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IMPACT OF SINGLE AND TWO-PHASE DISSOLVED OXYGEN TENSION CONTROL ON *Bacillus thuringiensis* CULTIVATION AND δ-ENDOTOXIN PRODUCTION

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ABSTRACT

Constant δ-endotoxin production is crucial in *Bacillus thuringiensis* cultivation with aeration strategy playing a significant role in the process. In this study, the impact of DOT control strategies on the cultivation performance of *B. thuringiensis* was investigated using a 5-L stirred tank bioreactor. Single-phase DOT control recorded low spore count (<5.0×10^{11} spore/mL) and low percentage of sporulation (<45%). Two-stage DOT control cultivation with DOT at 80% during the active growth phase, followed by a switch to 60% or 40% at mid-exponential growth phase contributed to enhanced sporulation (>60%), high maximum cell count (1.5 cfu/mL) with δ-endotoxin formed as early as 8 hr of cultivation. A high maximum specific growth rate, μ_{max} (>0.1 h^{-1}) was also essential in ensuring δ-endotoxin production. High cell count obtained in cultivation with DOT level of 80% saturation during active growth was concurrent with high cell count, high μ_{max}, fast generation time, high OUR, high CER value, and high respiratory quotient (RQ) value. During the two-phase DOT control cultivation, low RQ values were reported. The result showed that the exhaust gas data could be used to monitor *B. thuringiensis* cultivation performance.

Key words: Aeration strategy, dissolve oxygen tension, *Bacillus thuringiensis*, δ-endotoxin, high sporulation

INTRODUCTION

Bacillus thuringiensis is a Gram-positive bacterium with the ability to produce spore, parasporal crystal protein (ICPs), and δ-endotoxin. The toxin can be used to combat some agricultural pests and vectors of diseases (Bravo *et al*., 2011). Almost 90% of the microbial biopesticide products in the market are dominated by one entomopathogenic bacterium, which is *B. thuringiensis* (Kumar & Singh, 2015). Submerged culture is widely used for the industrial production of endotoxin-spore suspension of *B. thuringiensis*. A suitable aeration strategy during the cultivation of *B. thuringiensis* is required to enhance δ-endotoxin formation during sporulation (Sarrafzadeh *et al*., 2005). The importance of dissolved oxygen tension in the culture during *B. thuringiensis* cultivation has also been pointed out by several researchers (Rowe *et al*., 2003; Maldonado-Blanco *et al*., 2003; Sarrafzadeh & Navarro, 2006; Bravo *et al*., 2015).

A better understanding of the factors that affect oxygen transfer in aerobic cultivation is required to improve the production of the target products. Data on the respiration of microorganisms can be generated using non-proliferating suspensions and low viability suspensions in manometric apparatus. The use of the Warburg apparatus to analyze bacterial respiration has been reported (Stanbury *et al*., 2003; Lalke-Porczyk & Donderski, 2001). Another technique to measure gaseous exchange in microbial cultivation is by using a continuous-flow recording respirometer, which is a dissolved oxygen probe that senses the oxygen demand of the culture by constant-current electrolysis of water and is measured by recording the operating time of the power supply (Vanrolleghem, 2001; Kim *et al*., 2008; Zhang & Hughes, 2004). Several other techniques

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such as selective absorption of the component gases or gas chromatography (Cheung *et al*., 2009; Hildebrand, 2010) and continuous recording of pressure changes using manometers or pneumatic transducers (Kuphaldt, 2008) have also been used to measure gaseous exchange during the cultivation.

The effect of dissolved oxygen in the culture on the sporulation rate of *B. thuringiensis* and the synthesis of δ-endotoxin is not well understood and documented. *B. thuringiensis* is an aerobic bacterium, and it is practical to contemplate the level of dissolved oxygen present in the bioreactor as the main factor in the production of bioinsecticides (Amicarelli *et al*., 2010). It was reported that the most toxic product of *B. thuringiensis* subsp. *israelensis* was obtained in cultivation at high aeration rates (Maldonado-Blanco *et al*., 2003). High dissolved oxygen tension (DOT) of up to 100% saturation was required during the cultivation to increase the toxic activity of *B. thuringiensis* (Boniolo *et al*., 2012). Growth of *B. thuringiensis* subsp. H14 was enhanced in well-aerated culture, but the synthesis of δ-endotoxin was significantly repressed (Zuoari *et al*., 2002). It was also reported that in batch fermentation of *B. thuringiensis israelensis*, there was a marked increase in biomass production with the simultaneous increase of the larvicidal compound (Gopinathan *et al*., 2016). Significant effect of oxygen supply was reported not only on *B. thuringiensis*, but also on other *Bacillus* strains such as *Bacillus subtilis* (Ha *et al*., 2018). Appropriate control of DOT during the cultivation of *B. thuringiensis* has been reported as one of the crucial factors for the enhancement of δ-endotoxin synthesis (Avignone-Rossa *et al*., 1992; Aronson, 2002; Bravo *et al*., 2011).

The objective of this study is to investigate the impact of a single-phase and a two-phase DOT control on *B. thuringiensis* cultivation and δ-endotoxin production in a stirred tank bioreactor cultivation. The results obtained can be applied to up-scale *B. thuringiensis* fermentation with high sporulation and constant δ-endotoxin production.

MATERIALS AND METHODS

Microorganism

Bacillus thuringiensis MPK13 was obtained from the culture collection at Entomology and Integrated Pest Management Unit, Malaysian Palm Oil Board (MPOB) Headquarters, Malaysia. This bacterium was locally isolated from soil in the oil palm plantation and was cultivated three times from the dead lepidopteran larvae. This bacterium was isolated from the gut of the dead larvae of bagworm, *Metisa 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).

