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A Simple Technique to Enhance the Productivity of δ-endotoxin in Batch Culture

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Abstract

A technique to enhance productivity of *Bacillus thuringiensis* subsp. *kurstaki* SN5 based on inoculum size and productivity data assessment was established, using medium containing 2% GYS in a 2L bioreactor. Results show that a 10% v/v inoculum gave the highest productivity of 0.042 mg/ml/h at 12h compared with a 15% and 20% v/v inoculum that gave 0.022 and 0.013 mg/ml/h at 36h and 48h, respectively. When a 10% v/v inoculum was employed, spore percentage, at 12h was recorded at 35.9% with the highest concentration of δ-endotoxin of 0.50 mg/ml. The results showed that inoculum size have a significant effect on spore percentage, productivity of δ-endotoxin and harvesting time and lends the simple technique to enhance δ-endotoxin productivity. All yield data are the highest in the culture from a 10% v/v inoculum size.

Keywords: B. thuringiensis, productivity of δ-endotoxin and inoculum size

INTRODUCTION

Bacillus thuringiensis (Bt.) is a gram-positive spore forming bacterium and useful to man by its production of insecticidal crystal protein, evaluated as δ-endotoxin for biocontrol purposes. Once the bacteria are ingested, the spore and crystal released resulted in the degradation of the stomach lining of the insect pest. Currently, the practice to monitor for effective fermentation is when spore percentage is high; concentration of δ-endotoxin is assumed high. This may be due to no data for concentration of δ-endotoxin. This then led to the harvest time to be in the stationary phase, which is usually more than 48h. As large quantities of cells with high concentration of δ-endotoxin are required for commercial applications, the development and management of a simple technique capitalizing on existing technology that does not involve strain improvement; expensive bioreactor design and medium design are timely. The size of inoculum was reported as directly influences the duration of lag phase, biomass yield, sporulation and productivity (Sen and Swaminathan, 2004). The length of the lag phase and therefore productivity is also affected by the size of inoculum (Peter *et al*, 1995).

Other factor affecting the production of *B. thuringiensis* is oxygen concentration, which effect to δ-endotoxin concentration (Sachdeva *et al*, 1999). Its production will be low when oxygen supply is limited.

This paper evaluated the significant effect of inoculum size on productivity of δ-endotoxin in relation to harvest time.

MATERIALS AND METHODS

Microorganisms

B. thuringiensis subsp. *kurstaki* SN5 used in this study was obtained from the School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor. The strain was maintained on nutrient agar (NA) (Oxoid) slant.

Inoculum (14h vegetative cell) preparation

A loop full of *B. thuringiensis* subsp. *kurstaki* SN5 was transferred to a 500 ml Erlenmeyer flask containing 100 ml of GYS (Glucose-Yeast-Salt) medium. The GYS medium consisted of (w/v) $(NH_4)_2\ SO_4$ (Merck), 0.2%; yeast extract (Oxoid), 0.2%; K_2HPO_4 (Merck), 0.05%; glucose (Scharlau), 2%; $CaCl_2.2H_2O$ (Fisher Chemicals), 0.8%; $MgSO_4.7H_2O$ (Merck), 2% and $MnSO_4.H_2O$ (Univar), 0.5%. The flask was incubated using orbital shaker at 150 rpm and 30°C for 14 hours and used for inoculum (Wan Mohtar *et al*. 2002).

Batch cultivation with different inoculum percentage

Batch Cultivation: 10%, 15% and 20% inoculum were pipetted into a 2L bioreactor (Quick Fit, England) containing 1liter growth medium. Composition of the growth medium are as follows: glucose, 1.0 % (w/v); L-glutamate (Sigma), 0.02 %; HVP (hydrolyzed vegetable protein), 0.5%; $MnCl_2.4H_2O$, 0.005 %; $CaCl_2.2H_2O$, 0.0008 %; $ZnSO_4.7H_2O$ (Bendosen), 0.0005 %; $CuSO_4.5H_2O$ (Analar), 0.0005% and $MgSO_4.7H_2O$, 0.2%. The pH was adjusted to 7.2 with sterile 1M NaOH (Khovrychev *et al*., 1987). The culture was then incubated for 120 hours at agitation of 250 rpm, temperature of 30°C and aeration of 1v/v/m. HVP is a high protein substrate made from soybean and purchased from Ajinomoto Company Kuala Lumpur, Malaysia.

