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Effect of Cry+ *Bacillus thuringiensis* cells and fermentation condition on consistent production of δ-endotoxin

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Abstract

The method for preparation of the competent Cry+ *Bacillus thuringiensis* strain stock culture to overcome inconsistent production of δ-endotoxin in stirred tank bioreactor was investigated. The selection of Cry+, which has stable production of δ-endotoxin was achieved by repeated isolation of colonies until stable types of *B. thuringiensis* were obtained. The polymerase chain reaction (PCR) approach was used to analyse the presence of cry genes in the isolates. Cry+ cells were freeze-dried to prepare competent stock culture in a powderized form. Results indicate that inoculum prepared using the powderized form managed to achieve high maximum cell concentration (>1.0 x 10^{12} cfu/ml) and spore count (>3 x 10^{11} spore/ml), but a longer cultivation time (16 h) was required. The Cry+ strain was able to form larger aggregates during the growth phase contrary to the Cry- strain. Glucose concentration and dissolved oxygen tension (DOT) level in the culture significantly affected spore and δ-endotoxin formation during the cultivation of Cry+ *B. thuringiensis* in a 5 L stirred tank bioreactor. In comparison to cultivation without glucose supply and a high dissolved oxygen tension (DOT) level, cultivation with 8 g/L glucose and controlled DOT at 80% saturation produced the highest viable cells (1.3 x 10^{12} cfu/ml), with a high sporulation rate and the existence of δ-endotoxin.

Keywords: *Bacillus thuringiensis*, δ-endotoxin, Stock culture, Glucose, Dissolved oxygen tension (DOT)

1. Introduction

Bacillus thuringiensis is the most promising biological control agent and is widely used for agricultural and commercial applications. *B. thuringiensis* and its insecticidal toxins have been widely studied and are the most commercially used biological control agents over the last 40 years [1]. This protein crystal has allowed the use of *B. thuringiensis* as a natural biological control agent in agriculture and forestry for the elimination of pests and in human health for the elimination of disease vectors.

B. thuringiensis spores containing the insecticidal protein, known as δ-endotoxin, which can be mass-produced by either liquid or semi-solid fermentation**.** The production of δ-endotoxin in *B. thuringiensis* systems is regulated by the growth phases. During the stationary growth phase, the cells produce a spore and parasporal inclusion bodies [2]. Upon sporangial lysis, the crystal protein and the spore are released. The δ-endotoxin are not produced under conditions that do not permit sporulation or in certain sporulation mutants [3]. The regulation of δ-endotoxin synthesis may share common features with the regulation of sporulation genes in *Bacillus subtilis*. The expression of sporulation genes in *B. subtilis* is regulated, in part, by sporulation-specific sigma factors [4].

The associations between the production of δ-endotoxin and the existence of plasmids were reported in two major *B. thuringiensis* strains [5]. Plasmids contribute to essential functions in bacterial advancement and adaptation by interceding the exchange of genetic material. Frequently, plasmids give noticeable benefit to the host by encoding qualities that ensure their continuance and existence. The number of plasmids, their sizes and

copy numbers varies significantly in different bacterial strains. *B. thuringiensis* induced by heat shock has a high risk of losing six plasmids which were detected in the parental Cry+ strains of subspecies *kurstaki* [6]. Crystal (Cry) proteins derived from *B. thuringiensis* have been extensively used in transgenic crops due to their toxicity against insect pests [7]. The study on the identification of cry genes has been reported in many parts of the world including Azerbaijan province [8].

Consecutive cultivation of *B. thuringiensis* may cause sequential loss and exchange of plasmids, which in turn, may reduce yield and productivity for δ-endotoxin production. In order to ensure the cultivation process for bioinsecticides production is economically viable, problem-related to the loss of plasmids relevant with δ-endotoxin formation in large-scale production shall be overcome. One of the possible approaches to overcome this problem is to limit the number of transfers of the culture from the inoculum flask to the production bioreactor [9]. However, substantial loss of plasmid still occurred during the preparation of stock culture. Inappropriate stock culture preparation may result in the presence of a high percentage of the Cry- *B. thuringiensis* strain in the inoculum. Subsequently, the Cry- *B. thuringiensis* strain may monopolize in the production bioreactor with spores containing no δ-endotoxin.

The main objective of this study was to develop an appropriate method for the preparation of competent Cry+ *B. thuringiensis* in powderized form to prevent loss of plasmid during several stages of cultivation for δ-endotoxin production in a stirred tank bioreactor. The important fermentation process parameters in stirred tank bioreactor that affect the production of δ-endotoxin were also identified.

2. Materials and methods

2.1 Microorganism and inoculum preparation

The bacterium, *B. thuringiensis* MPK13, obtained from the culture collection unit at the Malaysian Palm Oil Board (MPOB), Bangi, Selangor, Malaysia, was used throughout this study [10]. The bacterium was grown on nutrient agar and stored at 4 ˚C as a stock culture prior to isolation of the Cry+ strain.

