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Effect of different fermentation strategies on *Bacillus thuringiensis* cultivation and its toxicity towards the bagworm, *Metisa plana* Walker (Lepidoptera: Psychidae)

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Abstract

The effect of batch and fed-batch fermentation on the cultivation performance of *Bacillus thuringiensis* was investigated using a 5-l stirred tank bioreactor. Significantly higher viable cell count (> 1.5×10^{12} CFU/ml) was obtained in the fed-batch compared to batch fermentation (1.4×10^{12} CFU/ml). Glucose feeding during the fermentation seemed to enhance cell growth but failed to enhance the sporulation rate. It was found that sporulation and δ-endotoxin synthesis in fed-batch fermentation could be enhanced by the application of optimal dissolved oxygen tension (DOT) control strategy without affecting the cell growth. Fed-batch cultivation with feeding at the exponential growth phase where the DOT was switched from 80 to 40% at 12 h of cultivation recorded the highest spore count of 7.1×10^{11} spore/ml. Cultures obtained from batch cultivation, as well as fed-batch cultivation with feeding at lag or exponential growth phase and the application of optimal DOT control strategy, recorded the presence of δ-endotoxin; however, none was detected in intermittent fed-batch fermentation. Bioassay data against the bagworm *Metisa plana* Walker (Lepidoptera: Psychidae) recorded the highest corrected mortality (80%) at 7 days of treatment (DAT), using the culture obtained from fed-batch cultivation with feeding during the exponential growth phase, and the DOT was switched from 80 to 40% at 12 h of cultivation. It is important to note that all cultures containing δ-endotoxin exhibited 100% mortality towards *M. plana* at 14 DAT.

Keywords: *Bacillus thuringiensis*, Batch, Fed-batch, Cell growth, Sporulation rate, *Metisa plana*

Background

Bacillus thuringiensis (*Bt*) is widely used to control insect pests in the order Lepidoptera, Diptera, and Coleoptera (Yury et al. 2019). This bacterium produces spores that contain a proteinaceous body known as crystal protein or δ-endotoxin that possesses insecticidal properties. These insecticidal proteins accumulate in the cell as crystal inclusions which constitute approximately 25% of the dry weight of the sporulated cells (Agaisse and Lereclus 1995). *Bt* is also very useful in controlling leaf defoliators such as bagworms (Noorhazwani et al. 2017). The currently recommended option to conserve the natural enemies is by using *Bt* for spraying against pest (Norman and Mazmira 2019).

Malaysian Palm Oil Board (MPOB) has established a local biopesticide product based on *Bt* known as Ecobac-1 (EC). The product has been used for ground and aerial spraying in smallholder areas and also plantations to combat the bagworm outbreak, especially in Perak and Johor (Mazmira et al. 2010). At least three consecutive aerial sprayings of *Bt* are required to control the bagworm population to below the threshold level (Noorhazwani et al. 2017). In Malaysia, severe economic losses are caused by two species of bagworm, namely *Metisa plana* Walker and *Pteroma pendula* Joannis (Lepidoptera: Psychidae) (Ramlah et al. 2007). The shortfalls due to bagworm attacks can cause up to 33–47% yield losses, especially in oil palm (Basri et al. 1994).

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From the mid-1960s, bagworm outbreak became less common but started to surge again with more severity in the 1990s (Brian and Norman 2019). The bagworm species known as *M. plana* was classified as the most economically significant insect pest of oil palm (Basri et al. 1988). Bagworm infestation has been a serious issue affecting the yield of oil palm due to procrastinated and incorrect control strategy (Tey and Cheong 2013). In 2018, the total hectarage of oil palm infested areas, especially in the smallholdings, reached up to more than 30,000 ha, and the use of *Bt* based biopesticides has been the best alternative to control the pest.

Batch cultivation mode is frequently used to produce *Bt* spores with δ-endotoxin (Rowe and Margaritis 1987; Avionone-Rossa and Mignone 1993; Adams et al. 1999). However, the kinetics of *Bt* in batch cultivation has not been studied extensively. A wide range of the maximum specific growth rate (0.4–1.9 h^{-1}) for *Bt* has been reported (Avionone-Rossa and Mignone 1993), indicating the lack of a systematic study on growth kinetics of *Bt.*

The final spore concentrations obtained in batch cultivation of *Bt* were relatively low and not exceeded 10^{11} spores/ml (Sarrafzadeh et al. 2005; Khodair et al. 2008; Vu et al. 2010). A mixture of *Bt* spores and crystals can be produced using different modes of cultivation (Aronson and Yechiel 2001). Many researchers have reported the use of fed-batch cultivation for the production of high-density cell culture (Stanbury et al. 2003; Krause et al. 2010; Warren et al. 2018). The maximum cell concentration obtained in fed-batch cultivation (53.7 g/l) of *Bt* subspecies *kurstaki* was ninefold higher as compared to that obtained in batch cultivation (5.9 g/l) (Liu et al. 1994). Kang et al. (1993) found that the fed-batch cultivation with a constant feeding did not produce sporulated cells even after cells were subsequently kept in the bioreactor and operated as batch mode.

Comprehensive reports on the effect of different modes of bioreactor operation on *Bt* cultivation for the production of spores with high entomotoxicity activities towards bagworm have not been reported in any literature. In this study, the cultivation performance was evaluated in terms of final cell concentration, percentage of sporulation, δ-endotoxin synthesis, and also its toxicity towards *M. plana*.