Cultivation media

The *B. thuringiensis* strains were cultivated in liquid media at the optimal conditions for δ-endotoxin production. The 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 was used as the basal medium (Jaquet *et al*., 1987; Içgen *et al*., 2002). Glucose at a concentration of 8 g/L was used as the main carbon source (Mazmira *et al*., 2013). The medium was sterilized at 110°C, 15 min at 15 psi. The initial pH of the medium was adjusted in the region of 6.5 – 7.0 using 1 M HCl.

Cultivation using 5-L stirred tank bioreactor

All cultivation experiments were conducted in a 5-L stirred tank bioreactor (BIOSTAT B-DCU, Sartorius Stedim, Germany). The bioreactor was equipped with dissolved oxygen, pH, temperature, and foam control systems. The standard six-bladed Rushton turbine impeller (diameter = 0.05 m) was used for bubble dispersion and mixing while the ring sparger was used for air sparging. The medium (3.6 L) in the bioreactor was heat sterilized at 121°C for 20 min. The sterilized bioreactor was inoculated with 400 mL of inoculum (11% v/v) to start the cultivation. The temperature within the bioreactor vessel was controlled at 30°C throughout the cultivation. The culture pH was not controlled but monitored throughout the cultivation. Silicone KM72FS (Shin-Etsu, Japan) at a concentration of 10% (v/v) was used as an antifoam agent to control foaming.

Different dissolved oxygen tension (DOT) control strategies were applied to the cultivation of *B. thuringiensis* MPK13 in the 5-L stirred tank bioreactor, as stated below:

Single-phase DOT control:

1) DOT was set at 80% throughout the cultivation
2) DOT was set at 60% throughout the cultivation
3) DOT was set at 40% throughout the cultivation

Two-phase DOT control:

4) DOT was initially set at 80% and subsequently set to 60% at 6 hr of cultivation
5) DOT was initially set at 80% and subsequently set to 40% at 6 hr of cultivation
6) DOT was initially set at 60% and subsequently set to 80% at 6 hr of cultivation
7) DOT was initially set at 40% and subsequently set to 80% at 6 hr of cultivation

The DOT profiles were decided upon the previous finding by Ghribi *et al*. (2007) and Kheder *et al*. (2014). Based on their findings, the level of dissolved oxygen in the culture medium plays a crucial role in the metabolism involved in biopesticide production. The DOT in the culture was measured using a polarographic dissolved oxygen electrode (Hamilton, Germany). In all cultivations, the airflow rate was fixed at 4 L/min (1 vvm). The DOT level was controlled at the required value by varying the agitation speed ranging from 50 to 500 rpm using a cascade model of the DOT control module.

Analysis of gaseous exchange during cultivation

The gaseous exchange during the cultivation was analyzed using the Exhaust Gas Analyser System (EGAS) (Sartorius Stedim, Germany). A gas analyzer type "EL3020", was included in the EGAS-L system to continuously analyze the composition of exhaust gas from the bioreactor. The gas sample was removed from the bioreactor exhaust gas line and fed through the multiplexer controller, which was then forwarded into the EGAS-L for the evaluation of the composition in a pressure-free condition.

The carbon dioxide evolution rate (CER) was calculated by analyzing the difference between the rate of carbon dioxide at the outlet and the inlet divided by the volume of the culture. Several assumptions made in O_2, CO_2, OUR, CER, and RQ analyses were as follows:

1) The ambient concentrations of O_2 and CO_2 were 20.9% and 0.03%, respectively.
2) The gases consumed and evolved during the cultivation were only O_2 and CO_2, respectively. Other gasses were considered inert.
3) Density and molecular weight of oxygen were 1.41 g/L and 32 g/mol, respectively
4) The density and molecular weight of carbon dioxide were 1.95 g/L and 44 g/mol, respectively.

Total Viable Cell Count

During the cultivation, culture samples were collected at different time intervals for analysis. The culture samples were serially diluted using 0.85% (v/v) saline buffer and plated on nutrient agar (NA) plates via the pour plate method. The plates were incubated at 30°C for 48 hr and the number of the single colonies formed was counted and expressed in cfu/mL. The plates were prepared in triplicates.

Spore Count

Culture samples were heated to 80°C for 15 min to eliminate vegetative cells before being serially diluted and plated on NA plates. The plates were incubated at 30°C for 48 hr and the number of the single colonies formed was counted and expressed as spore/mL. Spores were counted by the pour-plate counting technique after heat shock (Thompson and Stevenson, 1984) and expressed as spore/mL. The plates were prepared in triplicates.

The percentage of sporulation was expressed using the following formula:

$$[(\textit{Spore count} \mid \textit{cell number})] \times 100$$

Kinetic data analysis

The following simplified batch culture kinetic models for cell growth based on the logistic equation, which has been described elsewhere (Ariff *et al*., 1997), was used to evaluate the growth kinetics of *B. thuringiensis* MPK13,

Cell growth:

$$\frac{dX}{dt} = [\mu^{max}(1 - X/X^{max})] \quad (1)$$

where X is the cell concentration (g/L), X_{max} is the maximum cell concentration (g/L), μ_{max} is maximum specific growth rate (1/h) and t is the cultivation time (hr).

The growth kinetic model (Equation 1) was fitted to the experimental data by non-linear regression with a Marquardt algorithm using SIGMA PLOT 10 computer software. The model parameter values were first evaluated by solving equation 1 and then the computer program was used to minimize the sum of squares of the differences between the predicted and measured values (Weiss & Ollis, 1980; Mohamed *et al*., 2012). The predicted values were then used to simulate the profiles of the cell during the cultivation.