2.2 Isolation of Bt Cry+ strain

The culture from the stock was streaked on Petri dishes containing the nutrient agar (NA) (Oxoid, London) and incubated at 30 ˚C for 24 h. Every single colony developed on the agar was identified by PCR to select the Cry+ *B. thuringiensis* strains. Cry+ *B. thuringiensis* strains were subsequently cultivated in a 1000 ml Erlenmeyer flask containing 200 ml of basal medium for δ-endotoxin production. The basal medium consisted of (g/L): $(NH_4)_2SO_4$, 2.0: $K_2HPO_4.3H_2O$, 0.5: $MgSO_4.7H_2O$, 0.2: $MnSO_4.4H_2O$, 0.05; $CaCl_2.2H_2O$, 0.08 and yeast extract, 2.0 [11]. Glucose at a concentration of 8 g/L was also added into the medium. Initial pH of the medium was adjusted at 6.5 using 1 M HCl. The flask with the medium was sterilised at 121˚C for 15 min at 15 psi. The medium was inoculated with the single isolated colony and incubated at 30 ˚C in a rotary shaker, agitated at 200 rpm for 24 h. Samples were collected at 24 h, 48 h and 72 h of cultivation for PCR and SDS-PAGE analysis. The selection process was repeated for three cycles from Petri dishes containing NA. The spores of the Cry+ *B. thuringiensis* strains were recultivated in an Erlenmeyer flask for 24 h at 30 ˚C using similar medium and culture conditions. The flow chart for the isolation of the Cry+ *B. thuringiensis* strain and the preparation of stock culture in a powderized form is shown in Figure 1.

2.3 Preparation of culture in freeze-dried form

The selected Cry+ *B. thuringiensis* culture was dried using the freeze-drying method to prepare a stable stock culture. In brief, the harvested cells were washed with 60 mM phosphate buffer at pH 6.5. The cells (5 ml) were mixed with protective agents (5 ml). The efficiency of different protective agents such as skim milk, sucrose, fructose, glycerol, calcium carbonate and monosodium glutamate in protecting the cells during freeze-drying was evaluated. The concentration of the protective agents was varied according to the requirements of the experiments. The concentrations were as follows: skim milk (10% w/v), sucrose (10% w/v), fructose (10% w/v), glycerol (5% w/v), calcium carbonate (0.5% w/v) and monosodium glutamate (10% w/v). Distilled water was used as the control.

The cell suspension in protective agent was frozen at -80 ˚C and then lyophilized overnight at 50 ˚C using a 4.5 L freeze dryer (Labconco, USA). The freeze-dried Cry+ strain of *B. thuringiensis* MPK13 was stored in a bijou bottle at 4 ˚C as the competent stock culture for subsequent use in the cultivation experiments. All samples tested in this study were performed in triplicates. The viability of cells and spores in the samples was determined before and after freeze-drying in terms of percentage of survival. Freeze-dried samples were rehydrated to the original volume with distilled water. For viability and spore counts analysis, serial dilutions of each reconstituted sample (10^{-5} to 10^{-10} cfu/ml) were spread on NA by the pour plate technique and incubated at 30 ˚C for 48 h. The total

viable cell count performed on one mg of the freeze-dried Cry+ *B. thuringiensis* powder has productively attained beyond 1 x 10^{10} cfu/ml.

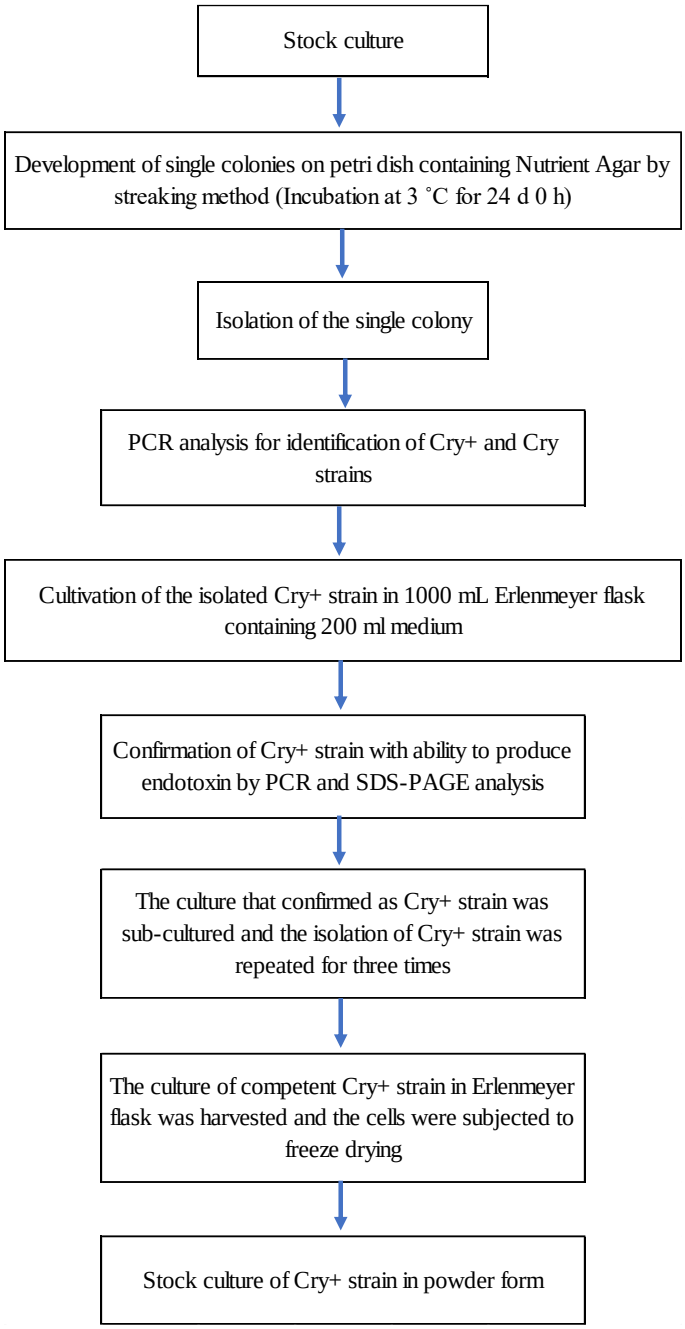

Figure 1 The flow and steps involved in the screening and isolation of *B. thuringiensis* Cry+ strain.