Materials and methods

Microorganism

Bacillus thuringiensis MPK13, obtained from the Malaysian Palm Oil Board (MPOB) culture collection, was used in this study (Mazmira et al. 2012). This bacterium was isolated from the gut of the dead larvae of bagworm *M. plana* through several isolation steps. The isolated bacterium was then grown on nutrient agar and stored at 4 °C as a stock culture (Mazmira et al. 2013).

Media and inoculum preparation

The preferred medium for the cultivation of *Bt* with high sporulation rate and δ-endotoxin production as described earlier (Içygen et al. 2002; Mazmira et al. 2012) was used in this study. The medium consisted of $(NH_4)_2SO_4$, 2.0 g/l; $K_2HPO_4.3H_2O$, 0.5 g/l; $MgSO_4.7H_2O$, 0.2 g/l; $MnSO_4.4H_2O$, 0.05 g/l; $CaCl_2.2H_2O$, 0.08 g/l; and yeast extract, 2.0 g/l. The initial pH was adjusted at 6.5. Glucose at a concentration of 8.0 g/l was added to the basal medium. Glucose needs to be separately sterilized at 110 °C for 10 min before being added to the medium. The feed medium used for fed-batch fermentation was similar to the original medium in all aspects. For inoculum preparation, the *Bt* colony from the stock was inoculated into 400 ml of sterile nutrient broth in 1 l Erlenmeyer flask. The flask was then incubated at 30 °C in rotary orbital shaker agitated at 150 rpm for 14 h. The culture was then used as a standard inoculum for all cultivations, using a 5-l stirred tank bioreactor.

Stirred tank bioreactor

All modes of cultivations investigated in this study were conducted using a 5-l stirred tank bioreactor (BIOSTAT B-DCU, Sartorius Stedim, Germany). The standard six-bladed Rushton turbine impeller (diameter = 0.05 m) was used for bubble dispersion and mixing while ring sparger was used for air sparging. The agitation speed was controlled in the range of 50 to 500 rpm, and the temperature was maintained at 30 °C throughout the cultivations. The control system provided the regulation of the mixing speed (50–500 rpm) as well as the regulation of the stirrer working time. The airflow was set at one v/v/m. Silicone KM72FS (Shin-Etsu, Japan) at 10% was used as an antifoam agent. The dissolved oxygen tension (DOT) regulation during the cultivation was obtained with variations of agitation speed. Samples (20 ml) were withdrawn every 4 h intervals to determine the total viable cell count, spore count, sporulation rate, and δ-endotoxin synthesis.

Batch cultivation

Actively growing seed from the inoculum was used to inoculate the bioreactor at 11% v/v. The medium (3.6 l) was sterilized at 121 °C, 15 psi for 15 min. The batch cultivation was started by inoculating the inoculum into the 5-l bioreactor. The temperature was maintained at 30 °C. The DOT level was controlled at 80% by variation in agitation speed ranging from 50 to 500 rpm using a cascade model of DOT control module.

Fed-batch cultivation

The schematic diagram of the equipment setup for fed-batch cultivation is shown in Fig. 1. Initial batch bioreactor operation conditions for subsequent fed-batch were

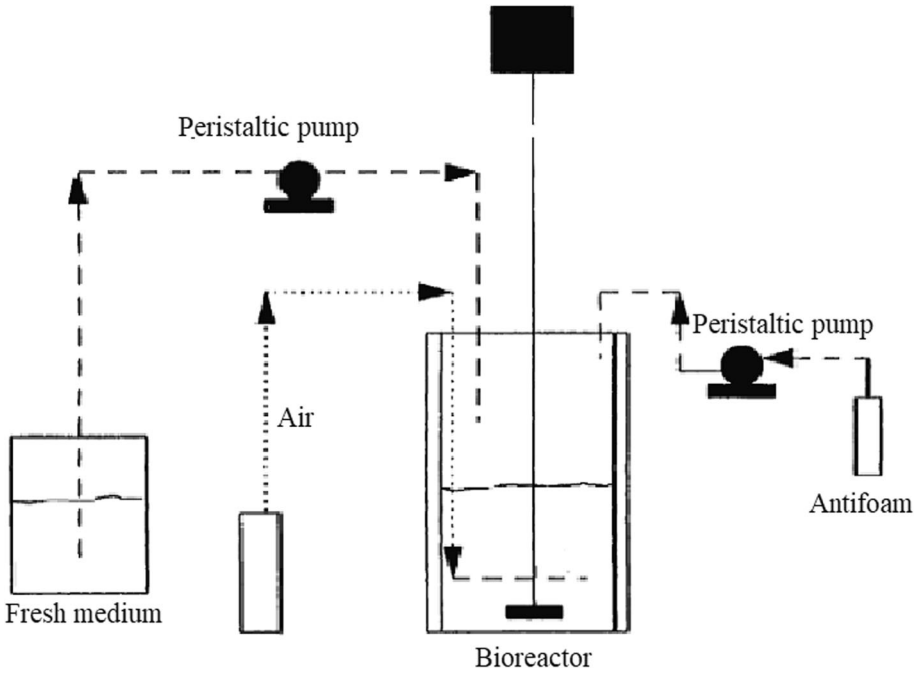

Fig. 1 Schematic diagram of the equipment setup for fed-batch cultivation

the same as batch cultivation, but the culture was reduced to 2 l. During fed-batch cultivation, a peristaltic pump (Watson-Marlow 101 U/R, England) was used to feed the fresh substrate into the bioreactor. The fed-batch feeding strategy was modified from the method reported by Rech and Ayub (2007).

