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Production of *Bacillus thuringiensis*-based biopesticides in batch and fed batch cultures using wastewater sludge as a raw material

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Abstract: ***Bacillus thuringiensis*** **subsp** ***kurstaki*** **was grown in batch and fed batch cultures using wastewater sludge as a raw material. A simple fed batch strategy based on dissolved oxygen measurement during the fermentation cycle was developed in this work. It was established that while shifting the process strategy from batch to fed batch, the maximum spore concentration was increased from 5.62×10^8 to 8.6×10^8 colony forming units per cm^3 and resulted in an increase of entomocidal activity from 13×10^9 to 18×10^9 spruce budworm potency units per dm^3. Higher entomotoxicity was recorded at low spore concentration using wastewater sludge as a raw material whereas low entomotoxicity was reported at high spore concentration in synthetic medium.**

Keywords: *Bacillus thuringiensis*; wastewater sludge; fed batch culture; entomotoxicity; protease activity; sporulation

1 INTRODUCTION

High protein (toxin) productivity or entomocidal activity is the goal for the process optimization of *Bacillus thuringiensis* (Bt) fermentation using synthetic medium or natural complex medium (such as wastewater sludge) as raw materials. In a batch culture, the optimization of different fermentation parameters, namely temperature, pH, agitation and aeration, volume and age of inoculum, sludge suspended solids concentration, C/N ratio and various pre-treatments methods of sludge undeniably allowed higher entomotoxicity (Tx) to be achieved in the final product.[1–6] In fact, all our previous studies on Bt fermentation using sludge as a raw material have been done in conventional batch process. The principal disadvantages of a batch process are the high proportion of unproductive time (down-time) between batches, comprising the charge and discharge of the fermenter vessel, the cleaning, calibration, and sterilization, and process re-starts. However, the cell and spore concentration and hence the Tx value can be further increased by adopting various operational process strategies.

Fed batch fermentation provides a valuable tool in order to increase productivity and a concomitant decrease in product manufacturing cost.[7–10] The outcome of fed batch culture depends on many variables such as fermentation time, feed rate profile, availability of nutrients in the medium, oxygen concentration and pH profile.[11] The main issue of such systems is to maintain an appropriate feeding strategy to prevent overfeeding or underfeeding.[12–15]

The fed batch operational strategy has been successfully studied to augment Bt cell and spore counts and the process productivity using conventional synthetic medium. Kuppusamy and Balaraman[14] established that high cell density of Bt H-14 could be achieved without any negative effect on sporulation and endotoxin expression by adopting a simple fed batch control based only on glucose limitation. The concentration of Bt cells could be improved from 6 g dry weight (dw) dm^{-3} in a batch system to well over 50 g dw dm^{-3} in a well-designed fed batch operation in a laboratory fermenter using synthetic medium.[16] Kang *et al*[17] obtained a spore concentration of 1.25×10^{10} spores cm^{-3} by employing intermittent fed batch culture in modified glucose–yeast extract–salt (GYS) medium. Using a fed batch strategy based on motile intensity, Chen *et al*[18] showed that the maximum cell concentration increased up to 50% compared with the batch culture. However, all these studies were carried out using synthetic medium and toxin yield or Tx

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value was not reported. Therefore, the aim of this work was to develop a fed batch strategy to find out how much cell and spore counts and the Tx value could be enhanced using wastewater sludge as a sole raw material.

2 MATERIALS AND METHODS

2.1 *Bacillus thuringiensis* strain

Bacillus thuringiensis var *kurstaki* HD-1 (ATCC 33 679) (Bt) was used in this study. An active culture was maintained by streaking nutrient agar slants plates, incubating at 30 °C for 48h and then storing at 4 °C for future use.

2.2 Inoculum preparation

The inoculum was prepared in a two-step process as reported by Lachhab *et al*.[3] All the media used for inoculum preparation were adjusted to pH 7 before autoclaving. Aliquots of bacteria from the slant were used to inoculate 500 cm^3 Erlenmeyer flasks containing 100 cm^3 of sterilized TSB (tryptic soy broth, 3% (w/v), Difco). After 8–12h incubation in a rotary shaker at 30 °C and 250 rpm (revolution per minute), a 2% (v/v) inoculum of this broth was used as seed culture to inoculate a 500 cm^3 Erlenmeyer flask containing 100 cm^3 of the same medium as used in the fermenter for Bt production. The flasks were then incubated in a rotary shaking incubator at 30 °C and 250 rpm for about 8–12h. Finally, a 2% (v/v) inoculum of the actively growing cells of the pre-culture was transferred to the fermenter.