2.4 Bioreactor and fermentation

The ability of the freeze-dried stock culture in producing endotoxin was further tested by cultivation in a 5 L stirred tank bioreactor (Biostat Sartorius, Germany). The inoculum was prepared by transferring a loop of Cry+ *B. thuringiensis* stock culture in powderized form into a 1 L Erlenmeyer flask containing 400 ml of nutrient broth. The flask was incubated at 30 ˚C in a rotary shaker, agitated at 200 rpm for 16 h.

The inoculum was then inoculated into the 5 L stirred tank bioreactor containing 3.6 L of basal medium and the temperature within the bioreactor vessel was controlled at 30 ˚C. The initial culture pH was set at 6.5 and was not controlled but monitored throughout the fermentation process. Foaming was controlled by the addition of an antifoaming agent (Shentzu, Japan) using a foam control module. The critical physical parameters, which were glucose and the dissolved oxygen tension (DOT) level that affected δ-endotoxin production in cultivation using a

5 L stirred tank bioreactor were identified in the preliminary experiment (data not shown). Cultivations were performed in medium with and without the presence of 8 g/L glucose. The second parameter investigated was the effect of the DOT level on the production of δ-endotoxin. The DOT level in the culture during the cultivation was controlled at 80% saturation by varying both agitation speed (from 50 to 500 rpm) and airflow rate (from 0.5 to 1.5 vvm) using a cascade control mode. For comparison, the cultivation was also performed at a constant airflow rate of 1 vvm and agitation speed at 200 rpm. Cultivations of *B. thuringiensis* using stock culture prepared in slant agar, with parameters similar to cultivations using freeze-dried Cry+ *B. thuringiensis* stock culture were also carried out.

The growth kinetics of Cry+ *B. thuringiensis* cells was made according to the Baranyi-Robert microbial propagation model [12] by the following equation:

$$X(t) = X_0 + \mu_{max}\, B(t) - ln\left(1 + \frac{exp^{\mu max .B^{(t)}} - 1}{exp^{(X_{max} - X_0)}}\right) \quad (1)$$

Where the coefficient, B:

$$B(t) = t + \frac{1}{\mu_{max}} ln\,(exp^{(-\mu_{max} .t)} + exp^{(-\mu_{max} .\lambda)} - exp^{(-\mu_{max} .(t+\lambda))}) \quad (2)$$

X_0: Initial cell concentration; X: Cell concentration; t: culture time; μ_{max}: Maximum specific growth rate and λ: Time during lag phase.

2.5 Analytical procedures

During the cultivations, samples were collected at 4 h intervals for analysis. The PCR amplification method was used for the detection of the *cry* genes. PCR is an extremely sensitive technique for quick detection and identification of target cry gene sequences as it only needs minimal volumes of DNA and permits simultaneous screening of many *B. thuringiensis* strains and classification of them according to their insecticidal activities [13].

The oligonucleotide primers used to amplify the *cry* gene were previously reported by Ben-Dov et al. [14]. In brief, amplifications were carried out in a thermocycler (Eppendorf, Germany) for 30 reaction cycles. Reactions were routinely carried out in 50 µl; 1 or 5 µl of template DNA which was mixed with reaction buffer, 0.2 mmol l-1 deoxynucleotide triphosphate, 0.2-0.5 mol l-1 primer, and 0.5 U of *Taq* DNA polymerase. Template DNA was denatured (1 min at 94 ˚C) and annealed to primers (50 s at 60 ˚C), and the extensions of PCR products were achieved at 72 ˚C for 90 s. DNA was analysed by agarose gel electrophoresis (0.8% agarose) in the presence of ethidium bromide. A DNA molecular weight marker (1 kb DNA ladder, Gibco-BRL, UK) was used to estimate the fragment lengths. SDS-PAGE analysis was used for the detection of 130 kD δ-endotoxin during sporulation [15].

For determination of total viable cell counts, the culture samples were serially diluted using 0**.**85% **(**v/v**)** saline buffers and plated on NA plates via the pour plate method**.** The plates were incubated at 30 ˚C for 48 h and the number of the single colonies developed was counted (triplicates) and expressed in cfu/ml. For spore count analysis, the culture samples were heated at 80 ˚C for 15 min to eliminate the vegetative cells before serially diluting them and then 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 spore/ml.

Spores were counted (triplicate determinations) by the pour-plate counting technique after heat shock and expressed as spore/ml. A phase-contrast microscope (Leica DM 3000, Germany) was used to monitor the morphology of the cells. Vegetative cells and spores were distinguished by the refractive nature of the spore.

3. Results

3.1 Isolation of Cry+ strain

In the preliminary isolation of the Cry+ strain, it was found that some of the Cry+ strains were unable to produce δ-endotoxin, though *cry* genes were detected by the PCR analysis (Figure 2). Among the six colonies selected from the preliminary isolation of the Cry+ strains, only four colonies were able to produce δ-endotoxin at 48 h of cultivation.

The remaining two colonies showed fade bands of the toxin. At 60 h of cultivation, pallid bands of δ-endotoxin were observed compared to samples withdrawn at 48 h (Figure 3A). In the second cycle of cultivation using the Cry+ and positive δ-endotoxin strain obtained from the first cycle of cultivation, the SDS-PAGE analysis showed a more competent result with only one colony unable to produce δ-endotoxin at 48 h and 60 h of cultivation (Figure 3B).