Two types of feeding strategies (constant and intermittent feeding) were applied in fed-batch cultivation. In constant fed-batch cultivations, fresh medium was fed to the bioreactor at a constant rate during three different growth phases: (1) lag growth phase, (2) exponential growth phase, and (3) stationary growth phase. In intermittent fed-batch cultivations, fresh medium was intermittently fed to the bioreactor at two different growth phases: (1) exponential growth phase (6 h of cultivation) and (2) stationary growth phase (24 h of cultivation). Cultivation conditions in all fed-batch were similar to batch cultivations and the DOT was not controlled but monitored throughout the process.

δ-Endotoxin synthesis by *Bt* could be enhanced in batch cultivation when the DOT was controlled at 80% saturation during the active growth phase and then switched to 40% saturation during the middle of the exponential growth phase. This DOT control strategy was also applied in fed-batch cultivation with feeding at lag and exponential growth phases. In fed-batch cultivation with feeding at lag phase (2 h of cultivation), the DOT was switched from 80 to 40% saturation at 8 h of cultivation. While in fed-batch with medium feeding at exponential growth phase (6 h of cultivation), the DOT was switched from 80 to 40% saturation at 12 h of cultivation.

Analytical procedures

During the cultivation, culture samples were collected at different time intervals for analysis. The culture samples were serially diluted using 0.85% (v/v) sterilized saline buffers and plated on nutrient agar (NA) plates. The plates were incubated at 30 °C for 48 h and the number of the single colonies developed was counted and expressed in CFU/ml. For spore count, the culture samples were heated at 80 °C for 15 min to kill the vegetative cells before serially diluted and plated on NA plates. The plates were incubated at 30 °C for 48 h and the number of the single colonies developed was counted and expressed as spores/milliliter (Thompson and Stevenson 1984).

Gel electrophoresis

SDS-PAGE analysis was conducted using the Laemmli method (Laemmli 1970). The Laemmli system is a discontinuous SDS system that is the most widely used electrophoretic system. The resolution in a Lemmli gel is excellent because the treated peptides are concentrates in a stacking gel before entering the separating gel. To set up two sets of gels for Hoefer unit, running gel consisting of 5 ml monomer solution (A:B), 15 ml 4 × running buffer 600 μl, 10% 0f SDS, and 29.1 ml of distilled water. The gel solution was vacuumed for 15 min and after that 300 μl of 10% ammonium persulfate and 20 μl

of Temed was added. The ammonium persulfate must be prepared fresh. The running gel solution was poured into the Hoefer unit. Stacking gel contains 2.6 ml monomer (A:B), an aliquot of 5 ml stacking gel buffer, and 200 μl 10% SDS. Before the samples were loaded into the gel, an aliquot of 2 × treatment buffer was added and incubated in a water bath at 100 °C for 90 s. Aliquot of 80 μl of each sample was loaded into each well of the gel. Aliquot of 10 μl of 10 kD marker was also loaded into the gel. After the samples were loaded into the wells, electric current was set up at 15 A and left overnight.

Laboratory bioassay towards *Metisa plana*

The efficacy of the *Bt* cells cultivated in different modes of bioreactor operation was carried out against early instar of *M. plana*. The spray suspensions were prepared by diluting the *Bt* culture samples obtained from the cultivation with sterile distilled water. Bioassay samples were taken at 48 h of cultivation from samples which contain δ-endotoxin. The control treatment was prepared by spraying sterile distilled water on the palm leaves. The *Bt* suspension was then sprayed uniformly on the palm leaves dipped in reverse osmosis water. After the spray became dry, five larvae (early instars) were placed on the sprayed leaves. Each experiment was performed in four replicates. The observation of larval mortality was recorded at different days (1, 3, 7, and until 13) after treatment (DAT). Data on mortality was converted to corrected mortality using the Abbot Formula (Abbott 1987):

$$\text{Corrected mortality} = [\%\text{treatment} - \%\text{control}/100 - \%\text{control}] \times 100\%$$

Results and discussion

Batch cultivation of *Bt* MPK13

The time course of batch cultivation of *Bt* MPK13 in a 5-l stirred tank bioreactor is shown in Fig. 2. The results showed that high cell growth and sporulation could be obtained during batch cultivation. The high cell growth (> 1.0×10^{11} CFU/ml) was achieved as early as 8 h of cultivation. The highest cell growth (1.4×10^{12} CFU/ml) and highest spore count (4.7×10^{11} CFU/ml) were recorded at 48 h of cultivation, respectively. During batch cultivation, log phase was recorded as early as 4 h of fermentation until 20 h of cultivation, which lasted for 16 h. The cells started to enter the stationary phase starting from 24 h of cultivation. The highest percentage of the sporulation rate recorded in batch cultivation was 37% (Table 1). Glucose was completely consumed at 28 h of cultivation (Fig. 2). *Bt* MPK13 cells efficiently consumed glucose to support the growth process. Maximum cell productivity (72×10^{11} CFU/l/h) and spore productivity (25×10^{11} spore/l/h) were recorded at 48 h of cultivation (Table 1).