Detection of δ-endotoxin

SDS-PAGE analysis was used for the detection of 130 kD δ-endotoxin during sporulation (Masri *et al*., 2020; 2013). The system used in this study is a discontinuous SDS system that is the most widely used electrophoretic system (Lemmli, 1970). 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 the Hoefer unit, running gel consisting of 5 mL monomer solution (A: B), 15 mL 4× running buffer 600 µL, 10% of SDS, and 29.1 mL of distilled water. The gel solution was vacuumed for 15 min and after that 300 µL of 10% ammonium persulphate and 20 µL of Temed were added. The ammonium persulphate 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. An 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, the electric current was set up at 15 amps and left overnight.

RESULTS AND DISCUSSION

B. thuringiensis cultivation without DOT control

The profile of cell growth and sporulation without DOT control throughout the cultivation is shown in Figure 1. The highest cell count (1.1×10^{12} cfu/mL), spore count (3.8×10^{11} spore/mL) and percentage of sporulation (35%) were recorded at 48 hr of cultivation (Table 1). Although substantially high cell count ($\geq 1.0 \times 10^{12}$ cfu/mL) was obtained in the cultivation without DOT control, the sporulation was low. The final spore count obtained in this cultivation was only 4.0×10^{11} spore/mL. A reduced maximum specific growth rate (0.05 h^{-1}) was also recorded during the exponential growth phase of the culture without DOT control. Low CO_2 value (0.08%), OUR (30 mM/L/h) and CER (1 mM/L/h) were also recorded at 6 hr of cultivation. Nonetheless, the RQ value was above 0.05 (Table 1). It is interesting to note that δ-endotoxin was not detected in the cultivation where DOT was left uncontrolled throughout the process.

Effect of single-phase DOT control on growth, sporulation, and d-endotoxin synthesis

The maximum viable cell count (ranging from 1.1×10^{12} cfu/mL and 1.4×10^{12} cfu/mL) was recorded at different DOT control strategies applied in this study (Figure 1A). However, a high maximum specific growth rate (μ_{max}) (above 0.1 h^{-1}) was observed in the cultivation where the DOT was controlled at a high level (80% saturation) during the active growth phase as compared to that in the cultivation where the DOT was controlled at lower levels (40% or 60% saturation).

Cultivation with the single-phase DOT control also recorded a low spore count ($<5.0 \times 10^{11}$ spore/mL) and a low percentage of sporulation (<45%). The degree of sporulation was greatly reduced when the DOT was controlled at 80% throughout the cultivation, which gave a final spore count of only 4.2×10^{11} spore/mL (Figure 1B). A higher sporulation rate (>35%) was obtained in the cultivation where the DOT was controlled at lower levels (60% or 40% saturation) throughout the process as compared to 80% saturation (Figure 1C). It is important to note that δ-endotoxin was only detected in the cultivation of *B. thuringiensis* MPK13 where the DOT was controlled at 80% saturation (Figure 2A), indicating that a high DOT was a crucial factor for δ-endotoxin synthesis.

We found that during the initial growth phase of *B. thuringiensis* MPK13, DOT of 80% saturation was required to enhance cell growth with a higher maximum specific growth rate exceeded 0.1 h^{-1} compared to lower aeration (DOT of 60% or 40% oxygen saturation) throughout the fermentation. In this study, lower aeration of 60% or 40% DOT did not record any production of δ-endotoxin throughout the cultivation. Production of δ-endotoxin was recorded only in cultivation where the initial DOT was set at 80%. This may be due to sufficient aeration during *B. thuringiensis* cultivation since the bacterium is a facultative microorganism and grows well in an aerobic environment. A similar observation was also reported by Khedher *et al*. (2014) where lower aeration (40% saturation) throughout the fermentation, significantly reduced the toxin synthesis.

A high dissolved oxygen concentration increased the cell's capacity to metabolize glucose and to produce delta-endotoxins. An adequate quantity of glucose supplied in the culture medium is required to promote high cell growth, sporulation, and formation of δ-endotoxin. Glucose also contributes to better cell morphology and crystalline protein production. The existence of δ-endotoxin was also observed in cultivation using a mixture of various sugars (fructose, sucrose, maltose, and lactose) with 8 g/L glucose, indicating that high glucose (>8 g/L) must be present in the culture to trigger δ-endotoxin formation in the spores of *B. thuringiensis* MPK13 (Mazmira *et al*., 2013). Besides glucose, the importance of optimizing the adequate concentration of dissolved oxygen has been reported as one of the crucial factors in the success of *B. thuringiensis* cultivation (Foda *et al*., 1985; Rossa & Mignone 1995; Morris *et al*., 1996). The level of DOT during *B. thuringiensis* cultivation significantly not only affects the cell density but also δ-endotoxin synthesis (Ghribi *et al*., 2007; Berbert-Molina *et al*., 2008). In this study, we found that the DOT of 80% saturation throughout the cultivation was required for optimized *B. thuringiensis* growth rate, however, the production of δ-endotoxin was slow and was recorded only at 48 hr of fermentation. A new cultivation strategy via aeration profiles must be exploited for faster production of δ-endotoxin during the cultivation.