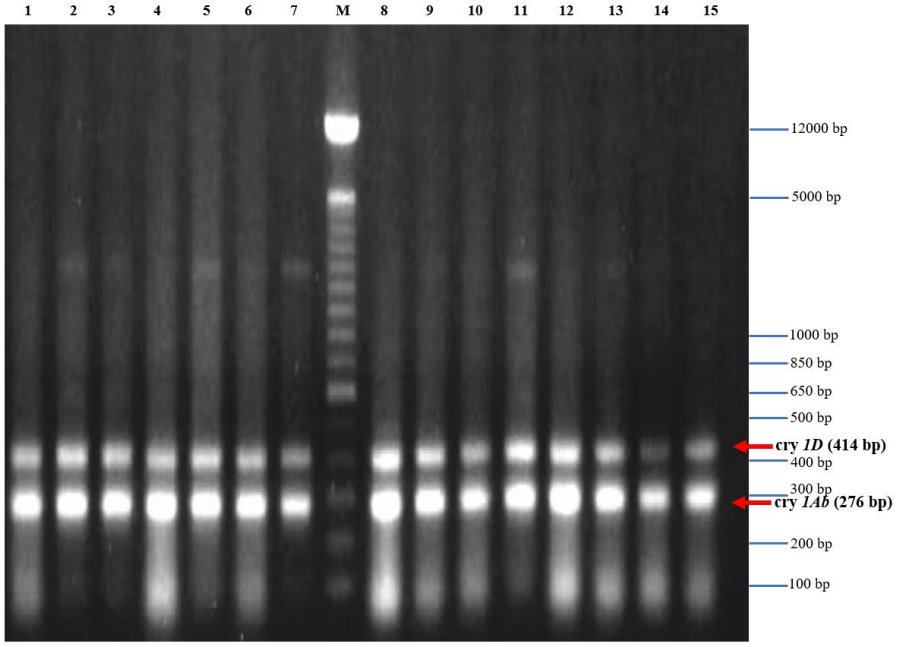

Figure 2 PCR profile of Bt MPK13 during the preliminary isolation of the Cry+ strain. Lane: M-1 kb DNA marker, Gibco Invitrogen, UK; 1 to 15-Bt MPK Cry+ strain showing bands at 276 bp and 414 bp.

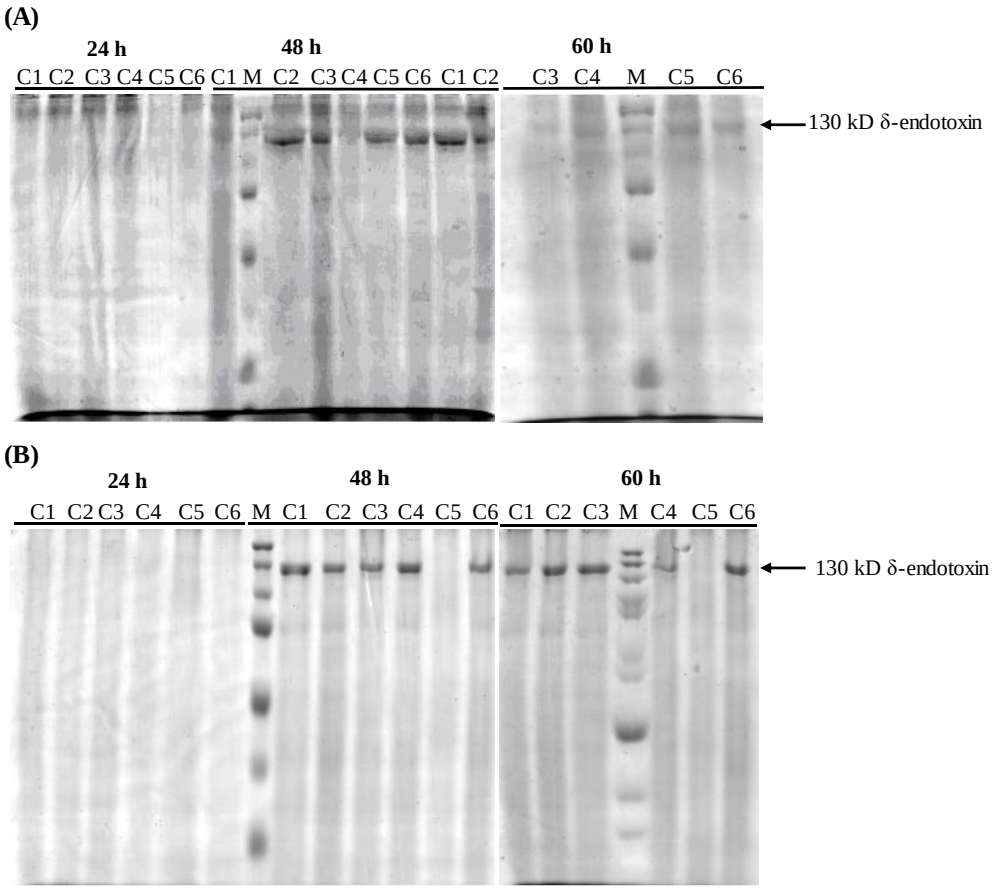

Figure 3 SDS-PAGE analysis of the Bt MPK1 Cry+ cells produced shake flask cultivation of the selected six colonies obtained from the (A) first cycle (B) second cycle of the isolation (M-marker).

From the microscopic observation of the culture, the condense cells growth was observed at 24 h cultivation. Both cultures with the Cry+ and Cry- strains showed the existence of spores when observed by phase- contrast microscopy. It was difficult to distinguish Cry+ and Cry- strain by observing only the cell morphology and spore production as both strains showed many resemblances. The main difference between Cry+ and Cry- strain was the appearance of cells aggregation. We found that the strain which formed aggregates was the Cry+ strain (Figure 4A). Aggregation was not observed with Cry- strain (Figure 4B).