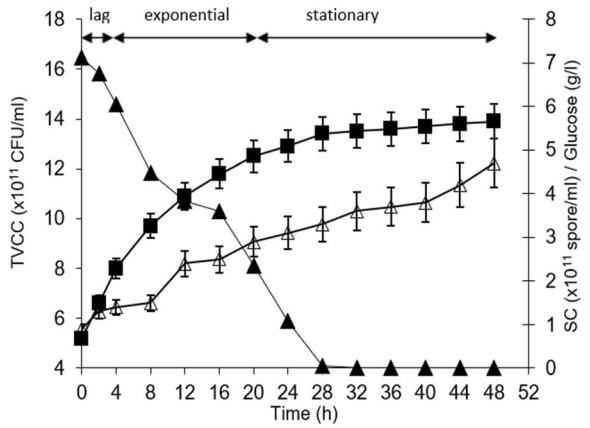

Fig. 2 Typical time course of batch cultivation of *Bacillus thuringiensis* MPK13. Black square denotes the total viable cell count (CFU/ml), white triangle spore count, and black triangle glucose concentration

Fed-batch cultivation of *Bt* MPK13

Feeding during the lag growth phase

The time course of fed-batch cultivation of *Bt* MPK13, with fresh medium feeding at 2 h of cultivation in a 5-l stirred tank bioreactor, is shown in Fig. 3a. The feeding time was initiated at 2 h of cultivation based on the lag phase of the growth profile obtained during batch cultivation. It was found that substrate feeding during the lag phase has substantially delayed the exponential phase for approximately 4 h. The highest cell count (14.7×10^{11} CFU/ml) in fed-batch cultivation with feeding during the lag phase and the highest spore count (3.7×10^{11} spore/ml) were recorded at 48 h of cultivation (Table 1). The exponential growth phase in fed-batch cultivation with feeding during lag phase lasted for 20 h as compared to 16 h in batch cultivation, as shown in Fig. 3a. The extension of the exponential growth phase may be due to the addition of fresh substrate during the lag growth phase. Glucose concentration in the culture was completely utilized by the cells at 44 h of cultivation. Maximum cell productivity (73×10^{11} CFU/l/h) and spore productivity (19×10^{11} spore/l/h) were recorded at 48 h of cultivation (Table 1).

Feeding during exponential growth phase

The highest cell count (15.8×10^{11} CFU/ml) and spore count (3.9×10^{11} spore/ml) were recorded at 40 and 48 h of cultivation, respectively (Table 1). Feeding of glucose during the exponential growth phase resulted in the extension of the phase (24 h) as compared to only 16 h for batch cultivation. As shown in Fig. 3b, glucose

Table 1 Comparison of cell growth, sporulation and δ-endotoxin production by *Bacillus thuringiensis* MPK13 in batch and fed-batch cultivations

Fermentation mode	Max cell count ($\times 10^{11}$ CFU/ml)	Max spore count ($\times 10^{11}$ spore/ml)	Increase in cell count (%)[a]	Decrease in spore count (%)[b]	δ-endotoxin	Max percentage of sporulation (%)	Max cell productivity ($\times 10^{8}$ CFU/l/h)	Max spore productivity ($\times 10^{8}$ spore/l/h)
Batch	13.9 (48 h)	5.2 (48 h)	0	0	Yes (36 h)	37 (48 h)	72.4	24.5
Fed-batch Feed during:								
1) Lag phase (2 h)	14.7 (48 h)	3.7 (48 h)	5.8	28.8	Yes (48 h)	25 (48 h)	73.4	19.3
2) Log phase (6 h)	15.8 (40 h)	3.9 (48 h)	13.7	25.0	Yes (48 h)	25 (48 h)	98.8	20.3
3) Stationary phase (24 h)	16.1 (48 h)	3.1 (48 h)	15.8	40.0	No	21.5 (24 h)	83.9	16.1
Intermittent fed-batch	17.2 (48 h)	2.6 (48 h)	23.7	50.0	No	15.2 (36 h)	89.6	13.5

[a]The increase (%) was calculated based on batch fermentation
[b]The decrease (%) was calculated in comparison with batch cultivation data

concentration in the culture was entirely utilized by the cells after 36 h of cultivation, which was at the stationary growth phase. Maximum sporulation rate, maximum cell productivity, and maximum spore productivity for this cultivation was 25%, 99 × 10^{11} CFU/l/h, and 20.3 × 10^{11} spore/l/h, respectively.

Feeding during stationary growth phase

The time course of fed-batch cultivation of *Bt* MPK13 with fresh medium feeding at 24 h of cultivation is shown in Fig. 3c. Feeding of glucose during the stationary phase resulted in the highest cell growth (16.1 × 10^{11} CFU/ml) and highest spore count (3.1 × 10^{11} spore/ml) at 48 h of cultivation. Maximum sporulation (22%) was recorded at 24 h of cultivation (Table 1). The final glucose concentration at 48 h of cultivation was 1.3 g/l (Fig. 3c). Maximum productivity for cells and spore during fed-batch cultivation with feeding during the stationary growth phase was 84 × 10^{11} CFU/l/h and 16 × 10^{11} CFU/l/h, respectively (Table 1).

Intermittent feeding during log and stationary phase

The time courses of intermittent fed-batch cultivation of *Bt* MPK13 with fresh medium feeding at 6 and 24 h of cultivation are shown in Fig. 4. The highest cell count (17.2 × 10^{11} CFU/ml) and spore count (2.6 × 10^{11} spore/ml) was recorded at 48 h of cultivation (Table 1). Glucose concentration at 48 h of cultivation was 1.0 g/l (Fig. 4). The maximum productivity for viable cells and spores for this cultivation was 90 × 10^{11} CFU/l/h and 13.5 × 10^{11} CFU/l/h, respectively (Table 1).