Effect of two-phase DOT control strategy on growth, sporulation and δ-endotoxin synthesis

The two-phase DOT control strategy provides a substantial improvement in the percentage of sporulation (57–61%) with a final spore count of $7.9 – 8.5 \times 10^{11}$ spore/mL was obtained in cultivation

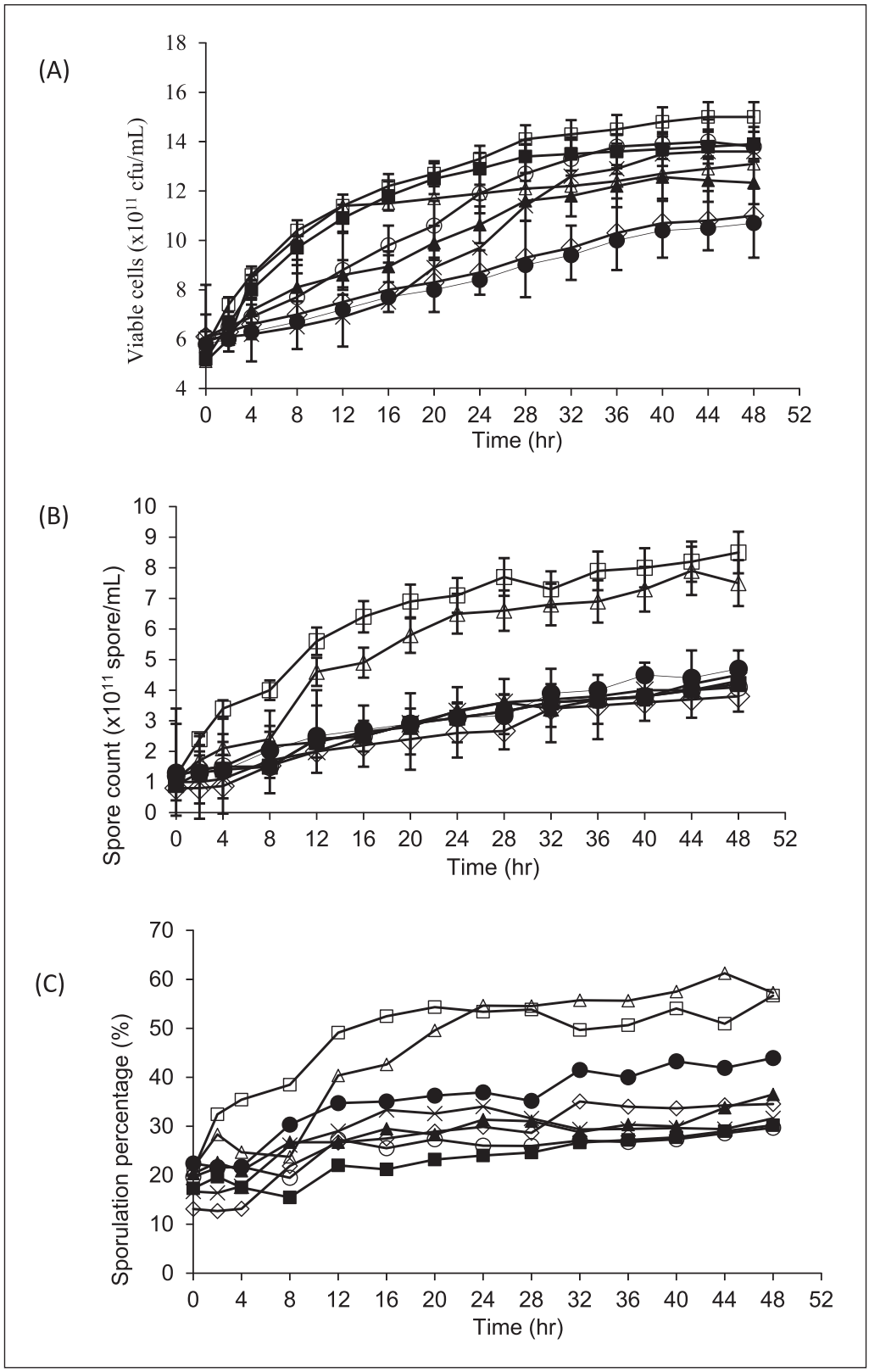

Fig. 1. Growth (A), sporulation (B) and sporulation percentage (C) profiles of Bt MPK13 cultivated in 5 L stirred tank bioreactor at different DOT control strategies. ■ Controlled DOT (80%); □ DOT was controlled at 80% and switch to 60% at 6 hr of cultivation; ▲ Controlled DOT (60%), △ DOT was controlled at 80% and switch to 40% at 6 hr of cultivation, ● Controlled DOT (40%), ○ DOT was controlled at 60% and switch to 80% at 6 hr of cultivation, × DOT was controlled at 40% and switch to 80% at 6 hr of cultivation, ◊ Without DOT control (aeration rate 1vvm, agitation speed fixed at 200 rpm.

Table 1. Effect of different DOT control strategies on growth, sporulation rate, and δ-endotoxin synthesis

DOT control strategies	C11	Max cell count ($\times 10^{12}$ cfu/mL	Max spore count ($\times 10^{11}$ spore/mL)	Max sporulation percentage (%)	δ-endotoxin synthesis	CO_2	OUR (Mm/L/h)	CER (Mm/L/h)	Respiratory Quotient (RQ.)
DOT was not controlled, aeration rate at 1 v/v/m, agitation fixed at 200 rpm	0.05	1.1	3.5	35	No	0.08	30	1	0.08
DOT was controlled at 80% throughout the cultivation	0.14	1.4	4.2	30	Yes (48 hr)	0.32	75	9	0.12
DOT was controlled at 60% throughout the cultivation	0.07	1.2	4.5	37	No	0.35	78	7	0.09
DOT was controlled at 40% throughout the cultivation	0.03	1.1	4.7	44	No	0.06	62	2	0.03
DOT was controlled at 80% and switched to 60% after 6 hr of cultivation	0.12	1.5	8.5	57	Yes (24 hr)	0.1	74	2	0.02
DOT was controlled at 80% and switched to 40% after 6 hr of cultivation	0.13	1.5	7.9	61	Yes (8 hr)	0.1	74	2	0.02
DOT was controlled at 60% and switched to 80% after 6 hr of cultivation	0.09	1.4	4.1	30	No	ND	ND	ND	ND
DOT was controlled at 40% and switched to 80% after 6 hr of cultivation	0.08	1.3	4.3	32	No	ND	ND	ND	ND