2.3 Fermentation medium

Batch and fed batch cultures were carried out using secondary wastewater sludge from a wastewater treatment plant in Quebec, Canada (Communauté Urbaine du Quèbec (CUQ)). The chemical and physical characteristics of the wastewater sludge used are presented in Table 1. The sludge was settled by gravity settling to increase the suspended solids concentration to 25 g dm^{-3}. The physical characteristics of wastewater sludge in terms of total sludge solids (TS), volatile solids (VS), suspended solids (SS) and volatile suspended solids (VSS) were determined according to the standard methods.[19] The total carbon and total nitrogen in wastewater sludge were analysed with an NA 1500-NCS analyser (Carlo Erba Instrument). Other chemical components in the sludge such as ammonia nitrogen, total phosphorus and orthophosphate as PO_4 were determined by a Technicon Analyser II (Technicon Instruments Corporation, New York). Different metal (Al, Cd, Cr, Cu, Fe, Mn, Pb, Zn, Mg, and Ca) concentrations were analysed by an atomic absorption spectrometer, Spectra AA-20 (Varian Techtron Pty Ltd, Australia).

2.4 Fermentation procedure

Fermentation experiments were conducted in two bench-scale bioreactors (15 dm^3 total capacity) equipped with accessories and automatic control systems for dissolved oxygen, pH, antifoam, impeller speed, aeration rate and temperature. The computer program (Fix 3.5, Intellution, USA) used allowed automatic set-point control and registration of all stated parameters. The two bioreactors were operated in parallel, one in batch mode and the other in fed batch mode.

Wastewater sludge was filled in the fermenter (10 dm^3 for batch mode and 8 dm^3 for fed batch mode) and polypropylene glycol (PPG, Sigma-Canada) (0.1% v/v) solution was added to control the foam during sterilization. The fermenters were sterilized *in situ* at 121 °C for 30min. After sterilization, fermenters were cooled to 30 °C. The fermenters were then inoculated (2% v/v inoculum) with actively growing cells of the pre-culture. The agitation speed (300–500 rpm) and aeration rate (0.3–0.5 vvm—dm^3 of air dm^{-3} of fermenter content min^{-1}) were varied in order to keep the dissolved oxygen (DO) values above 30% of saturation, which ensured the oxygen concentration was above the critical level.[20] The pH was controlled at 7.0 ± 0.1 using either 4 N NaOH or 6 N H_2SO_4 through computer-controlled peristaltic pumps.

Table 1. Physical and chemical characteristics of secondary wastewater sludge

Characteristic	Concentration
Physical	
Total solids (TS) (g dm^{-3})	28
Volatile solids (VS) (g dm^{-3})	17
Suspended solids (SS) (g dm^{-3})	25
Volatile suspended solids (VSS) (g dm^{-3})	16
pH	5.5
Chemical	
Total carbon (% w/w of dry TS)	41.02
Total nitrogen (% w/w of dry TS)	4.95
Ammonia nitrogen (mgN kg^{-1})	673
Total phosphorus (mgP kg^{-1})	12 231
Orthophosphates (mgP kg^{-1})	7 356
Metals (*mg* kg^{-1})	
Al^{3+}	13 323
Ca^{2+}	16 090
Cd^{2+}	3.4 (3–10)[a]
Cr^{2+}	89 (210)[a]
Cu^{+}	252 (400)[a]
Fe^{2+}	10 471
Mg^{2+}	1 796
Mn^{2+}	194
K^{+}	1 761
Pb^{2+}	62 (150)[a]
Zn^{2+}	578 (700)[a]

Values in parenthesis represent concentrations of metals prescribed by the Quebec Environment Ministry[41] for agriculture application.

2.5 Fed batch operation

A proper feed rate of substrate is required during fed batch culture to achieve maximum productivity. In order to establish a fed batch strategy, many

parameters (feeding time, feed volume, feed number, feed composition) should be optimized. Thus, preliminary work was performed in shake flasks in order to identify the proper feeding strategy. This showed that, when fermentation started at two-thirds the working volume, it offered a cell count two times higher than that obtained from total working volume.

