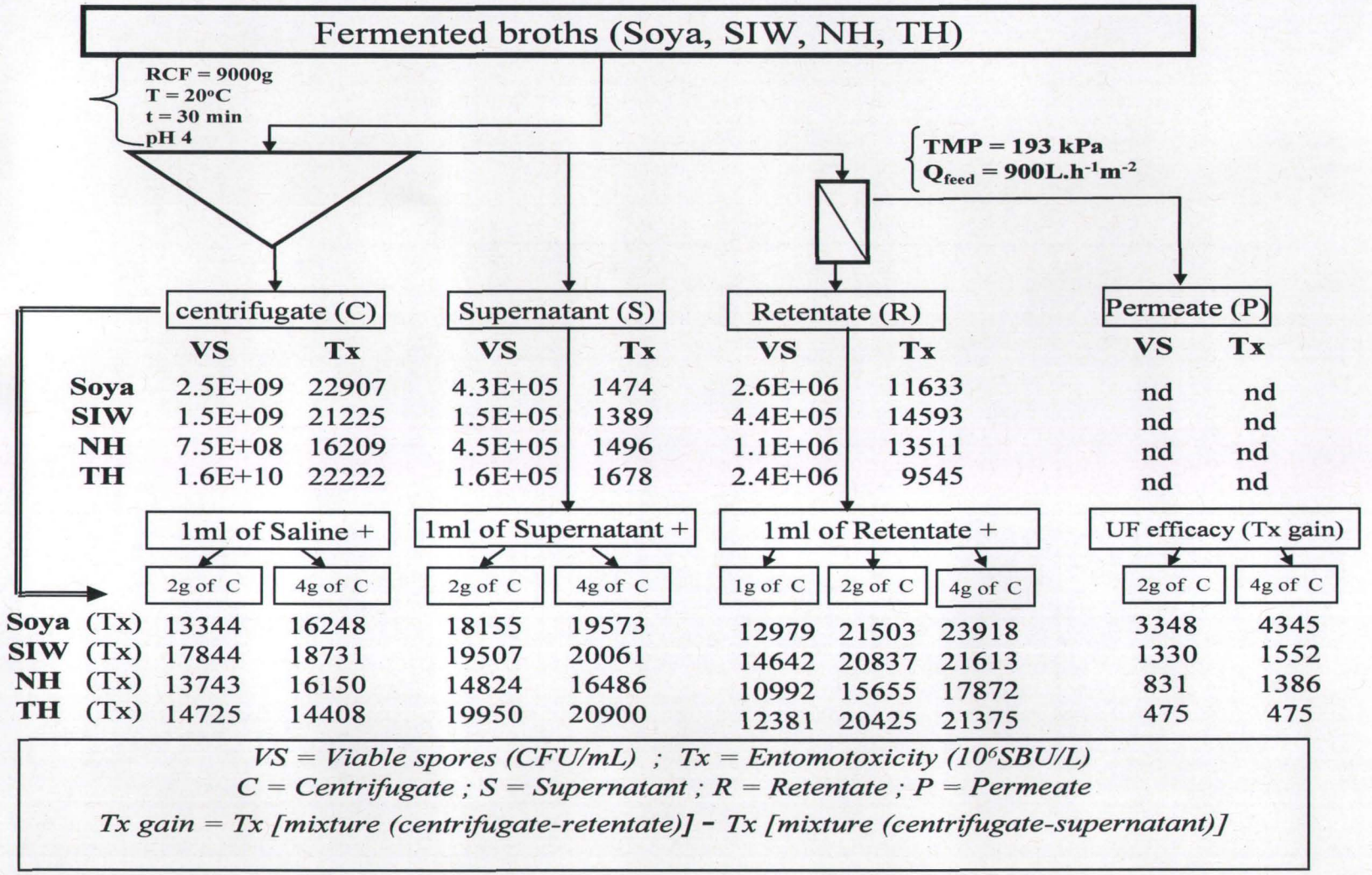

Fig 5. Analysis of results of centrifugation and ultrafiltration (summary of centrifugation and ultrafiltration of four culture media

PARTIE II

(Résultats de l'objectif 3)

RECOVERY OF ENTOMOTOXICITY COMPONENTS OF *BACILLUS THURINGIENSIS* FERMENTED WASTEWATER AND SLUDGE: ULTRAFILTRATION SCALE-UP APPROACH

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RÉSUMÉ

Cette étude rapporte les résultats sur la possibilité de mise à l'échelle du procédé d'ultrafiltration pour la récupération des composants actifs de *Bacillus thuringiensis* des bouillons fermenté des eaux usées d'industrie d'amidon et des boues d'épuration hydrolysées. Cette étude a permis de déterminer les valeurs optimales des paramètres opératoires de la pression transmembranaire et du flux d'alimentation d'ultrafiltration des deux milieux. Ainsi, avec les eaux usées d'industrie d'amidon, la pression transmembranaire et flux d'alimentation sont 90 kPa et 550 $L.h^{-1}.m^{-2}$ respectivement. Pour les boues hydrolysées ces valeurs sont 110 kPa et 720 $L.h^{-1}.m^{-2}$. La perte des composants actifs biologiques mesurée en termes de spores viables et les protéines solubles est plus élevée pour les boues hydrolysées à cause de sa viscosité élevée due aux tailles des ses particules (très petites). Pour ce qui concerne la possibilité de mise à l'échelle, la résistance dynamique est considérée comme un paramètre indicateur important pour l'évaluation du colmatage. Cette résistance est plus élevée dans le cas du surnageant des boues hydrolysées par rapport à celui du surnageant des eaux usées d'industrie d'amidon. Ainsi, l'ultrafiltration du surnageant des eaux usées d'industrie d'amidon présenterait une facilité de mise en échelle par rapport au surnageant des boues hydrolysées.

Mots-clés : *Bacillus thuringiensis*, eaux usées/boue hydrolysée, ultrafiltration, colmatage mise en échelle

ABSTRACT

This study reports results on the possibility of scale-up of ultrafiltration system for recovery of entomotoxicity components from *Bacillus thuringiensis* fermented wastewater/wastewater sludge broths. The wastewater/wastewater sludge comprised starch industry wastewater and hydrolyzed sludge. The study demonstrated that the optimal operational parameters, namely transmembrane pressure and feed flux for starch industry wastewater were 90 kPa and 550 $L.h^{-1}.m^{-2}$, respectively. Likewise, the respective values for hydrolyzed sludge were 110 kPa and 720 $L.h^{-1}.m^{-2}$. The loss in biological activity reported in terms of viable spores and soluble proteins was higher for hydrolyzed sludge due to higher viscosity and lower particle size. In the context of scale-up, dynamic resistance can serve as a key parameter which was reported to be higher for hydrolyzed sludge when compared to starch industry wastewater. Thus, starch industry wastewater will impose relatively less power input requirements and can be easily scaled-up.

Keywords: *Bacillus thuringiensis,* wastewater/wastewater sludge, ultrafiltration, fouling, scale-up.

1. INTRODUCTION

The processes of membrane separation are the most prevalent in the field of biotechnology. Among the various membrane separation processes, there are processes that function under pressure gradient, such as ultrafiltration which is mainly used for bio-separation to concentrate or purify proteins and ionic species in solution [1, 2] or to recover microbial products (cells, spores, etc.) present in a culture medium [3, 4, 5]. However, in spite of the encouraging results, the applications of the membrane processes encounter in general, some specific problems often indicated by "fouling". The fouling is an obstruction of the membrane pores caused by adsorption and/or solids deposited on the membrane. This phenomenon causes a reduction of filtration flux, resulting in an increase in the time of filtration and possibly degradation of the product with eventual recovery involving high production cost.

The work reported in this study is a follow-up on the study reported by Adjallé *et al.* [5], which comprised recovery of active components of *Bacillus thuringiensis*, (namely crystal proteins, cells, spores, enzymes, and other bioactive components) by ultrafiltration from different fermented media (starch industry wastewater and wastewater sludge). The work demonstrated that for same operational conditions, losses of the active components (cells spores, crystals proteins, etc.) caused by fouling of solids on the membrane depended on the physical-chemical constitution of different media. Additionally, other parameters, such as initial concentration of the medium, transmembrane pressure, feed flow rate and viscosity of the supernatant of fermented broths have strong effects on the filtration flux, in particular, by altering its performance. Thus, there is a need to better understand the mechanism of decrease of permeate flux during ultrafiltration of these alternative broths.

In this context, the objectives of the present study are: (1) to determine the optimal values of the operational parameters for an effective recovery of the active components of various supernatants of fermented broths of *Bacillus thuringiensis*; (2) to study the phenomenon of reduction in the permeate flux and fouling of the membrane by various supernatants caused by the permeability and resistance of the fouled layer; (3) to evaluate the variability of the losses of certain active

components (viable spores and soluble proteins) on the membrane by using mass balance; and (4) to analyze the possibilities of scale-up of the ultrafiltration process of the supernatants used.

2. THEORY

2.1. Tangential flow ultrafiltration

According to the principles of tangential flow ultrafiltration (UF) using pressure gradient principle, the direction of the feed is tangential to that of the membrane. A difference in pressure (transmembrane pressure) applied perpendicular to the membrane makes a portion of the medium of feed (filtrate or permeate) travel across the membrane. The portion retained by the membrane (the retentate) circulates tangentially across the membrane. Taking into account the characteristics and the nature of membrane used on one hand and those of the medium of feed on the other hand, certain physical, chemical and biological phenomena can occur at the membrane – medium interface. These phenomena thus affect variation of the permeability and selectivity of the membrane [6]. This phenomenon known as fouling of the membranes depends on several factors, such as the process, characteristics and interactions of the particles in the media for which the membrane system is used [7, 8]. This phenomenon of fouling of the membrane involves a reduction in the permeate flux through the membrane [9], which sometimes requires an increase in the transmembrane pressure to maintain constant flux to limit the increase in the time of filtration. The fouling is inevitable in the membrane processes, and the optimization of the operating conditions is the only way to limit or reduce these phenomena. Depending on the types of the processes, one can have reversible fouling (easily eliminated by suppression of the transmembrane pressure) and an irreversible fouling which requires total cleaning of the membrane. Within the framework of this study, taking into account the process used (UF with tangential flow) and the characteristics of the membrane (spiral cellulosic membrane), it is actually physical and irreversible fouling which necessitates adequate optimization of the process compared to the characteristics of the medium of the feed (total and suspended solids concentration).

2.2. Permeability and resistance of the fouling layer

During UF, the flux of permeate through the membrane varies according to the transmembrane pressure, the total solids concentration of the feed or its viscosity, the proper resistance of the membrane and the dynamic resistance of the fouling layer. Wijmans *et al.* [10] proposed a model (Equation 1) on the variation of permeate flux according to the following parameters:

$$J_p = \frac{\Delta(TMP)}{\mu(R_m + R_c)} \tag{1}$$

Where, Δ(TMP), R_m, R_C and μ are average transmembrane pressure, resistance of the membrane, dynamic resistance of the concentration of the fouling layer and viscosity of solvent, respectively. R_c varies according to the Equation (2) as described below:

$$R_c = \int_0^{\varepsilon} \frac{1}{L_c(x)} dx \tag{2}$$

Where, L_c is the permeability of the aqueous solutions through the fouling layer of thickness,"ε" and concentration, C as denoted by:

$$L_c = \frac{\mu s}{(1 - V_s / V_w)C} \tag{3}$$

where μ, and V_S is the viscosity and partial molar volume of the solvent, respectively. V_w is the partial molar volume of the solvent and S is the coefficient of sedimentation of the aqueous solutions of partial molar volume V_S. It is observed from these Equations that the concentration of solids in the fouling layer is a function of thickness of the fouling which increases as a function of the filtration time, concentration and volume of the feed. Thus, on applying the conventional theory of filtration [11, 12, 13] and by taking into account the approximation of Russotti *et al.* [14] between tangential flow and normal flow which relates to the formation of the fouled layer, one can estimate apparent resistance, r_c of the fouling layer by the following Equation:

$$\frac{t-t_s}{V-V_s}=\frac{r_c\,\mu C}{2A^2\Delta(TMP)}(V+V_s)+\frac{\mu R_m}{A\Delta(TMP)} \qquad (4)$$

Where, t is filtration time; V is volume of the permeate, t_S and V_S are time and volume of the permeate, respectively at the beginning of the stationary phase of filtration; A, R_m being surface and proper resistance of the membrane, respectively and Δ(TMP) is the average transmembrane pressure. The information gained from the slope of the curve, "$(t-t_S)$ /$(V-V_S)$" versus "V" will make it possible to evaluate the resistance of the layer of fouling, and the possibilities of scale-up of UF process.

3. MATERIALS AND METHODS

3.1. Feed media

The media used in this study of ultrafiltration (UF) are the supernatants obtained by centrifugation of the fermented broths of *Bacillus thuringiensis* var. kurstaki HD-1. These are the fermented broths of four media which include: semi-synthetic soya, starch industry wastewater, non-hydrolyzed and thermo-alkaline hydrolyzed secondary sludges. The conditions of hydrolysis and centrifugation have been already described by Adjallé *et al.* [5].

3.2. Ultrafiltration

The process of UF comprises the tangential flow system (PREP/SCALE-TFF, Cartridges Millipore) with recirculation. The principle of UF, the operating conditions, the procedure of optimization of the operational parameters and the cleaning methods of the membrane have been already described and discussed by Adjallé *et al.* [5]. The characteristics of the membrane used in this study are presented in **Table 1.** A 100L volume of supernatant was concentrated to 12.5 L of retentate (12.5 %). A first series of experiments of UF using 1 L of supernatant of various fermented broths was conducted to determine the optimal values of the feed flux and the transmembrane pressure of each supernatant. To evaluate the effect of fouling, a volume of the supernatant (2 and 4 liters) was filtered and the average permeate flux was given by measuring the permeate volume, "V", obtained during ultrafiltration and corresponding time "t". The permeate flux is calculated based on the surface ''A'' of the membrane by Equation (5).

In parallel, sampling of the retentate and permeate was carried out for the calculation of mass balance of total solids and active components (soluble proteins and viable spores of Bt) in order to evaluate the losses on the membrane. The values so obtained will enable calculation of the apparent resistance of the fouling layer for various volumes (2L and 4L) of supernatant to evaluate the possibilities of scale-up of the process.

$$J_p = \frac{V}{tA} \tag{5}$$

3.3. Measured parameters: total solids (TS), viable spores (VS) and soluble proteins (SP)

The total solids of the samples were measured by drying 50 ml volume at 105±1^{O} C [15]. The standard deviation of measurements was 5 to 7 %. The counting of VS was conducted according to the procedure of Vidyarthi et al [16]. It comprised heating the sample at 80 ^{o}C for 10 min in an oil bath (Thermo-lift, Buchler instrument, USA). The sample was finally cooled for 5 min before spreading on the tryptic soya agar plate. The standard deviation was 7 to 8 %. The determination of soluble protein (SP) concentration of the samples was carried out according to the method of Bradford [17] with a spectroscopic measurement of the absorbance at 595 nm. The standard deviation was 6 to 7%. The determination of the mass balance which also takes into account the volumes of the retentates during filtration is given by the relation:

$$C_{feed}V_{feed} = C_{retentate}V_{retentate} + \sum C_{sampling}V_{sampling} + C_{permeate}V_{permeate} + M_{membrane} \tag{6}$$

4. RESULTS AND DISCUSSION

4.1. Optimization of the parameters of UF of various media

Figure 1 presents the values of the concentration of viable spores in the retentate of the four media in relation to the flux of the feed. This allows the determination of optimal feed flux for each supernatant, which is given by the maximum values of viable spores in the retentate. Figure 1 illustrates that soya, SIW and NH have the same optimal value of the feed flux of 550 L h^{-1} m^{-2} whereas for TH sludge, the optimal flux was 720 L h^{-1} m^{-2}. The corresponding values of the concentrations of the viable spores in the retentate of soya, SIW, NH and TH were 4.3 x 10^6; 6.1 x 10^6; 6.0 x 10^5 and 7.1 x 10^6 CFU/ml, respectively. It should be noted that during the UF and for each feed flux value, the pressure of the retentate was adjusted in order to obtain the same transmembrane pressure (TMP) of 110 kPa. The similar optimal feed flux values of SIW, NH and soya are justified by the fact that the supernatants have more or less the same viscosity (± 1.3 mPa.s), whereas the viscosity of TH was higher (1.8 mPa.s); and thus exhibited a higher probability of fouling than the other media. In other words, at equivalent transmembrane pressure, a higher feed flux is required in the case of TH (in this specific process of tangential flow UF) to limit the phenomenon of fouling.

The supernatants of SIW and NH possess more or less similar values of total solid content (13.5 g/L), viscosity (± 1.3 mpa.s) and same optimal value of feed flux (550 L h^{-1} m^{-2}) but different from that of TH (total solid content and viscosity are 16.2 g/L and 1.8 mPa.s respectively). For this reason, further study of optimization of TMP of ultrafiltration was only conducted on the supernatant of SIW and TH broths with the respective feed fluxes of 550 and 720 L h^{-1} m^{-2}. In fact, TMP is a function of the pressure of the retentate and that of the feed (variable with the flow rate of the feed) according to the relation:

$$\Delta(TMP) = \left|(P_{feed} + P_{ret})/2\right| - P_{feed} \qquad (7)$$

The results of the optimization of the transmembrane pressure of SIW and TH are given in Figure 2. According to these results, the optimal value of TMP of SIW and TH are 90 and 100 kPa, respectively. These values of TMP give higher concentration of viable spores in the retentate of SIW and TH (6.3×10^6 and 7.1×10^6 CFU/ml, respectively). This difference in values of the transmembrane pressure can also be explained by the difference in viscosity, as mentioned earlier. TH sludge is more prone to fouling due to its higher viscosity, which lends high resistance to the fouling layer. Thus, one needs higher transmembrane pressure so as to make the permeate pass easily through this layer and the membrane.

4.2. Variation of the permeate flux and loss of total solids

The variation of the permeate flux in relation to the volume of the permeate collected for media of SIW and TH with the optimal conditions (flow of feed and TMP) as defined earlier is presented in Figure 3. This variation of the permeate flux can be subdivided into three phases for the two media: initial first phase with a comparatively rapid reduction of the permeate flux until 500 ml (12.5 % of the initial volume of the supernatant) of permate was obtained, which corresponded to a filtration time of 16.48 min and 18.39 min, respectively for SIW and TH. This reduction was explained by the obstruction of the pores of the membrane caused by the deposit of the particles on the internal wall of the pores, thus decreasing the diameter of the pores (Hermia, 1982). In the second phase, the reduction of permeate flux is relatively slower and corresponding permeate volume which is collected is between 500 ml and 1000 ml (25 % of the initial volume of the supernatant). The filtration time for the second phase is 20.50 min for SIW and 24.30 min for TH. The slower phase is due to the beginning of the formation of the first layers of fouling on the membrane and subsequently increasing the thickness of the membrane and thus causing the dynamic resistance. The third phase, more or less stationary, is due to the fact that fouling reaches a thickness limited by the force of the tangential flow which further reduces fouling. This stationary phase (corresponding to the permeate volume collected between 1000 ml and 2900 ml is much longer (1h 19 min for SIW and 1h 57 min for TH), and it seems to be more constant in the case of SIW than TH.

The total resistance of the membrane, which is the sum of proper (intrinsic) resistance of the membrane and dynamic resistance of the fouling layer, is well explained by the Poiseuille law which expresses the total resistance of the membrane by Equation (8):

$$R_m = \frac{8\alpha}{n_p \pi r_p^4} \tag{8}$$

Where, α, n_p and r_p are thickness of the active layer of the membrane, number of pores/m^2 and pore radius, respectively.

It should, however, be noted that it is in the third phase that resistance due to the fouling layer is higher due to the increase of the losses of the solids during fouling. The losses of the solids in relation to the permeate volume collected in case of SIW and TH under the optimal feed flux and TMP defined earlier, is also presented in Figure 3. In contrast to permeates fluxes which decrease, the losses in total solids on the membrane increase. These losses are higher in the case of TH than that of SIW (TH - 76 % and SIW - 63 % of initial solids in supernatant), which justifies the fact that the values of permeate flux of TH are lower than those of SIW.

4.3. Losses of active components

The losses of the active components on the membrane are evaluated by intermediate losses of soluble proteins and viable spores via mass balance. Figure 4 shows the results of variations of the losses of proteins and viable spores on the membrane during filtration. There is linear increase in the losses of the viable spores and soluble proteins for the two media. Similar to the losses of the total solids, the values of the losses of the viable spores and soluble proteins are higher in the case of TH than that of SIW. The maximum viable spore losses were 36 and 42% for supernatants of SIW and TH, respectively; whereas the soluble proteins losses were 32 and 39% for SIW and TH, respectively. Loss of viable spores and soluble proteins can be explained by the fact that these two components are adsorbed onto the total solids.

4.4. Resistance of the fouling layer and scale - up possibility

The variability of the resistance of the fouling layer, explained theoretically by Equation (2) is determined based on the Equation (4). Figures 5 illustrates the curve $(t-t_S)/(V-V_S)$ versus V (volume of the permeate) for SIW and TH. In order to evaluate the value of apparent resistance of the fouling layer r_c based on the equation of profile curve and Equation (4), one can estimate the value of the expression $r_C \mu C/A^2 \Delta(TMP)$.

With the filtration carried out using a volume of 4L of each of the supernatants of SIW and TH, the apparent values of the resistances of the fouling layer calculated from the profile of Figure 5 a) and b) are 1.2×10^{12} m^{-1} for SIW and 2.1×10^{13} m^{-1} for TH. The high value of apparent resistance in the case of TH is explained by the difficulty of filtration of supernatant of TH due to easy fouling and high loss of solids on the membrane of this medium. It is due to the small particle size of TH (effect of hydrolysis) and the viscosity of this complex medium when compared to SIW. The high apparent resistance of TH can also be explained by the fact that, during the filtration of 4 liters of supernatant, the third phase (stationary phase) of SIW is more stable than that of TH (Figure 3), with the result that the regression coefficient value is higher for SIW than TH ($R^2 = 0.990$ for SIW and 0.927 for TH). This low regression coefficient limits the applicability of approximations of the Equation (4) in the case of TH.

However, similar filtration experiments carried out with a volume of 2 liters of supernatant (Figures 5 c) and d)) for SIW and TH gives a value of apparent resistance of the fouling layer of 3.5×10^{12} m^{-1} (with $R^2 = 0.992$) for SIW and 1.8×10^{12} m^{-1} (with $R^2 = 0.996$) for TH. These results showed that, with a membrane area of 0.2 m^2, a filtration of 2 and 4 liters of supernatant of SIW give the same order of power (10^{12}) of the apparent resistance of the fouling layer. In contrast, for the same surface of membrane and in the case of TH supernatant, the power of the apparent resistance of the fouling layer is multiplied by 10 when volume of the supernatant increases from 2L to 4L. This difference is not due to the nature of the membrane nor due to its surface, but due to the particle size and composition of the two media. In fact, the composition of the supernatant of hydrolyzed sludge (TH) is more complex (comprising humic acid and fulvic

acid) with a viscosity higher than that of the supernatant of starch industry waste water (SIW) which comprises soluble proteins.