Fed-batch with optimal DOT control strategy

In fed-batch cultivation with fresh medium, feeding at lag growth phase and the DOT was switched from 80 to 40% at 8 h of cultivation, and the highest cell growth (14.7 × 10^{11} CFU/ml) and the highest spore count (6.6 × 10^{11} spore/ml) were recorded at 48 h of cultivation (Table 2). The highest sporulation percentage (45.9%) was also recorded at 48 h of cultivation. The cell and spore productivity for this cultivation was 77 × 10^{11} CFU/ml/h and 30 × 10^{11} spore/ml/h, respectively.

In addition, fed-batch cultivation with fresh medium feeding at exponential growth phase and DOT was switched from 80 to 40% at 12 h of cultivation, and the highest cell growth (14.5 × 10^{11} CFU/ml) and the highest spore count (7.1 × 10^{11} spore/ml) were recorded at 48 h of cultivation (Table 2). The highest percentage of sporulation (49.0%) was also recorded at 48 h of cultivation. The cell and spore productivity for this cultivation was 75.5 × 10^{11} CFU/ml/h and 37 × 10^{11} spore/ml/h, respectively. *Bt* MPK13 cells in fed-batch cultivation with feeding at exponential growth phase and DOT were switched at 12 h had a high capability to sporulate than the cells in fed-batch cultivation with feeding at lag growth phase, and DOT was switched at 8 h of cultivation.

Comparison of cultivation performance in different modes of bioreactor operation

The cultivation performance of *Bt* MPK13 in different modes of bioreactor operation was presented in Table 1. The lowest cell count (1.4 × 10^{12} CFU/ml) and the lowest cell productivity (72 × 10^{8} CFU/l/h) was obtained in batch cultivation, though considerably high spore productivity was achieved (25 × 10^{8} CFU/l/h). Increased cell count by about 6% was obtained in fed-batch cultivation by feeding during lag growth phase than in the batch cultivation. However, the percentage of sporulation (25 %) was lower than that obtained in

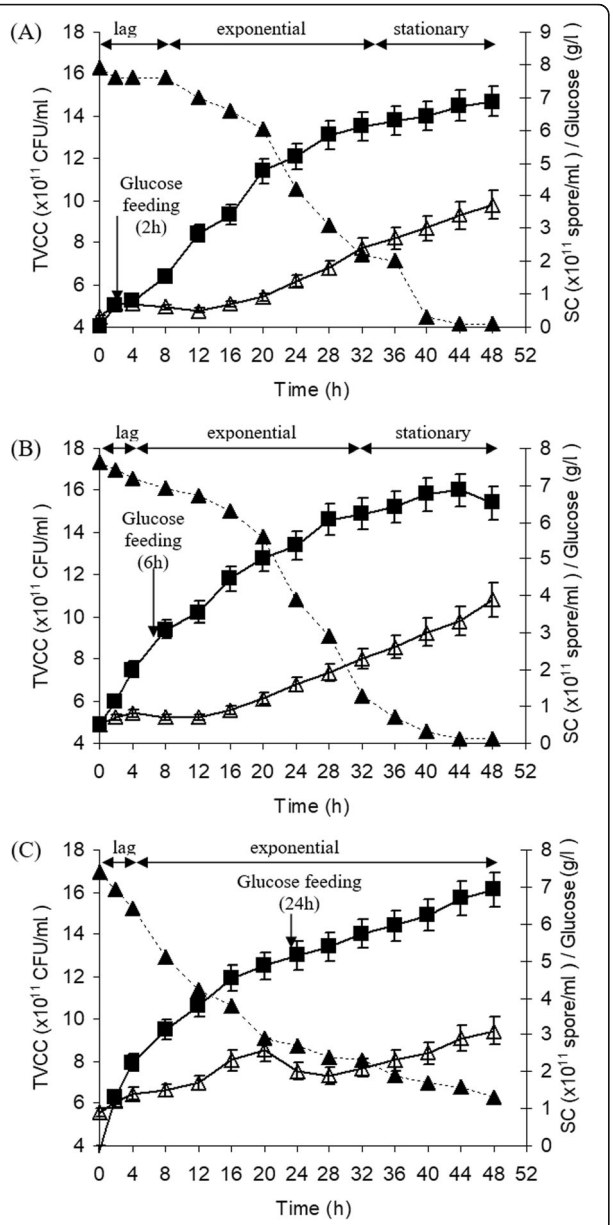

Fig. 3 Typical time course of fed-batch cultivation of *Bacillus thuringiensis* MPK13 with a constant feeding of fresh medium (**a** feeding at lag growth phase; **b** feeding at exponential growth phase; **c** feeding at stationary growth phase). Black square denotes the total viable cell count (CFU/ml), white triangle spore count, and black triangle glucose concentration