In this work, two feeds were added and the feeding time was based on DO measurement during the fermentation cycle. Fermentation was started in batch mode with a working volume of 8 dm^3. Following inoculation, the DO concentration began to drop until the end of exponential growth. After that, DO started increasing. When DO approached towards stabilization, a fresh pre-sterilized sludge (2 dm^3) with similar suspended solids concentration (25 g dm^{-3}) was added. The DO decreased, followed by an increase. Once the DO reached near maximum, 3 dm^3 of fermenter contents was withdrawn and replaced by the same amount of sterilized fresh sludge. Afterwards, the fermentation process was continued in batch mode until 72 h. The samples were drawn from the fermenters at regular intervals to determine cell and spore counts, protease activity and entomotoxicity (Tx).

2.6 Estimation of cell and spore count

To determine cell and spore count the samples were serially diluted with sterile saline solution (0.85% w/v NaCl). The appropriately diluted samples (0.1 cm^3) were plated on tryptic soy agar (TSA) plates and incubated at 30 °C for 24h to form fully developed colonies. For spore count (SC), the appropriately diluted samples were heated in a silicone bath at 80 °C for 10 min and then chilled on ice for 5 min. This heat/cold shock lysed the vegetative cells and liberated those spores already formed in the bacterial cells. The titres of cell and spore counts were estimated by counting colonies grown on nutrition media. For all counts, an average of at least three replicate plates was used for each tested dilution. For enumeration, 30–300 colonies were counted per plate. The results were expressed in colony forming units per cm^3 (cfu cm^{-3}). The statistical treatment of the results showed maximum deviations of 6%.

2.7 Bioassay

The entomotoxicity (Tx) of the samples was measured by bioassay as the relative mortality to eastern spruce budworm larvae (*Choristoneura fumiferana*) responsible for the destruction of conifer forests compared with the mortality induced by the commercial forestry formulation Foray 76B (Abbott Labs, Chicago, USA) and expressed in terms of relative spruce budworm potency units (SBU). This product contains a mixture of spores and crystals of *Bacillus thuringiensis* var *kurstaki* at a potency of 20.1×10^9 IU dm^{-3} (International Unit) measured against cabbage looper (*Trichoplusia ni*). Comparative bioassays were conducted on Bt-fermented sludge samples using spruce budworm larvae and cabbage looper. It was found that the SBU values reported in this research were 20–25% higher than the IU values.

The bioassays were carried out using the diet incorporation method.[21] In this technique 1.5 cm^3 of serially decimal-diluted sample was mixed into 30 cm^3 molten (usually 55 °C) agar-based diet and distributed into 20 bioassay tubes (1 cm^3 in each tube). Three sets of controls (diet with sterilized production medium) were also included in the procedure to correct the mortality of larvae due to the sludge. One third-instar larvae of eastern spruce budworm was placed in each vial after the diet solidified. The vials were incubated at ambient temperature for one week and the mortality of the larvae was counted. Samples having mortality more than 10% in controls were discarded and the whole procedure was repeated. The standard deviation for entomotoxicity measurement was less than 5%.

2.8 Proteolytic activity assay

Proteolytic activity was determined according to Kunitz[22] with minor modifications. Samples collected from fermenters were centrifuged at 8000 rpm for 20 min at 4 °C. The supernatant thus obtained was properly diluted with borate buffer, pH 8.2. Alkaline protease activity was assayed by incubating 1 cm^3 of properly diluted enzyme solution with 5 cm^3 of casein (1.2% w/v, Sigma-Aldrich Canada Inc) for 10 min at 37 °C in a constant temperature water bath. The reaction was terminated by adding 5 cm^3 of 10% (w/v) trichloroacetic acid (TCA). This mixture was incubated for 30 min in order to precipitate the total non-hydrolysed casein. Parallel blanks were prepared with inactivated casein. At the end of the incubation period, samples as well as blanks were filtered using Whatman paper, 934-AH. The absorbance of the filtrate was measured at 275 rpm. The validation of the results was established by treating a standard enzyme solution where activity was known, in the same way and under the same conditions. One international proteolytic activity unit (IU) was defined as the amount of enzyme preparation required to liberate 1 μmole (181 μg) of tyrosine from casein per minute at pH 8.2 and 37 °C. The statistical treatment of the results showed maximum deviations of 5%.

