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GROWTH AND SPORULATION OF *BACILLUS THURINGIENSIS* SUBSP. *AIZAWAI* SN2 IN A TWO-STAGE CONTINUOUS CULTURE

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RINGKASAN: *Kajian terhadap pembiakan dan pensporaan Bacillus thuringiensis di dalam sistem kultur selanjar dua tahap dengan medium tertakrif pada tahap mantap telah dijalankan. Kesan nisbah kadar pencairan (D1/D2) di dalam reaktor 1 (K1) dan reaktor 2 (K2) serta kesan pH awal di dalam K2 yang memberikan nilai bilangan sel hidup viabel (TVCC) tertinggi dalam K1 dan nilai peratus spora tertinggi dalam K2 telah ditentukan. Data yang diperolehi menunjukkan peratus spora di dalam K2 adalah lebih tinggi daripada K1 manakala bilangan sel hidup viabel (TVCC) di dalam K2 pula adalah kurang daripada di dalam K1. Keadaan operasi seperti yang berikut iaitu pada kadar alir = 0.45 h^{-1} dan nisbah kadar alir D1/D2 = 4 memberikan nilai tertinggi untuk bilangan sel hidup viabel iaitu sebanyak 32x10^9 CFU/ml di dalam K1 dan peratus spora sebanyak 50% di dalam K2. Kesan pH (5.5 - 8.5) di dalam K2 ke atas peratus spora dan bilangan spora (SC) juga turut dilaporkan. Daripada data yang diperolehi, didapati medium dengan pH awal 7.5 memberikan nilai peratus spora tertinggi iaitu sebanyak 65%. Nilai ini dicapai dengan nilai TVCC sebanyak 6x10^9 CFU/ml dan SC sebanyak 4x10^9 CFU/ml. Sementara itu di dalam eksperimen yang lain, nilai peratus spora tertinggi yang dicatatkan di dalam K1 adalah hanya sebanyak 46%, dengan TVCC sebanyak 24x10^9 CFU dan SC sebanyak 11x10^9 CFU/ml. Hasil daripada kajian kami menyokong bahawa sistem selanjar dua tahap boleh dioperasikan dengan mudah pada tahap mantap untuk mengoptimumkan pembiakan di dalam reaktor pertama dan kemudian mengoptimumkan pensporaan di dalam reaktor yang kedua.*

ABSTRACT: Growth and sporulation of *Bacillus thuringiensis* in a two-stage continuous culture at steady-state conditions using a defined medium was investigated. The effect of dilution rate ratio (D1/D2) in reactor 1 (K1) and reactor 2 (K2) and initial pH in K2 which gives the highest value for total viable cell count (TVCC) in K1 and spore percentage in K2 was ascertained. Result showed that spore percentage in K2 was higher than in K1 whereas the total viable cell count in K2 was lower than in K1. The following operating conditions, namely, dilution rate = 0.45 h^{-1} and the ratio of dilution rate D1/D2= 4 gave the highest value for total viable cell count (TVCC) of 32x10^9 CFU/ml in K1 and a spore percentage of 50% in K2. The effect of pH (5.5 - 8.5) in K2 on spore percentage, TVCC and spore count (SC) are also reported. Our results showed that medium in K2 with initial pH 7.5 gave the highest spore percentage of 65%. This was achieved with TVCC of 6x10^9 CFU/ml and spore count of 4x10^9 CFU/ml. Whilst in another experiment, the highest spore percentage recorded in K1 was only 46%, with TVCC of 24x10^9 CFU/ml and spore count of 11x10^9 CFU/ml. Our result supports the idea that a two-stage continuous culture could be easily operated at steady-state conditions to optimize growth in the first reactor and then to optimize sporulation in the second reactor.

KEYWORDS: *Bioinsecticide, B. thuringiensis,* two-stage continuous culture.