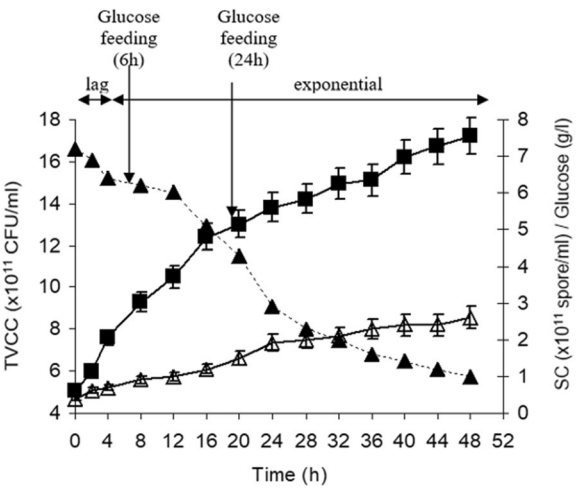

Fig. 4 Typical time course of fed-batch cultivation of *Bacillus thuringiensis* MPK13 with intermittent feeding of fresh medium (feeding at exponential and stationary growth phase). Black square denotes the total viable cell count (CFU/ml), white triangle spore count, and black triangle glucose concentration

batch cultivation (37 %). In fed-batch cultivation with feeding at the exponential growth phase, a 14% increase in cell growth was recorded than in the batch cultivation. However, spore count decreased by about 25% than that obtained in batch cultivation (Table 1). In fed-batch cultivation with feeding at the stationary growth phase, a 16% increase in cell count was recorded. However, a substantial decrease in spore count (40%) was recorded compared to that obtained in the batch cultivation. In addition, glucose was not fully consumed in fed-batch cultivation, fed during the stationary growth phase (Fig. 3c), suggesting that glucose was not required for sporulation.

Among all cultivation modes tested in this study, the highest viable cell count (1.7×10^{12} CFU/ml) was obtained in intermittent fed-batch cultivation. In comparison with batch cultivation, approximately 24% increase in cell count was recorded in intermittent fed-batch cultivation (Table 1). However, a substantial reduction in spore count (2.6×10^{11} spore/ml) was obtained in this cultivation. Substantial enhancement in the percentage of sporulation was achieved in fed-batch cultivation when the optimal DOT control strategy was applied. The highest sporulation percentage (49%), spore productivity (37×10^{11} spore/l/h), and spore count (7.1×10^{11} spore/ml) were recorded in fed-batch cultivation with medium at exponential growth phase, where DOT was switched from 80 to 40% at 12 h of cultivation (Table 2). Fed-batch cultivation of *Bt*, without appropriate DOT control strategy, enhanced cell growth but not the percentage of sporulation.

In the cultivation of *Bt* MPK13, glucose was identified as the most critical nutrient that supports both viable cell growth and also sporulation (Mazmira et al. 2012). Reports on the empirical feeding policies have been developed to achieve high cell density culture (Khodair et al. 2008). In this experiment, the excess feeding of glucose seemed to decrease sporulation and also blocked the synthesis of δ-endotoxin. Intermittent feeding of glucose at exponential and stationary growth phase, as well

Table 2 Comparison of cell growth, sporulation, and δ-endotoxin production by *Bacillus thuringiensis* MPK13 in fed-batch cultivation with the optimal DOT control strategy

Fed-batch with aeration strategy	Max cell count (× 10^{11} CFU/ml)	Max spore count (× 10^{11} spore/ml)	Increase in cell count (%)[a]	Increase in spore count (%)[b]	δ-endotoxin	Max percentage of sporulation (%)	Max cell productivity (× 10^{11} CFU/l/h)	Max spore productivity (× 10^{11} spore/l/h)
Medium feeding at lag growth phase (2 h) (DOT was switched from 80% to 40% at 8 h of cultivation)	14.7 (48 h)	6.6 (48 h)	5.8	26.9	Yes (24–48 h)	45.9 (48 h)	76.6	30.2
Medium feeding at exponential growth phase (6 h) (DOT was switched from 80% to 40% at 12 h of cultivation)	14.5 (48 h)	7.1 (48 h)	4.3	36.5	Yes (28–48 h)	49.0 (48 h)	75.5	37.0

[a]The increase (%) was calculated based on batch fermentation
[b]The decrease (%) was calculated in comparison with batch cultivation data

as continuous feeding of glucose throughout the cultivation, successfully promoted high cell growth (≥ 1.6 × 10^{11} CFU/ml). However, sporulation was reduced by a spore count of less than < 3.5 × 10^{11} CFU/ml). Although the existence of glucose is crucial for sporulation, high concentration in the culture may disturb the initiation of the sporulation process. It is well noted that sporulation and germination in bacilli are dependent on the nutritional status of the microorganisms (Rajalakshmi and Shethna 1980).

Sporulation and cry protein yields are usually low in fed-batch cultivation (López and de la Torre 2005). Liu et al. (1994) studied the effect of several feeding strategies on vegetative cell growth, spore formation, crystal protein content, carbon dioxide production, and oxygen consumption in fed-batch cultivation of *Bt* subspecies *kurstaki.* They found that spore and crystals were not formed in fed-batch cultivation. During fed-batch cultivation, there was a redirection of bacterial metabolism which takes place during the feeding.

In *Bt*, the setup of transition state was also reported during feeding in fed-batch cultivation. The physiological changes indicated that the transition state was set up during feeding, and it seemed to give a negative effect on sporulation and cry gene expression. Reduced spore count decreased in the percentage of sporulation in fed-batch cultivation with feeding during stationary phase, intermittent feeding, and continuous cultivation as demonstrated in this study could be explained by this mechanism.

Glucose feeding during fed-batch or continuous cultivation also made the glucose not be entirely metabolized in time and resulted in the mass accumulation of organic acids (Wen et al. 2007). Thus, the Krebs cycle activity decreases and the cell is unable to produce sufficient ATP, which in turn reduces the power and biosynthetic intermediates for spore formation (Kim et al. 2003). Nonetheless, fed-batch cultivation of *Bt* subsp. *darmstadiensis* 032 with an improved pH and glucose control strategy improved *thuringiensis* yield significantly (Zhou et al. 2007), though cell growth and sporulation performance were not analyzed. Results from this study demonstrated that the feeding strategy during fed-batch cultivation is crucial and greatly influenced the synthesis of δ-endotoxin.