2.9 Determination of K_La, OUR and OTR

The volumetric oxygen transfer coefficient (K_La) measurement was based on a dynamic method.[23] This technique consists of interrupting the air input. Afterwards, the aeration is re-established. The decrease and the increase in DO concentration were recorded. K_La was determined from the mass balance on DO just after sampling of fermentation broth.

During batch fermentation, the mass balance of the DO concentrations could be written as:

$$\frac{dC_L}{dt} = OTR - OUR$$

where:

OTR: oxygen transfer rate from gas phase to liquid phase: $OTR = K_L a(C^* - C_L)$
OUR: oxygen uptake rate : $OUR = Q_{O_2} X$
$K_L a$: volumetric oxygen transfer coefficient
C^*: saturated oxygen concentration
C_L: dissolved oxygen concentration in the medium
Q_{O_2}: specific oxygen uptake rate
X: cell concentration

Oxygen concentration in the fermentation broth was converted from % air saturation to mmol $O_2\,dm^{-3}$ as follows: the DO electrode was calibrated in medium at 30 °C and then transferred to air-saturated distilled water at known temperature and ambient pressure. This reading was used, with the known saturation concentration of oxygen in distilled water (0.07 559 mmol dm^{-3}) (100%), to estimate the saturation concentration of oxygen in the cultivation media at 30 °C.

3 RESULTS

3.1 Effect of fed batch culture on process performance

The evolution of fermentation parameters, $K_L a$, OTR and OUR, during the proposed fed batch strategy is illustrated in Fig 1. The initial period of cultivation (approximately, first 10h) was a critical period for the overall process performance. A significant decrease in dissolved oxygen occurred after inoculation until the end of exponential growth (9h fermentation). This was due to higher oxygen uptake rate during active cell growth. Afterwards, the oxygen consumption rate decreased and DO increased (9–15h growth). A similar DO drop was observed at the beginning and after the addition of fresh sludge (Fig 1).

Peaks of $K_L a$, OTR and OUR were observed when DO was minimum, ie towards the end of exponential growth (at 9h growth) and after addition of fresh sludge (at 30h growth). It could be stated that fed batch culture required a higher oxygen transfer rate, especially when the second feed was added. This could be attributed to higher cell density, which required high oxygen transfer rates to support growth.

Initially, two modes of fermentation (batch and fed batch culture) started with the same cell and spore count and operated under similar conditions, a higher maximum specific growth rate (μ_{max}) was observed when the fermenter was filled with only 8 dm^3 (0.567 h^{-1}) of wastewater sludge (fermenter for fed batch culture) compared with 10 dm^3 working volume (0.436 h^{-1}) (fermenter for batch mode) (Table 2). It could be due to a better oxygen transfer in a smaller working volume which is also evident from higher values of $K_L a$, OTR and OUR for 8 dm^3 working volume (Fig 1). Higher μ_{max} could also explain the higher cell and spore counts obtained after 9 h in the fermenter for fed batch culture (Fig 2). This finding also supported the observations of Foda *et al*[24] who reported a marked increase in cell and

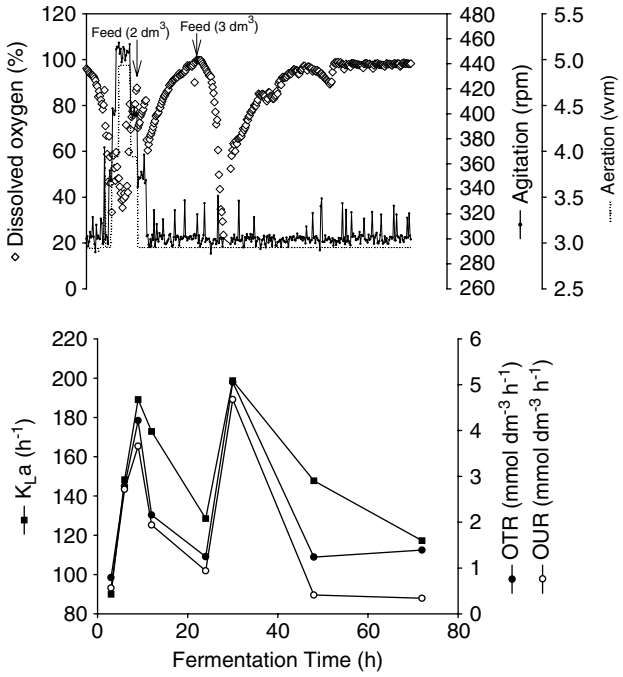

Figure 1. Profiles of dissolved oxygen, stirrer speed, aeration, $K_L a$, OTR and OUR in fed batch culture.