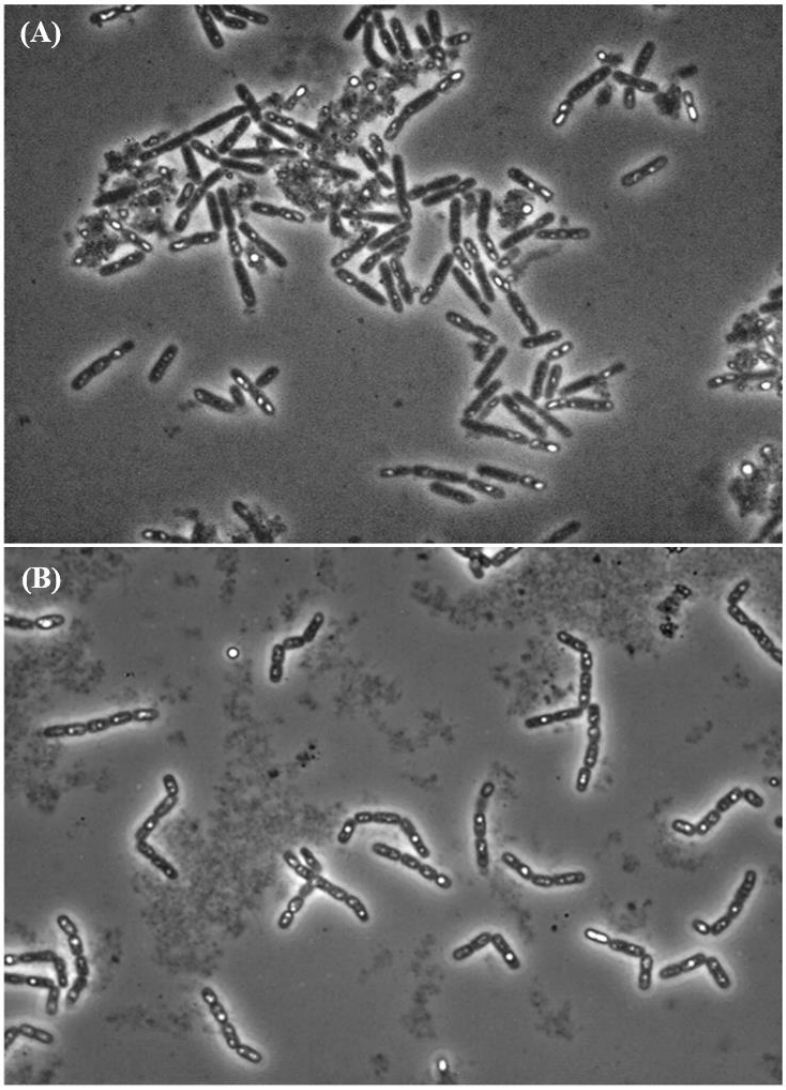

Figure 4 Cells aggregation properties of *B. thuringiensis* Cry+ strain (A) and Cry- strain (B). The Cry+ strain formed larger aggregates which were not observed in Cry- strain.

3.2 Effect of different protective agent on the survival of the cells before and after freeze-drying

The effect of six formulations of protective agents on the survival rate of *B. thuringiensis* was studied and the results are shown in Table 1. The results showed that the highest survival rate (11.43%) of Cry+ *B. thuringiensis* cells during the freeze-drying process was obtained when skimmed milk was used as a protective agent, followed by fructose (9.11%), monosodium glutamate (9%) and sucrose (8.33%). A very low survival rate was obtained when calcium carbonate (1.1%) and glycerol (0.12%) was used as protective agents during freeze-drying of *B. thuringiensis* cells. The cell without the addition of any protective agent (control) was recorded as the lowest survival rate (0.03%). This result shows that the addition of protective agents during freeze-drying resulted in higher viability, especially pronounced in cells suspended in more effective cryoprotectors (skimmed milk, fructose and monosodium glutamate). This effect was smaller in poorly or nonprotected cells (cells suspended in calcium carbonate, glycerol and water).

Table 1 Effects of various protective agents on the survival rates of ***B. thuringiensis*** cells after the freeze-drying process.

Protective agent	Total viable cell count (CFU/ml)		% of survival
	Before freeze-drying	After freeze-drying	
Distilled water	$1.5x10^{11}$	$4.0 x10^{7}$	0.03
Sucrose (10% w/v)	$1.2 x10^{11}$	$1.0 x10^{10}$	8.33
Calcium carbonate (0.5% w/v)	$9.0 x10^{10}$	$1.0 x10^{9}$	1.11
Glycerol (5% w/v)	$8.0 x10^{10}$	$9.3 x10^{7}$	0.12
Skim milk (10% w/v)	$1.4 x10^{11}$	$1.6 x10^{10}$	11.43
Fructose (10% w/v)	$1.2 x10^{11}$	$1.1 x10^{10}$	9.17
Monosodium glutamate (10% w/v)	$1.0 x10^{11}$	$9.0 x10^{9}$	9.00

3.3 Effect of fermentation condition (glucose in medium and DOT control strategies) on the synthesis of endotoxin by B. thuringiensis Cry+ strain

The effect of the addition of glucose into the basal medium on growth and sporulation of Cry+ *B. thuringiensis* MPK13 is shown in Table 2. The addition of 8 g/L glucose in the basal medium greatly enhanced growth and sporulation of *B. thuringiensis* MPK13 Cry+ strain in cultivation with and without DOT control strategies. The maximum cell growth and spore count obtained in cultivation using the medium with 8 g/L glucose and DOT controlled at 80% were $1.3x10^{12}$ cfu/ml and $3.9x10^{11}$ spore/ml, respectively. Both maximum viable cells and spore count were obtained at 48 h. In cultivation with medium containing 8 g/L glucose but without DOT controlled at 80%, the maximum viable cells ($8.3x10^{11}$ cfu/ml) and spore count ($2.1x10^{11}$ spore/ml) were achieved at 48 h. Although 8 g/L of glucose was presence in the medium, growth and sporulation rate were greatly reduced in cultivation without DOT at a high level during the cultivation, suggesting that sufficient oxygen supply was crucial to support growth and sporulation of *B. thuringiensis* MPK13. On the other hand, reduced growth and sporulation rates were observed in cultivation without the addition of glucose in the medium. In the absence of glucose, the maximum viable cells and spore number obtained in cultivation with the DOT controlled at 80% saturation were $1.7x10^{11}$ cfu/ml and $5x10^{10}$ spore/ml, respectively. On the other hand, maximum viable cells ($1.0x10^{11}$ cfu/ml) and spore count ($2x10^{10}$ spore/ml) obtained in cultivation without DOT control were the lowest amongst all cultivations tested in this study.