High cell densities are favorable in fed-batch and continuous cultivations, but yields of spores and cry proteins synthesis were significantly reduced (Arcas et al. 1987; Liu et al. 1994). The reason of why sporulation was affected

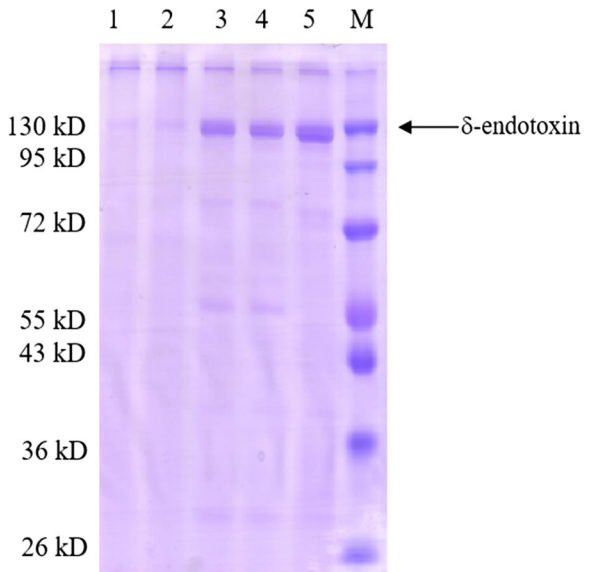

Fig. 5 The SDS-PAGE analysis of δ-endotoxin (130 kD) for *Bacillus thuringiensis* culture obtained from different modes of cultivation. Cultures, collected at 48 h of cultivation, were applied to 10% polyacrylamide gel and stained with Coomassie brilliant blue. M, standard marker; 1, intermittent fed-batch cultivation with feeding at exponential growth phase and stationary growth phase; 2, fed-batch cultivation with feeding at stationary growth phase; 3, fed-batch cultivation with feeding at lag growth phase; 4, fed-batch cultivation with feeding at exponential growth phase; and 5, batch cultivation

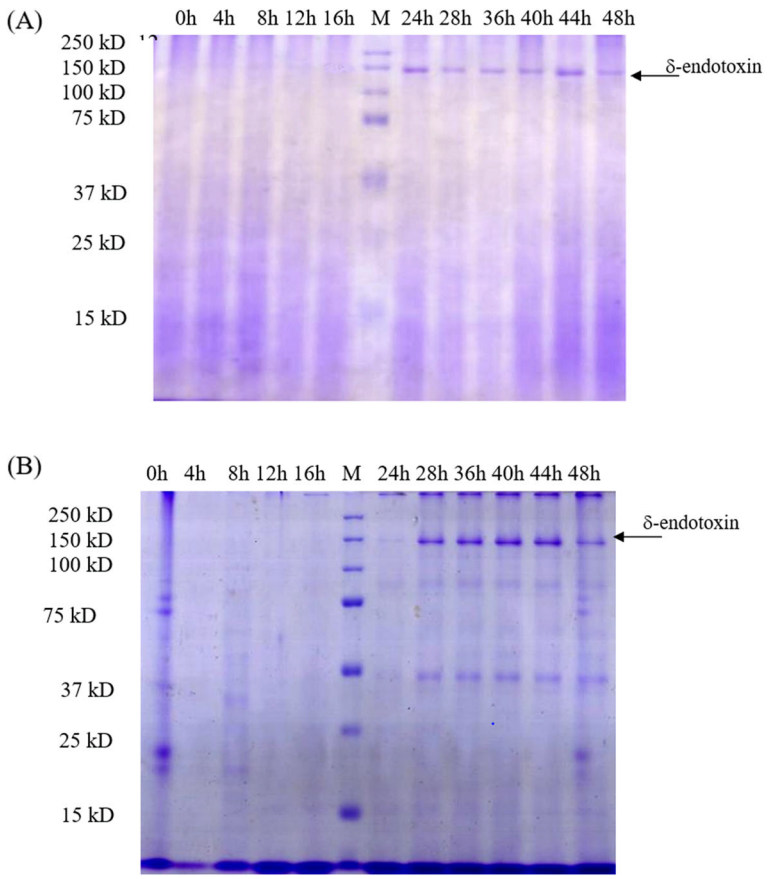

Fig. 6 SDS-PAGE analysis of δ-endotoxin (130 kD) in the cultures of *Bacillus thuringiensis* MPK13 obtained from **a** fed-batch cultivation with feeding at lag growth phase, DOT was switched from 80 to 40% at 8 h, and **b** fed-batch cultivation with feeding at exponential growth phase, DOT was switched from 80 to 40% at 12 h. The cultures were applied to 10% polyacrylamide gel and stained with Coomassie brilliant blue. M, standard marker

during feeding of a medium can be explained by the transition state regulators that might be overproduced during feeding in the fed-batch or continuous cultivation. Occurrence of catabolite repression is another possible explanation, where excess carbon source in the medium not only causes catabolite repression but also represses the expression of the SpoOA fusion gene that affects sporulation (Yamashita et al. 1989; Lereclus et al. 2000; Sonenshein 2000). Obtained results indicated that feeding the culture with glucose as the carbon source in order to fit the nutrient demand with nutrient availability as a way to obtain high cell densities was not sufficient for the success of higher cell sporulation and better δ-endotoxin production.