Note: Value of CO_2, O_2, OUR, CER, and RQ were recorded at 6 hr of cultivation
ND – not determined

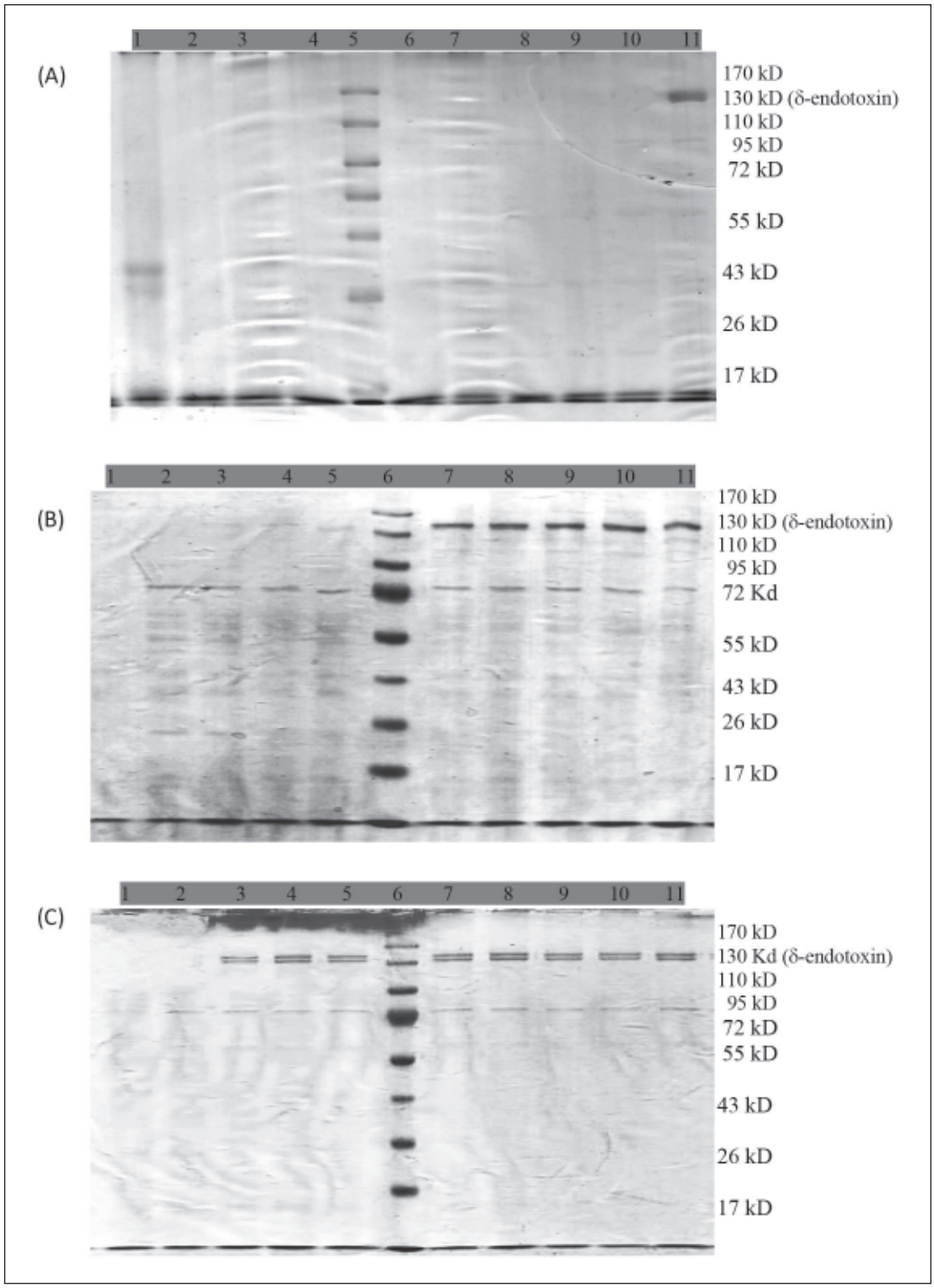

Fig. 2. SDS-PAGE analysis of Bt MPK13 samples from culture with (A) Controlled DOT of 80% saturation throughout the cultivation (Lane 1: 0 hr; Lane 2: 4 hr; Lane 3: 8 hr; Lane 4: 12 hr; Lane 5: broad range protein marker, MBiotech, Korea; Lane 6: 16 hr; Lane 7: 24 hr; Lane 8: 28 hr; Lane 9: 36 hr; Lane 10: 40 hr; Lane 11: 48 hr); (B) DOT was controlled at 80% and switch to 60% at 6 hr of cultivation (Lane 1: 0 hr; Lane 2: 4 hr; Lane 3: 8 hr; Lane 4: 12 hr; Lane 5: 16 hr; Lane 6: broad range protein marker, MBiotech, Korea; Lane 7: 24 hr; Lane 8: 28 hr; Lane 9: 36 hr; Lane 10: 40 hr; Lane 11: 48 hr); (C) DOT was controlled at 80% and switch to 40% at 6 hr of cultivation (Lane 1: 0 hr; Lane 2: 4 hr; Lane 3: 8 hr; Lane 4: 12 hr: Lane 5: 16 hr; Lane 6: broad range protein marker, MBiotech, Korea; Lane 7: 24 hr; Lane 8: 28 hr; Lane 9: 36 hr; Lane 10: 40 hr; Lane 11: 48 hr). *Note*: kD = kilodalton.