In the case of tangential filtration, the increase in transmembrane pressure increases the initial permeate flux and the level in the third phase [18]. However, in certain cases, a high transmembrane pressure accompanied by an initial high flux of permeate can result in fast reduction in the flux of permeate with a low stationary level of flux [19]. This is justified by the fact that the effect of transmembrane pressure on the flux of permeate depends especially on the compressibility of the fouling layer (thus of its resistance) which is a function of the particle size and the viscosity of the medium and the nature of the membrane. In the case of TH, given that one has an optimal value of the transmembrane pressure, evaluated from the maximum recovery of the viable spores (Figure 2), an increase in this pressure can result in increased losses on the membrane.

Thus, it can be recommended that the scale-up of SIW will be more feasible compared with TH sludge for an ultrafiltration membrane of similar surface area. In fact, the dynamic resistance increases very slowly with the increase in filtration volume of the feed for SIW and on the other hand, the same increases rapidly (varies by a factor of 10) for TH sludge. This difference in dynamic resistance profiles will affect the feed flux and the filtration time will be prolonged in the case of TH sludge which will translate into high power requirements and hence higher ultrafiltration cost.

CONCLUSIONS

This study of ultrafiltration of the supernatants of SIW and TH demonstrates that:

1 The optimal transmembrane pressures of SIW and TH are more or less close at values of 90 and 100 kPa, whereas the optimal values of flux are 550 L h-1 m-2 and 720 L h-1 m-2, respectively.

2. The reduction in the flow of permeate is faster for SIW and TH in the first fifteen minutes after filtration of a volume of permeate of 500 ml (12.5 % of the initial volume of the supernatant). The maximum losses of the active components after filtration of 4L of supernatant for SIW and TH were 36 and 42 %, respectively for viable spores, and 32 and 39 %, respectively in the case of soluble proteins.

3. The apparent resistance of the stationary phase during filtration slightly varied in the case of SIW when one passes from a filtration of 2L to 4L, whereas this variation was multiplied by a factor of 10, in the case of TH.

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Table 1. Characteristics of the membrane

Description of membrane	Prep/Scale Spiral Wound TFF-1
Filter type	Ultrafiltration
Length, cm (in)	15.2 (6)
Diameter, cm (in)	5.8 (2.3)
Minimum working volume, mL	100
pH range	2.0 - 13.0
Configuration	Spiral Wound Cartridge
Filtration area, m^2	0.2
Opérating temperature range, ^{o}C	4 - 50
Filter material	Regenerated Cellulose
Maximum Inlet pressure, bar (psi)	0 - 5.5 (0 - 80)
Recirculation rate, L/min	1.0 - 6.0
Molecular weight cut off (MWCO), kDa	5

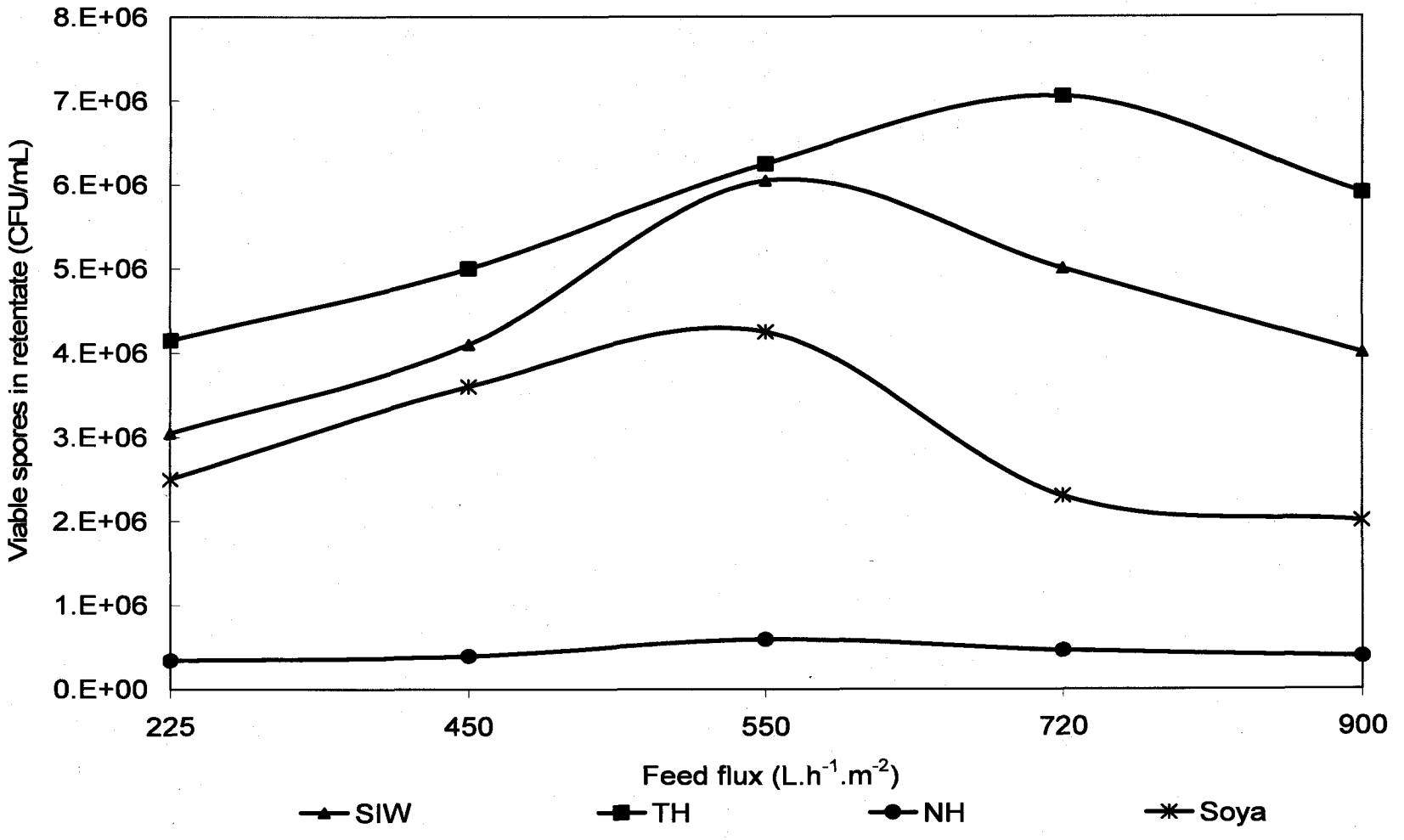

Fig. 1. Concentration of viable spores in the retentate of soya, SIW, TH and NH in relation to the feed flux.

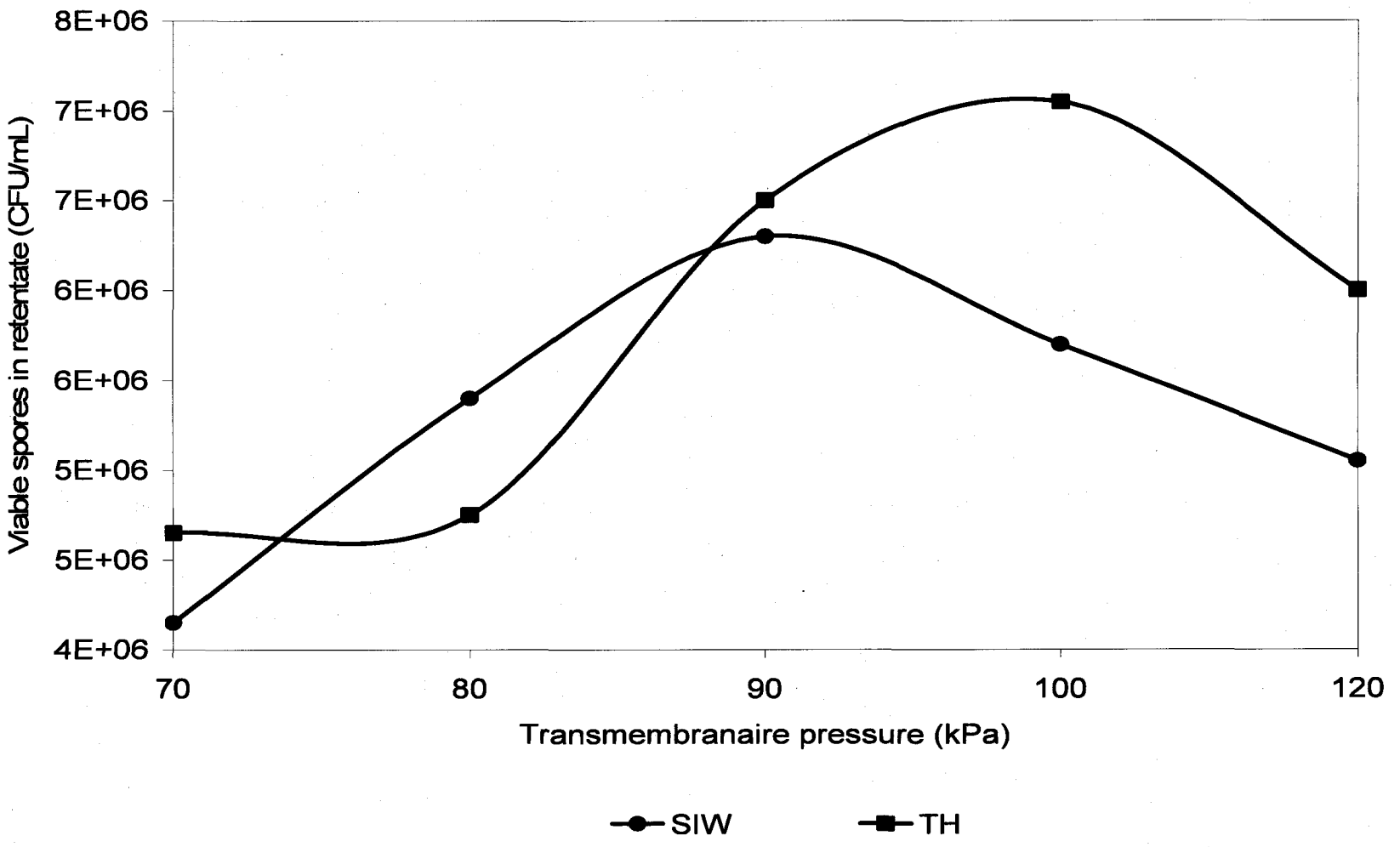

Fig. 2. Concentration of viable spores in the retentate of SIW and TH in relation to transmembrane pressure of ultrafiltration.

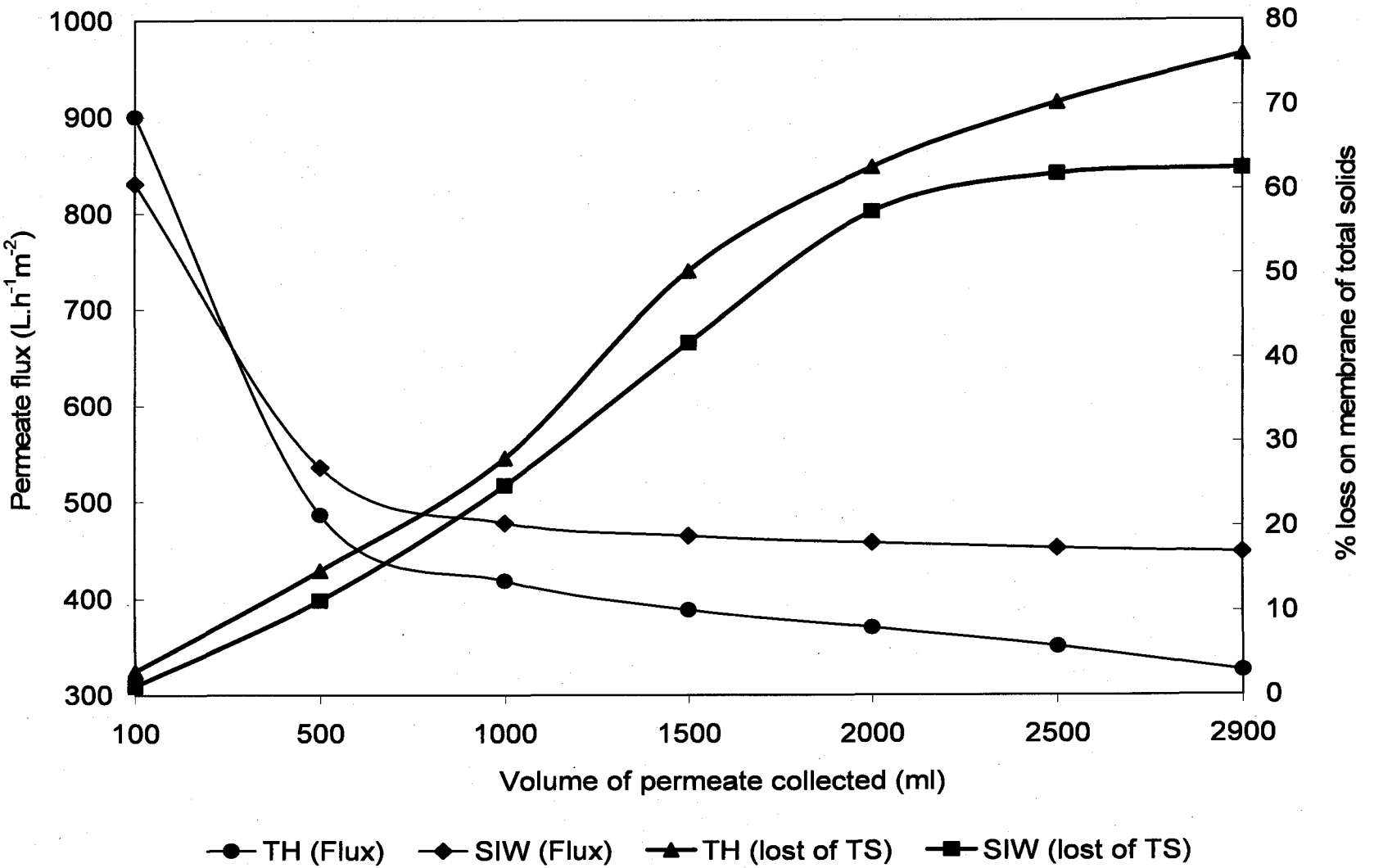

Fig. 3. Variation of permeate flux and loss on the membrane of total solids SIW and TH versus volume of permeate.

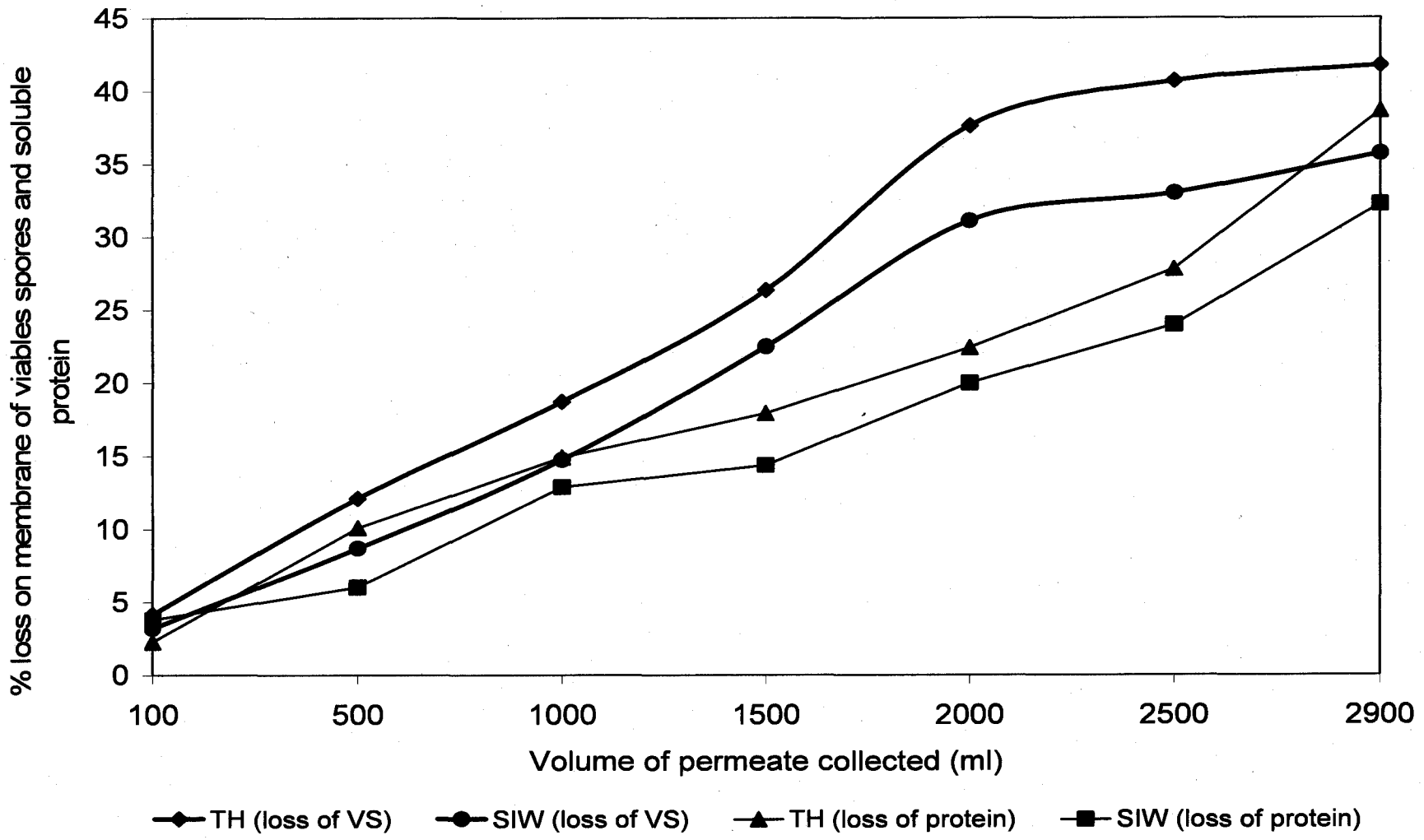

Fig. 4. Variation of losses of soluble proteins and viable spores of TH and SIW on the membrane versus the volume of the permeate

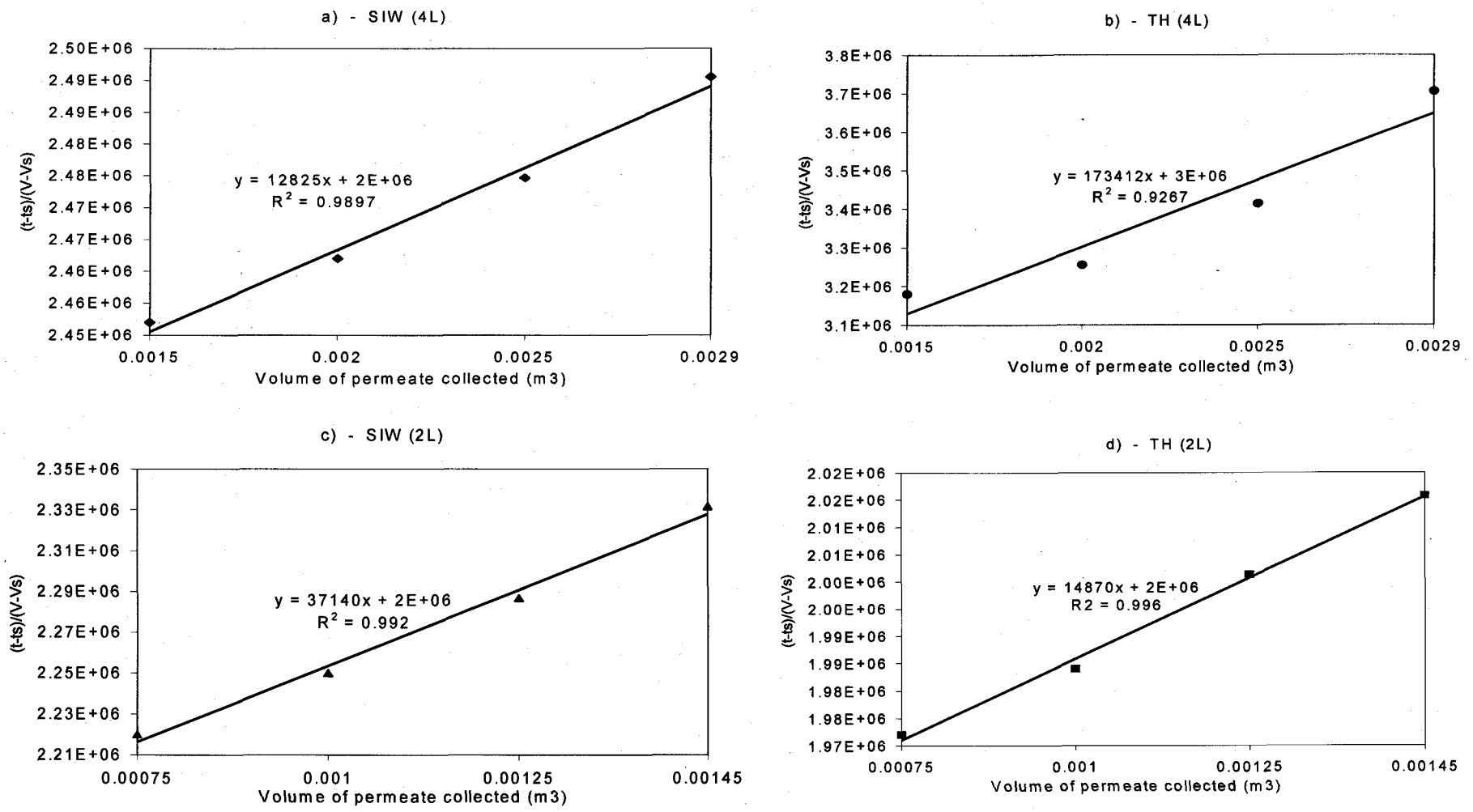

Fig. 5. Variation of $(t-t_S)/(V-V_S)$ versus volume of permeate during filtration of 2 an 4 liters of supernatant. a) case of SIW (4L); b) case of TH (4L); c) case SIW (2L) and d) case TH (2L).

Chapitre 3. Formulation

PARTIE I

(Résultats de l'objectif 4)

PHOTOSTABILIZATION OF *BACILLUS THURINGIENSIS* FERMENTED WASTEWATER AND WASTEWATER SLUDGE BASED BIOPESTICIDES USING ADDITIVES

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RÉSUMÉ

Cette étude porte sur la photoprotection contre des radiations UV-A et UV-B des composants actifs du *Bacillus thuringiensis* variété *kurstaki* obtenus à partir de la fermentation des différents milieux de culture. Il s'agit des milieux des eaux usées d'industries d'amidon, des boues secondaires (hydrolysées et non-hydrolysées) et le milieu de soya (utilisé comme référence). La photoprotection se fait à travers l'utilisation des additifs de protection contre les effets des radiations ultraviolets. Il s'agit de l'acide para-amino-benzoique, l'acide lignosulfonique et la mélasse à différentes concentrations (0.1%, 0.15%, et 0.2% p/p). Les résultats de cette étude montrent qu'en absence des additifs de protection, les boues secondaires présentent une protection naturelle contre les radiations UV avec des demi-vies d'entomotoxicité de 3.25 et 3.40 jours. Ces valeurs sont 1.9 et 1.8 jours pour les milieux de soya et des eaux usées d'industrie d'amidon respectivement. Avec les additifs de protection, l'acide p-amino benzoique à 0.20% p/p donne une meilleure protection avec les milieux de soya et des eaux usées d'industrie d'amidon avec des demi-vies d'entomotoxicité respectives de 5.9 et 7.0 jours. L'acide lignosulfonique à 0.20% p/p est plus efficace pour les boues secondaires hydrolysées et non-hydrolysées avec respectivement 7.25 et 8 jours de demi-vies d'entomotoxicité. On peut conclure que pour une même concentration des additifs, on a de meilleurs résultats avec les milieux alternatifs qu'avec les milieux conventionnels.