Sampling: Samples (10 ml) are taken every 12 hours starting from the beginning of fermentation (0 hr).

Analysis of sample

Cell growth and spore production were determined by total viable cell count (TVCC) and spore count (SC) respectively. Samples for spore count (SC) were heated at 70°C for 10 minutes. Samples at appropriate dilutions with physiological solution (0.85% NaCl) for estimation of TVCC and SC were plated on nutrient agar by the spread plate method. The plates were then incubated in an incubator at 30°C for 24h. The colonies counted per plate were between 30 and 300. Spores percentages were calculated as follows:

$$\textbf{Spores percentage} = SC/TVCC \times 100$$

δ-endotoxin assay

δ-endotoxin concentration was determined using the Bio-Rad Bradford reagent (Munich, Germany). 1 ml of each sample was centrifuged for 10 min at 1000 x g, the pellet was washed twice with 1M NaCl and twice with distilled water. The pellet was then suspended in 200 µl of 50 mM NaOH (pH 12.5) in order to solubilise the δ-endotoxin. After incubation for 3 hours at 30 °C, the total protein in the supernatant was measured by Bio-Rad reagent and absorbance spectrophotometer at 595 nm. (Zouri *et al*, 1999).

Calculation productivity of δ-endotoxin

δ-endotoxin productivity in batch culture (mg/ml/h) was calculated using (Naritomi *et al*, 2002)

RESULTS AND DISCUSSION

Effect of inoculum size on TVCC, SC and Spore percentage

Results on growth measured as TVCC, SC and Spore percentage with different inoculum size are presented in Figures 1, 2 and 3 respectively. At 12 and 24 hrs, the 20% v/v inoculum gave the highest (11.6 and 16.4×10^8 cell/ml) TVCC whilst the lowest (3.8 and 4.9×10^8 cell/ml) TVCC was obtained using 10% v/v inoculum. The corresponding SC at 12 and 24 hrs was also lowest (1.4 and 2.7×10^8 spore/ml) when the 10% v/v inoculum was used. But the highest of spore percentage for all inoculum (10%,15% and 20% v/v inoculum that gave 77.78% at 48 hrs, 77.41% at 36h and 74.40% at 48h, respectively) was not significant different and the lowest spore percentage was when a 20% inoculum was used.

Our results indicates that based on spore percentage, concentration of δ-endotoxin could be highest using a 15% inoculum (0.777 mg/ml) compared to a 10% inoculum (0.658 mg/ml). High initial cell concentration (when 20% v/v inoculum was used) in the production medium may result in a rapid consumption of oxygen and other nutrients resulting in the limitation of dissolved oxygen and nutrients (Lachhab *et al*, 2001) may occur too early in the exponential phase. This resulted in high specific growth rate (µ) and may also result in low concentration of δ-endotoxin (0.607 mg/ml) when 20% v/v inoculum was used**.** On the other hand, with a low initial cell concentration (10% v/v inoculum) there was no rapid consumption of oxygen in the early exponential phase indicated by the gradient of the growth curve, representing µ**.** This is evident from figure 1 that µ for a 10% inoculum is lowest (0.1288 h^{-1}) compare to 15% and 20% inoculum (0.3598 and 0.4027 h^{-1}). This further indicates that a 10% v/v inoculum could be best suited for the synthesis of δ-endotoxin. This idea is further discussed using Table1 in the next section.