where the DOT was controlled at 80% saturation during active growth and then switched to either 60% or 40% saturation after 6 hr (Figure 1B). A higher maximum specific growth rate, ranging from 0.12 to 0.13 h^{-1}, was also recorded in cultivation using the two-phase DOT control (Table 1). Faster production of δ-endotoxin was also detected in these cultivations.

In cultivation where the DOT profile was switched from lower to higher saturation after 6 hr of fermentation, a reduced sporulation percentage from 30 – 32% was recorded. A reduced maximum specific growth rate (<0.1 h^{-1}) was also observed in these cultivations. It was observed that δ-endotoxin synthesis was not detected in cultivations where the DOT was controlled at a value lower than 80% saturation during the initial stages of the cultivation (Table 1).

During the two-phase DOT control strategy, where DOT was initially controlled at a high level (80% saturation) and then switched to a lower level (40% or 60% saturation) after 6 hr, thick bands of δ-endotoxin at the molecular weight of 130 kD was detected at 24 to 48 hr of cultivation (Figure 2B). Synthesis of δ-endotoxin was obtained at 8 hr of cultivation where the DOT was controlled at 80% and switched to 40% saturation. The intensity of the bands remained constant until 48 hr of cultivation (Figure 2C). The synthesis of δ-endotoxin at 8 hr of cultivation was a response to drastic changes in DOT changes at 6 h of cultivation. Results from this study indicated that high DOT level during active growth was required not only to support high cell growth but also to enhance sporulation and δ-endotoxin. The interruption of DOT level in the culture with actively growing cells may change the cell metabolism, which in turn, sporulation and δ-endotoxin synthesis were further enhanced.

An aeration strategy that supports both high cell count and spore count in the culture is vital to trigger the δ-endotoxin production. Taking into consideration all effects of aeration, it can be concluded that carrying out high aeration level during the first 6 hr of culture followed by a moderate oxygenation rate up to the end of fermentation could induce an increase in delta-endotoxin production and an improvement in *B. thuringiensis* toxicity (Khedher *et al*., 2014). High cell count and also high spore count are crucial for *B. thuringiensis* cultivation as it is coupled with the production of δ-endotoxin (Vu *et al*., 2012). High dissolved oxygen during the exponential growth phase is important for the production of *B. thuringiensis* spores (Silveira & Molina *et al*., 2005). However, a very high DOT level (100% saturation) was not preferred for *B. thuringiensis* cultivation (Sarrafzadeh *et al*., 2006), in which, saturated rich oxygen condition would affect cell growth and cease the onset of sporulation. A rich oxygen environment in the culture is not suitable for sporulation since it can stunt the process (Saraffzadeh *et al*., 2007). Changes in DOT level during the cultivation have been proven to manipulate the spore production (Ghribi *et al*., 2007; Berbert-Molina *et al*., 2008). The toxicity of *Bacillus* spp. was also affected by the DOT level, where a high DOT level (100%) causes a reduction in toxicity (Ismail *et al*., 1996). Low metabolism is one of the criteria required for the cells to sporulate and produce δ-endotoxin. In this study, we found that the presence of high viable cells in the culture is needed for high spore count and the right aeration profiles from high to lower DOT must be applied at 6 hr of fermentation for faster production of δ-endotoxin.

The two-phase DOT control strategy (80% saturation during the initial active growth phase and then switched to 40 – 60% saturation after 6 hr) as proposed in this study may be applied in the industrial process for enhancement of sporulation rate of *B. thuringiensis* and δ-endotoxin.

Relationship between exhaust gas analysis and *B. thuringiensis* cultivation performance

The profile of OUR, CER, and RQ during the cultivation of *B. thuringiensis* MPK13 at different DOT control strategies are shown in Figures 3, 4, and 5, respectively. The highest OUR (78 mM/L/h) was observed in cultivations with a single-phase DOT control strategy (80% and 60% saturation) (Table 1). Reduced OUR (65 mM/L/h) was observed in cultivation where the DOT was controlled in a single-phase at 40% saturation.

In cultivation with a two-phase DOT control strategy, higher OUR ranging from 71 mM/Lh and 78 mM/Lh, were observed when the DOT was controlled at a high level (80% saturation) during the initial active growth phase (Figure 3). Reduced OUR value was also observed in the two-phase control strategy with DOT lower than 80% during the initial growth phase. In all cultivations, the OUR value became level off when growth reached a stationary growth phase. It was evident that the high initial DOT level (80% saturation) during initial active growth contributed to high OUR.