Mots clés: *Bacillus thuringiensis*, Entomotoxicité, Boues secondaires, Eaux usées d'industry d'amidon, Radiations UV

ABSTRACT

Photoprotection (against UV-A and UV-B radiations) of the active components of *Bacillus thuringiensis* var. *kurstaki* obtained from the fermentation of various culture media was investigated. The culture media comprised: starch industry wastewater; secondary wastewater sludge (non-hydrolyzed and hydrolyzed) and soya (used as a reference). Photoprotection was carried out by using various UV-protection additives, namely, para-amino-benzoic acid, lignosulfonic acid and molasses at different concentrations (0.1%, 0.15% and 0.2% w/w). In the absence of UV-protection agents, secondary sludge demonstrated natural UV protection with half-lives ranging from 3.25 to 3.4 d. The half life for soya and starch industry wastewater was 1.9 and 1.8 d, respectively. Para-amino-benzoic acid as a UV-protection agent at 0.20% w/w gave excellent UV-protection for soya and starch industry wastewater with half-lives being 5.9 and 7 d, respectively. Likewise, lignosulfonic acid at 0.20% w/w was an effective photostabilizer for hydrolyzed and non-hydrolyzed secondary sludge with half-lives of 7.25 and 8 d, respectively. Hence, when similar concentration of the UV-protection additives was used, photoprotection was higher for the alternative media than the conventional soya medium, validating the technical feasibility of using three additives.

Keywords: *Bacillus thuringiensis*; Entomotoxicity; Secondary sludge; Starch industry wastewater; UV radiations.

NOMENCLATURE

LSA	LIGNOSULFONIC ACID
M	MOLASSES
M	ARITHMETIC MEAN = $(\sum X_I)/N$
M_1	LIMIT LOWER THAN THE CONFIDENCE INTERVAL AT 95% ($M - T_{0.025} X\Sigma(N)^{1/2}$)
M_2	LIMIT HIGHER THAN THE CONFIDENCE INTERVAL AT 95% ($M + T_{0.025} X\Sigma(N)^{1/2}$)
$[M_1 - M_2]$	WIDTH OF THE CONFIDENCE INTERVAL AT 95%
N	NUMBER OF VARIABLES
NH	NON HYDROLYZED SLUDGE
PABA	PARA-AMINO BENZOIC ACID
SIW	STARCH INDUSTRY WASTEWATER
TH	THERMAL HYDROLYZED SLUDGE
T_X	ENTOMOTOXICITY
$T_{0.025}$	VALUE OF STUDENT T-TEST ON THE LEVEL OF CONFIDENCE OF 95% WITH 5 DEGREES OF FREEDOM (2.57)
UV	ULTRAVIOLET
E	COEFFICIENT OF EXTINCTION ($L.MOL^{-1}.CM^{-1}$)
Σ	ESTIMATE OF STANDARD DEVIATION = $(\sum(X_I - M)^2/(N - 1))^{1/2}$

1. INTRODUCTION

The potential adverse impacts of chemical insecticides on the environment have led to the use of biological control. Highly employed biopesticides namely, *Bacillus thuringiensis* (Bt) occupy 95 % share of the world biopesticide market (Powell, 1993). In spite of the beneficial effects of Bt, high cost of the synthetic culture media accounting for 35 59% of the production cost, limits the wide accessibility of these biological pesticides (Lisansky *et al.*, 1993; Stambury *et al.*, 1993). Hence, high production cost stimulated research on the use of residual matter for Bt production (Saksinchai *et al.*, 2001). In the same context, research was also carried out on the use of wastewater and wastewater sludge as substrates (Yezza *et al.* 2006) for the production of Bt biopesticides yielding encouraging results.

Field application of Bt biopesticides is marred by the short duration of insecticidal action caused by UV exposure. In fact, different mechanisms have been suggested for the inactivation of Bt protoxins and spores (major biopesticidal Bt components of Bt) by UV radiations: probable generation of free radicals following the oxidation of amino acids and destruction of two amino acids, namely, tryptophan and histidine (Ignoffo and Garcia, 1978; Pozsgay *et al.*, 1987; Becker *et al.*, 1992).

Several studies have been reported on various techniques of UV protection of the biopesticidal components. The principal techniques include: microcapsules (Murat and Attila, 1994), granular formulations (Ahmed *et al.*, 1973), or formulations with UV-protection additives (Dunkle and Shasha, 1989). In the formulation containing UV-protection additives, the photoprotection of the active components was obtained by absorption of UV radiation by the chromophores of additives. Cohen *et al.* (1991) studied the interaction between *B. thuringiensis* and acriflavin additive. In fact the effectiveness of these additives is a function of their concentration and synergism (interaction and charge or energy transfer) and rheology of the culture medium (Burges, 1998, Brar *et al.*, 2005, 2006a).

Various low and high product yielding culture media have been studied for the production of biopesticides in our laboratory (starch industry wastewater; non-hydrolyzed and hydrolyzed sludge and soya). The individual fermented broths have demonstrated different rheological behaviour necessitating specific formulation requirements (Brar *et al.* 2005). Further, earlier formulation studies mainly concentrated on qualitative selection of UV-protection additives (molasses, p-aminobenzoic acid, lignosulfonic acid, folic acid, Congo red, benzilidine sulphonic acid) (Brar *et al.*, 2006a). The reported study lacked the quantitative optimisation of UV-screen agents which will be more pertinent in terms of formulations and future field application. Hence, in this study, three additives (p-amino benzoic acid, molasses and lignosulfonic acid) were selected among the six screened earlier, according to the efficacy/cost factor. The principal objectives of this study are to: (1) evaluate the effect of UV radiations on the insecticidal potential of Bt fermented broth obtained with different media namely, wastewater, wastewater sludge and soya; and (2) quantify the efficacy of the selected UV protection additives.

2. MATERIALS AND METHODS

2.1. Bacterial strain - culture medium – fermentation

The bacterial strain used in this study was *Bacillus thuringiensis* var. *kurstaki* HD-1 (ATCC 33679). The media investigated included: (i) synthetic soya medium (used as a control) which comprised (g/l): soybean meal, 15.0; glucose, 5.0; starch, 5.0; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.02; $CaCO_3$, 1.0; (ii) secondary sludge from wastewater treatment plant of Communauté Urbaine de Quebec, Ste-Foy, Quebec and; (ii) starch industry wastewater from ADM-Ogilvie (Candiac, Québec, Canada). The characteristics of wastewater and wastewater sludge are presented in Table 1 and was carried out using standard methods (APHA *et al.*, 1998)

Starch industry wastewater and soya medium were directly used for fermentation after sterilization. The secondary sludge was either directly utilized for fermentation after sterilization or after hydrolysis followed by sterilization before fermentation. The hydrolysis was performed according to the pre-optimized conditions: 140±1 ^{O}C under a pressure of 40 psig for 30 min (Barnabé, 2004). Sterilization was carried out *in situ* at 121±1 ^{O}C under a pressure of 15 psig for 30 minutes. Fermentation was executed in a bioreactor with accessories, connected to a computer with *iFix 3.5, intellution* software equipped with controls of pH, temperature, air flow rate, agitation and, anti-foam addition (Vidyarthi *et al.* 2002). Henceforth, hydrolyzed, non-hydrolyzed secondary sludge, and starch industry wastewater will be designated as TH, NH, and SIW, respectively.

2.2. Recovery of active components from Bt fermented broths

After fermentation, the active components of Bt (protein crystals, cells, spores, enzymes, vegetative insecticidal proteins, etc. which are responsible for composite entomotoxicity) fermented broths were recovered by centrifugation and ultrafiltration of the supernatant after

centrifugation. Bt fermented broths (Soya, SIW, NH, TH) at pH 4.5 were aseptically centrifuged by using the optimal conditions (9000xg, 30 min, 25°C) established by Brar *et al.* (2006b). The active components lost in the supernatant during centrifugation were aseptically concentrated by optimized ultrafiltration process (Adjallé *et al.*, 2007).

2.3. Anti-UV formulations

2.3.1. Physical and spectroscopic characteristics of the media

The sample to be formulated was prepared by mixing the centrifugate and the retentate of ultrafiltration in the optimal proportion (25% v/w) (Adjallé *et al.*, 2007). The concentrations of dry matter and water content (moisture) of each mixture of the four culture media, namely, SIW, NH, TH and soya (control) were obtained by drying samples at 105 ° C for 24 h and by moisture measurement using HR 83 Halogen Moisture analyzer (Mettler Toledo, Ontario, Canada), respectively. The moisture content will permit comparison of the dissolution of UV-protection additives in the different mixtures. In fact, for each medium, a study of UV absorbance (280 – 400 nm) was carried out with suspensions (10^3 dilution) of centrifugate + retentate mixture. The absorbance of the dilutions was measured using UV-visible spectrophotometer (Varian/CaryWinUV 50 ConC Spectrophotometer, Mississauga, Ontario, Canada). The experiment compared the absorbance of different media without UV-protection additives and to ascertain the inherent UV-protection, if any.

2.3.2. Properties and characteristics of UV-protection additives

UV-protection additives used in this study were: p-aminobenzoic acid (Fisher Scientific Company, NJ, USA); lignosulfonic acid (Aldrich Chemical Company, Inc., Milwaukee, WI, US) and molasses (M) procured from the local grocery store. The characteristics of p-aminobenzoic acid and lignosulfonic acid are given in Table 2. Spectroscopic absorbance of the three additives at concentration of 1 g/l was obtained between the wavelengths 280 and 400 nm (using

Varian/Cary WinUV 50 ConC UV-visible Spectrophotometer). Henceforth, para-amino benzoic acid, lignosulfonic acid and molasses will be designated as PABA, LSA and M, respectively.

2.3.3. Source of UV-A and UV-B radiations

UV-A and UV-B radiations were emitted simultaneously and uninterrupted by lamps, L_1 and L_2, respectively. The samples were placed on the same height at 20 cm distance from the lamps. L_1 was a source of UV Hand Lamps model 3UV-36 (115V, 60Hz, 0.16A) emitting UV-A radiation of wavelength 365 nm with a surface power density of 0.12 mW/cm^2. L_2 was a UV source from Benchtop UV transilluminator, model 26, (115 V, 60 Hz, 0.8 A) which emitted UV-B radiation with a wavelength of 302 nm and surface power density of 0.81 mW/cm^2.

2.3.4. Tests of exposure to UV radiations

In total, four samples were prepared: (1) centrifugate + retentate mixture at 0.10% w/w; (2) centrifugate + retentate mixture at 0.15% w/w; (3) centrifugate + retentate mixture at 0.20% w/w; and (4) centrifugate + retentate mixture without UV protection additive (control). The centrifugate + retentate + additive mixtures thus, formulated were well mixed and stored in dark for 24 h to allow dissolution of the additives.

For each centrifugate + retentate + additive mixture, an initial suspension was prepared at 1/10 dilution of the starting mixture. The principal objective of this dilution was to obtain a mixture with concentration more or less equal to the dilution of Bt biopesticides, generally utilized during the field application. The dilution so obtained would allow easy passage of UV radiations vis-à-vis the centrifugate + retentate mixture which is highly concentrated. Subsequently, 2 ml of suspension was introduced into each of the five identical transparent methacrylate cuvettes (Fisher Scientific, Mississauga, Ontario, Canada) numbered from 1 to 5. The methacrylate cuvettes comprised 4.5 ml of volume with the passage of UV radiation in the wavelengths between 285 and 400 nm. Finally, the five cuvettes containing the Bt suspensions mixed with the additives were exposed to combined UV-A and UV-B radiations for the same time period and

samples were withdrawn after 8, 16, 24, 32 and 48 h of exposure. The samples were exposed to the UV-A radiations at energy equivalents of 34560, 69120, 103680, 138240 and 207360 J/m^2 for 8, 16, 24, 32 and 48 h of exposure, respectively. Likewise, the energy equivalents for UV-B radiations at 8, 16, 24, 32 and 48 h of exposure were 233280, 466560, 699140, 933120, 1399680 J/m^2, respectively. The exposure was carried out in a closed compartment with the interior lined by an aluminium foil to avoid loss of stray radiations. Additionally, in order to limit the effect of evaporation, sample cuvettes were covered with parafilm prior to UV exposure. The exposed samples were kept in suspension by slow agitation (every 2 h) of the cuvettes. After the exposure, the suspensions in various cuvettes were evaluated for number of spores and the biopesticidal potential (entomotoxicity).

2.4. Measurement of biological parameters

2.4.1. Spores count

The counting of the spores was carried out according to the procedure described by Vidyarthi *et al.* (2002). It comprised heating of the sample to 80 ±1°C for 10 min in an oil bath (Thermo-lift, Buchler instrument, USA) followed by rapid cooling in a cold water bath before spreading on the tryptic soya agar plates (17g Tryptone, 3g Soytone - enzymatic digest of soybean meal, 2.5g Dextrose, 5g Sodium Chloride, 2.5g K_2HPO_4, 15g Agar). The standard deviation of counting was 7-8%.

2.4.2. Entomotoxicity

Entomotoxicity was evaluated by the bioassay method using 2nd instar spruce budworm larvae (*Choristoneura fumiferana*) furnished by Natural Resources Canada (Sault Ste-Marie, Ontario. Canada). The larvae were reared on the artificial diet for 4 days to obtain third and fourth instar larvae. The bioassay method was adopted from Beegle, (1990). For a given sample, five dilutions were prepared on a saline solution of 8.5% (w/v), and the last three dilutions were used for

entomotoxicity measurements. About 0.75 ml of each dilution was mixed with 15 ml of diet comprising different diet components (Tirado-Montiel *et al.*, 2001). The mixture thus obtained was distributed in a stack of 10 glass tubes of dimensions 15 mm x 45 mm (VWR, Canlab, Mississauga, Ontario, Canada). Each of the 30 tubes containing 1 ml of diet was used as a control. Other controls were prepared by mixing 1 ml of sterilized medium of soya, SIW, NH and TH with 30 ml of diet. Once the diet solidified and cooled, a larva was introduced into each tube. The tubes were capped by perforated caps and kept at 25 ±1 ^{O}C for 7 d. Consequently, mortality was evaluated in each stack of 10 tubes. Calculations were performed using ANOVA tests. The standard deviation of counting was 7-8%.

2.5. Data analysis

The distribution of the entomotoxicity values at various exposure times (0, 8, 16, 24, 36 and 48 h) was analyzed by normal distribution using mean and standard deviation. The calculation of the confidence interval at 95 % was carried out using Student ($t_{0.025}$) "t" value established in Table 3. This confidence interval allows evaluation of the distribution of entomotoxicity values around the average. The efficacy of various UV-protection additives against UV-A and UV-B radiations was measured through the determination of half-lives of the biopesticidal potential (entomotoxicity), losses of entomotoxicity and residual spores after 6 d of exposure. The half-life (time at the end of which half of the Bt entomotoxicity remained due to exposure to UV radiations) is denoted by the values of residual entomotoxicity at the end of various exposure times by adopting the method of Herman *et al.* (2002). If for a given sample, the half-life was higher than 48 h, the suspension was exposed for a period higher than 48 h (for example, 64 h). The results were evaluated by taking into account the fact that 8 h of UV exposure under laboratory conditions was equivalent to 1 day of field exposure (Shasha *et al.* 1998).

3. RESULTS AND DISCUSSION

3.1. Physical and spectroscopic properties of centrifugate + retentate mixture

The total solids content of centrifugate + retentate mixtures of soya, SIW, TH and NH were 7.8, 9.0, 9.0 and 8.8 (% w/w), respectively with the corresponding moisture content being: soya-92 %, SIW-91%, TH-92%, NH-92%. The results showed that the four media possessed more or less the same water content. Thus, the quantities of various UV additives added per gram of mixture were equal and that the moisture (water content) necessary for their dissolution was almost similar. In fact, it was possible to have similar base of comparisons between the UV protection additives and the media used.

The most popular method of analysis of photoprotection is the spectroscopic method which plays an important role in the characterization of biological compounds (Coohill, 1992). In fact, spectroscopic study in biology is often based on monochromatic radiations, and is explained by the absorption of radiations. The UV absorbance of suspensions of soya, SIW, TH and NH was measured at total solids concentrations of 0.1% (w/v) (Fig 1). The four media absorbed in the UV radiation region and the absorbance varied with the media. In the near and mid-UV region (200-400 nm), absorption is mainly due to the presence of unsaturated groupings (π electrons, similar case in benzene rings) and free doublets ("n" electrons) on heteroatoms viz. O and N; (Arnaud, 1997). The absorption of UV radiations by ``π' ' electrons results in the interactions of photons with the electrons which participate in the formation of bonds. The absorption wavelengths of a molecule or a compound depend on the binding energy of various electrons. Thus, electrons involved in the double bonds of the molecules are not as strongly dependent as the single bonds, and are thus easily excitable by the near and mid-UV radiations (Skoog *et al.*, 1997). The above mentioned unsaturated organic functional groups absorbing in the UV and visible region of electromagnetic spectrum are called the chromophores. The crystal proteins, spores and other media components such as, the chromophoric compounds absorb in the UV region (Manka *et al.*, 1974; Manasherob *et al.* 2002). The fact that the four media absorb between 280 and 400 nm makes it possible to study the effect of higher absorbance (long-term exposure) on the active

components of Bt, as well as the inherent property of natural protection of the various media investigated in this study.

The absorbance of NH medium was relatively lower compared to that of soya, SIW and TH. The lower absorbance of NH could be due to the lower concentration of dissolved solids and the presence of flocs and particles in suspension (which can reflect the UV radiations) in comparison to higher concentration of dissolved solids in soya, SIW and TH. Moreover, according to Beer Lambert's law (Eq. (1)), the absorbance strongly depends on concentration of dissolved solids rather than suspended solids. Hence, it is possible that, at lower total solids concentrations (< 0.01M), certain large ions, molecules or particles do not follow Beer Lambert's law due to the optical phenomena related to reflection, diffusion and diffraction.

$$\text{Absorbance } (A) = \varepsilon LC \qquad (1)$$

where,

ε = coefficient of extinction (l mol^{-1} cm);

C = concentration ($mol.l^{-1}$) of dissolved solids of exposed sample;

L = length (cm) of cuvette containing the sample.

3.2. Spectroscopic properties of various UV-protection additives

The spectroscopic absorbance study of the three UV-protection additives -molasses (M), p-amino benzoic acid (PABA) and lignosulfonic acid (LSA)-dissolved in distilled water, is presented in Fig. 2. It was evident that the molasses (M) and lignosulfonic acid (LSA) absorb in the entire UV-A and UV-B radiation range with higher absorbance for lignosulfonic acid (LSA) and relatively low for the molasses. As for PABA, it absorbed primarily in UV-B region (280 – 320 nm) with a very high absorbance and also in small part of the UV-A region (320-340 nm). The higher absorbance of LSA and PABA is due to the presence of aromatic rings, and ''π'' bonds in their structures (Table 3), which is not the case for ''M'' which is a syrup

derived from sugar, and small percentage of aromatic unsaturations in terms of vitamins. In fact, complex structure of LSA can offer photoprotection by absorption caused by cleavage of unsaturated bonds and formation of free radicals. Also, as the formulations were prepared at pH 4.5, where the bacteria have neutral charge and can be effectively caged in the branched complex structure of LSA which is negatively charged. Thus, the steric effect was positive in the sense that the bacteria can be incorporated in the complex structure leading to photoprotection. Moreover, the branching of LSA will result in steric protection from adverse impacts of UV radiations. Likewise, PABA with its unsaturation groupings can offer a resonance stabilization effect which can protect the spores and crystal proteins. The efficacy of these additives to protect the Bt crystal proteins from UV action was measured by evaluating the half-lives and entomotoxicity losses after 6 days of exposure.

3.3. UV exposure tests

At the end of 48 h exposure, temperature in the exposure compartment is only 36 ^{O} C. This temperature is far from destroying the spores and toxins of Bt which are stable at this temperature (Chen *et al.*, 2005). Additionally, the measured evaporation is 0.1ml which is 5% of the total volume. Since control (sample without additive) is also exposed under the same conditions as the samples with additives, the effects of the temperature and evaporation will have small influence on the effectiveness of the additives, which is one of the objectives of the present study.

Table 3 presents the statistical analysis of entomotoxicity values (obtained at different time of UV exposure) at different concentrations of additives. This statistical treatment allows verification of coherence of raw values of measure of entomotoxicity. Moreover, for a given medium, the width of confidence intervals decreased as concentrations of UV-protection additives increased. Consequently, the distribution (and thus the variance) of the entomotoxicity values decreased as concentration of UV-protection additives increased. This justifies the protective character of additives, as increase in the concentration of additives mitigates the effect of UV irradiation resulting in a highly stable sample.

3.3.1. Natural protection of culture media against UV radiations

Fig. 3 and Table 4 present respectively, the half-lives based on the entomotoxicity losses of various centrifugatee – retentate and centrifugate – retentate – additives mixtures of the four media; SIW, TH, NH and soya. The half-lives depended on the rate of reduction of the entomotoxicity in each medium due to absorption of UV radiation by the active components. In fact, UV radiation not only caused a modification or destruction of the amino acids (main component of crystal protein), but also the inactivation/denaturation of proteins and related enzymes (Prinsze *et al.*, 1990). The inactivation of proteins and enzymes would be directly caused by UV photolysis of the aromatic amino acids or disulfide groups and presence of the active sites (Hollosy, 2002).

According to Cohen *et al.* (1991), two principal mechanisms can explain the photoprotection of Bt toxins: (i) effect of the photostabilization additives and; (ii) specific interaction between the chromophores and active site of Bt. "Active sites" of Bt are sites which are photosensitive and contain photosensitive amino acids (histidine and tryptophan). In this light, Fig 3 points out that the half-lives of secondary sludge used as Bt production media (TH and NH) were higher than SIW and soya, and followed the order: NH (3.4 d) > TH(3.25 d) > SIW(1.9 d) > soya (1.8 d). The difference in half-live values between secondary sludges and soya and SIW could be explained by the difference in their physical (Table 1) and rheological characteristics (Brar *et al.*, 2005). Moreover, UV absorption within the matrix of the molecule can cause the damage further away from the actual absorption site via energy migration to functionally important active sites (Jordan, 1993). This involves degradation of the components, and the rate usually depends on the nature of solutes and solvent (Guingamp and Alais, 1974). For this reason, in addition to the active components of Bt, all other absorbing components present in secondary sludge degrade slowly in the presence of UV radiations than those in the case of soya and SIW. This is attributed to the chromophoric compounds and auxochromes such as, melanin, fulvic and humic acids present in sludge which absorb in the UV region (Manka *et al.*, 1974).