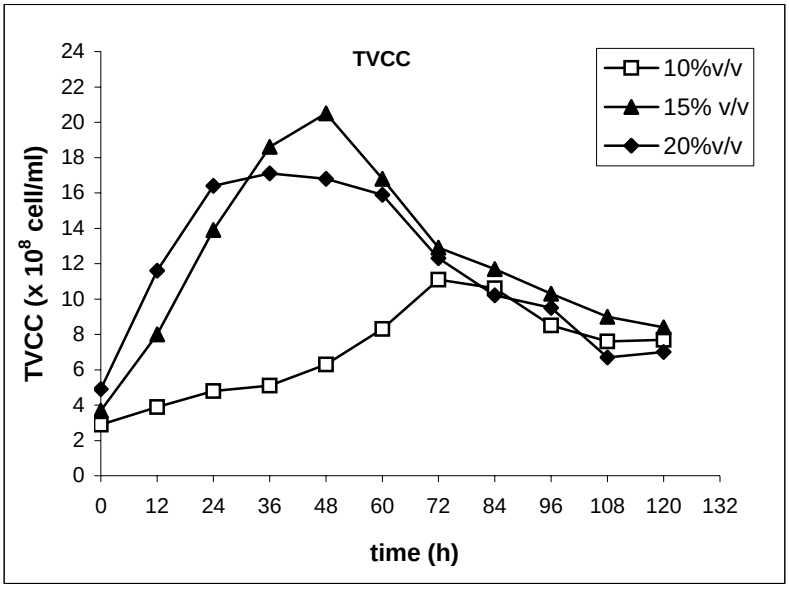

Figure 1. Variation of total variable cell count (TVCC) of *B. thuringiensis* subsp. *kurstaki* SN5 in batch culture with different inoculum sizes (v/v).

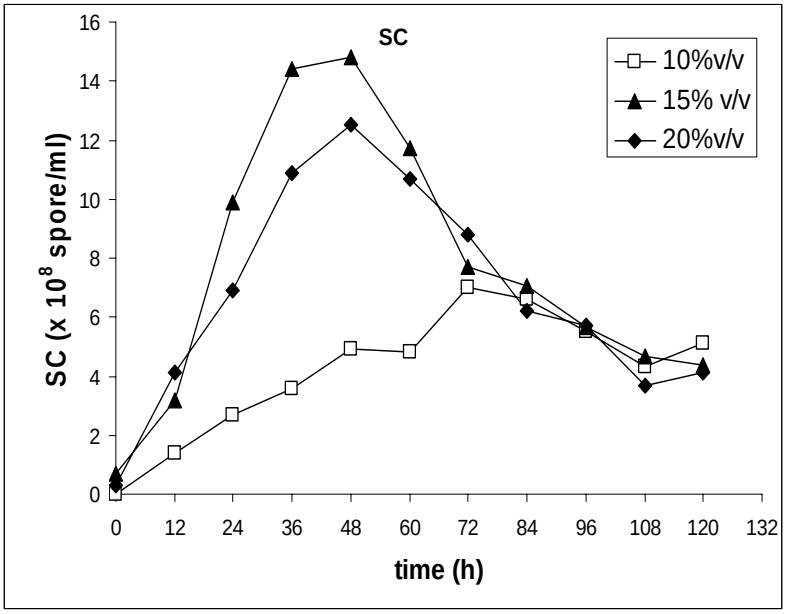

Figure 2. Variation of spore count (SC) of *B. thuringiensis* subsp. *kurstaki* SN5 in batch culture with different inoculum sizes (v/v).

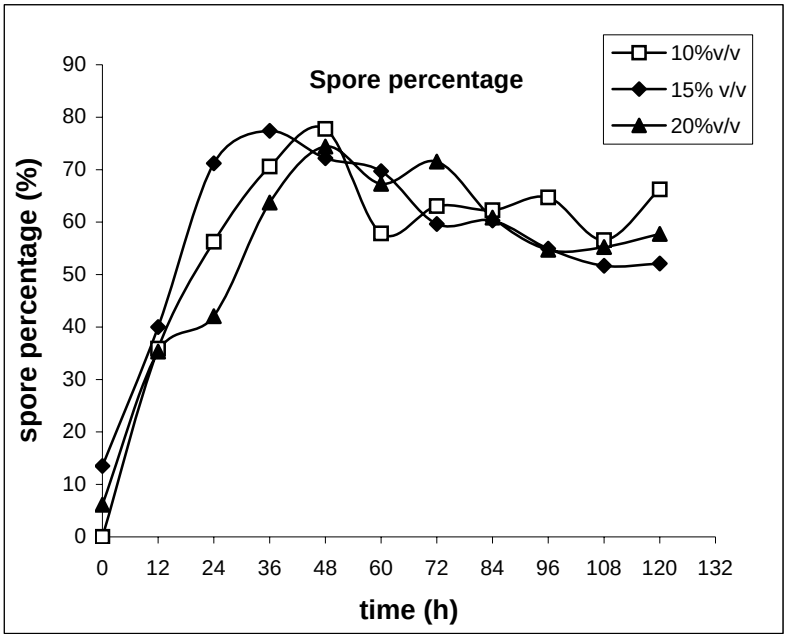

Figure 3. Profiles of spore percentage of *B. thuringiensis* subsp. *kurstaki* SN5 in batch culture with different inoculum sizes (v/v).