Table 2. Performance of the Bt process in batch and fed batch cultures after 72h growth

Parameter	Culture mode	
	Batch	Fed batch
μ_{max} (h^{-1})	0.436	0.567
Cell count (cfu cm^{-3})	6.17×10^8	9.07×10^8
Spore count (cfu cm^{-3})	5.62×10^8	8.60×10^8
% sporulation	91	95
Entomotoxicity (10^9 SBU dm^{-3})	13	18

spore concentrations when the ratio of total volume to operational volume was decreased in shake flask experiments.

Furthermore, foam formation at the beginning of fermentation (exponential growth) in the fermenter started with 8 dm^3 working volume (fed batch culture process) was low as compared with 10 dm^3 working volume (batch mode) and no major foam problem was observed after or during two feeds. However, Vidyarthi *et al*[25] observed a higher foam production during the initial batch fermentation period due to higher amounts of non-digested sludge proteins. Actively growing cells need a high oxygen uptake, which in turn requires a higher oxygen transfer rate furnished by higher aeration and agitation rates. This resulted in enhanced foam formation.

Batch culture of Bt using sludge as a raw material produced a maximum cell count of 6.17×10^8 cfu cm^{-3} and a spore count of 5.62×10^8 cfu cm^{-3}, whereas the fed batch culture yielded a 9.07×10^8 cfu cm^{-3} cell count and a 8.6×10^8 cfu cm^{-3} spore count (Table 2, Fig 2). Sporulation percentage (spore count/total cell count) observed