INTRODUCTION

Microbial insecticides are used to control pests in agriculture and forestry as an alternative to chemical insecticide (Khachatourians, 1986; Kang *et al.*, 1993). *Bacillus thuringiensis* is the most widely employed bioinsecticide (Feitelson *et al.*, 1992); hence, the importance of *B. thuringiensis* production in many undeveloped and developing countries (Jason *et al.*, 1984). Commercial production of *B. thuringiensis* spores usually employs batch culture. However, Dawes & Mandelstam (1970) proposed that continuous culture was better than batch culture in producing *B. thuringiensis.* Theoretical considerations (Pirt, 1974) also support the idea of enhanced production of biomass in continuous culture. Dawson (1972) further strengthens the idea in his work on continuous culture and reported an increase in the production of *B. thuringiensis.* Other researchers (Khovreychev *et al.*, 1986, 1990; Kang *et al.*, 1993) have also reported enhanced production of *B. thuringiensis* in a two-stage continuous culture. In this article, we report the effect of dilution rate and pH in the production of *Bacillus thuringiensis* using a two-stage continuous culture.

MATERIALS AND METHODS

Organism and Inoculum Medium

A locally isolated strain, *Bacillus thuringiensis* subsp. *aizawai* SN2, was used in this study. The bacterium was grown at 30°C and maintained on nutrient agar (NA: SIGMA) at 4°C. For inoculum medium, a modified glucose-yeast extract-salt (GYS) medium was used (Bechtel *et al.*, 1975). The GYS medium consisted of (w/v) $(NH_4)_2$ SO_4, 0.2%; yeast extract, 0.2%; K_2HPO_4, 0.05%; glucose, 2%; $CaCl_2.H_2O$, 0.8%; $MgSO_4.7H_2O$, 2% and $MnSO_4.H_2O$, 0.5%.

Cultivation and Cultivation Medium

A loopful of *B. thuringiensis* subsp. *aizawai* SN2 was used to inoculate 100ml of GYS medium in 250 ml Erlenmeyer flasks. The flasks were placed on an orbital shaker at 200 rpm and incubated at 30±2°C for 14 hours. After the cultivation, 10 ml of cells were transferred into a 500 ml Erlenmeyer flask containing 100ml of cultivation medium with the following composition (w/v): soluble starch, 2%; glutamate, 0.02%; hydrolysed vegetable protein (HVP) obtained from Ajinomoto (M) Sdn. Bhd., 0.5%; $MnCl_2.4H_2O$, 0.005%; $CaCl_2.2H_2O$, 0.0008%; $ZnSO_4.7H_2O$, 0.0005%; $CuSO_4.5H_2O$, 0.0005% and $MgSO_4.7H_2O$, 0.2%. Cultivation of the bacterium was carried out in a shaking incubator overnight at 200 rpm, 30°C (Khovreychev *et al.*, 1989). The pH of the medium was adjusted to 7.2 with sterile 1M NaOH.

Two-stage continuous culture operation

10 ml of culture from the inoculum medium (SC= $5x10^8$ spores/ml) were transferred into the first fermentor (K1) in each set (A & B) of experiment before starting the two-stage continuous operation. The system consisted of two cylindrical glass vessels with volume of 500 ml for K1 and 1000 ml for K2. There are four glass tubes connected via rubber stopper to each vessel for aeration (input and output), medium supply and product line. Silicon rubber tubes with inside diameter of 0.4 cm were used to connect K1 and K2. Foaming was prevented by the addition of 1.0 ml olive oil per 100 ml culture medium. Magnetic stirrers (4.5cm length) were used for agitation in K1 and K2. Aeration in K1 and K2 was set at 3 v/v/m and 0.75 v/v/m respectively. Operation temperature was kept at $25\pm2^{o}C$ for K1 and $35\pm2^{o}C$ for K2. Temperature in K2 was set at $35\pm2^{o}C$ in order to provide a suitable condition for *B. thuringiensis* sporulation. Sampling and pH measurements were carried out at 12 ± 2 hour interval 48 ± 2 hour after inoculation of batch culture. Fresh medium supply and harvesting of product were carried out using three peristaltic pumps (Watson-Marlow).