Table 2 Viable cells and spore number of *B. thuringiensis* cultivated in 5 L stirred tank bioreactor with different medium formulations and DOT control strategies.

Cultivation strategies			Performance/Kinetic parameter value				
DOT level (%)	Glucose (g/L)	Fermentation time (h)	Maximum viable cell concentration, Actual ($x10^{11}$ cfu/ml)	Maximum viable cell concentration, Model ($x10^{11}$ cfu/ml)	Maximum spore count ($x10^{11}$ spore/ml)	Specific growth rate, μ (h^{-1})	Lag time (h)
80	8	48	13.0	12.33	3.9	0.46	0
80	n/a	44	1.7	1.72	0.5	0.04	6.8
n/c	8	44	8.3	8.48	2.1	0.23	14.5
n/c	n/a	40	1.0	0.97	0.2	0.03	9.6

*n/c is DOT was not controlled; n/a is no presence of glucose. When DOT was not controlled, agitation speed was set at 200 rpm.

The SDS-PAGE analysis of δ-endotoxin produced by *B. thuringiensis* MPK13 spores cultivated in basal medium with or without the presence of glucose in combination of two different DOT control strategies during the cultivation are shown in Figure 5. The presence of δ-endotoxin was only detected in spores obtained from the cultivation with 8 g/L glucose and controlled DOT at 80% saturation throughout the cultivation. The decrease in cell growth not only led to a low rate of sporulation but also produced spores without the presence of δ-endotoxin, as observed in cultivation without glucose and without DOT control at a high level. Results from this study indicated that the formation of δ-endotoxin by *B. thuringiensis* MPK13 Cry+ cells required sufficient supply of glucose and also oxygen in the culture.

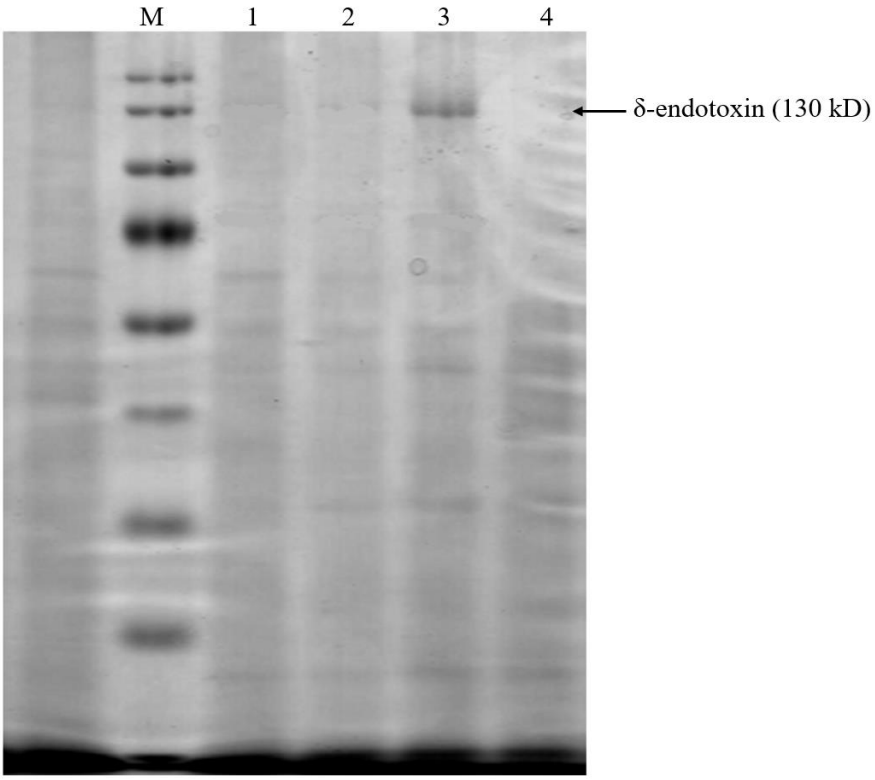

Figure 5 SDS-PAGE analysis of Bt MPK13 spores cultivated in medium with or without the presence of glucose and different DOT control strategies (M-marker; 1-DOT was controlled at 80% saturation and without glucose in medium; 2-DOT was not controlled and without glucose in medium; 3-DOT was controlled at 80% saturation and with 8 g/L glucose in medium; 4-DOT was not controlled and with 8 g/L glucose in medium.