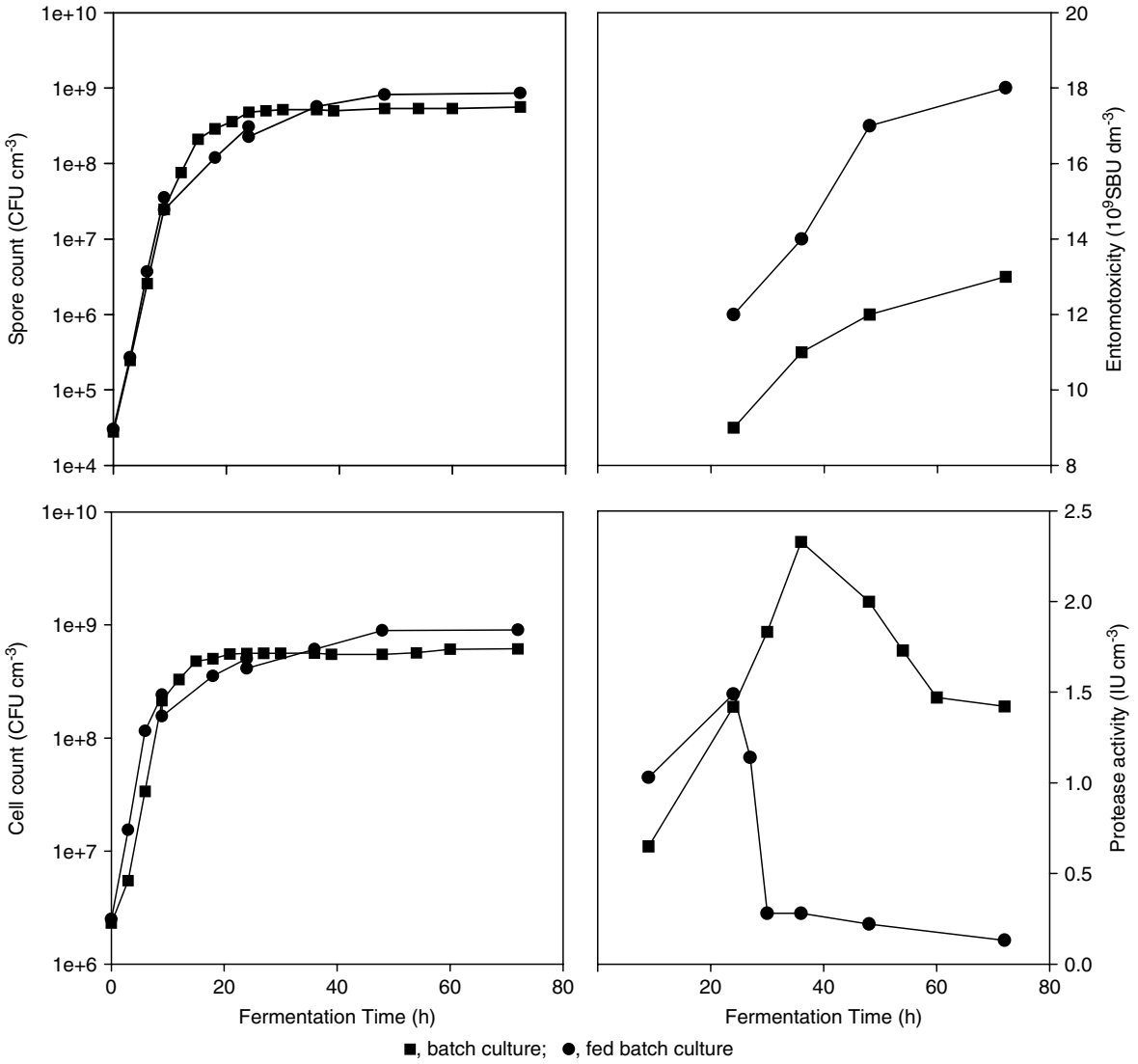

Figure 2. Cell and spore count, protease activity and entomotoxicity of *Bacillus thuringiensis* var *kurstaki* in batch and fed batch culture using secondary wastewater sludge (SS = 25 g dm^{-3}).

at the end of fermentation (72h growth) was 95% and 91% in batch and fed batch processes, respectively (Table 2). These results confirmed that the proposed fed batch strategy could be employed to obtain high cell count and spore count on sludge medium as stated in previous reports on synthetic media.[17,18,26] The spore count of the final broth increased proportionally with the final cell count. The high final spore count obtained in fed batch culture could be attributed to a faster cell growth during the fed batch operation as has been suggested by other workers.[17,18,26] It was likely that faster growing cells contained higher energy and other factors necessary for sporulation, while slowly growing cells did not.

3.2 Evolution of entomotocidal activity during fed batch culture

The Tx value increased with time (Fig 2). At 24 and 36 h fermentation, spore counts were higher in batch mode, but the Tx values were higher in fed batch culture than in batch mode. An increase of 20 and 25% in Tx value was observed in fed batch culture at 24 and 36h, respectively (Fig 2). This suggested that spore count was not the only indicator of entomotoxicity and was in line with the conclusions of many other workers.[27–30] Endotoxin and some other pathogenic factors such as vegetative insecticidal protein secreted during vegetative growth also play an important role. It is well known that Bt has complex growth characteristics. During batch growth, Bt cells change from vegetative to sporulated cells, and during this process they undergo significant metabolic, physiological and kinetic changes.[31] These changes seemed to become more and more complicated when fresh medium was added during fed batch culture and affected the cell growth and production of metabolites. It must be also stated here that a heterogeneous population was found in the fed batch culture after addition of fresh feed: cells in dividing state, cells in sporulation process, spore maturation, crystal release, cells lysis and spore germination. In fact, when fresh sludge was added, spores could germinate (due to availability of fresh nutrients), producing more cells and consequently more spores and toxin crystals. Also,

fresh sludge could have provided more energy for spore maturation, leading to higher Tx values.

After 48h, no substantial increase in Tx or in the counts of spores and cells was observed in fed batch culture (Fig 2). This could be due to the fact that feeding of fresh sludge was stopped. This warrants further extensive studies to optimize the feeding strategy (optimization of feed numbers, cultivation time and concentration of suspended solids in the feed) and thus develop a suitable and viable Bt process using sludge as raw material.

3.3 Enhancement of entomotoxicity in fed batch process

After 72h fermentation, the improvement of both final cell and spore counts in fed batch over the batch process was 1.5 times superior, and consequently an increase of the final Tx value was 38% (Table 2). It was assumed here that a large number of cells produced a correspondingly large number of spores, and consequently a large amount of insecticidal crystal proteins or high Tx value. This also corroborated with the results of other workers.[32,33] These workers found that δ-endotoxin production was related to spore formation in the fed batch process.