Set A experiment

In set A experiment, the effect of dilution rate was investigated by varying the flow rate (F) as follows; 0.42, 0.50, 0.75 and 0.90 ml/min. The working volumes of the first and second reactors were 100 ml and 400 ml, respectively. The dilution rate ratio was kept constant at D1/D2= 4. The dilution rate in reactor 1 (D1) or in reactor 2 (D2) was defined as the rate of cell bleeding (F) divided by the volume of the reactor.

Set B experiment

In this experiment, comparison between dilution rate ratio D1/D2= 4 and D1/D2= 2 was the main focus. In the first experiment (D1/D2=4), the working volume in K1 and K2 was 100 ml and 400 ml respectively. In this experiment the flow rate (F) was kept constant at 0.75 ml/min whilst the working volume in K2 was decreased to 200 ml and the working volume in K1 remains at 100 ml.

Set C experiment

Experiment C was carried out to study the effect of initial pH in the second reactor (K2) on growth and sporulation of *B. thuringiensis*. Working volumes of K1 and K2 was set at 100 ml and 500 ml respectively (D1/D2=5). The flow rate (F) of feed to both fermentors was kept constant at 0.75 ml/min. The effect of the following pH: 5.5, 6.5, 7.5 and 8.5 were elucidated in this study. Other environmental factors such as agitation, aeration and temperature were the same as in set A and B experiments.

Total viable cell count and spore count

Total viable cell count and spore count were determined every 12 hours (after 24 hours continuous culture) by spread plate method (Perry *et al.*, 1997). Serial dilution of the samples were made in sterile 0.85% NaCl (physiological saline). Plates were incubated at 30°C for 24 to 48 h. Plates with 30 to 300 colonies were selected to count the colony. For spore count, cultures were heated at 65°C for 20 min before serial dilutions were made.

RESULTS AND DISCUSSION

Set A experiment: The effect of dilution rate on total viable cell count and spore percentage in a two-stage continuous culture (ratio D1/D2= 4)

The results obtained (total viable cell count, spore count and spore percentage at steady-state conditions after 120 hours) are shown in Table 1. In the first stage, the highest spore percentage (25%) was at D1= 0.45h^{-1} while the lowest spore percentage (18%) was at D1= 0.54h^{-1}. The highest spore percentage (50%) was obtained in the second reactor at D2= 0.11h^{-1} and the lowest (28%) was obtained at D2= 0.14h^{-1}. Data shows that a small change in D2 (i.e. specific growth rate in K2 at steady-state) from 0.14h^{-1} to 0.11h^{-1} corresponds to an increase in spore percentage from 28% to 50%. In the first reactor (K1) only 25% maximum value for spore percentage was recorded. The highest TVCC at steady-state in the first reactor (K1) was 37x10^9 CFU/ml (D1= 0.30h^{-1}) and the lowest TVCC was 23x10^9 CFU/ml (D1=0.54h^{-1}). The highest TVCC at steady-state in the second reactor (K2) was 27x10^9 CFU/ml (D2=0.75h^{-1}) and the lowest was 18x10^9 CFU/ml (D2= 0.14h^{-1}). The effect of dilution rate on *B. thuringiensis* growth and sporulation are shown in Figure 1 and Figure 2.