Synthesis of δ-endotoxin

The synthesis of δ-endotoxin at 48 h of cultivation in batch, fed-batch with feeding at lag, and the exponential growth phase is shown in Fig. 5. No detection of δ-

Table 3 Corrected mortality of *Bacillus thuringiensis* MPK13 δ-endotoxin against *Metisa plana* at 7 and 14 DAT

	Corrected mortality (%)	
	7 DAT	14 DAT
Batch cultivation	56	100
Fed-batch cultivation		
Feeding at lag growth phase	63	100
Feeding at exponential growth phase	67	100
Feeding at lag growth phase; DOT was switched from 80 to 40% at 8 h of cultivation	75	100
Feeding at exponential growth phase; DOT was switched from 80 to 40% at 12 h of cultivation	80	100

endotoxin was recorded in intermittent fed-batch cultivation, fed-batch cultivation with feeding at a stationary growth phase, and also continuous cultivation at all dilution rates. The advantage of batch cultivation can be clearly observed from the time δ-endotoxin was produced (28 h of cultivation). The toxin was synthesized 20 h earlier than the fed-batch cultivation with feeding at lag and exponential growth phase, where the existence of δ-endotoxin was only detected at 48 h of cultivation (Table 1). The lack of δ-endotoxin synthesis in the respective culture corresponded to the lowest spore count, the low percentage of sporulation, and also a substantial reduction in spore count (≥ 40%) than those obtained in batch cultivation. Feeding of glucose at stationary growth phase, intermittent feeding and continuous feeding throughout the cultivation seemed to promote a high cell growth, thus invading the cells to sporulate. However, with the right DOT control strategy during fed-batch cultivation, enhancement and early synthesis of δ-endotoxin were observed. As shown in Fig. 6a, fed-batch cultivation with fresh medium feeding at lag phase and DOT was switched from 80 to 40% at 8 h of cultivation recorded early synthesis (24 h of cultivation) of δ-endotoxin while fed-batch cultivation with fresh medium feeding at exponential growth phase and DOT was switched from 80 to 40% at 12 h of cultivation recorded thick 130 kD δ-endotoxin bands starting from 28 to 48 h of cultivation (Fig. 6b).

The absence of δ-endotoxin in intermittent fed-batch cultivation has also been reported. Although intermittent fed-batch cultivation enhanced the growth of *Bt* cell, the sporulation and δ-endotoxin synthesis were significantly reduced (Vu et al. 2010). Sasaki et al. (1998) claimed that high cell concentration (16.1 g/l) could be obtained in fed-batch cultivation, using sodium acetate-yeast extract (AYE) as a feeding medium, which was fed twice during the cultivation. Nevertheless, a deficient percentage of sporulation was observed after 55 h of cultivation. In this study, the optimal DOT control strategy was successfully applied in fed-batch cultivation to enhance δ-endotoxin synthesis. Bodizs et al. (2007) reported the importance of DOT regulation using a cascade model in enhancing the industrial pilot-scale fed-batch fungal fermentation. In this study, the change of DOT from a high level (80% saturation) to a low level (60% saturation) promoted sporulation rate and triggered δ-endotoxin synthesis without significantly affecting the cell growth.

Toxicity against *Metisa plana*

The toxicity of δ-endotoxin, obtained from *Bt* MPK13 cultures, was tested against *M. plana*. The highest corrected mortality (80%) at 7 DAT (days of treatment) and 14 DAT (100% mortality) was recorded by the culture obtained from fed-batch cultivation with feeding during the exponential growth phase, and the DOT was switched from 80 to 40% at 12 h of cultivation (Table 3). Culture from fed-batch cultivation with feeding during the lag growth phase and the DOT was switched from 80 to 40% at 8 h of cultivation exhibited the second highest corrected mortality (75%) at 7 DAT. For culture obtained from batch cultivation, the corrected mortality recorded at 7 DAT was 56%. For cultures obtained from fed-batch cultivation without DOT control strategy, either feeding at lag or exponential growth phase, the corrected mortality recorded at 7 DAT was not more than 67%. It is important to note that all cultures containing δ-endotoxin exhibited 100% mortality towards *M. plana* at 14 DAT (Table 3).

All cultures that contain δ-endotoxin during the cultivation recorded a high corrected mortality rate (≥ 55% mortality) towards the bagworm *M. plana* at 7 DAT. The 100% mortality of the bagworm at 14 DAT after exposure to the δ-endotoxin further confirms the high-efficacy effect of *Bt* MPK13 on the lepidopteran pest.

Conclusion

The results of this study demonstrated that the fed-batch had the potency to increase *Bt* cell growth than the batch cultivation. However, the fed-batch cultivation, with feeding during the stationary growth phase and intermittent feeding, did not support high spore production as the system was supplied with the highest concentration of glucose during the cultivation. The synthesis of δ-endotoxin, with a molecular weight of 130 kD, was detected in batch and constant fed-batch cultivations with feeding at lag or exponential growth phase. The capability of fed-batch cultivation, with feeding during lag or exponential growth phase, can be enhanced significantly with the application of optimal DOT control strategy.

Abbreviations

CFU: Colony-forming unit; DAT: Day after treatment; DOT: Dissolved oxygen tension; EC: Emulsified concentrate; rpm: Rotation per minuteATPAdenosine triphosphate

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Authors' contributions

MMMM and ABA designed the experiment. MMMM conducted the experiment and drafted the manuscript. ABA helped in data analysis and added inputs in the drafted manuscript. Both authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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