Apart from a synergistic spore effect, the higher Tx value at the end of the process in the fed batch culture compared with batch culture could be due to other factors that might have contributed to a better plasmid expression. These factors include:

(i) Larger parasporal crystal inclusion and then a production of a larger amount of δ-endotoxin. The study of Feng *et al*[34] showed that cells and endotoxin crystals produced in the fed batch mode appeared larger than those from batch mode.

(ii) Secretion of large amounts of chitinase. This enzyme is produced by Bt itself and is likely to assist pathogenesis of insects by hydrolysing the chitin present in the mid-gut wall.[35]

(iii) In fed batch culture, the cells were thin and motile.[36] Motile intensity acts as an indicator of cellular activity during the course of fermentation; higher motile intensity indicates higher nutrient consumption by Bt cells.[18]

(iv) Lesser proteolytic hydrolysis of the protein inclusion in fed batch culture due to lower protease activity compared with the batch process. Protease activity gradually increased in both cultivation modes and reached a maximum of 1.5 and 2.3 IU cm^{-3} at 24 and 36h in batch and fed batch modes, respectively (Fig 2). In fact, morphological characteristics of δ-endotoxin crystals released from the cell in batch and fed batch cultures could be altered by proteases.[37] Moreover, observations by phase contrast and electron microscopy indicated that sporulation and parasporal crystal release occurred earlier in the batch than in the fed batch process.[34] This earlier crystal release could be detrimental to the final Tx value, since higher production of proteases in the batch process and the longer contact time (72 h fermentation) could induce a loss of Tx potential.

4 DISCUSSION

The fed batch process is a well known process to increase the cell/spores' concentration using synthetic medium. However, the results obtained on one raw material cannot be extrapolated to another. Therefore, one of the goals of this research was to evaluate the gain in cell and spore count and Tx value using wastewater sludge as a raw material through slug feeding or operating the process in a fed batch mode. During the course of this work, it was established that while shifting the process strategy from batch to fed batch, the maximum spore concentration was increased from 5.62×10^8 to 8.6×10^8 cfu cm^{-3} and that resulted in an increase of Tx value from 13×10^9 to 18×10^9 SBU dm^{-3} (a gain of 38%).

It is also important to mention here that most of the work carried out on fed batch culture has been limited to measurement of Bt cells and spores' concentration without measurement of Tx value, and thus predicting a higher Tx value based on high spore count. For example, Kang *et al*[17] using a glucose medium in a fed batch culture reported a spore count of 1.25×10^{10} cfu cm^{-3}. In order to achieve a high spore concentration, Bt growth studies were conducted on wastewater sludge with and without fortification of glucose in shake flask experiments.[38] The spore count was increased from 5.63×10^8 without glucose to 1.5×10^{10} cfu cm^{-3} (which is of the same order as that obtained by Kang *et al*[17]) with glucose fortification, whereas the Tx value was increased from 10×10^9 (without glucose) to 12.7×10^9 SBU dm^{-3} (with glucose).[38] Thus an increase of spore concentration by 26.6 times due to addition of glucose to sludge in the batch process resulted in an increase of Tx by 2.7×10^9 SBU dm^{-3}, however, increase in spore count by 1.52 times (8.68×10^8 cfu cm^{-3}) while shifting from batch to fed batch culture using sludge as a raw material resulted in an increase of Tx by 5×10^9 SBU dm^{-3}.

Further, a lower spore concentration (2.2×10^8 cfu cm^{-3}) was obtained using wastewater sludge as a sole raw material than using the synthetic soybean medium (3.5×10^9 cfu cm^{-3}) in shake flask experiments.[39] However, a higher Tx value was recorded in the wastewater sludge (9×10^9 SBU dm^{-3}) than in the synthetic medium ($6-8 \times 10^9$ SBU dm^{-3}). This indicated that the toxin crystals produced by Bt while growing in wastewater sludge were more toxic than those produced in the synthetic medium or glucose-fortified sludge. Thus, an increase of Tx value with increase in spore count was evident from this work, as has been done in the past

by many researchers. However, it is difficult to predict the change in the magnitude of Tx value based on spore concentration and it is highly dependent on the characteristics of the raw material used and the process strategy applied. Thus, the decision regarding entomotoxicity value based on merely spore count, as has been proposed by many researchers, could be highly misleading.