Table 1. Total viable cell count, spore count, spore percentage and productivity at steady-state (D1/D2= 4) at different flow rate

Exp.	F (ml/min)	D (h^{-1})	Retention time (h)	TVCC (CFU/ml)	SC (spores/ml)	Spore percentage (%)	Productivity (CFU $ml^{-1}h^{-1}$)
1	0.42	D1/D2= 4					
		D1= 0.250	4.0	36X10^9	7x10^9	19%	30x10^8
		D2= 0.063	15.9	26x10^9	8x10^9	32%	5x10^8
2	0.5	D1/D2= 4					
		D1= 0.300	3.3	37x10^9	8x10^9	20%	38x10^8
		D2= 0.075	13.3	27x10^9	9x10^9	33%	7x10^8
3	0.75	D1/D2= 4					
		D1= 0.450	2.2	32x10^9	8x10^9	25%	48x10^8
		D2=0.113	8.8	20x10^9	10x10^9	50%	8x10^8
4	0.90	D1/D2= 4					
		D1= 0.540	1.9	23x10^9	4x10^9	18%	41x10^8
		D2= 0.135	7.4	18x10^9	5x10^9	28%	8x10^8

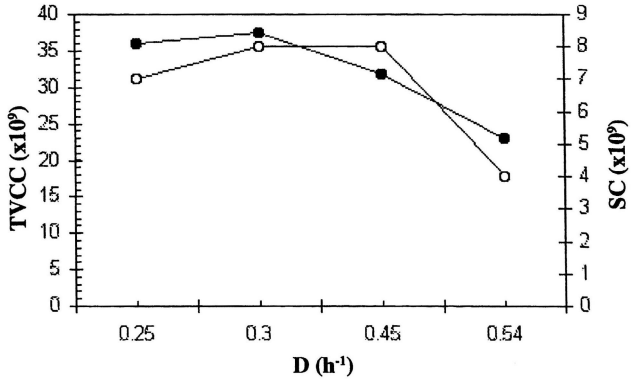

Figure 1. *Effect of dilution rate on total viable cell count (TVCC) and spore count (SC) in K1.*
Symbols: TVCC (•), *SC* (o).

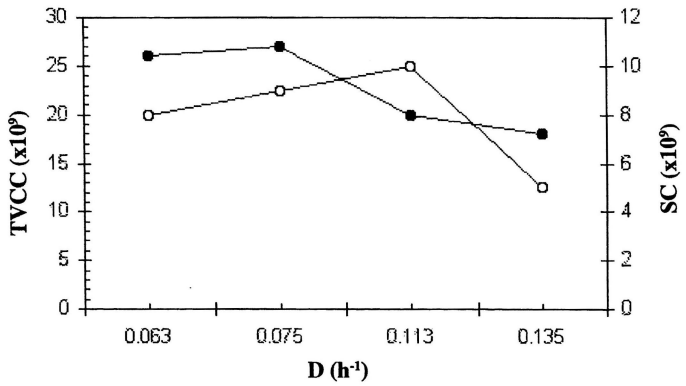

Figure 2. *Effect of dilution rate on total viable cell count (TVCC) and spore count (SC) in K2.*
Symbols: TVCC (•), *SC* (o).

The total viable cell count in K1 was higher than in K2 might be due to the fact that the retention time in K2 was higher (four times) than in K1 (Table 1). This supports the idea that a two-stage continuous culture is easy to operate for optimizing cell cultivation and sporulation separately. It can be seen that pH in K2 was slightly higher than in K1 (Figure 3) suggesting that the condition in K2 can be established to enhance sporulation as compared to the condition in K1. A decrease in retention time in K2 corresponds to an increase in spore percentage up to a certain critical time (8.8h) below which spore percentage decreases (7.4h). Similar relationship was also observed in K1. In all sets of experiments, the steady-state was detected after 120 hours of cultivation. The steady-state was maintained for 60 hours until the experiments were terminated at 180 hours.