NH sludge possesses higher half-life than TH sludge (Fig. 3). This was explained by two principal reasons: (a) low absorbance of UV radiation by NH (compared to TH) (Fig. 2) caused by low concentration of dissolved solids and lower inactivation of crystal-spore complex; and (b) presence of flocs in NH sludge partly protected the spore-crystal complex by reflection or dispersion of some UV radiations. Additionally, the flocs can exert masking effect on the spore-crystal complex. Moreover, it has been already reported that the particle size (μm) of the four media varied according to the order: NH (35) > TH (25) > SIW (6) > soya (3) (Brar *et al.*, 2004). Hence, larger particle size of NH sludge ensured protection of active components of Bt as compared to TH, SIW and soya.

The entomotoxicity losses at the end of 48 h of exposure to UV radiations (equivalent to 6 days of field exposure) are presented in Table 4 and were found to be (in %): 90, 90, 67 and 67, for soya, SIW, NH and TH, respectively. In the absence of UV protection additives, the entomotoxicity losses were pertinent, necessitating the addition of UV-protection agents to increase the half-lives and eventually decrease the entomotoxicity losses.

3.3.2. Effects of UV-A and UV-B radiations on spore count

Table 4 presents the spore count at the beginning and at the end of 48 h. The spore count remaining after 48 h strongly depended on the additives and their concentrations. The spore count after UV exposure remained very low. Nevertheless, the residual spores increased with the concentration of UV-protection additives and were in agreement with the half-lives. Meanwhile, spore count cannot be taken as a true indicator of photoprotection of Bt as they do not represent the whole biopesticidal potential (Luthy *et al.*, 1985).

3.3.3. Efficacy of UV-protection additives

Fig. 3 presents the half-lives of formulated (centrifugate + retentate + additive) mixtures of the four media for each UV-protection additive at different concentrations. The general observation was that the UV-protection additives increased the half-lives based on entomotoxicity, and

absorbance followed the Beer-Lambert's law. This also justified the increase in the half-lives with the concentration of the additives. However, the half-live values obtained with M were lowest as it showed lowest absorbance in the UV region (280 – 400 nm) (Fig. 2). For PABA and LSA, the UV protection was relatively higher depending on the media.

For SIW, Fig. 3(a) showed that PABA gave higher UV-protection than LSA. In fact, with PABA at 0.20 % (w/w), the half-life was 3.7 times higher than the control, whereas it was 3.1 times higher than the control for LSA. The entomotoxicity losses (after 6 days of exposure) were 44% and 50% for PABA and LSA, respectively as against 90 % in the absence of UV-protection additives. Thus, PABA gave good synergy with SIW and superior UV protection (absorption at 302 nm, Fig. 2) of the active components of Bt compared to M and LSA. Moreover, earlier studies have demonstrated that starch form complexes in solution with PABA (Goudah and Gurth, 1965). Hence, Bt crystal proteins and spores may be entrapped in this complex and better protected against action of UV. The lamps, L_1 and L_2 used in this study emit a maximum wavelength of 365 and 302 nm, respectively. Thus, lower the wavelength of radiations, higher is the transport of energy so that the UV radiation becomes more destructive.

Likewise, results obtained with soya (Fig. 3(b) showed that PABA at 0.20% (w/w) gave good synergy with soya and better UV protection of the active components of Bt compared to other two additives. For PABA at 0.20% (w/w), the half-life of the mixture (centrifugate + retentate + additive) was 3.3 times higher than the control (centrifugate+retentate) as against 2.7 times for LSA. The entomotoxicity losses (after 6 days of exposure) for PABA and LSA were 53% and 56%, respectively as against 90% in the absence of UV-protection additives. The reasons for better performance of PABA in soya were similar as in the case of SIW. However, lower half-life in the case of soya compared to SIW can be explained by the higher absorbance of SIW compared to soya.

Figs. 3(c) and 3(d) show the performance of different UV-protection additives for secondary NH and TH sludge. It was observed that LSA gave better protection against UV-A and UV-B radiations. In fact, with LSA at 0.20% w/w, the half-lives in the mixtures (centrifugate + retentate + additive) of NH and TH were 2.4 and 2.2 times higher than the respective controls with

entomotoxicity losses of 47% and 45% as against 67% in the absence of UV-protection additives. For secondary sludges, due to the presence of chromophoric compounds and auxochromes, there was higher absorbance in the UV-B region which attenuated the efficacy of UV absorption by PABA in these media. LSA, by virtue of its structure and the fact that it absorbs at 302 and 365 nm, it seems to be more effective and highly compatible with the chromophoric compounds of secondary sludge.

Thus, the efficacy of UV-protection additives also depends on the culture medium in which they are added. And for a given medium, the level of protection of the active components of Bt against UV radiations is a function of the concentration of the additive. These results are in agreement with earlier studies for synthetic media (Burges, 1998). Among the three additives used in this study, PABA is suitable for SIW and soya at a concentration 0.20% (w/w), and LSA at 0.20% (w/w) is appropriate for TH and NH.

The half-lives of soya and SIW while using PABA were 5.9 and 7.0 days, respectively. The half-lives were 8 and 7.3 d, for NH and TH, respectively with LSA. However, taking into account the entomotoxicity losses (and thus the residual entomotoxicity), 0.20% (w/w) was sufficient concentration for sustained protection of the active components of Bt during field application. PABA and LSA gave higher UV-protection for the majority of media. While, considering the higher cost and taking into account the regulations associated with the use of PABA compared to that of LSA, in certain formulations, LSA is a universally recommended UV-protection agent at 0.20% (w/w). Thus, LSA was retained as a universal UV-protection agent henceforth for all studies.

CONCLUSIONS

The study on photoprotection of active components of *B. thuringiensis* in different fermented media led to following conclusions:

1. UV-protection additives are mandatory for UV-protection of B. thuringiensis fermented alternative media namely, non-hydrolyzed sludge, hydrolyzed sludge and starch industry wastewater.

2. The biopesticides obtained using secondary sludge (non-hydrolyzed and hydrolyzed) as raw material demonstrated a natural resistance against UV-A and UV-B radiations compared to soya and starch industry wastewater with half-lives (in days) in the order: non-hydrolyzed (3.4) > hydrolyzed (3.25) > starch industry wastewater (1.9) > soya (1.8).

3. p-amino benzoic acid as UV-protection additive increased the half-life of starch industry wastewater (7.0 d) and soya (5.9 d) whereas lignosulfonic acid gave higher UV protection in the case of hydrolyzed (7.3 d) and non-hydrolyzed (8.0 d) sludge.

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Table 1. Characteristics of secondary wastewater sludge and starch industry wastewater

Parameter (s)	Secondary sludge		Starch industry wastewater	
TS (g/l)	18	±1.5	17	±1.1
TVS (g/l)	14	±1.1	14	±1.0
SS (g/l)	15	±1.0	2.2	±0.8
VSS (g/l)	13	±2	2.2	±0.7
pH	5.5	±0.1	3.3	±0.1
Concentration (mg/kg TS)				
C	301097	±5987	700345	±6986
N_t	42307	±500	37089	±1578
P_t	7987	±203	340176	±3001
$N\text{-}NH_3$	889	±198.4	109.8	±59.8
$N\text{-}NO_2$, $N\text{-}NO_3$	14.7	±1.1	4.8	±1.2
$P\text{-}PO_4$	4988	±402	14987	±2801
Al	4999	±437	56987	±3798
Ca	14011	±511	11567	±402
Cd	3.01	±0.9	0.54 (3-10)	±0.1
Cr	27.9	±1.1	1.3 (210)	±0.04
Cu	401	±157	338 (400)	±171.2
Fe	11987	±603	7986.4	±803.4
K	998	±371	23056	±3124
Pb	27	±4.3	27.4 (150)	±4.7
S	4369	±538	2301.4	±62.5
Zn	325	±197	250 (700)	±77
Na	1456	±408	2189.4	±231
Ni	10.7	±3.9	-	-

± refers to the standard error

Values in parentheses represent concentrations of metals prescribed by the Quebec Environment Ministry (MENV, 2004) for agriculture application.

Table 2. Characteristics of UV-protection additives

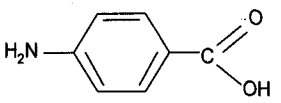
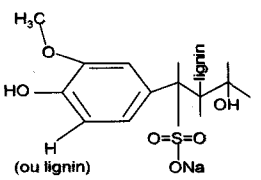
Additives	Generic name	Molecular weight (g)	Empirical formula	Structural formula	Physical form	Purity (%)	Boiling point (°C)	Solubility
p- Aminobenzoic acid	PABA	137.13	$C_7H_7NO_2$		Crystalline powder	> 98%	187-189	6.1 g/l at 25°C in water
Lignosulfonic acid	LSA	Average $M_{w\sim 52,000}$	-		Brown powder	-	-	400 g/l at 20°C in water

Table 3. Statistics analysis of entomotoxicity values at different concentrations of additives.

		Concentration of additives (%, w/w)	Data analysis					
			$m = (\sum x_i)/n$	σ	$s = \sigma/(n)^{1/2}$	$m_1 = m - s(t_{0.025})$	$m_2 = m + s(t_{0.025})$	$[m_2 - m_1]$
SIW	Control	0.00	10492	7127	2910	3015	17970	14955
	PABA	0.10	10435	4245	1733	5982	14889	8907
		0.15	13257	3156	1288	9946	16568	6622
		0.20	**13922**	**3319**	**1355**	**10440**	**17403**	**6964**
	M	0.10	11256	6777	2767	4145	18367	14222
		0.15	12943	5098	2081	7594	18292	10698
		0.20	**14403**	**4496**	**1836**	**9685**	**19120**	**9435**
	LSA	0.10	9987	6331	2584	3345	16629	13284
		0.15	13522	4135	1688	9183	17860	8677
		0.20	**15086**	**4081**	**1666**	**10804**	**19367**	**8564**
TH	Control	0.00	14563	6284	2566	7969	21156	13187
	PABA	0.10	12961	4530	1849	8208	17714	9506
		0.15	14285	4995	2039	9045	19526	10481
		0.20	**14685**	**4191**	**1711**	**10289**	**19082**	**8794**
	M	0.10	13531	5712	2332	7537	19524	11987
		0.15	14147	5175	2113	8717	19576	10860
		0.20	**14855**	**4858**	**1983**	**9757**	**19952**	**10195**
	LSA	0.10	11484	3701	1511	7601	15366	7765
		0.15	12900	3643	1487	9078	16722	7644
		0.20	**13177**	**3326**	**1358**	**9687**	**16667**	**6980**
NH	Control	0.00	10862	4236	1729	6418	15306	8888
	PABA	0.10	9864	3801	1552	5877	13852	7975
		0.15	10640	3951	1613	6495	14786	8291
		0.20	**10917**	**3639**	**1486**	**7099**	**14735**	**7636**
	M	0.10	12053	4592	1875	7235	16871	9635
		0.15	12813	4369	1784	8228	17397	9168
		0.20	**13151**	**3983**	**1626**	**8972**	**17330**	**8359**
	LSA	0.10	11090	3485	1423	7434	14746	7312
		0.15	12468	3429	1400	8871	16066	7195
		0.20	**12714**	**3317**	**1354**	**9234**	**16194**	**6960**
Soya	Control	0.00	9537	6175	2521	3058	16015	12957
	PABA	0.10	13592	4317	1762	9063	18121	9059
		0.15	12547	3305	1349	9079	16015	6936
		0.20	**11785**	**3595**	**1468**	**8013**	**15557**	**7543**
	M	0.10	9331	6219	2539	2806	15856	13049
		0.15	10946	5817	2375	4843	17050	12207
		0.20	**13947**	**4714**	**1925**	**9001**	**18893**	**9893**
	LSA	0.10	11435	5645	2305	5512	17358	11846
		0.15	12277	4587	1873	7464	17089	9626
		0.20	**11951**	**3881**	**1584**	**7879**	**16023**	**8144**

Table 4. Half-lives based on entomotoxicity and viable spores at 0 h and after 48 h of exposure to UV-A and UV-B radiations.

			p-amino-benzoic acid (PABA)			Molasses (M)			Lignosulfonic acid (LSA)		
		Control	(% w/w)			(% w/w)			(% w/w)		
			0.10	0.15	0.2	0.10	0.15	0.2	0.10	0.15	0.2
SIW	0 h	3.8E+08	3.7E+08	3.0E+08	2.7E+08	1.1E+08	1.6E+08	1.9E+08	2.2E+08	1.9E+08	2.0E+08
	48 h	1.1E+03	1.3E+05	8.3E+05	3.9E+06	1.4E+03	8.5E+03	2.1E+04	1.9E+04	9.1E+04	2.3E+05
	Tx half -lives (d)	**1.9**	**2.5**	**6.0**	**7.0**	**2.4**	**3.0**	**4.0**	**2.3**	**4.5**	**6.0**
	Losses of Tx	**90**	**63**	**49**	**48**	**82**	**63**	**53**	**83**	**57**	**50**
TH	0 h	2.3E+09	2.2E+09	2.0E+09	1.8E+09	1.8E+09	2.0E+09	1.9E+09	2.2E+09	1.6E+09	1.2E+09
	48 h	4.0E+07	4.8E+07	5.5E+07	6.3E+07	3.8E+07	4.5E+07	5.7E+07	3.3E+07	1.5E+07	4.6E+07
	Tx half- lives (days)	**3.3**	**3.8**	**4.0**	**5.0**	**3.3**	**3.5**	**3.8**	**4.0**	**5.25**	**7.3**
	Losses of Tx	**67**	**62**	**59**	**53**	**68**	**64**	**55**	**59**	**53**	**45**
NH	0 h	4.9E+07	4.1E+07	4.5E+07	4.2E+07	4.3E+07	4.4E+07	4.4E+07	4.5E+07	4.5E+07	4.3E+07
	48 h	1.2E+06	2.2E+06	2.4E+06	2.5E+06	1.8E+06	2.1E+06	2.3E+06	2.1E+06	2.6E+06	2.8E+06
	Tx half- lives (days)	**3.4**	**3.4**	**3.5**	**3.5**	**3.4**	**3.7**	**4.9**	**4.0**	**5.5**	**8.0**
	Losses of Tx	**67**	**66**	**62**	**57**	**65**	**60**	**53**	**59**	**53**	**48**
Soya	0 h	2.5E+08	2.2E+08	2.5E+08	2.5E+08	2.3E+08	2.5E+08	2.5E+08	2.4E+08	2.5E+08	2.5E+08
	48 h	4.5E+03	3.9E+05	7.6E+05	3.3E+06	4.9E+04	5.6E+04	2.8E+05	4.1E+05	8.6E+05	4.4E+06
	Tx half- lives (days)	**1.8**	**3.8**	**5.6**	**5.9**	**1.6**	**1.9**	**4.0**	**2.3**	**3.0**	**4.8**
	Losses of Tx	**90**	**54**	**51**	**53**	**89**	**73**	**65**	**77**	**61**	**56**

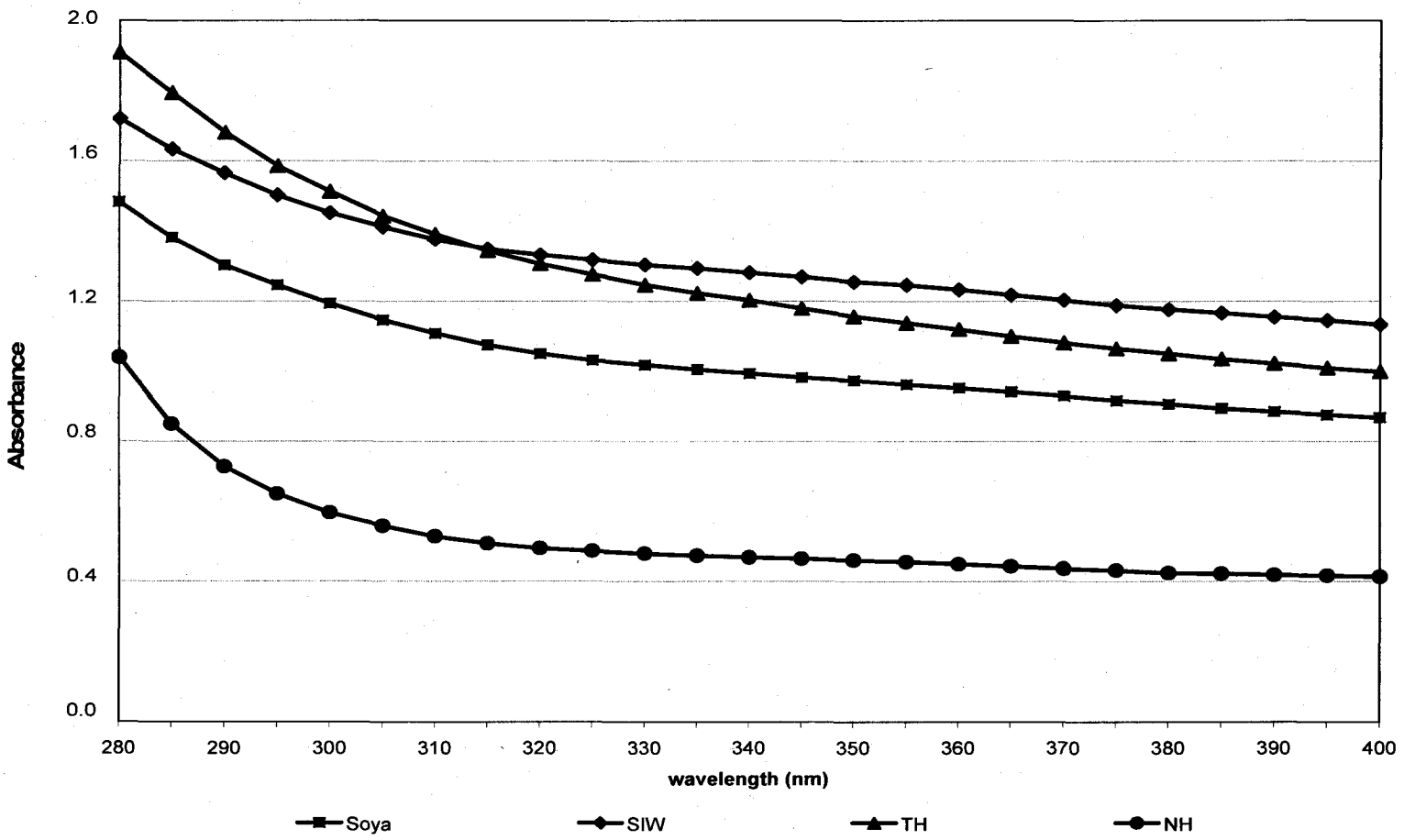


Fig.1. Absorbance profiles of suspensions of soya, starch industry wastewater (SIW), hydrolyzed sludge (TH), and non-hydrolyzed sludge (NH) in the UV region.

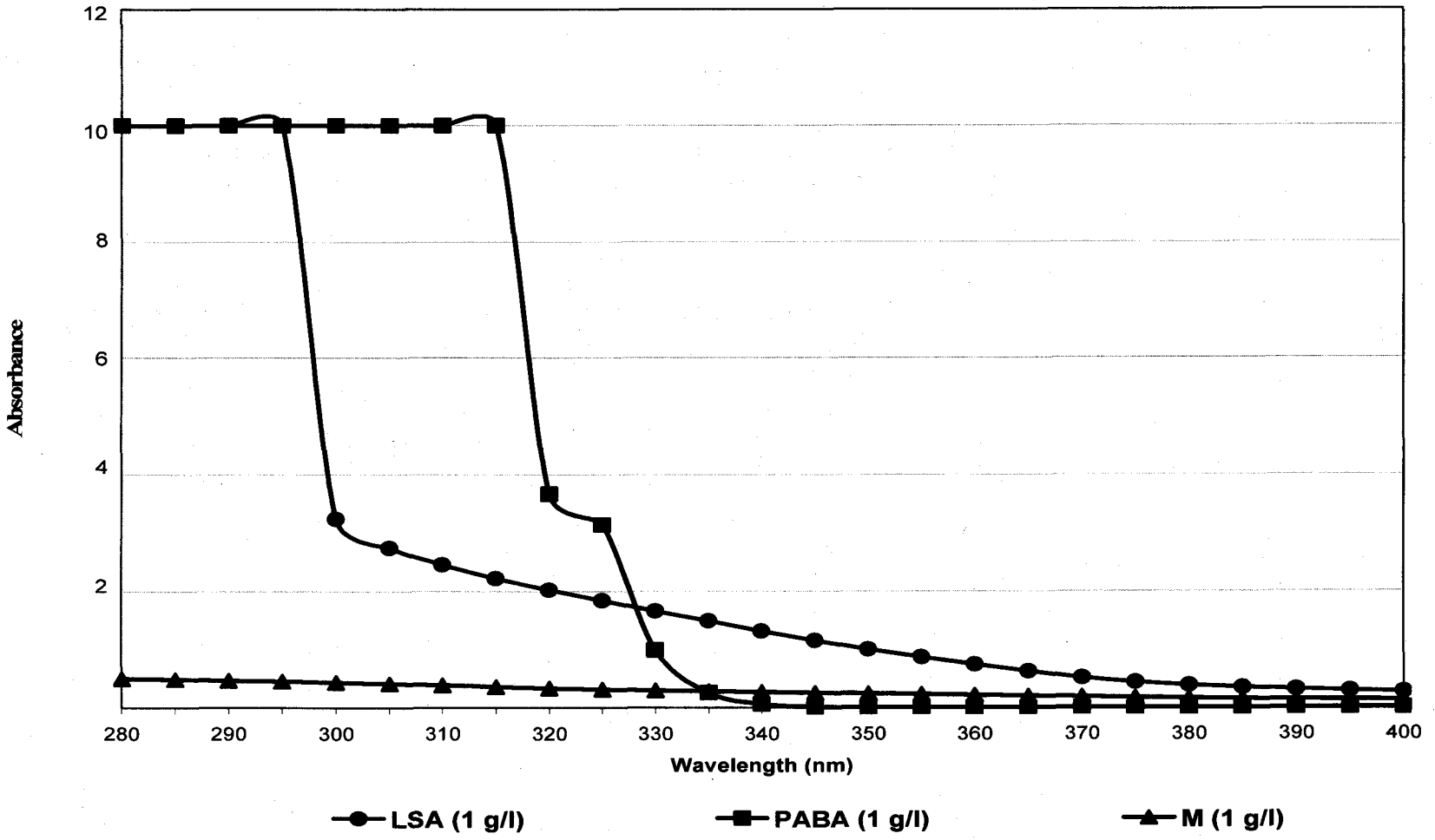


Fig. 2. UV Absorbance (in distilled water) profiles of lignosulfonic acid (LSA), p-aminobenzoic acid (PABA), and molasses (M)