The performance of the Bt fermentation process could be directly related to the available protein and carbohydrates in the medium, which could change with sludge origin, type of wastewater treated, method of wastewater treatment and operating conditions in a wastewater treatment plant.[2,4] The nutrients required for growth of Bt and synthesis of toxin crystals are embedded in the sludge's suspended solids (which is mostly bacterial cells). During sterilization, the bacterial cell wall is broken and the intracellular material is released, which in turn is available for Bt metabolism. The effect of variability of sludge composition on Bt growth and toxin synthesis was verified by collecting the sludge samples from the CUQ wastewater treatment plant at different times (to cover seasonal, day and night, and other possible variations) for a period of one year from July 1999 to July 2000. These samples were used to grow Bt under similar conditions. It was found that utilizing the optimum suspended solids concentration, $20-25\,g\,dm^{-3}$, the variation of Tx value was between +9% and −13% (non-published data). Thus, sludge composition does not affect seriously the Bt process performance as long as the optimum suspended solids concentration is used.

Two principal problems associated with the use of wastewater sludge as a sole raw material are the presence of toxic heavy metals and pathogens. For pathogens, before Bt fermentation the sludge is sterilized, where all types of pathogens are eliminated. As far as metals are concerned, there are abundant federal and provincial laws regulating metal concentration in sludge for agriculture, forestry, and other uses. Based on these laws sludge has been classified as Class A, B or C according to USEPA.[40] Class A sludge has been allowed with an unlimited use without any constraint, with the result that if the sludge used for Bt fermentation follows these regulations, there is no danger. The sludge used in the present experiments (Table 1) for Bt production was the one that met the regulatory criteria prescribed by the Quebec Environment Ministry.[41] It is worth mentioning here that almost 50% of total sludge (1million tons of dry sludge solids per year) produced in Canada meets the regulatory criteria.[42]

Another aspect that must also be specified in relation to toxic metals is the actual quantity of sludge required that could be used for Bt growth and subsequently for application in forestry or agriculture to control insects. The current application rate of the formulated Bt product to control spruce budworm in Canadian forests is about 30BIU (billions of IU) per hectare (or $1.5\,dm^3$ of the product with potency 76B or 76BIU per gallon).[43] To produce 30 BIU, according to the present results ($19 \times 10^9\,SBU\,dm^{-3}$ which is equivalent to $15.2\,BIU\,dm^{-3}$ at $25\,g\,dm^{-3}$ sludge suspended solids), approximately 50g of sludge would be required. In other words, the application rate of sludge to control spruce budworm is approximately 50 g of Bt fermented sludge per hectare. This quantity is very low compared with the amount of sludge permitted for agriculture land application (15–30 tons per hectare depending on N, P, K content) and hence the risk of metal contamination is almost nil.

Table 1 provides the total carbon present in the medium at suspended solids concentration of $25\,g\,dm^{-3}$. It is indeed very difficult to find how much carbon is available for Bt growth and toxin synthesis. However, on an average, the biodegradable solids present in secondary sludge (the type of sludge used in these experiments) vary between 30 and 40% (w/w).[1] Biodegradability of sludge also depends on the operating conditions in an individual wastewater treatment plant; the most important factor is the sludge age or mean solids retention time. Therefore, one could safely argue that 30–40% (w/w) carbon could be available for Bt metabolism. As sludge is a semi-solid medium, it is also difficult to exactly measure how much carbon will be used by Bt. Moreover, at the end of fermentation, Bt cells, spores and toxin crystals are mixed with the residual solids of sludge, therefore, under present circumstances it is not possible to measure separately the non-Bt carbon.

Moreover, Tx value achieved using sludge as a raw material is much higher than those obtained in synthetic medium.[39] Fed batch culture using same sludge solids concentration ($25\,g\,dm^{-3}$) further increased the value of Tx and cell and spore counts. Further, the sludge is a low cost raw material (or a negative cost material—needs disposal cost) and is available year round everywhere on the globe. The sludge also contains all required necessary nutrient elements to sustain growth, sporulation and toxin synthesis by Bt. The cost of Bt production using sludge as a raw material is much lower than that using soy-based synthetic raw material.[6] Increase in entomotoxicity during the fed batch process will further help in lowering the cost of Bt biopesticide production with wastewater sludge as a raw material. Apart from a substantial reduction of final production cost, use of wastewater sludge for the production of value added products (such as biopesticides) contributes towards a sustainable solution for sludge disposal/recycle, which is a socially useful, economically viable and environmentally benign strategy.

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