3.4 Effect of different stock culture preparations on endotoxin production by B. thuringinesis

The cultivation of *B. thuringiensis* MPK13 using two different types of stock culture, Cry+ in powderized and slant agar, were repeated quadruplicates in 5 L stirred tank bioreactor using the basal medium with 8 g/L glucose and DOT was controlled at 80% saturation throughout the cultivation. The typical time courses for both cultivations are shown in Table 3. The maximum viable cells for cultivation using slant agar stock culture ($1.5x10^{12}$ cfu/ml) was slightly higher than the cultivation using Cry+ stock culture in powderized form ($1.2x10^{12}$ cfu/ml). Cultivation using the stock culture of Cry+ *B. thuringiensis* MPK13 in powderized form needed more time (28 h) to achieve maximum cell growth as compared to the slant stock culture (24 h), indicating that the cells required a longer time to adapt to a new culture environment. A higher sporulation rate was also observed for cultivation using slant agar stock culture as compared to the cultivation using Cry+ stock culture in powderized form, though the final spore count (SC) obtained in cultivation using Cry+ stock culture in powderized form ($3.8x\ 0^{12}$ spore/ml) was slightly higher than that obtained in cultivation using slant agar stock culture ($3.5x10^{12}$ spore/ml).

Table 3 Growth and sporulation profiles of *B. thuringiensis* cultivation using powderized form stock and slant agar stock in 5 L stirred tank bioreactor. All cultivations were performed using medium with the addition of 8 g/L glucose and DOT was controlled at 80%, saturation.

Stock culture	Fermentation time (h)	Maximum viable cell concentration, Actual ($x10^{11}$ cfu/ml)	Maximum viable cell concentration, Model ($x10^{11}$ cfu/ml)	Maximum spore count ($x10^{11}$ spore/ml)	Specific growth rate, μ (h^{-1})	Lag time (h)
Powderized	28	12±2.1	11.83	38±3.5	0.31	0
Slant agar	24	15±3.0	14.56	35±4.1	0.53	0

Consistent productions of δ-endotoxin were recorded, as analysed by SDS-PAGE for spores collected at 48 h, for all four cultivations using Cry+ stock culture in powderized form (Figure 6). On the other hand, δ-endotoxin was only detected in only one out of four cultivation runs using slant agar stock culture, indicating an inconsistency in δ-endotoxin formation in the spores.

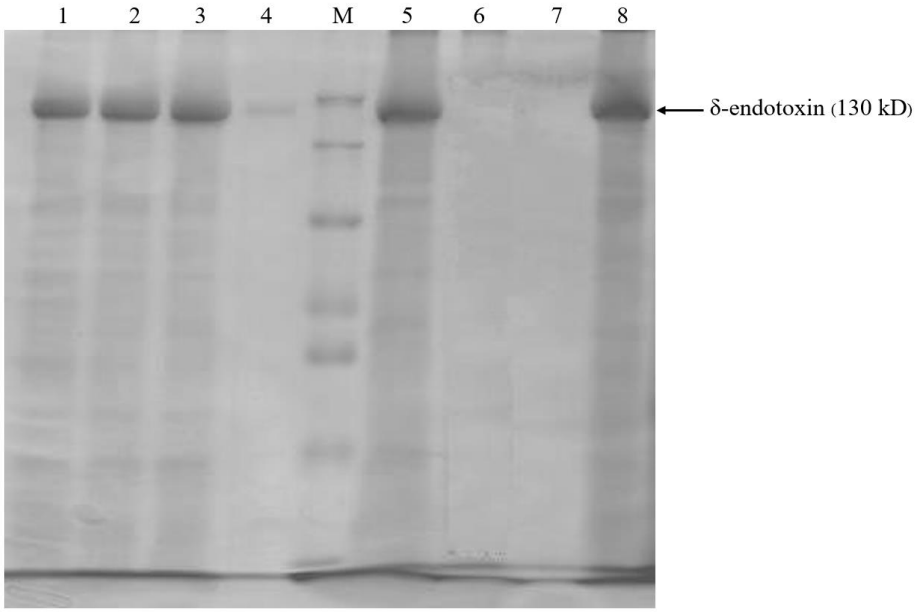

Figure 6 SDS-PAGE analysis of *B. thuringiensis* MPK13 spores obtained from cultivation using two different types of stock culture, Cy+ in powderized form and slant agar, repeated for four times. Lane: M-marker; 1-Powderized Bt MPK13 stock (1st cultivation); 2-Slant agar Bt MPK13 stock (1st cultivation); 3-Powderized stock (2nd cultivation); 4-Slant agar stock (2nd cultivation); 5-Powderize stock (3rd cultivation); 6-Slant agar B stock (3rd cultivation); 7-Slant agar stock (4th cultivation); 8-Powderized stock (4th cultivation).

4. Discussion

Repeated isolation of Cry+ *B. thuringiensis* MPK13 colonies, for at least 3 cycles, was required to obtain the most resistant Cry+ strains with consistent ability in producing δ-endotoxin. PCR and SDS-PAGE analysis during the first cycle of the isolation was insufficient to decide whether the Cry+ strain was truly resilient and possessed a low risk of plasmid loss during the cultivation. Most *B. thuringiensis* strains have the potential to lose their plasmids after several multiplications during subculturing and transfers in the cultivation process for the production of spores with endotoxin [9]. Results from this study have demonstrated that repeated isolation steps with an appropriate analyses technique using PCR and SDS-PAGE has been successfully used to obtain the strong Cry+ strain of *B. thuringiensis* MPK13 with the consistent ability to produce δ-endotoxin.