The highest CER values for all cultivations were recorded during the initial stage of the exponential growth phase (4 hr). Cultivation with a single-phase DOT control strategy (80% and 60% saturation) recorded the highest CER (11 mM/L/h). In comparison, the cultivation with a two-phase DOT control strategy (80% to 40 – 60%) recorded the highest CER value (10 mM/L/h) during the exponential growth phase (Figure 4). Cultivation with a single-phase DOT control strategy at 40%

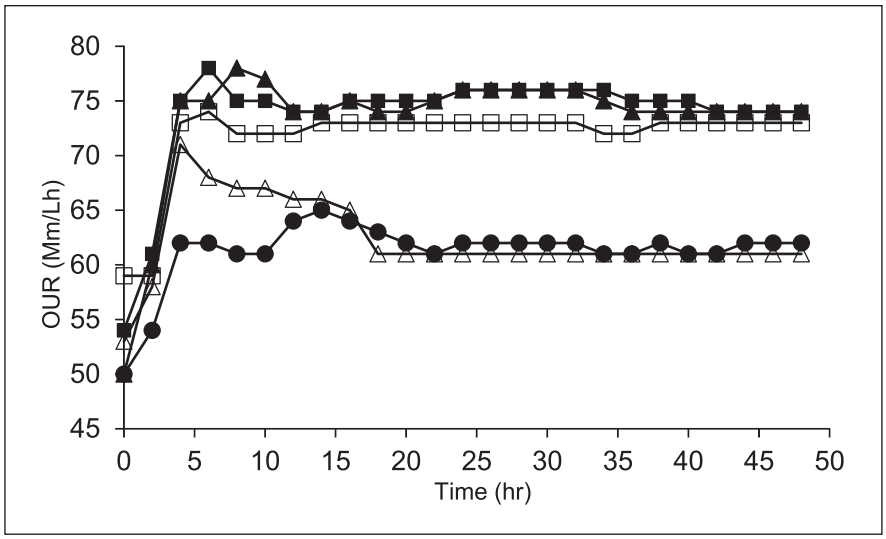

Fig. 3. Oxygen uptake rate (OUR) profile of Bt MPK13 cultivated in 5 L stirred tank bioreactor at different DOT control strategies. ■ Controlled DOT (80%); □ DOT was controlled at 80% and switch to 60% at 6 hr of cultivation; ▲ Controlled DOT (60%), △ DOT was controlled at 80% and switch to 40% at 6 hr of cultivation, ● Controlled DOT (40%).

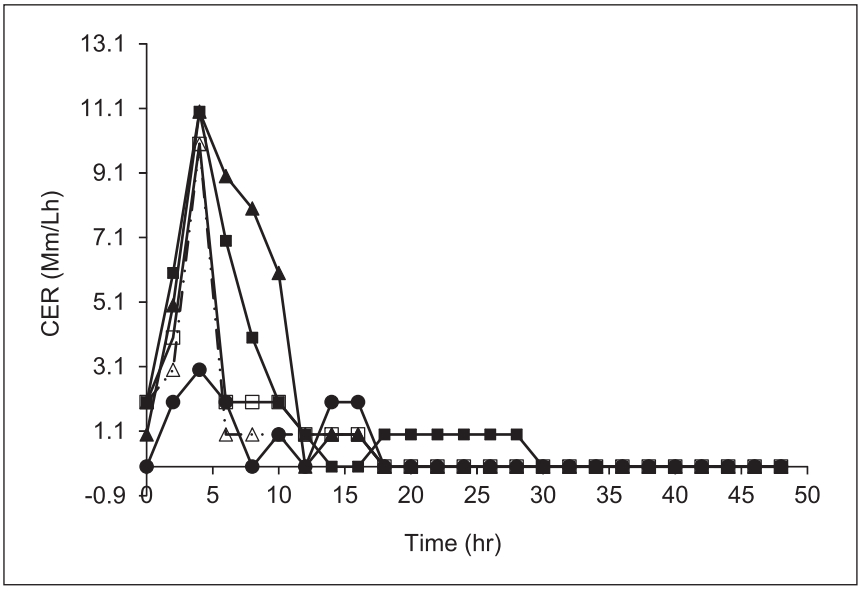

Fig. 4. Carbon dioxide extraction rate (CER) profile of Bt MPK13 cultivated in 5 L stirred tank bioreactor at different DOT control strategies. ■ Controlled DOT (80%); □ DOT was controlled at 80% and switch to 60% at 6 hr of cultivation; ▲ Controlled DOT (60%), △ DOT was controlled at 80% and switch to 40% at 6 hr of cultivation, ● Controlled DOT (40%).

saturation recorded low CER (ranging from 3 mM/L/h to 10 mM/L/h). In all cultivations, CER values were decreased to 0 mM/Lh after 30 hr of cultivation.

The highest value of respiratory quotient (RQ) in all cultivations was recorded during the initial stage of the exponential growth phase (Figure 5). The profile of RQ was quite similar to the CER profile where all the highest values were recorded at 4 hr of cultivation. The microbial respiratory quotient (RQ), defined as the ratio of mol CO_2 evolution per mol O_2 uptake. In this study, the highest value of CER and RQ recorded at 4 hr of cultivation refers to the active growth phase of the cell where the respiration and amount of CO_2 emitted by the microbes was higher than the available O_2 in the vessel. All cultivations where the DOT was controlled at a high level (80% saturation) during the initial active growth phase recorded the highest value of RQ, ranging from 0.14