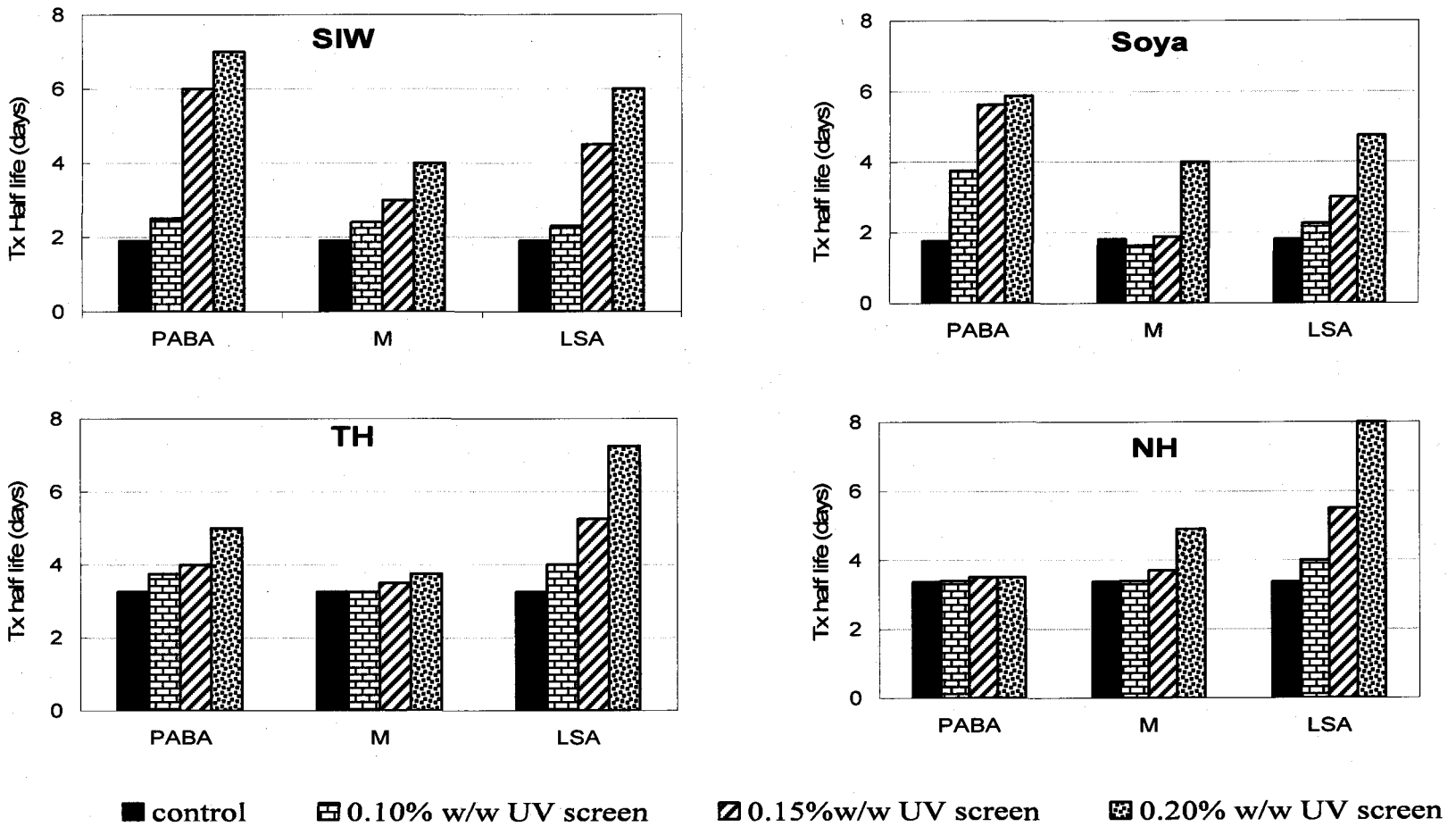


Fig. 3. Entomotoxicity half-lives of Bt at various concentrations of selected UV screens (p-amino benzoic acid-PABA, molasses-M and lignosulfonic acid-LSA) of different Bt fermented media: (a) starch industry wastewater; (b) soya; (c) hydrolyzed sludge; (d) non hydrolyzed sludge.

PARTIE II

(Résultats de l'objectif 5)

PROTECTION OF *BACILLUS THURINGIENSIS* BIOPESTICIDAL FORMULATIONS PRODUCED FROM WASTEWATER AND WASTEWATER SLUDGE AGAINST MICROBIAL DEGRADATION

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RÉSUMÉ

Cette étude porte sur l'optimisation des concentrations des différents additifs anti-microbiens des biopesticides à base de *Bacillus thuringiensis* obtenus avec des milieux résiduels. Il s'agit de déterminer pour chaque milieu de culture, le meilleur additif ainsi que leurs concentrations. Les différents milieux utilisés sont les eaux usées d'industrie d'amidon, les boues secondaires (hydrolysées et non hydrolysées) et le milieu synthétique de soya considéré comme milieu de référence. Les additifs anti-microbiens utilisés sont l'acide propionique, le métabisulfite de sodium et l'acide ascorbique, avec des concentrations de 0.1, 0.3 et 0.5 (% w/w).

Au bout de trois ans de conservation des formulations préparées, aucune contamination par les microorganismes visés n'a été décelée dans les formulations préparées avec chacun des trois additifs avec des concentrations de 0.3 et 0.5% (w/w). Cependant, dans certaines formulations servant de temoins ainsi que certaines formulations avec 0.1% (w/w) d'additifs, on note un début de formation de moisissures. En se basant sur les valeurs de l'entomotoxicité et surtout sur celles des spores viables, on peut conclure que l'acide propionique avec des concentrations de 0.5%, 0.5% et 0.3% (w/w) est plus efficace pour la formulation des milieux de soya, des eaux usées d'amidon et des boues non hydrolysées respectivement, et que le métabisulfite de sodium paraît le plus efficace à 0.3% w/w pour la formulation des boues hydrolysées.

Mots clés: Additifs, antimicrobiens, *Bacillus thuringiensis*, eaux usées/Boues d'épuration

ABSTRACT

Three anti-microbial additives were tested for the conservation of *Bacillus thuringiensis* formulations derived from fermented wastewater and wastewater sludge. Experiments were designed to determine, for each culture medium, the best additive and their optimal concentrations for improved protection against degradation. The various formulated media were starch industry wastewater, hydrolyzed, non-hydrolyzed secondary sludge and of soya synthetic medium considered as medium of reference. Three additives were propionic acid, sodium metabisulfite and ascorbic acid. For each additive, different concentrations of 0.1, 0.3 and 0.5 (% w/w) were tested.

At the end of three years conservation, no contamination by selected micro-organisms was detected in all formulations with 0.3 and 0.5% (w/w) of all the additives used. However, in the controls and formulations with 0.1% (w/w) of additives, onset of mould growth was observed. Based on the values of entomotoxicity and especially of those of viable spores, it was concluded that the formulation with additive of propionic acid of 0.5%, 0.5% and 0.3% (w/w) concentration was more effective for soya, starch industry wastewater and non-hydrolyzed secondary sludge, respectively. Sodium metabisulfite appeared to be the most effective at 0.3% w/w for the formulation with hydrolyzed secondary sludge.

Keywords: Additives, anti-microbial, *Bacillus thuringiensis*, wastewater/wastewater sludge

INTRODUCTION

Biopesticides are being used widely to replace the chemical pesticides that encompass adverse health and environmental impacts. In the field of pest control in forests and agriculture, such as spruce budworm (*Choristoneura fumiferan*), cabbage looper (*Trichoplusia ni*), and others, commonly used biopesticide includes *Bacillus thuringiensis* (Bt) (Knowles *et al.*, 1987; Powell, 1993,). In fact, residual matter has been proven to be an effective and economical medium to produce Bt based biopesticides (Alves *et al.*, 1997; Rojas *et al.*, 1999; Saksinchai *et al.*, 2001). However, as the formulation of the biopesticides depends on rheology and characteristics of the media used for fermentation (Burges, 1998), there is a need to establish complete formulation for a given residual medium. Thus, it is important to search for suitable additives, and their optimal concentration to obtain an effective formulation of biopesticides with the necessary properties so as to result in test efficacy on the field. It is within the framework of this study that determine action of suitable additives for an anti-microbial formulation of the biopesticides containing *Bacillus thuringiensis* while using starch industry wastewater and wastewater sludge as substrates of fermentation is important.

During the transport and/or storage of Bt formulations, the product mixture can be prone to contamination by foreign micro-organisms. In order to prevent the development of these micro-organisms other than Bt and to increase the lifespan of the formulated product, several techniques during formulation development have been used. According to Burges (1998), the formulations in the forms of granules, briquettes and/or microcapsules possess a structural bond that makes it possible to keep the integrity of the formulation for a longer duration and thus prevent all types of contamination. Date (1970), proposed a formulation making it possible to obtain an optimal pH (4 to 6) of storage that increased the lifespan of Bt formulations, thus protecting against the possible contamination. Additionally, at this pH, the insecticidal crystal proteins are completely insulated from any proteolytic solubilization, as these crystals are solubilized in basic medium (Dubois et al. 1993). Nevertheless, too high or too low pH can inactivate the active ingredients of Bt product (Griffiths, 1982; Salama and Moris, 1993).

Thus, in this study, various anti-microbial additives were tested for the conservation of Bt formulations derived from fermented wastewater and wastewater sludge media. Specifically, this study focused on: (1) study of synergism between the anti-microbial additives and different media used (starch industry wastewater, hydrolyzed and non-hydrolyzed secondary sludge) and choice of best additives, such as concentration for each medium; and (2) to evaluate the impacts of the additives on the entomotoxic potential of the formulated product and also the conservation capacity based on determination of various contaminants.

2. MATERIALS AND METHODS

2.1. *Bacillus thuringiensis* (Bt), culture medium and pre-treatment

The bacterial strain used is *Bacillus thuringiensis* variety *kurstaki* HD-1 (ATCC 33679). The various residual culture media used for Bt fermentation are: (1) starch industry wastewater from ADM-Ogilvie (Candiac, Québec, Canada); (2) secondary sludge from wastewater treatment plant of Communauté Urbaine de Québec, Ste-Foy. The semi-synthetic medium of soya is also used as a reference medium. The characteristics of these media are presented in **Table 1.**

2.2. Pre-treatment, fermentation and recovery of active components of Bt

Starch industry wastewater and soya medium are directly used for fermentation after sterilization. For secondary sludge, it is divided into two portions where one portion is directly used for fermentation after sterilization. The other portion is hydrolyzed and then sterilized prior to fermentation. Sterilization was carried out in situ at 121±1°C at a pressure of 15 psig for 30 min. The hydrolysis was performed according to already established optimal conditions: 140±1°C, pressure of 40 psig for 30 min (Barnabé, 2005). Fermentation was performed in a fermented well-equipped with various accessories, connected to a computer using the software *iFix 3.5, intellution* (Massachusetts, USA) for the control of pH, temperature, aeration rate, agitation, and anti-foam addition. The active components of the fermented broth are recovered by centrifugation according to the conditions already established by Brar *et al.* (2006). The residual components that remained in the supernatant of centrifugation process are aseptically recovered by ultrafiltration in the form of retentate by using the optimal conditions established by Adjallé *et al.* (2007). Henceforth, all through the article, non-hydrolyzed secondary sludge, hydrolyzed secondary sludge, and starch industry wastewater will be designated as, NH, TH and SIW respectively. The soya semi-synthetic medium will be refered to ''soya''.

2.3. Anti-microbial Formulation

The sample to be formulated was obtained by mixing 4 g of the centrifugate of each medium and 1mL of the respective retentates after ultrafiltration (Adjallé *et al.* 2007). The quantity of dry matter and water content of each mixture was determined by HR 83 Halogen Moisture analyzer (Mettler Toledo, Ontario, Canada). The anti-microbial additives of formulation were selected according to the studies of Brar *et al.* (2005) which comprised following additives: ascorbic acid, citric acid, propionic acid, para methyl benzoate, and sodium metabisulfite. For the development of formulations and optimization of the concentrations, three additives were selected as per the results of Brar *et al.* (2005) and depending the cost of these additives. The additives were propionic acid, ascorbic acid and sodium metabisulfite. Starting from the centrifugate-retentate mixture of a given medium, three replicates of the same mass of samples were prepared and same additive was added to these samples at different concentrations: 0.1, 0.3, and 0.5 (% w/w). Thus, for each medium, there were three additives and in all nine samples. A tenth sample without additive was regarded as control sample thus prepared and was kept at $4\pm1^{O}C$ under ordinary conditions (without being closed air-tight) for three year period.

2.4. Control micro-organisms and measured parameters

Tests of contamination control by micro-organisms other than Bt were regularly carried out (every two months during the first year, and every six months in the second year) according to IUPAC recommendations as presented in **Table 2**. The target micro-organisms were: Salmonella, fecal Streptococci, fecal coliforms, Staphylococcus, yeasts and moulds. The pH of the samples was regularly measured by using pH paper (Hydrion Papers, Micro Essential laboratory, B'klyn, N.Y. 11210, USA).

The entomotoxicity measurements were carried out by using 2^{nd} instar spruce budworm larvae (*Choristoneura fumiferana*). In fact, for each sample of soya, SIW, NH and TH, five dilutions were performed in saline water and the last three dilutions were used for the entomotoxicty measurements as described by Adjallé *et al.* (2009), based on the method of Beegle (1990). In

fact, about 0.75 ml of each dilution was mixed with 15 ml of diet comprising different diet components (Tirado-Montiel *et al.*, 2001). The mixture thus obtained was distributed in a stack of 10 glass tubes of dimensions 15 mm x 45 mm (VWR, Canlab, Mississauga, Ontario, Canada). Each of the 30 tubes containing 1 ml of diet was used as a control. Other controls were prepared by mixing 1 ml of sterilized medium of soya, SIW, NH and TH with 30 ml of diet. Once the diet solidified and cooled, a larva was introduced into each tube. The tubes were capped by perforated caps and kept at 25 $\pm 1^{\circ}C$ for 7 days. Consequently, mortality was evaluated in each stack of 10 tubes. Calculations were performed using ANOVA tests and the standard deviation of counting was 7 to 8%.

3. RESULTS

3.1. Tests of contamination

Couch and Ignoffo (1981) estimated that a lifespan of 18 months was the minimum practical period for microbial pesticides. However, it should be noted that some commercial chemical pesticides can have a minimal lifespan of one to two years. Thus, Rhodos (1993) suggested that a lifespan of four years would be desirable for biopesticides in order to support their marketing compared to chemical pesticides. In this study, at the end of three years of storage, no contamination of the concerned micro-organisms (Salmonella, fecal Streptococci, fecal coliforms, Staphylococcus, yeasts and moulds) were detected in the formulations at 0.3 and 0.5% (w/w) concentration for each of the three additives used (propionic acid, ascorbic acid, sodium metabisulfite).

However, for the formulations being used as controls (centrifugate-retentate mixture without additives) for NH, TH, SIW and soya formulations containing 0.1% (w/w) of sodium metabisulfite and 0.1% (w/w) of ascorbic acid, there was onset of mould formation. In fact for NH and TH controls, 67 and 55 respective colonies of moulds per gram of formulation were counted. For the formulations of SIW (0.1% (w/w) of sodium metabisulfite) and soya (0.1% (w/w) of ascorbic acid), 61 and 44 respective colonies of moulds per gram of formulation were counted. Although these values are lower than the allowed limit (100/g), the presence of these moulds can be justified by the fact that, according to the results of the pH **(Table 3),** the addition of 0.1% (w/w) of concentration of the three additives did not vary the pH of the mixtures compared to those of controls. One can thus conclude that the concentration of 0.1% (w/w) of additive is not effective for protection against the contaminant micro-organisms. The pH of the formulations with 0.3% (w/w) and 0.5% (w/w) of concentrations of additives vary between 5.5 and 6, and are highly protective against the microbial contaminants. As concentrations of 0.3% (w/w) and 0.5% (w/w) additives gave an optimal pH for storage, the selection of better additives will also depend on the values of viable spores and entomotoxicity.

3.2. Effect of additives on viable spores and entomotoxicity

The results of entomotoxicity during a period of three years are presented in **Table 3**. It shows a loss of entomotoxic potential in all formulations of the four media as in their controls. In fact, the initial values of entomotoxicity (at the beginning of three years) for different controls of the four media (soya, SIW, TH and NH) were 22.9, 21.3, 23.2 and 18.3 (x10 ^{9} SBU/L), respectively. Compared with these values, losses observed after three years in the respective controls (42%, 23%, 29% and 50% for soya, SIW, TH and NH, respectively) and some of the formulations are same. Thus the formulation containing anti-microbial agents does not actually preserve the entomotoxic potential of the biopesticides. However, for certain concentration of additives, the values of entomotoxicity are higher in the formulations than their respective controls. The conservation is improved for formulations of soya, SIW and NH by using propionic acid at concentrations of 0.5%, 0.5% and 0.3% (w/w) respectively, and formulation of TH with sodium metabisulfite at 0.3% (w/w) concentration. In parallel, it is noted that for the same formulations, higher values of viable spores are obtained. This confirms the importance of spores for the entomotoxic potential of Bt, and the role of the anti-microbial additives in a formulation.

Thus, based on the values of the entomotoxicity and especially on those of the viable spores, one can conclude that propionic acid at concentrations of 0.5%, 0.5% and 0.3% (w/w) is more effective for the formulation of media of soya, SIW and NH, respectively, and sodium metabisulfite appears to be most effective at 0.3% w/w in the case of TH.

4. CONCLUSION

Propionic acid and sodium metabisulfite gave the best results as anti-microbial agents in the formulation of biopesticides produced using starch industry wastewater and wastewater sludge. This protection was accompanied in general by a reduction in the entomotoxic potential and number of viable spores.

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Table 1. Characteristics of starch industry wastewater and secondary sludge

Parameter (s)	Starch industry wastewater		Secondary sludge	
TS (g/l)	17	±1.1	18	±1.5
TVS (g/l)	14	±1.0	14	±1.1
SS (g/l)	2.2	±0.8	15	±1.0
VSS (g/l)	2.2	±0.7	13	±2
pH	3.3	±0.1	5.5	±0.1
	Concentration (mg/kg TS)			
C	700345	±6986	301097	±5987
N_t	37089	±1578	42307	±500
P_t	340176	±3001	7987	±203
$N\text{-}NH_3$	109.8	±59.8	889	±198.4
$N\text{-}NO_2^-, N\text{-}NO_3^-$	4.8	±1.2	14.7	±1.1
$P\text{-}PO_4^{3-}$	14987	±2801	4988	±402
Al	56987	±3798	4999	±437
Ca	11567	±402	14011	±511
Cd	0.54	±0.1	3.01	±0.9
Cr	1.3	±0.04	27.9	±1.1
Cu	338	±171.2	401	±157
Fe	7986.4	±803.4	11987	±603
K	23056	±3124	998	±371
Pb	27.4	±4.7	27	±4.3
S	2301.4	±62.5	4369	±538
Zn	250	±77	325	±197
Na	2189.4	±231	1456	±408
Ni	-	-	10.7	±3.9

Table 2. Microorganisms and identification tests

Microorganisms	Medium	Environmental conditions	Incubation time	Inference Test	Upper limits of concentration (1)
Yeast et Mold	Malt Extract Agar	pH = 3.5; T = 30 ± 1°C	4-7 days	Colonial morphology (dumb bell sharped under 100X magnification)	< 100/g
Feccal streptoccoci	Slanetz et Bartley Agar	T = 37 ± 1°C	48 h	Pink/dark red with white border	< 1 x 104/g
Coliforms fecal	MFC Agar/Endo Agar	T = 44.5 ± 1°C	24 h	Pink or blue colonies(''Chain-link" under the microscope)	< 10/g
Staphylococcus aureus	TSB; Plating on Baird Parker Agar + 5% Egg Tellurite	T = 35-37 ± 1°C	48 h (check at 24 h &46h)	Gram stain positive colonies (black and glossy)- resemble ''bunch of grapes'' under the microscope)	< 1/g
Salmonella	Lactose Broth and added Selenite (10 ml) and Tetrathionate Broth (10 ml)	T = 35 ± 1°C	18 – 24 h	Blackening with sheen (rod shaped without spores or crystal protein and characteristics flagella under the microscope	< 1/10g

IUPAC (Quinlan, 1990)

Table 3. Results of viable spores and entomotoxicity of different anti microbial formulations

Media	Parameters	Control (t=0)	Control (t=3 years)	Sodium metatbisulfite (% w/w)			Propionic acid (% w/w)			Sorbic acid (% w/w)		
				0.10	0.30	0.50	0.10	0.30	0.50	0.10	0.30	0.50
	pH	7.0	7.0	7.0	6.5	6.0	7.0	6.0	6.0	7.0	6.0	6.0
Soya	Entomotoxicity (10^9 SBU/L)	22.9	13.3		14.3	14.2		13.3	17.7		10.0	11.1
	Viable spores (10^8 CFU/mL)	22	6.4	4.1	9.2	11.1	7.3	12.8	15	6.6	10.3	12.2
	pH	7.5	7.5	7.0	6.0	6.0	7.0	6.0	5.5	6.0	6.0	5.5
SIW	Entomotoxicity (10^9 SBU/L)	21.3	16.4		15.0	17.7		17.6	19.3		11.6	13.3
	Viable spores (10^8 CFU/mL)	12.0	7.2	5.8	7.2	6.9	6.1	9.1	10.2	7.7	8.5	8.9
	pH	7.0	6.5	6.5	6.0	6.0	6.5	6.0	5.5	7.0	6.5	6.0
TH	Entomotoxicity (10^9 SBU/L)	23.2	16.3		18.3	17.7		13.3	14.4		11.8	11.8
	Viable spores (10^8 CFU/mL)	128.0	79.0	82.0	109.0	114.0	65.0	84.0	89.0	59.0	77.0	93.0
	pH	7.0	7.5	7.0	6.0	6.0	6.5	6.0	6.0	7.0	6.0	6.0
NH	Entomotoxicity (10^9 SBU/L)	18.3	9.1		11.0	9.8		13.0	13.5		10.3	13.7
	Viable spores (10^8 CFU/mL)	6.0	2	2.5	2.4	3.1	4.2	5.3	4.6	3.3	3.9	3.8

PARTIE III

(Résultats de l'objectif 6)

OPTIMIZATION OF SPRAY DRYING BY RESPONSE SURFACE METHODOLOGY FOR THE PRODUCTION OF *BACILLUS THURINGIENSIS* DRY BIOPESTICIDES BY USING WASTEWATER/WASTEWATER SLUDGE

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(MANUSCRIPT TO BE SUBMITED)

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Résumé

La méthode de réponse en surface (RSM) est utilisée pour optimiser un procédé de séchage par pulvérisation pour la production des poudres de biopesticides à base de *Bacillus thuringiensis* à partir des bouillons fermentés des eaux usées d'industrie d'amidon et des boues d'épuration de concentration respective en solides totaux de 15 g/L et 25 g/L. L'analyse statistique des résultats est faite par la méthode ANOVA et la RSM avec comme variables dépendantes le nombre de spores viables dans la poudre obtenue. Les coefficients de détermination des modèles obtenus sont de 92% et 94 % respectivement pour les bouillons fermentés des eaux usées d'industrie d'amidon et des boues d'épuration. Ainsi, Le débit d'alimentation de 0.29 g/min, le débit d'aspiration d'air chaud 51 m^3/min, la température d'entrée 180°C et la pression d'atomisation d'air 0.10 MPa sont les valeurs optimales des variables indépendantes dans le cas des eaux usées d'industrie d'amidon donnant un nombre de spores viables élevé de 2.2 x10^8 CFU/mg. Ces valeurs optimales sont 0.45 g/min, 0.49 m^3/min, 170°C, et 0.096 MPa dans le cas du bouillon fermenté de boues d'épuration avec un nombre de spores viables élevé de 1.3 x10^8 CFU/mg. On note alors une perte de spores viables de 18% et 13% par rapport aux bouillons fermenté initiaux des eaux usées des industries d'amidon et des boues d'épuration respectivement. Les valeurs d'entomotoxicité des poudres obtenues dans ces conditions optimales ont donné une perte de 28% et 18% par rapport aux bouillons fermentés des eaux usées des industries d'amidon et des boues d'épuration respectivement.