Effect of inoculum size on time-profile concentration and productivity of δ-endotoxin

Table 1 shows that the highest concentration of δ-endotoxin was recorded when 15% v/v inoculum was 0.78 mg/ml at 36h with productivity 0.022 mg/ml/h. On the other hand, when based on productivity of δ-endotoxin, a 10% v/v inoculum gave a doubling in productivity (0.042 mg/ml/h) and concentration of δ-endotoxin at 0.500 mg/ml at 12h of fermentation. When compared at the same fermentation time (12h), the 15% and 20% v/v inoculums gave the concentration of δ-endotoxin at 0.183 and 0.115 mg/ml and productivity at 0.007 and 0.001 mg/ml/h respectively. It seems that the synthesis of δ-endotoxin was greater at 12h using the 10% v/v inoculum at the μ corresponds to μ at stationary growth phase. Therefore, we focused on fermentation at low μ in the earliest possible time.

Several reports (Zouari *et al*. 2002), states their focuses the occurrence of δ-endotoxin is at the late stage of growth. Our results show that the occurrence of δ-endotoxin seems to be growth dependent very early in the batch process and is effect by inoculum sizes employed. This lends us a means to establish for the most suitable inoculum size that gave the highest productivity fastest during the fermentation. This is supported by Bech *et al*, 1976 reported that sporulation and crystal protein were completed at 12h of fermentation.

Table 1 Effect of size inoculum on concentration and productivity of δ-endotoxin

Time of fermentation (h)	10% v/v		15% v/v		20%v/v	
	(mg/ml)	(mg/ml/h)	(mg/ml)	(mg/ml/h)	(mg/ml)	(mg/ml/h)
0	0.025	0.002	0.051	0.001	0.080	0.001
12	0.500	**0.042**	0.183	0.007	0.115	0.001
24	0.602	0.025	0.525	**0.022**	0.233	0.001
36	0.613	0.017	**0.777**	0.022	0.374	0.001
48	**0.658**	0.014	0.667	0.015	**0.607**	**0.013**
60	0.583	0.010	0.468	0.008	0.396	0.007
72	0.595	0.010	0.326	0.005	0.522	0.007
84	0.312	0.004	0.402	0.005	0.311	0.004
96	0.332	0.003	0.257	0.003	0.106	0.001
108	0.473	0.004	0.219	0.002	0.204	0.002
120	0.503	0.004	0.194	0.002	0.241	0.002

Effect of inoculum size on yield (Y p/x)

Table 2 showed that a 10% v/v inoculum size gave the highest yield, 0.172 mg/ $x10^8$ cell at 12 hrs compared with the 15% and 20% v/v inoculum size of just 0.042 and 0.036 mg/ $x10^8$ cell at 36 h and 48 h, respectively. Every samples of 10% v/v inoculum size gave higher yield compared with 15% and 20% v/v size inoculum. One of the main problems in high-density fermentation is that the accumulation of some of the metabolites represses the growth of cells or the formation of desired product (Yang X.M. & Wang S.S., 1998. A 10% v/v inoculum may result in low accumulation of metabolite for cell growth but it improved δ-endotoxin synthesis (Table 1) and led to the highest on yield (Table 2).

Table 2 Value of Y p/x

Time of fermentation (hr)	10%	15%	20%
	(mg/$x10^8$ cell)		
0	0.009	0.040	0.002
12	**0.172**	0.010	0.001
24	0.125	0.038	0.002
36	0.120	**0.042**	0.022
48	0.100	0.033	**0.036**
60	0.070	0.028	0.025
72	0.054	0.025	0.042
84	0.030	0.034	0.030
96	0.040	0.025	0.011
108	0.060	0.024	0.030
120	0.070	0.023	0.030

CONCLUSIONS

A 10% v/v inoculum gave the highest productivity of 0.042 mg/ml/h with the shortest harvest time of 12 h.

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