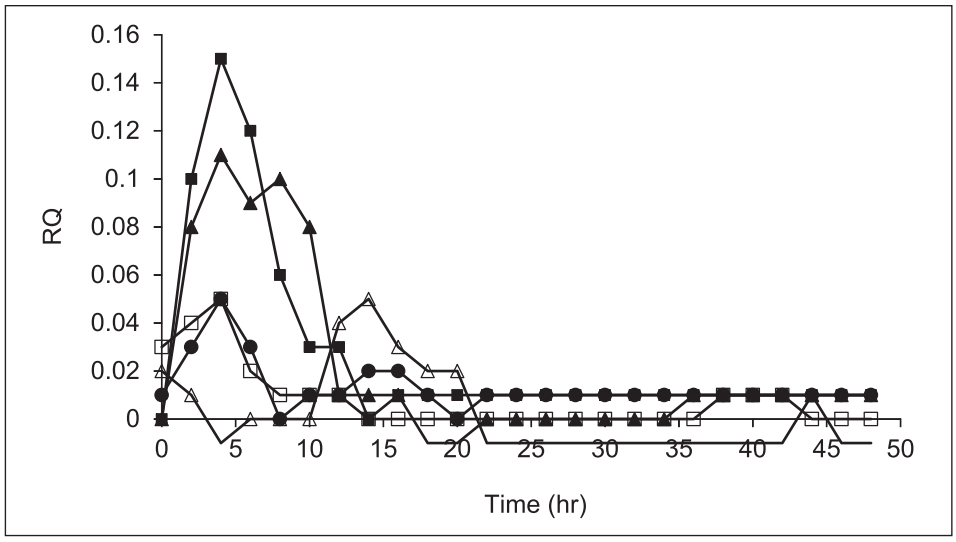

Fig. 5. Respiration quotient (RQ) profile of Bt MPK13 cultivated in 5 L stirred tank bioreactor at different DOT control strategies. ■ Controlled DOT (80%); □ DOT was controlled at 80% and switch to 60% at 6 hr of cultivation; ▲ Controlled DOT (60%), △ DOT was controlled at 80% and switch to 40% at 6 hr of cultivation, ● Controlled DOT (40%).

to 0.15. The highest RQ value for cultivation with a single-phase DOT control strategy at 60% saturation and 40% saturation was 0.12 and 0.05, respectively (Figure 5). In all cultivations, the reduced value of RQ (0.01) was observed after 20 hr. It is interesting to note that the CER and RQ values for cultivation with a two-phase DOT control strategy decreased drastically after 6 hr, as compared to those recorded in cultivations with a single-phase DOT control strategy. These results showed that in a stirred tank bioreactor, the requirement of dissolved oxygen during each growth phase played a crucial role in producing δ-endotoxin. It was found that the dissolved oxygen was required during the first 6 hr of vegetative growth for regulating the carbon source assimilation rate. The requirement to decrease oxygen saturation after 6 hr of vegetative growth could be explained by additional time for cry gene expression and crystal formation (Zouari & Jaoua, 1999).

The relationship between OUR and CER in *B. thuringiensis* cultivation and other microbes has not been well documented. The only report available in the literature on the relationship between OUR and CER was on aerobic thermophilic sludge digestion (Kovacs *et al*., 2007). In this study, the CER and RQ values in cultivation with a two-phase DOT control strategy (80% to 40 – 60% saturation) greatly varied as compared to the values recorded in a single-phase DOT control strategy. The OUR value did not significantly vary in all cultivation except for cultivation where the DOT was controlled at a low level (40% saturation) during the initial active growth phase. High CER indicated that the rate of CO_2 exiting the bioreactor was high. As respiration produces CO_2, this meant that the cells were actively growing. High cell count obtained in cultivation with high DOT level (80% saturation) during active growth was concurrent with high cell count, high μ_{max}, fast generation time, high OUR, high CER value, and high RQ value. High OUR value recorded during the one-phase DOT control strategy of 80% saturation correlates to high cell count during the exponential phase. Rowe *et al*. (2003) also reported a similar situation with high OUR value that was achieved during inoculation and throughout the exponential growth phase of batch cultivation.

Low respiratory quotient (RQ) values were also reported during the two-phase DOT control strategy. The low RQ values in the cultures contribute to higher sporulation and δ-endotoxin production. RQ value is particularly useful for understanding cell metabolism. It can be defined as the ratio of the number of CO_2 molecules produced by an organism to the number of O_2 molecules consumed. It means that cell metabolism can be compared at different conditions by referring to the RQ value. Detail study on the relationship of RQ value with the results from this study indicated that the RQ value for the cultivation where the DOT was controlled at 80% and 60% was higher than the values recorded in cultivation where the DOT was controlled at a low level (40% saturation) during the initial active growth phase. The evaluation of exhaust gas during the cultivation enables the calculation of several parameters (OUR, CER, and RQ), which can be used for on-line monitoring of the *B. thuringiensis* cultivation performance. However, for this study, a further study on mechanistic (physiological and metabolic) details should be carried out and analyzed for δ-endotoxin production at lower DOT levels.

CONCLUSION

Results from this study demonstrated that cell growth, sporulation rate, and δ-endotoxin synthesis of *B. thuringiensis* were greatly influenced by different DOT control strategies during the cultivation. The optimal DOT control strategy for enhancement of sporulation (up to 61%) and δ-endotoxin synthesis recorded as early as 8 hr of cultivation was obtained in the two-phase DOT strategy with 80% saturation and then switched to 40% after 6 hr. On the contrary, δ-endotoxin in single-phase DOT control at 80% saturation was recorded only after 48 hr of the cultivation, and disrupt the *B. thuringiensis* sporulation potential. It is essential to achieve a maximum specific growth rate, μ_{max} values exceeded 0.1 h^{-1} for constant δ-endotoxin production during *B. thuringiensis* MPK13 cultivation. Results from this study also demonstrated the potential of exhaust gas analysis that could be used for quick monitoring of *B. thuringiensis* cultivation performance for the production of spores with high bioinsecticidal activity.

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