Mots clés: *Bacillus thuringiensis*; eaux usées; boue d'épuration; séchage par pulvérisation; méthode de réponse en surface

ABSTRACT

Response surface methodology (RSM) was used to optimize spray drying to produce powders of *Bacillus thuringiensis* based biopesticides using fermented broth of starch industry wastewater and wastewater sludge at total solids of 15 g/L and 25 g/L, respectively. The provisional optimal values of the independent variables (determined by steepest ascent method) were useful to design a central composite plan. The statistical analysis of the results was carried out by ANOVA and RSM using dependent variables, such as number of viable spores in the powder obtained. The coefficients of correlation obtained for the models were 92% and 94 % for the fermented broth of starch industry wastewater and wastewater sludge, respectively. Thus, at a feed rate of 0.29 g/min, the air inflow rate was at 51 m^3/min, the inlet temperature was 180°C and the pressure of atomization of air was 0.10 MPa as the optimal values of the independent variables for starch industry wastewater gave a viable number of spores as 2.2 x 10^8 CFU/mg. The optimal values were 0.45 g/min, 0.49 m^3/ min, 170°C, and 0.096 MPa for fermented wastewater sludge with a viable number of spores at 1.3 x 10^8 CFU/mg. Hence, there was a loss of viable spores of 18% and 13% for starch industry wastewater and wastewater sludge, respectively. The entomotoxicity (measured by the bioassay method by using 2nd instar spruce budworn larvae) of the powders obtained under these optimal conditions gave a loss of 28% and 18% for fermented starch industry wastewater and wastewater sludge, respectively.

Keywords: *Bacillus thuringiensis*; wastewater; sludge; spray drying, response surface methodology

1. INTRODUCTION

Biopesticides derived from bacteria, fungi, viruses and protozoa are gaining wide interest due to the adverse impacts of chemical pesticides. Among the biopesticides, *Bacillus thuringiensis* (Bt), var. k*urstaki,* is a widely employed biocontrol agent (Andrews *et al.,* 1987; Prabhakar and Bishop, 2009). Final product formulations of Bt comprise mixture of spores and insecticidal crystal proteins obtained after grown and sporulation in a bioreactor and settling of fermented broth. The biopesticides of Bt are usually marketed as solid and liquid formulations.

Compared with the liquid formulations, the solid formulations have the advantages of longer shelf-life, and ease of transport (Burges, 1998). Among the solid formulations, wettable powders obtained by drying the fermented broths containing the active components (spores, insecticidal crystal proteins, enzymes) are widely used. Drying is regarded as the suitable method of conservation to prevent contamination by micro-organisms (Kim and Bhowmik, 1990; Wan-Yin *et al.,* 1994). Among the industrial methods of production of wet powder by drying, the process of spray drying seems to be suitable process (Bertrand *et al.,* 2003; Xueyong *et al.,* 2008). However, the conditions of drying affect the viability of the spores and the efficacy of the insecticidal crystal protein. The drying parameters which affect the viability of the cells and spores include air inlet and outlet temperatures, type of atomization and/or pulverization, the strain of organism (Johnson and Etzel, 1993; Lievense *et al.*, 1994; Boza *et al.*, 2004; Xueyong *et al.,* 2008). Similarly, the drying process affects the size of the particles which is an important parameter in the efficacy of action during field application of the product (Walton, 2000; Brar *et al.* 2004). For these reasons, it is important to optimize the operational drying parameters in order to limit the negative impacts of drying on the active components of Bt (spores, insecticidal crystal proteins, enzymes, etc.).

The originality of this work lies in the fact that the fermented broth of Btk used for the spray drying process was fermented using wastewater and wastewater sludge that poses specific problems due to the complex nature of the substrates. Initial studies carried out in our laboratory on development of liquid formulations from fermented wastewater and wastewater sludge have yielded encouraging results (Brar *et al.,* 2005) leading to dry powder formulations. Looking at

the importance of dry formulations, and taking into account the rheology of starch industry wastewater and wastewater sludge, it would be interesting to evaluate the possibility and feasibility of the production of powders from *Bacillus thuringiensis* fermented broths. Thus, in this study, following aspects of Bt formulation will be addressed: (1) determination of optimal condition of drying (values of inlet temperature, spraying pressure, hot air flow and feed rate which give high viable spores concentration) of fermented broths of these two media by using the central composite design (CCD) and response surface methodology (RSM); and (2) evaluation of the impacts of these viable parameters on spores and entomotoxicity of Bt.

2. MATERIALS AND METHODS

2.1. Bacterial strain, culture medium and fermentation

The bacterial strain used in this study was *Bacillus thuringiensis* var. kurstaki (Btk) HD-1 (ATCC 33679). The media used for spray drying in this study were the fermented broths of Btk obtained using different substrates, namely, (i) secondary sludge from wastewater treatment plant of Communauté Urbaine de Québec, Ste Foy and (ii) starch industry wastewater from ADM-Ogilvie (Candiac, Quebec, Canada). Starch industry wastewater was directly used for fermentation after *in-situ* sterilization at 121±1°C, at a pressure of 15 psig for 30 min, whereas secondary sludges were pre-treated (hydrolyzed) at 140±1°C, at 40 psig pressure for 30 min. Fermentation was conducted in a bioreactor of 15 l (Biogénie Inc., Québec, Quebec) with accessories, connected to a computer with the *iFix 3.5, intellution* software, (Massachusetts, USA) for the control of pH, temperature, air flow, agitation and anti-foam. At the end of fermentation, the pH was lowered from 7 to 4.5 and the fermented broth was aspetically collected in a sterilized container for eventual drying by spray dryer. Henceforth, the fermented starch industry wastewater and hydrolyzed wastewater sludge will be referred to as SIW and TH, respectively.

2.2. Spray drying process

Yamato spray dryer, Model Pulvis GB 22, of Yamato Scientific America, Inc (South San Francisco, CA, USA) was used for the drying of different fermented broths. The principle of operation is detailed in a process scheme in Figure 1. The fermented broth sample was conveyed to the spray dryer nozzle by means of a peristaltic pump. In this study, because of the large different of concentration between the two fermented broths, two different diameter of nozzle were used (0.711 mm for SIW and 1 mm for TH). At the entry of nozzle, sample was mixed with air under pressure (controlled by a needle valve) and the mixture was spray dried in a ``drying chamber''. The diameter of liquid droplets obtained was approximately 20 μm with a surface area of 3.00 $cm^2$ per ml of the sample (as per Yamato instructions manual). The droplets were instantaneously dried by hot air in the ``drying chamber''. If the surface of contact of the droplets

and the hot air is large, more than 90% of the moisture is evaporated in the ``drying chamber" (Yamato manual). Finally, the dried powder particles were collected in the ''product vessel'' after separated from the vapor of moisture through the "cyclone". The characteristic of powders (moisture, particles sizes, solubility, etc.) obtained, and the viability and potential of actif components (cells, spores, insecticidal crystal proteins, etc.) depend of the suitable operational parameters, which require optimization. The important parameters of spray dryer are as follows: (1) inlet temperature which measures the degree of hot air; (2) rate of feed which defines the concentration of the sample and determines the water content in the air-sample mixture; (3) pressure of atomization of air defining the size and surface of the droplets; and (4) flow rate of aspiration of hot air which determines the duration of contact between the hot air and droplets of pulverized sample-air mixture.

By applying the response surface methodology (RSM), and covering a large interval of the parameters as described above, it was possible to determine the optimal values of these parameters. These parameters make it possible to obtain low moisture content, such as outlet temperature not affecting the viable spores and thus the crystal proteins, high number of viable spores and high entomotoxicity. Additionally, this statistical RSM will make it possible, on one hand, to precisely evaluate the significant effects of various parameters on the moisture content, viable spores, entomotoxicity; and on the other hand, to evaluate the interactions if there is any, between these drying operational parameters.

2.3. Experimental design

2.3.1. Independent and dependent variables

In the statistical response surface method, the operational parameters, such as feed rate of the pump (X_1), flow rate of hot air (X_2), inlet air temperature (X_3) and the pressure of atomization of air (X_4) are regarded as independent variables. The outlet air temperature (T_{outlet}) (which can be controlled by inlet temperature and feed rate), moisture content (M), viable spores count (S_v) and entomotoxicity (T_X) are dependent variables. A study of determination of the provisional optimal values of these variables was carried out by using the steepest ascent method.

2.3.2. Method of ascending slope

The method of the ascending slope makes it possible to follow the direction of the response while varying the independent variables (Chen *et al.,* 2009). The orientation of the slope follows the direction so that it increases while the dependent variable is decreased and the independent variables are increased.

2.3.3. Central composite design (CCD) and response surface methodology (RSM)

In continuation of the experiments to determine the provisional optimal values of the independent variables, a central composite plan will be drawn to cover the relevant values of the independent variables. The results of the CCD experiments and measurements of the dependent variables (moisture content, outlet temperature, number of viable spores and entomotoxicity) will be analyzed by STATISTICA 6 of STATSOFT Inc. (Thulsa, U.S.) by surface response methodology. The effects of independent variables on the Y response of the dependent variables were analyzed according to a polynomial model of second order of surface response given by the following general Equation:

$$Y = A_o + \sum_{i=1}^{n} A_i X_i + \sum_{j \leq i}^{n} B_{ij} X_i X_j \qquad (1)$$

Where, Y = predicted response and A_O = intercept, X_i and X_J = values of various levels of the independent variables, A_i = values of linear coefficients, B_{ij} = values of quadratic coefficients.

2.4. Measurement of dependent variables

2.4.1. Outlet temperature and moisture content

The outlet temperature is directly measured at the exit port of the powders by the corresponding probe (Figure 1). The water content (moisture) of different dry powders was obtained by drying the samples by using HR 83 Halogen Moisture analyzer (Mettler 120 Toledo, Ontario, Canada).

2.4.1. Viable spores and entomotoxicity

For the counting of viable spores and the entomotoxicity measurements, a suspension containing 0.075 g of SIW and 0.125 g of TH powder in 5 mL sterilized water. Thus, the mixture prepared possesses the same concentration as the fermented broths of SIW and TH. The mixtures so obtained were diluted in sterilized saline water (0.85% w/v) before the counting of viable spores which was carried out according to the procedure described by Vidyarthi *et al.* (2000). It comprised heating of the sample to 80°C for 10 min in an oil bath (Thermo-lift, Buchler instrument, USA) followed by rapid cooling in a cold water bath before spreading on the tryptic soya agar plates. The plates were incubated at 30°C, for 24 h, and after, the count of viable spores was done. The standard deviation of counting was 7 to 8%.

The entomotoxicity measurements were carried out by using 2nd instars spruce budworm larvae (*Choristoneura fumiferana*). In fact, for the given suspensions of 5mL prepared with powder of SIW and TH, five dilutions were performed in saline water and the last three dilutions were used for the entomotoxicty measurements as described by Adjallé *et al.* (2009), based on the method of Beegle (1990). In fact, about 0.75 ml of each dilution was mixed with 15 ml of diet comprising different diet components (Tirado-Montiel *et al.*, 2001). The mixture thus obtained was distributed in a stack of 10 glass tubes of dimensions 15 mm x 45 mm (VWR, Canlab, Mississauga, Ontario, Canada). Each of the 30 tubes containing 1 ml of diet was used as a control. Other controls were prepared by mixing 1 ml of sterilized medium of soya, SIW and TH with 30 ml of diet. Once the diet solidified and cooled, a larva was introduced into each tube. The tubes were capped by perforated caps and kept at 25 ±1°C for 7 d. Consequently, mortality was evaluated in each stack of 10 tubes. Data were subjected to ANOVA for determination of significant differences.

3. RESULTS AND DISCUSSION

3.1. Composition of the medium and concentration of the fermented broths

The characteristics of starch industry wastewater and wastewater sludge used in this study are presented in Table 1. The concentrations of the fermented broths of starch industry wastewater and wastewater sludge used to feed the spray dryer were 15 g/L and 25 g/L, respectively. The solids concentration affected the final spray drying process which has been discussed in details later.

3.2. Steepest ascent method

The results of the experiments of the method of the steepest ascent for the two media are presented in Table 2. When optimized, spray drying removes more than 90% of moisture (Alamilla *et al.,* 2005), the optimal values of the independent variables should make it possible to obtain a moisture lower than 11% (p/p) for the biopesticide powders. For the two media, the values of moisture content were lower than 11%. Thus, the high values of viable spores which will make it possible to determine the provisional optimal values for the central composite design (CCD).

For SIW, the experimental plan and the results showed that experiment n° 5 gave a high value of viable spores (3.90×10^7 CFU/mg) with a moisture content of 4.2% (g/g). Provisional optimal values of the independent variables, X_1, X_2, X_3, and X_4 were 0.29 g/min, 0.52 m^3/min, 180°C and 0.11 MPa, respectively. In the case of TH, the condition that made it possible to determine the optimal provisional values was given by experiment n° 4, and the provisional optimal values of the independent variables, X_1, X_2, X_3, and X_4 were 0.45 g/min, 0.59 m^3/min, 170°C and 0.095 MPa, respectively with response for viable spores of 1.40×10^7 CFU/mg. These values will make it possible to establish the central composite design plan of the experiments.

3.3. Central composite design (CCD) and response surface methodology (RSM)

The CCD consists of an experiment, except for the central point where there can be replications. For the two media, Table 3 presents the principal values at five levels (-2, -1, 0, 1, 2) allotted to the four independent variables. Tables 4 and 5 present the experimental plan with 7 replicates at the center, as well as results of the dependent variables (outlet temperature (T_{outlet}), moisture content (M), viable spores (Sv) and entomotoxicity (T_X)). In the light of these results, following analyses and interpretations were drawn:

3.3.1. Outlet temperature and moisture content

It was observed that, for SIW and TH, the values of outlet temperatures were relatively lower than the temperature used for determination of the count of viable spores (80°C), and were far from largely affecting the spores and the insecticidal crystal proteins which can tolerate temperatures higher than these values. These outlet temperatures were slightly lower than those obtained by Xueyong *et al.* (2008) who used the method of spray drying for Bt powders. Likewise, except for experiment n° 18 in the case of SIW, moisture values were lower than 10%, similar to the ones obtained by Alamilla *et al.* (2005). This high value of moisture content for experiment n° 18 (13.75%) in the case of SIW, was probably due to the high feed rate (0.35 g/min). This experiment also gave lower value of entomotoxicity (1.11×10^8 SBU/g). This can be explained by the fact that high moisture content decreases the quantity of dry matter contained in 0.075 g of powder used for the measurement of Tx and that of the number of viable spores.

3.3.2. Viable spores

An analysis of the variance is carried out to determine the ``lack of fit'' and the significant effect of the independent variables on the viable spores (S_v) of the two media (SIW and TH). The lack of fit test is a measure of the failure of the model to represent data in the experimental region in which points were not included in the regression (Varnalis *et al.* 2004). The coefficient of

determination, R^2 of the model represents the percentage of variation in the response which can be explained by the model. It is important that this percentage is at least higher than 80%. In this study, the analysis of the results on the basis of Equation (1) by ANOVA method showed that the regression model resulted in coefficients of determination, R^2 of 0.92 and 0.94 for SIW and TH, respectively. The variability in the response with the supposition of 92% for SIW and 94% for TH can be explained by this model. The statistical analysis, ANOVA for the two media demonstrated that the quadratic effects (X_1^2, X_2^2, X_3^2, X_4^2) of the four independent variables were all negative and significant. The linear effects and the interactions were not significant in the case of two media.

In the case of SIW, the statistical analysis of the coefficients of regression showed that: (i) linear coefficients of the variables X_1, X_2, X_3 produced statistically significant positive effects (P<0.0002), (ii) quadratic coefficients of X_1^2, X_2^2, X_3^2 and X_4^2 have statistically significant negative effects (P = 0.0002) and; (iii) coefficient of interactions were not statistically significant (P>0.7). Thus, the Equation for SIW will be:

$$S_v\,(SIW) = -2.7 \times 10^{10} + 3.5 \times 10^{10} X_1 - 5.6 \times 10^{10} X_1^2 + 2.0 \times 10^{10} X_2 - 1.9 \times 10^{10} X_2^2 + 1.7 \times 10^{8} X_3 - 4.8 \times 10^{5} X_3^2 - 6.9 \times 10^{10} X_4^2 \quad (2)$$

Where, S_v = viable spore count in the SIW medium; X_1 = feed rate; X_2 = aspiration rate; X_3 = inlet temperature; X_4 = pressure of air atomization.

The method of surface response was used to determine the optimum conditions for drying that gave high viable spore counts. The independent effects of feed rate (X_1), hot air rate (X_2), inlet temperature (X_3) and pressure of air atomization (X_4) on the number of spores are presented in Figures 2a and 2b. Thus, X_1 = 0.29 g/min, X_2 = 0.51 m^3/min, X_3 = 180°C and X_4= 0.10 MPa are the optimal values of independent variables for high spore count in the case of SIW. This optimal condition gave a spore count of 2.2 x 10^8 CFU/mg as compared to that of 2.7x 10^8 CFU/mg in the initial fermented broth which gave a relatively high loss of 18%. This is

explained by the combined negative effects of the independent variables which affect the viability of the spores and thus reduced the viable spores count.

In the case of TH, the regression model showed the same configuration as noted for the quadratic effects (negative and significant with $p<0.0002$). However, the linear coefficients, while being positive, produced less significant effects than in the case of SIW ($0.002 = P = 0.02$). Hence, the Equation for TH will be:

$$S_v\,(TH) = -4.0 \times 10^{10} + 1.2 \times 10^{10} X_1 - 1.2 \times 10^{10} X_1^2 + 1.1 \times 10^{10} X_2 - 1.2 \times 10^{10} X_2^2 + 3.9 \times 10^8 X_3 - 1.2 \times 10^6 X_3^2 + 3.17 \times 10^{10} X_4 - 1.8 \times 10^{11} X_4^2 \quad (3)$$

where, S_v = viable spore count in TH medium; X_1 = feed rate; X_2 = aspiration rate; X_3 = inlet temperature; X_4 = pressure of air atomization.

With this model, following optimal values were obtained: $X_1 = 0.45$ g/min, $X_2 = 0.49$ m^3/min, $X_3 = 170^oC$ and $X_4 = 0.096$ MPa. This condition gave high viable spore count of 1.3×10^8 CFU/mg (Figures 3a and 3b) as compared with 1.5×10^8 CFU/mg in the initial fermented broth of TH, which gave a relatively high loss of 13%.

3.3.3. Entomotoxicity

The analysis of the results of entomotoxicity for the two media on the basis of Equation (1) by ANOVA method showed that the regression models demonstrated lower coefficient of determination (R^2) of 57% and 58% for SIW and TH, respectively. These low coefficients can be explained by the fact that the measurement of entomotoxicity involved other parameters, such as the larval environment, diet and conditions (temperature and external moisture) which can influence the actual obtained values. As the coefficients were poor, it was not necessary to determine the optimal conditions using these models. However, with the given optimal values by statistical analyses of the results of the spores, entomotoxicity of 7.20×10^8 SBU/g for SIW and 5.99×10^8 SBU/g for TH were obtained when compared with 9.98×10^8 SBU/g and

7.31 x 10^8 SBU/g in their respective fermented broths. Hence, there was an entomotoxicity loss of 28% in the case of SIW and 18% in the case of TH. This loss can be explained by the reduction in viability of various active components of Bt (spores, insecticidal crystal proteins, enzymes) which are affected by the inlet temperature and pressure during spray drying. The fact that this loss is higher in the case of SIW was explained by lower concentration of total solids in the fermented broth. In fact, lower concentration of the fermented broth will comprise low concentration of active components and high moisture in the water droplet, which takes more time to evaporate. Thus at high temperature, the active components in the medium with low concentration consume more heat and require longer duration to dry than a medium of higher concentration.

In the light of the results of this work, it was observed that there was not a large difference between the optimal conditions of two media (SIW - 15 g/L and TH - 25 g/L). In fact, according to Vu *et al.* (2009), the increase in the concentration of SIW up to 30 g/L enhances the viable spores count and the entomotoxicity of the fermented broth. Thus, one can increase viable spores and entomotoxicity obtained in the powders of SIW by increasing the solids concentration of the fermentation medium.

3.4. Estimation of fermented broth volume for powder formulation and field application

By using these optimized results of spray-drying, the volume of fermented broth required to produce powder product in order to treat 1 ha of balsam fir was calculated and compared with the volume required for liquid product. Field efficacy of Btk based biopesticides to specify application prescriptions for optimal protection of balsam fir, *Abies balsamea* (L.), healthy stands against the spruce budworm was determined over a period of 6 years (1996-2001) in southwestern Québec (Bauce *et al.*, 2004). The recommended dosage of 30 BIU could be applied in lower application volumes (1.5 L/ha) by using a high-potency product (20 BIU/L) or higher application volumes (2.37L/ha) of a lower potency product (12.7 BIU/L) without affecting the field efficacy (Bauce *et al.*, 2004). Commercial Btk biopesticides products are normally based on

International Unit (IU) determined using cabbage looper larvae as target insect for bioassay (Dulmage *et al.*, 1971). In our earlier research, we found that SBU (spruce budworm unit) determined against spruce budworm is 20-25% higher than IU (Yezza *et al.*, 2006). Therefore, the SBU measured in this research was converted to IU by using the above factor of conversion.

Table 6 demonstrates the volume of fermented broth of SIW and TH required for producing powder formulation which can be applied to treat 1 ha of balsam fir. Following assumptions were considered in the calculation: (1) Loss of Tx in dried powders of SIW and TH was 28 and 18%, respectively after spray-drying due to the destruction of bioactive compounds during spray drying; (2) 11% of the powder will be lost during industrial spray drying process in drying chamber wall and in transfer pipes etc. of spray-drier (R. Lewandoskii, personal communication); (3) Tx value of dried powder will be reduced by 25 % due to the conversion from SBU into IU (Yezza *et al.*, 2006); (4) Tx obtained after powder formulation will be reduced by about 10% due to the addition of additives required for formulation (dilution) (Brar *et al.*, 2006b); (5) standard application of Bt based biopesticides is 30 BIU/ha of balsam fir (Bauce *et al.*, 2004).