The Cry+ *B. thuringiensis* culture can be preserved for a longer period as the competent master stock by the freeze-drying method, which provides a long-term advantage for *B. thuringiensis* production. Freeze-drying is a standard procedure to prepare a starter culture in dairy and food industries. Nevertheless, freeze-drying and the ensuing storage usually recorded a decline in cell viability. The degree of cell viability loss depends on the properties of the strain, the growth conditions and growth state. Cryoprotectives are normally used to avoid or decrease cell death during freeze-drying and subsequent storage. In this study, skimmed milk (10% w/v) has effectively provided the highest degree of protection to *B. thuringiensis* cells during freeze-drying as compared to other protective agents. Furthermore, skimmed milk has been reported as the best protective agents for many microorganisms. Freeze drying of *Lactobacillus lactis* with protectants based on skimmed milk was the most effective (survival levels ~60%) [16]. The best survival rates (~85%) for *Candida sake* was obtained when skimmed milk was used as the protective agents during freeze-drying [17]. The highest survival rates (47.63%) during freeze-drying of *Bifidobacterium infantis* 20088 was also obtained when skimmed milk was used as the protective agent [18]. Skimmed milk has also been used as a protective agent in the freeze-drying of many probiotic bacteria [19] and *Geotrichum candidum* [20]. Moreover, the application of a variety of chemical compounds either alone or in addition to skimmed milk has been tested [21]. The use of saccharides (sucrose and fructose) during freeze-drying of a bacterial strain has also been proved effective as protective agents [22]. Monosodium glutamate has also been reported as a good protective agent for *lactobacillus* species [23]. In this study, although sucrose, fructose and monosodium glutamate provided comparable survival rates, the market price for these compounds is expensive compared to skimmed milk. Thus, based on the economic factor and the higher survival rates of cells after freeze-drying, skimmed milk was the preferred protective agent for *B. thuringiensis* MPK13 Cry+ strain.

Besides freeze-drying, immobilization in alginate was reported as another technique for long-term preservation of *B. thuringiensis* israelensis preservation [24]. The spores count of freeze-dried cell powder was significantly

higher than the oven-dried cell powder but close to the spray-dried cell powder, which was performed with inlet and outlet air temperatures of 200 ˚C and 75 ˚C, respectively [25]. Nevertheless, the potency of stock culture prepared by the freeze-drying method during large-scale preparation of *B. thuringiensis* needs to be fully explored for long term perception.

Aggregation of cells in the *B. thuringiensis* H14 Cry+ strain was also observed by Saffrazadeh *et al.* [26]. The aggregation of cells was signified as a strong indication of plasmid loss during *B. thuringiensis* cultivation. The aggregated characteristic of the *B. thuringiensis* cells is associated with a conjugation-like plasmid transfer. The phenomena usually occurred when the bacteria socialized during the log phase. It was also reported that a large aggregation of *B. thuringiensis* cells correlated with the existence of the large (250 kb) self-transmissible plasmid pXO16 [27]. The loss of pBtoxis plasmid, which is also the plasmid responsible for the production of toxin in *B. thuringiensis*, is usually associated with the loss of pXO16 plasmid [27].

It is notable that cells and spores morphology obtained in the Cry+ strain is similar to the Cry- strain. However, the Cry+ strains tend to form aggregates. Thus it is impossible to differentiate Cry+ and Cry- strain solely based on the morphology of the cells and spores. Although it is easy to habitually confirm the existence of δ-endotoxin encoding plasmid just by observing the cell aggregation, the method is still not practical in industry as the occurrence of cell aggregation as reported in this study can only be observed after 24 h of cultivation. As a consequence, a simple and effective technique in the preparation of stock culture shall be considered as the first step in the process to avoid plasmid loss in *B. thuringiensis* bioinsecticides production. Nevertheless, the result showing the Cry+ strain's ability to aggregate during the cultivation as reported here is important in developing a simple forecasting technique for plasmid loss during the cultivation.

Aeration was reported as the most crucial factor that can significantly affect the whole process of *B. thuringiensis* cultivation [28,29]. Thus, research on the fundamental effect of oxygen on *B. thuringiensis* cell growth, sporulation rate and δ-endotoxin synthesis need to be investigated in detail. The carbon source was also reported to be an important component to support better growth of *B. thuringiensis* [14,30]. Information on the effect of different types of monosaccharides and disaccharides is significant, as it provides novel and specific details on carbon sources consequences to the production of *B. thuringiensis* based bioinsecticides.

A repeated isolation cycle with appropriate analysis of the isolated colonies using PCR and SDS-PAGE can be applied to obtain the resistant Cry+ strain of *B. thuringiensis* MPK13 with a consistent ability to produce δ-endotoxin, that would not easily lose its plasmid during the cultivation. The preparation technique of the *B. thuringiensis* Cry+ stock culture, based on freeze-drying, has also been successfully developed to overcome the problem related to plasmid loss during *B. thuringiensis* cultivation that leads to competent production of spores containing δ-endotoxin. Consistent production of *B. thuringiensis* spores containing δ-endotoxin using the competent Cry+ stock culture in powderized form has also been achieved by cultivation in a 5 L stirred tank bioreactor. However, the synthesis of δ -endotoxin from the Cry+ strain can only be obtained using growth medium with 8 g/L glucose and an appropriate aeration strategy (the DOT level was controlled at above 80% saturation) during the cultivation.

5. Conclusion

A repeated isolation cycle with appropriate analysis of the isolated colonies using PCR and SDS-PAGE can be applied to obtain the resistant Cry+ strain of *B. thuringiensis* MPK13 with the consistent ability to produce δ-endotoxin, that would not easily lose its plasmid during the cultivation. The preparation technique of *B. thuringiensis* Cry+ stock culture, based on freeze-drying, has also been successfully developed to overcome the problem related to plasmid loss during *B. thuringiensis* cultivation that leads to competent production of spores containing δ-endotoxin. Consistent production of *B. thuringiensis* spores containing δ-endotoxin using the competent Cry+ stock culture in powderized form has also been achieved by cultivation in a 5 L stirred tank bioreactor, where high cell growth and a spore count exceeding $1x10^{12}$ cfu/ml and $3.0x10^{11}$ spore/ml were obtained, respectively. However, the synthesis of δ-endotoxin from the Cry+ strain can only be obtained using growth medium with 8 g/L glucose and an appropriate aeration strategy (DOT level was controlled at above 80% saturation) during the cultivation.

6. References

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