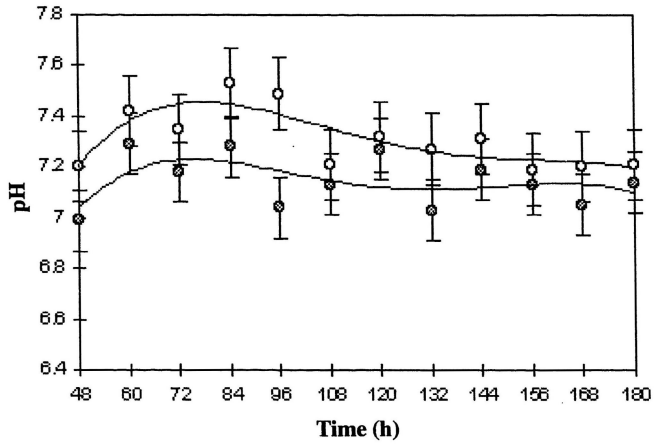

Figure 3. *pH profile at F= 0.50 ml/min (D1/D2=4). Symbols: reactor 1 (K1)* (•), *reactor 2 (K2)* (o).

Set B experiment: Effect of dilution rate ratio comparison (D1/D2) on Bacillus thuringiensis spore percentage (F=0.75 ml/min)

From Table 2 (D1/D2= 2), total viable cell count in the first reactor (K1) was higher than in the second reactor (K2). However, spore percentage in the first reactor (K1) was lower than in the second reactor (K2). The results obtained are similar to set A experiment. However, there should be an increase in spore concentration from K1 to K2 because of temperature differences from 25°C to 35°C. We found that at D1/D2= 2, spore concentration in the second reactor (K2) was $7x10^9$ spores/ml. However at D1/D2= 4, spore concentration in K2 was $10x10^9$ spores/ml. A possible explanation for this is that the retention time in the second reactor for D1/D2= 4 (8.8 hours) was longer than D1/D2= 2 (4.4 hours). Therefore, the time for maximum sporulation cannot be achieved (not enough time for sporulation) in D1/D2=2.

Table 2. TVCC, spore count, spore percentage and productivity at steady-state condition (D1/D2= 2)

F (ml/min)	D (h^{-1})	Retention time (h)	TVCC (CFU/ml)	SC (spores/ml)	Spore percentage (%)	Productivity (CFU $ml^{-1}h^{-1}$)
0.75	D1/D2=2					
	D1= 0.451	2.2	$30x10^9$	$8x10^9$	26%	$48x10^8$
	D2= 0.225	4.4	$16x10^9$	$7x10^9$	40%	$12x10^8$
0.75	D1/D2=4					
	D1=0.450	2.2	$32x10^9$	$8x10^9$	25%	$48x10^8$
	D2=0.113	8.8	$20x10^9$	$10x10^9$	50%	$8x10^8$

Set C experiment: Effect of pH in the second bioreactor (K2)

The results obtained from set C experiment are shown in Table 3. The initial pH of 7.5 in the second reactor (K2) was found to be the most suitable for spore production. The highest spore percentage recorded at this pH was 65%. Sporulation was detected during the early eight hours of operation. Meanwhile at initial pH 6.5 and 5.5, there were not much differences in spore percentage between these two pH. Spore percentage at initial pH 6.5 and 5.5 was 54% and 51% respectively. The acidic environment in K2 may affect *Bacillus thuringiensis* vegetative phase and also sporulation because sporulation only occurs after vegetative phase (Luthy, 1982). Initial pH 8.5 in K2 was found to be the most unsuitable pH for sporulation where the spore percentage was only 33%. It can be seen that a small increase in alkaline environment in K2 significantly decreased sporulation as compared to acidic environment.

Table 3: The effect of pH on total viable cell count, spore percentage at steady-state condition in K2

Exp	pH	TVCC ($x10^9$)	SC ($x10^9$)	Spore percentage (%)
1	8.5	6.2	2.2	33
2	7.5	5.5	3.7	65
3	6.5	3.4	1.9	54
4	5.5	2.8	1.4	51

Two-stage continuous culture system of *B. thuringiensis* subsp. *azawai* SN2 under certain conditions are able to support high cell mass as well as high degree of sporulation. The first stage can be easily optimized to increase cell mass and the second stage can be optimized to increase spore percentage.

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