In the case of liquid formulation, the IU values of formulated product were reduced to 22% due to the fortification of additives (antimicrobial agents, preservatives, phagostimulants, stickers, etc.) for formulation (Brar *et al.*, 2006b). Based on standard application of 30 BIU/ha, our previous research demonstrated 21-22 L of fermented broth of SIW (15 g/L total solids) was required to produce the final liquid formulation (2.1-2.2 L of suspension) to treat 1 ha of balsam fir (Vu *et al.*, 2009).

The volume of fermented broth required for powder formulation is less than that of liquid formulation (Table 6). In fact, it is known that centrifugation of fermented broth could not recover all bioactive compounds, such as residual delta-endotoxin, spores, enzymes, vegetative insecticidal proteins (Vips) (Brar *et al.*, 2006; Adjallé *et al.*, 2007). These compounds are soluble and present in the supernatant of centrifuged fermented broth (Brar *et al.*, 2006a; Adjallé *et al.*, 2007) which was usually discarded. In powder formulation, these bioactive compounds were recovered by spray-drying. For example, with 10 L of fermented broth (15 g/L total solids) used for production of liquid or powder formulation, the obtained products will have different total

solids concentration. In liquid formulation, after centrifugation and mixing the pellet with supernatant, 1 L of suspended pellet with total solids concentration of 50 g/L and total Tx of 20.9 x1 0^9 SBU was obtained (Vu *et al.*, 2009). In powder formulation, after spray drying, about 133.5 g of total solids with total Tx of 96.1 x 10^9 SBU was obtained. Thus, about 83.5 g of solids were lost after centrifugation of fermented broth in liquid formulation. The lost solids probably contained bioactive compounds incurring higher volumes of fermented broth for liquid formulation.

It is clear that powder formulation have some advantages as compared with liquid formulation: (1) requires less volume of fermented broth to produce formulated product to treat per ha of balsam fir; (2) requires less space for storage; (3) can be stored at room temperature; (4) less chance of contamination due to less humidity; (4) spray-drying can convert the entire fermented broth into solid form and doesn't require centrifugation step; and (5) supernatant obtained after centrifugation of fermented broth (in the case of liquid formulation) requires further treatment (wastewater treatment).

CONCLUSIONS

Optimization of spray drying processes employing surface response method for the production of Bt powders from the fermented broths, namely starch industry wastewater and hydrolyzed sludge, led to following conclusions:

1. Optimal values of the independent variables allowed high number of viable spores with coefficients of determination, R2 acceptable and higher than 90% for the two media. The optimal conditions for feed rate, dry air flow rate, inlet temperature and pressure of air atomization were as follows: 29 g/min, 51 m3/min, 180oC, 0.10 MPa for SIW and 45 g/min, 49 m3/min, 170oC, 0.096 MPa for TH sludge.

2. Under the optimal conditions, the number of viable spores decreased by 18% in the case of starch industry wastewater and 13% for hydrolyzed sludge due to the negative effects of the variables of process of spray drying.

3. The application of the response surface method to the results of the entomotoxicity gave lower coefficients of determination of R2 < 60%. However, the optimal condition values for spores resulted in a loss of entomotoxicity of 28% and 18% for the fermented broths of starch industry wastewater and hydrolyzed sludge, respectively.

4. Lower loss of viable spores and entomotoxicity observed in the case of starch industry wastewater compared to hydrolyzed sludge were due to the lower solids concentration of starch industry wastewater fermented broth (15 g/L) as compared to 25 g/L for hydrolyzed sludge.

5. The volume of fermented broth required to produce powder formulation for application to treat 1 ha of balsam fir is very less compared to that required for a liquid formulation obtained with the centrifugation pellet.

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Table 1. Characteristics of starch industry wastewater and secondary sludge

Parameter (s)	Starch industry wastewater	Secondary sludge
TS (g/l)	17 ±1.1	18 ±1.5
TVS (g/l)	14 ±1.0	14 ±1.1
SS (g/l)	2.2 ±0.8	15 ±1.0
VSS (g/l)	2.2 ±0.7	13 ±2
pH	3.3 ±0.1	5.5 ±0.1
	Concentration (mg/kg TS)	
C	700345 ±6986	301097 ±5987
N_t	37089 ±1578	42307 ±500
P_t	340176 ±3001	7987 ±203
$N\text{-}NH_3$	109.8 ±59.8	889 ±198.4
$N\text{-}NO_2$, $N\text{-}NO_3$	4.8 ±1.2	14.7 ±1.1
$P\text{-}PO_4$	14987 ±2801	4988 ±402
Al	56987 ±3798	4999 ±437
Ca	11567 ±402	14011 ±511
Cd	0.54 ±0.1	3.01 ±0.9
Cr	1.3 ±0.04	27.9 ±1.1
Cu	338 ±171.2	401 ±157
Fe	7986.4 ±803.4	11987 ±603
K	23056 ±3124	998 ±371
Pb	27.4 ±4.7	27 ±4.3
S	2301.4 ±62.5	4369 ±538
Zn	250 ±77	325 ±197
Na	2189.4 ±231	1456 ±408
Ni	- -	10.7 ±3.9

Table 2. Results of the steepest ascent method.

Starch industry wastewater						
n°	X_1 (g/min)	X_2 (m^3/min)	X_3 (°C)	X_4 (MPa)	M (%)	Sv (CFU/mg)
1	0.23	0.40	140	0.050	10.4	1.65E+07
2	0.24	0.43	150	0.065	8.9	1.80E+07
3	0.26	0.46	160	0.080	7.2	1.65E+07
4	0.27	0.49	170	0.095	5.6	1.75E+07
5	0.29	0.52	180	0.110	4.2	3.90E+07
6	0.30	0.55	190	0.125	3.9	8.90E+06
7	0.32	0.58	200	0.140	3.5	8.30E+06
8	0.33	0.61	210	0.155	3.5	8.45E+06
Secondary hydrolyzed sludge						
n°	X_1 (g/min)	X_2 (m^3/min)	X_3 (°C)	X_4 (MPa)	M (%)	Sv (CFU/mg)
1	0.38	0.40	140	0.050	9.2	7.60E+06
2	0.40	0.43	150	0.065	8.8	8.90E+06
3	0.43	0.46	160	0.080	7.4	9.80E+06
4	0.45	0.49	170	0.095	5.9	1.40E+07
5	0.48	0.52	180	0.110	4.4	1.20E+07
6	0.50	0.55	190	0.125	4.1	1.20E+07
7	0.53	0.58	200	0.140	3.8	1.10E+07
8	0.55	0.61	210	0.155	3.8	1.06E+07

(X_1) feed rate of the pump; (X_2) flow rate of hot air; (X_3) inlet air temperature and (X_4) pressure of atomization of air

Table 3. Levels of the factors tested in the central composite design

Starch industry wastewater

Independent variables	Symbol	Levels of factors				
		-2	-1	0	1	2
Feed flow rate	X_1 (g/min)	0.23	0.26	0.29	0.32	0.35
Hot air aspiration flow rate	X_2 (m^3/min)	0.42	0.47	0.52	0.57	0.62
Inlet temperature	X_3 (°C)	160	170	180	190	200
Pulverization Pressure	X_4 (MPa)	0.060	0.075	0.110	0.125	0.16

Secondary hydrolyzed sludge

Independent variables	Symbol	Levels of factors				
		-2	-1	0	1	2
Feed flow rate	X_1 (g/min)	0.35	0.40	0.45	0.50	0.55
Hot air aspiration flow rate	X_2 (m^3/min)	0.39	0.44	0.49	0.54	0.59
Inlet temperature	X_3 (°C)	160	165	170	175	180
Pulverization Pressure	X_4 (MPa)	0.070	0.085	0.095	0.110	0.120

Table 4. Results of experimental plan by central composite design for starch industry wastewater.

n°	Independent variables				dependent variables			
	X_1 (g/min)	X_2 (m^3/min)	X_3 (°C)	X_4 (MPa)	Toutlet (oC)	M (%)	Sv (CFU/mg)	Tx ($x10^6$ SBU/g)
1	0.26	0.47	170	0.075	44	4.14	1.1E+07	554
2	0.26	0.47	170	0.125	44	4.67	1.1E+07	610
3	0.26	0.47	190	0.075	52	4.12	2.1E+07	499
4	0.26	0.47	190	0.125	52	4.12	2.3E+07	480
5	0.26	0.57	170	0.075	65	5.65	1.6E+07	443
6	0.26	0.57	170	0.125	70	5.36	1.7E+07	671
7	0.26	0.57	190	0.075	73	5.25	2.7E+07	234
8	0.26	0.57	190	0.125	75	5.25	2.7E+07	222
9	0.32	0.47	170	0.075	42	5.59	3.2E+07	610
10	0.32	0.47	170	0.125	43	9.60	3.2E+07	720
11	0.32	0.47	190	0.075	45	5.57	3.1E+07	554
12	0.32	0.47	190	0.125	46	4.29	3.1E+07	530
13	0.32	0.57	170	0.075	51	8.01	3.1E+07	554
14	0.32	0.57	170	0.125	53	5.13	3.1E+07	523
15	0.32	0.57	190	0.075	71	4.52	2.9E+07	582
16	0.32	0.57	190	0.125	73	4.30	3.0E+07	388
17	0.23	0.52	180	0.110	58	4.99	2.6E+07	831
18	0.35	0.52	180	0.110	45	13.75	2.3E+07	111
19	0.29	0.42	180	0.110	46	4.29	4.4E+07	659
20	0.29	0.62	180	0.110	62	5.29	2.6E+07	610
21	0.29	0.52	160	0.110	40	8.42	3.4E+07	554
22	0.29	0.52	200	0.110	58	4.87	3.5E+07	388
23	0.29	0.52	180	0.060	56	3.92	4.0E+07	610
24	0.29	0.52	180	0.160	55	3.27	3.0E+07	610
25	0.29	0.52	180	0.110	54	3.45	2.3E+08	665
26	0.29	0.52	180	0.110	53	3.46	1.9E+08	665
27	0.29	0.52	180	0.110	53	3.53	2.2E+08	720
28	0.29	0.52	180	0.110	53	3.68	1.6E+08	655
29	0.29	0.52	180	0.110	53	3.62	2.4E+08	637
30	0.29	0.52	180	0.110	54	3.84	2.8E+08	776
31	0.29	0.52	180	0.110	55	3.84	2.4E+08	677

(X_1) feed rate of the pump; (X_2) flow rate of hot air; (X_3) inlet air temperature and (X_4) pressure of atomization of air

Table 5. Results of experimental plan by central composite design for secondary hydrolyzed sludge.

	Independentvariables				dependent variables			
n°	X_1 (g/min)	X_2 (m^3/min)	X_3 (°C)	X_4 (MPa)	Toutlet (oC)	M (%)	Sv (CFU/mg)	Tx (x10^6 SBU/g)
1	0.40	0.44	165	0.085	45	3.99	1.5E+07	499
2	0.40	0.44	165	0.110	46	4.37	6.6E+06	466
3	0.40	0.44	175	0.085	49	4.25	1.3E+07	432
4	0.40	0.44	175	0.110	53	3.45	1.4E+07	399
5	0.40	0.54	165	0.085	67	4.59	9.6E+06	424
6	0.40	0.54	165	0.110	73	4.89	1.0E+07	451
7	0.40	0.54	175	0.085	74	4.97	1.6E+07	366
8	0.40	0.54	175	0.110	77	5.12	1.6E+07	563
9	0.50	0.44	165	0.085	45	4.95	1.9E+07	499
10	0.50	0.44	165	0.110	44	7.25	1.9E+07	447
11	0.50	0.44	175	0.085	46	5.75	1.9E+07	432
12	0.50	0.44	175	0.110	49	4.75	1.9E+07	451
13	0.50	0.54	165	0.085	52	6.13	1.9E+07	488
14	0.50	0.54	165	0.110	54	4.93	1.9E+07	432
15	0.50	0.54	175	0.085	72	4.35	1.7E+07	466
16	0.50	0.54	175	0.110	70	4.51	1.8E+07	399
17	0.35	0.49	170	0.095	61	4.67	1.5E+07	599
18	0.55	0.49	170	0.095	46	9.54	1.7E+07	565
19	0.45	0.39	170	0.095	47	4.73	2.7E+07	528
20	0.45	0.59	170	0.095	63	5.19	1.6E+07	499
21	0.45	0.49	160	0.095	42	6.99	2.0E+07	466
22	0.45	0.49	180	0.095	61	4.11	2.1E+07	521
23	0.45	0.49	170	0.070	58	3.15	2.4E+07	480
24	0.45	0.49	170	0.120	56	3.74	1.8E+07	532
25	0.45	0.49	170	0.095	55	3.45	1.4E+08	532
26	0.45	0.49	170	0.095	55	3.56	1.1E+08	665
27	0.45	0.49	170	0.095	56	3,67	1.3E+08	543
28	0.45	0.49	170	0.095	54	3.71	9.4E+07	576
29	0.45	0.49	170	0.095	55	3.65	1.4E+08	565
30	0.45	0.49	170	0.095	57	3.49	1.7E+08	599
31	0.45	0.49	170	0.095	57	3.79	1.4E+08	599

(X_1) feed rate of the pump; (X_2) flow rate of hot air; (X_3) inlet air temperature and (X_4) pressure of atomization of air

Table 6. Volume of fermented broth required to produce powder formulation which can be applied to 1 ha of balsam fir (30 BIU/ha)

Fermentation medium	SIW	TH
Total solids concentration of 1 L of fermented broth (g)	15	25
Tx of fermented broth (10^9 SBU/g)	1	0.73
Tx of dried powder (10^9 SBU/g)	0.72	0.6
Loss of Tx during spray-drying (%) [1]	28	18
Powder loss in the chamber wall of spray-drier (%)	11	11
Total dried powder obtained from 1 L of fermented broth (g) [2]	13.35	22.25
Total Tx obtained from 1 L of fermented broth after spray-drying (10^9 SBU) [3]	9.6	13.4
Total Tx obtained from 1 L of fermented broth after spray-drying (10^9 BIU) [4]	7.2	10.1
Total Tx of formulated powder from 1 L of fermented broth (BIU) [5]	6.5	9.1
Volume of fermented broth required to produce formulated powder to treat 1 ha [6]	4.62	3.3

1 Due to destruction of bioactive compounds during spray-drying

[2] Calculated based on the loss of powder during spray-drying

[3] Multiplying Tx of dried powder with total dried powder obtained from 1 L of fermented broth

[4] Conversion of SBU to BIU (Yezza *et al.*, 2006)

[5] Tx will be reduced to 10 % due to formulation of dried powder with formulated agents

[6] Based on standard application of Bt biopesticides (30 BIU/ha) (Bauce *et al.*, 2004)

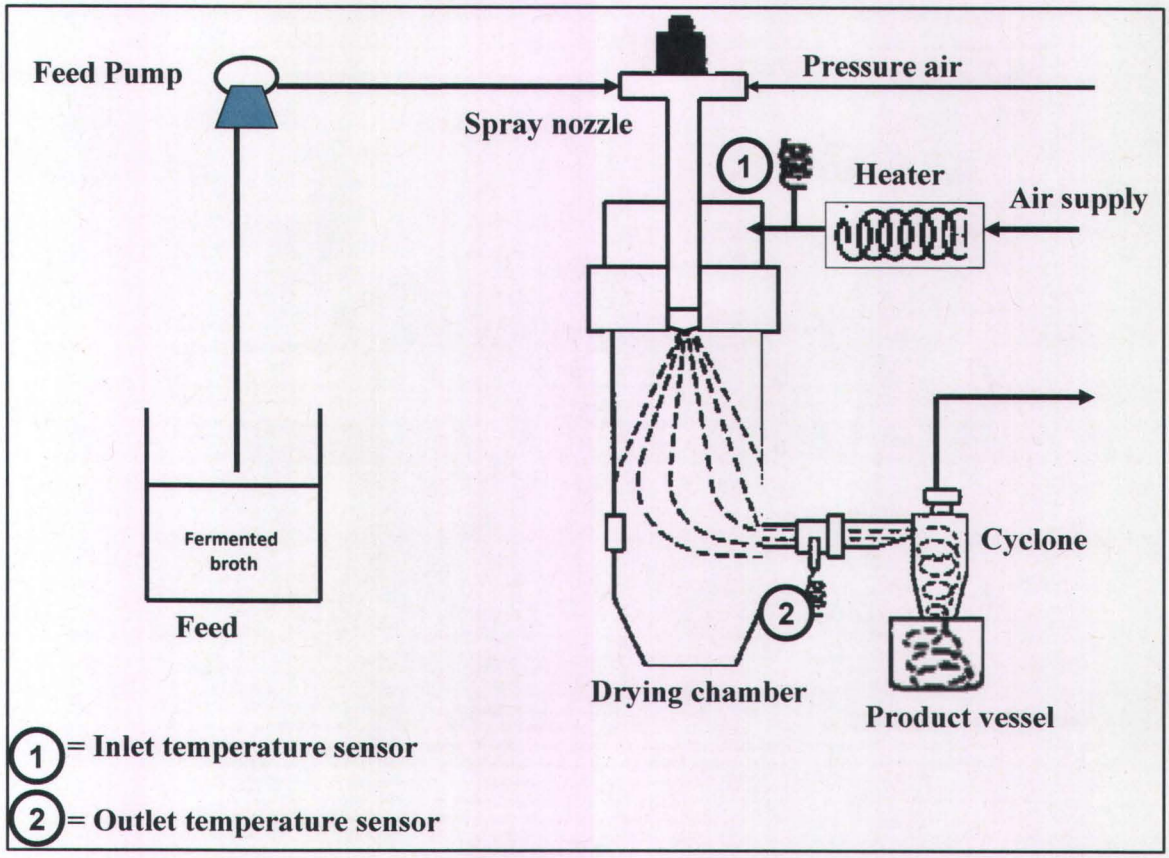

Fig.1. Schematic of spray dryer

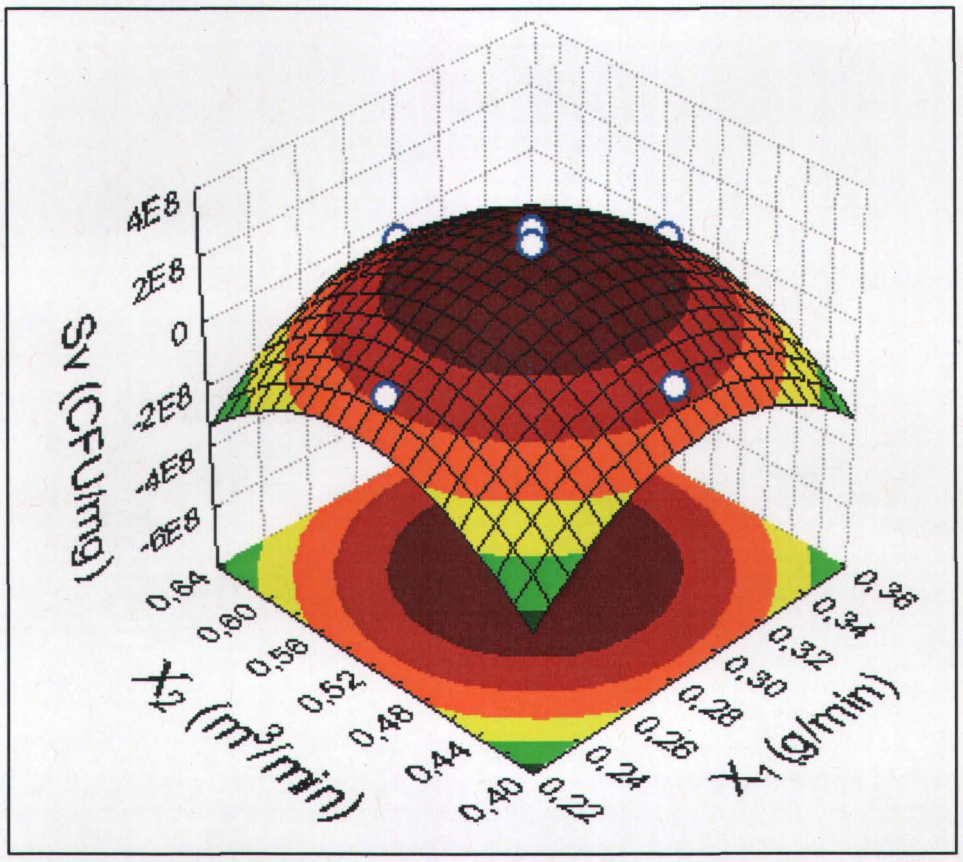

Fig. 2a. Response surface of viable spores obtained by varying feed flow (X_1) rate and hot air flow rate (X_2) and keeping inlet temperature (X_3) and air atomization pressure (X_4) constant (case of SIW)

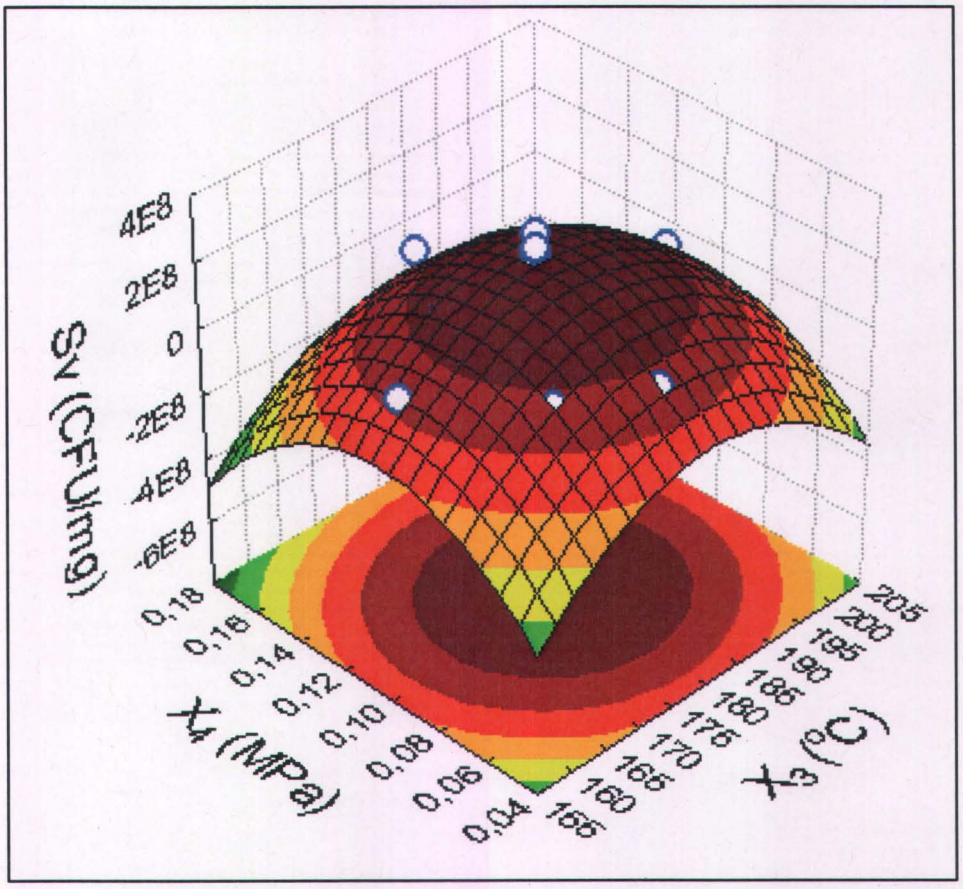

Fig. 2b. Response surface of viable spores obtained by varying the inlet temperature (X_3) and air atomization pressure (X_4) and keeping feed flow (X_1) and hot air flow rate (X_2) constant (case of SIW)

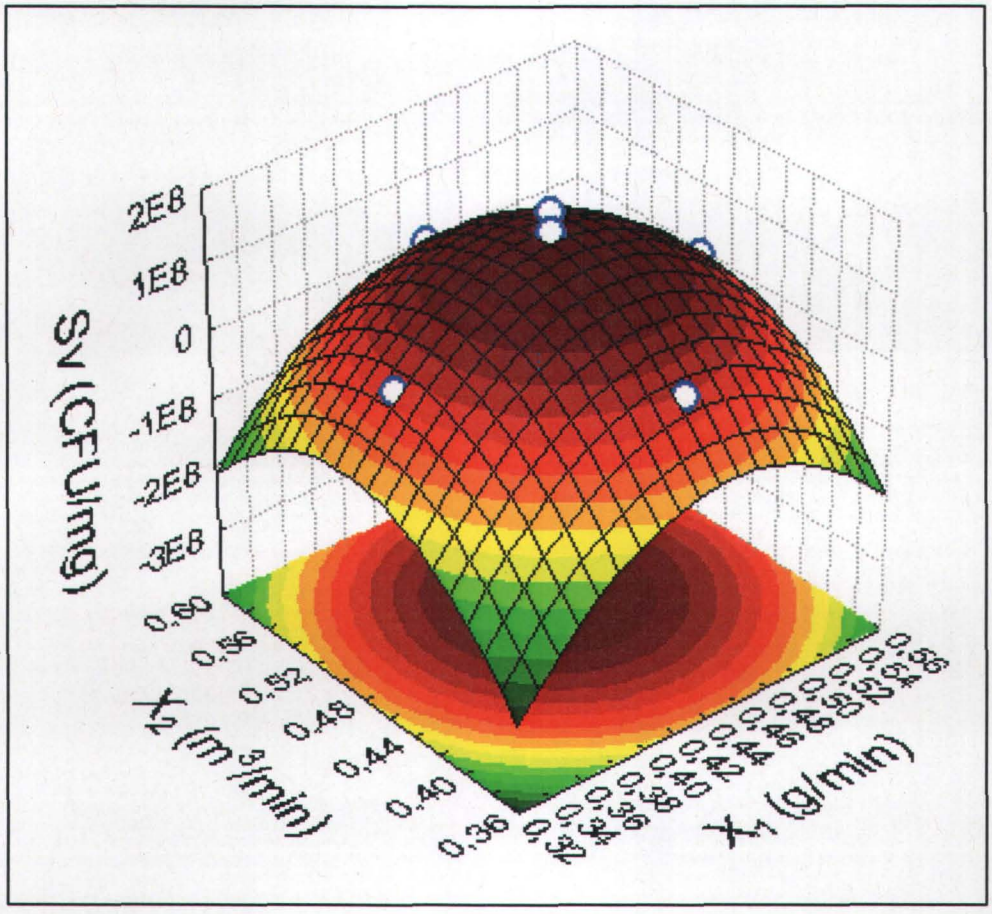

Fig 3a. Response surface of viable spores obtained by varying feed flow (X_1) rate and hot air flow rate (X_2) and keeping inlet temperature (X_3) and air atomization pressure (X_4) constant (case of TH)

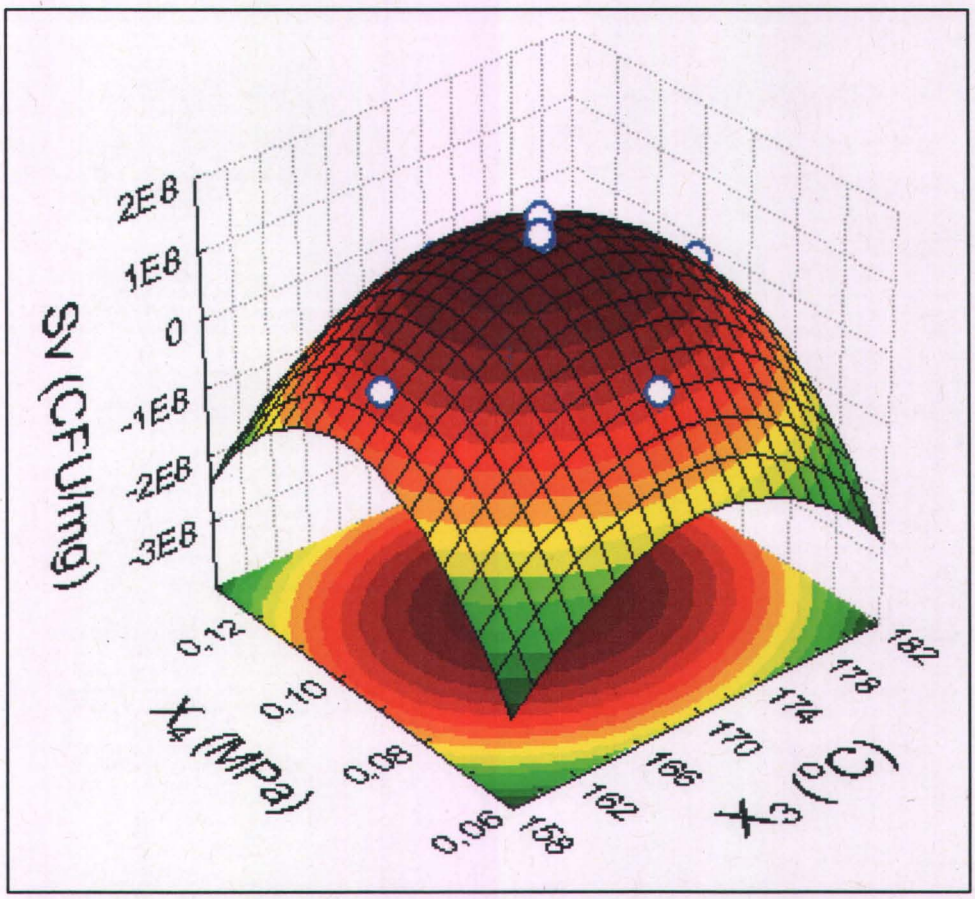

Fig. 3b. Response surface of viable spores obtained by varying the inlet temperature (X_3) and air atomization pressure (X_4) and keeping feed flow (X_1) and drying hot air flow rate (X_2) constant (case of TH)

Chapitre 4. Conclusions - Recommandations

4. CONCLUSION

Cette étude démontre que les techniques de récupération (centrifugation et ultrafiltration) et les différents procédés de formulation avec les additifs habituellement utilisés dans la production des biopesticides à base de *Bacillus thuringiensis* avec les milieux conventionnels de soja, peuvent être appliqués pour les milieux résiduels après une optimisation de certains paramètres requis. L'analyse et les interprétations des résultats obtenus ont permis d'aboutir aux conclusions et recommandations suivantes:

4.1. Augmentation de l'entomotoxicité par l'étape de l'ultrafiltration

L'ajout de l'étape d'ultrafiltration à la suite centrifugation dans le procédé de production des biopesticides à base de Bt, en utilisant les milieux résiduels, a permis d'augmenter l'entomotoxicité du concentré de la centrifugation et évidemment du produit formulé. L'étude réalisée au laboratoire a montré que cette augmentation, essentiellement due aux effets des composants solubles du rétentat d'ultrafiltration, dont les protéases et les chitinases, n'est pas aussi élevée qu'attendue. Cela est dû aux pertes des composants bioactifs de Bt par colmatage sur la membrane d'ultrafiltration. Ce colmatage varie suivant la nature et la rhéologie du milieu résiduel. Cependant, l'étude d'approche de mise en échelle de l'ultrafiltration a montré que cette perte par colmatage peut être réduite si on utilise un grand volume de surnageant et une bonne optimisation des paramètres opératoires d'ultrafiltration (la pression transmembranaire et le flux d'alimentation).

Aussi, une étude energétique préliminaire du procédé d'ultrafiltration, basée uniquement sur la consommation énergétique de la pompe et la durée de filtration a donné des valeurs de 1069 et 1651 (KWh/m^3) respectivement pour les milieux des eaux usées d'industrie d'amidon et des boues hydrolysées. Cette valeur est de 891 KWh/m^3 dans le cas du milieu synthétique de soja. Étant donné que le but principal de cette étude est de réduire le coût de production des biopesticides, pour que l'étape d'ultrafiltration soit économique, il faut associer son optimisation avec celle de la centrifugation et ensuite faire une analyse technico – économique du procédé combiné de centrifugation – ultrafiltration.

4.2. Formulation anti – UV et antimicrobienne

Les travaux sur la formulation anti-UV ont montré que, outre la réduction du coût des biopesticides à base de Bt produits avec les milieux résiduels, ces milieux ont en plus une capacité naturelle de protection contre les radiations UV contrairement au milieu conventionnel de soja. L'ordre de protection évalué en terme de demi-vies d'entomotoxicité est: boue non hydrolysée (3.4 j) > boue hydrolysée (3.25 j)> eau usée d'industrie d'amidon (1.9 j)> soja (1.8 j). Avec les additifs de protection contre les radiations UV-A et UV-B, l'acide p-amino benzoique (PABA) a augmenté la demi-vie d'entomotoxicité des milieux de soja et eaux usées d'amidon (demi-vies d'entomotoxicité 5.9 et 7.0 jours respectivement). L'acide lignosulfonique (LSA) donne un meilleur résultat dans le cas des boues hydrolysées et des boues non hydrolysées avec respectivement 7.3 et 8 jours de demi-vie d'entomotoxicité. Les valeurs des demi-vies d'entomotoxicité obtenues avec chacun des deux additifs dans les quatre milieux sont intéressantes. Cependant, vu le coût élevé de l'additif PABA par rapport à celui de LSA d'une part, et compte tenu des réglementations et certains effets secondaires dans l'utilisation de PABA d'autre part, on peut utiliser l'additif LSA pour les milieux de soja et eaux usées d'industrie d'amidon avec une concentration de 0.25% p/p. Pour la formulation antimicrobienne pour la protection des biopesticides contre les contaminations extérieures, l'acide propionique et le métabisulfite de sodium ont donné de meilleurs résultats au bout de trois années de conservation des formulations préparées avec les eaux usées d'industrie d'amidon et les boues d'épuration.

4.3. Production de poudres humides de Bt

La production des poudres humides des biopesticides Bt à partir des bouillons fermentés des boues d'épuration et des eaux usées d'industrie d'amidon par un procédé de séchage par pulvérisation, a conduit à deux résultats. Premièrement, les valeurs optimales des variables indépendantes déterminées par la méthode de réponse en surface (avec le nombre de spores comme réponse) sont obtenues avec des coefficients de détermination R^2 supérieures à 90% pour les deux milieux. Ces conditions optimales sont : 29 g/min, 51 m^3/min, 180°C, 0.10 MPa pour les eaux usées d'industrie d'amidon et 45 g/min, 49 m^3/min, 170°C, 0.096 MPa pour les

boues hydrolysées. Deuxièmement, avec ces conditions optimales, on note des pertes des composants bioactifs à travers les valeurs des spores viables et celles d'entomotoxicité. Ainsi, les pertes d'entomotoxicité sont de 28% et 18% pour les eaux usées d'industrie d'amidon et les boues hydrolysées respectivement par rapport aux valeurs de leurs bouillons fermentés respectifs.

4.4. Recommandations

À travers les objectifs de cette étude et les résultats obtenus, les recommandations suivantes peuvent être formulées pour des travaux de R&D qui devraient être effectués :

4.4.1. Ultrafiltration

Brar et al (2005) ont optimisé le procédé de la centrifugation des bouillons fermentés de Bt. D'après les résultats de cette étude sur l'ultrafiltration, il est important pour l'analyse techno-économique du procédé en aval, de faire une étude d'optimisation du procédé combiné de centrifugation – ultrafiltration. Il s'agira:

a) *de réaliser plusieurs centrifugations avec différentes forces centrifuges;*

b) *d'évaluer le pourcentage de colmatage pendant l'ultrafiltration d'un grand volume de surnageant obtenu pour chaque valeur de la force centrifuge;*

c) *de faire l'analyse techno – économique du procédé centrifugation – ultrafiltration pour chaque valeur de la force centrifuge;*

4.4.2. Formulation

Vu la revue de littérature sur la formulation, les recommandations suivantes sont formulées:

1). Étudier la suspension des formulations de biopesticides obtenus avec les eaux usées d'industrie d'amidon et les boues d'épuration. Compte tenu de la particularité de ces milieux résiduels (avec des charges), il serait intéressant de coupler la mesure de la suspension avec celle du potentiel zêta. Cela permettra de contrôler et de prévoir la stabilité de la dispersion/suspension des produits formulés de Bt par la mesure du potentiel zêta. En effet, il s'agira :

   a) *de déterminer comment les interactions entre les particules influencent la stabilité physique de la suspension afin de prévoir l'évolution de cette suspension dans le temps.*
   b) *de trouver une corrélation entre la mesure de la suspension par la méthode standard (traditionnelle) et la mesure du potentiel zêta.*
   c) *Et si c'est possible, d'utiliser les résultats de cette corrélation pour étudier la sélection et l'efficacité des différents additifs de suspension/dispersion.*

2. Sélectionner différents additifs de phagostimulation et d'adhérence aux feuilles en tenant compte de la rhéologie des milieux de culture. La procédure de détermination de ces additifs de phagostimulation peut être la même que celle utilisée dans le cas des radiations UV. Pour chacun des quatre milieux, plusieurs additifs de phagostimulants tel que mélasse, farine de soya et de maïs seront étudiés à de différentes concentrations. L'efficacité des phagostimulants sera évaluée en termes d'entomotoxicité et de croissance des larves dans les contrôles. Pour ce qui concerne l'étude d'adhérence aux feuilles par les biopesticides formulés, un test de simulation de pluie sera effectué pour déterminer l'efficacité des différents additifs tels que mélasses, carboxy-methyl cellulose, gomme ghatti, gomme xanthane, etc. avec des concentrations différentes.

4.4.3. Formulation des poudres humides de biopesticides

Étudier la taille des particules des poudres ainsi que la dispersion des poudres avec et sans additifs de dispersion et réaliser une formulation complète des poudres. Faire une étude technico-économique comparative du procédé d'obtention des poudres humides par séchage et celui produisant une formulation en suspension concentré par la combinaison de la centrifugation et ultrafiltration.

Les Annexes

ANNEXE I

ULTRAFILTRATION RECOVERY OF ENTOMOTOXICITY FROM SUPERNATANT OF *BACILLUS THURINGIENSIS* FERMENTED WASTEWATER AND WASTEWATER SLUDGE

Figure 2. Variation of turbidity, cells and viable spores in the retentate of soya semi synthetic medium at an optimal value of transmembrane pressure (193 kPa).

Turbidity, viables spores et total cells			
Flux($L.h^{-1}m^{-2}$)	Turbidity	Viable spores (CFU/mL)	Total Cells (CFU/mL)
450	620	1.1E+06	2.4E+06
900	855	2.5E+06	3.6E+06
1440	770	1.5E+06	2.2E+06
1800	790	1.6E+06	2.0E+06

Figure 3. Mass balance of suspended solids for different supernatants of Bt fermented centrifuged media after UF (percentage of mass of the suspended matter in the retentate and permeate were compared to the mass of suspended matter in the supernatants of various media).

Mass balance				
Media	% Supernatant	% Retentate	%Permeate	% retention on the membrane
Soya	100	85.0	0.0	15.0
SIW	100	81.6	6.4	12.0
NH	100	92.6	0.0	7.4
TH	100	31.9	0.0	68.1

Figure 4. Profiles of turbidity in supernatant, retentate and permeate of different media

Turbidity (NTU)			
Media	Supernatant	Retentate	Permeate
Soya	51	855	0.12
SIW	10	16	0.24
NH	119	750	0.20
TH	806	610	0.29

ANNEXE II

ULTRAFILTRATION SCALE-UP OF RECOVERY OF ENTOMOTOXICITY COMPONENTS OF *BACILLUS THURINGIENSIS* FERMENTED WASTEWATER/WASTEWATER SLUDGE BROTH

Figure 1. Concentration of viable spores in the retentate of soya, SIW, TH and NH in relation to the feed flux.

Viable spores = f (flux)				
flux ($L.h^{-1}m^{-2}$)	Soya	SIW	NH	TH
225	2.5E+06	3.1E+06	3.5E+05	4.2E+06
450	3.6E+06	4.1E+06	4.0E+05	5.0E+06
550	4.3E+06	6.1E+06	6.0E+05	6.3E+06
720	2.3E+06	5.0E+06	4.7E+05	7.1E+06
900	2.0E+06	4.0E+06	3.9E+05	5.9E+06

Figure 2. Concentration of viable spores in the retentate of SIW and TH in relation to transmembrane pressure of Ultrafiltration

TMP (kPa)	SIW (550 $L.h^{-1}m^{-2}$)	TH (720 $L.h^{-1}m^{-2}$)
70	4.2E+06	4.7E+06
80	5.4E+06	4.8E+06
90	6.3E+06	6.5E+06
100	5.7E+06	7.1E+06
120	5.1E+06	6.0E+06

Figure 3. Variation of permeate flux and loss on the membrane of total solids SIW and TH versus volume of permeate

Flux and % of retention of total solids on the membrane in relation of volume to permeate				
Total volume of permeate recovery (mL)	Flux of SIW ($L.m^{-2}.h^{-1}$)	% retention of total solids of SIW on the membrane	Flux of SIW ($L.m^{-2}.h^{-1}$)	% retention of total solids of TH on the membrane
100	830.8	1.1	900.0	2.8
500	535.7	11.2	486.5	14.7
1000	478.3	24.8	418.6	28.0
1500	465.0	41.7	388.5	50.3
2000	457.6	57.3	370.2	62.6
2500	452.3	61.9	350.6	70.3
2900	448.1	62.5	325.6	76.0

Figure 4. Variation of losses of soluble proteins and viable spores of TH and SIW on the membrane versus the volume of the permeate

Losses of viable spores and soluble proteins on the membrane in relation of volume to permeate				
Total volume of permeate recovery (mL)	% of losses of Viable spores of SIW on the membrane	% of losses of soluble proteins of SIW on the membrane	% of losses of Viable spores of TH on the membrane	% of losses of soluble proteins of TH on the membrane
100	3.2	3.8	4.2	2.3
500	8.7	6.0	12.1	10.1
1000	14.8	12.9	18.7	14.9
1500	22.5	14.4	26.4	17.9
2000	31.1	20.0	37.6	22.4
2500	33.0	24.0	40.7	27.8
2900	35.7	32.2	41.7	38.6

ANNEXE III

PHOTOSTABILIZATION OF *BACILLUS THURINGIENSIS* FERMENTED WASTEWATER AND WASTEWATER SLUDGE BASED BIOPESTICIDES USING ADDITIVES

Figure 1. Absorbance profiles of suspensions of soya, starch industry wastewater (SIW), hydrolyzed sludge (TH), and non-hydrolyzed sludge (NH) in the UV region.

Media absorbance				
λ (nm)	Abs Soya	Abs SIW	Abs NH	Abs TH
400	0.868	1.134	0.413	0.999
395	0.876	1.146	0.416	1.01
390	0.885	1.155	0.418	1.023
385	0.894	1.167	0.422	1.035
380	0.907	1.177	0.424	1.051
375	0.917	1.189	0.431	1.065
370	0.93	1.203	0.436	1.082
365	0.942	1.218	0.443	1.10
360	0.953	1.233	0.449	1.12
355	0.963	1.246	0.455	1.138
350	0.974	1.255	0.46	1.157
345	0.983	1.269	0.464	1.18
340	0.995	1.282	0.468	1.203
335	1.006	1.294	0.473	1.223
330	1.019	1.304	0.479	1.247
325	1.032	1.318	0.487	1.276
320	1.051	1.332	0.494	1.307
315	1.076	1.35	0.508	1.345
310	1.109	1.377	0.528	1.392
305	1.147	1.413	0.558	1.444
300	1.194	1.453	0.596	1.514
295	1.246	1.503	0.65	1.586
290	1.303	1.566	0.728	1.681
285	1.383	1.634	0.85	1.794
280	1.484	1.72	1.04	1.908

Figure 2. UV- Absorbance (in distilled water) profiles of lignosulfonic acid (LSA), p-aminobenzoic acid (PABA), and molasses (M)

Absorbance of the additives			
λ (nm)	LSA (1 g/l)	PABA (1 g/l)	M (1 g/l)
400	0.27	0.01	0.13
395	0.29	0.00	0.13
390	0.32	0.00	0.14
385	0.34	0.00	0.15
380	0.38	0.00	0.16
375	0.44	0.00	0.17
370	0.52	0.00	0.19
365	0.63	0.01	0.20
360	0.74	0.01	0.22
355	0.87	0.01	0.23
350	1.00	0.01	0.24
345	1.16	0.02	0.26
340	1.32	0.06	0.27
335	1.49	0.26	0.28
330	1.66	0.99	0.30
325	1.85	3.14	0.32
320	2.02	3.67	0.34
315	2.22	10.00	0.36
310	2.46	10.00	0.39
305	2.75	10.00	0.41
300	3.24	10.00	0.44
295	10.00	10.00	0.45
290	10.00	10.00	0.47
285	10.00	10.00	0.49
280	10.00	10.00	0.50

Figure 3. Entomotoxicity half-lives of Bt at various concentrations of selected UV screens (p-amino benzoic acid-PABA, molasses-M and lignosulfonic acid-LSA) of different Bt fermented media: a) starch industry wastewater; b) soya; c) hydrolyzed sludge; d) non-hydrolyzed sludge.

SIW

	Tx - Half life (Days)				% losses of Tx (after 48 hours)			
Additives	control	0.10%	0.15%	0.20%	contrôle	0.10%	0.15%	0.20%
PABA	1.90	2.50	6.00	7.00	90	63	49	44
M	1.90	2.40	3.00	4.00	90	82	63	53
LSA	1.90	2.30	4.50	6.00	90	83	57	50

TH

	Tx - Half life (Days)				% losses of Tx (after 48 hours)			
	control	0.10%	0.15%	0.20%	contrôle	0.10%	0.15%	0.20%
PABA	3.25	3.75	4.00	5.00	67	62	59	53
M	3.25	3.25	3.50	3.75	67	68	64	55
LSA	3.25	4.00	5.25	7.25	67	59	53	45

NH

	Tx - Half life (Days)				% losses of Tx (after 48 hours)			
	control	0.10%	0.15%	0.20%	contrôle	0.10%	0.15%	0.20%
PABA	3.38	3.40	3.50	3.50	67	66	62	57
M	3.38	3.40	3.70	4.90	67	65	60	53
LSA	3.38	4.00	5.50	8.00	67	59	53	47

Soya

	Tx - Half life (Days)				% losses of Tx (after 48 hours)			
	control	0.10%	0.15%	0.20%	contrôle	0.10%	0.15%	0.20%
PABA	1.75	3.75	5.63	5.88	90	54	51	53
M	1.80	1.63	1.88	4.00	90	89	73	65
LSA	1.80	2.25	3.00	4.75	90	77	61	56