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Bioprocess development for enhanced spore production in shake flask and pilot scale bioreactors of *Bacillus thuringiensis* var. *israelensis* in submerged culture

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Abstract: *Bacillus thuringiensis subsp. Israelensis (Bti), has proven to be a safe and effective larvicide for controlling mosquito and black fly larvae. The effect of cultivation and bioprocess development on Bti growth and sporulation was investigated in shake flask level and batch cultivation in the semi-industrial scale 16-L stirred tank bioreactor. For industrial production of biocontrol microorganism, it is necessary to obtain high cell mass and spore production in a short time with low cost cultivation media. In this study, the new composition of production media was optimized which composed of (g L^{-1}): glucose, 10; yeast extract, 30; KH_2PO_4, 5; K_2HPO_4, 5; $MgSO_4$. $7H_20$, 0.005; $MnSO_4.H_2O$, 0.03; $FeSO_4$, 0.01; $CaCl_2.7\ H_2O$, 0.05; NaH_2PO_4, 1.5; $NH_4H_2PO_4$, 1.5. The maximal cell dry mass and spore production, $Spore_{max}$ for shake flask study were 4.26 gL^{-1} at 36 h and 3.29×10^6 spore mL^{-1}, respectively. Furthermore, studies of the cultivation conditions under controlled and uncontrolled pH in the 16L-bioreactor was performed. The growth of Bti under uncontrolled pH cultivation showing decreased of glucose and total protein concentration in the media was correlated with the vegetative cell growth and sporulation. The maximal cell dry mass and $Spore_{max}$ for uncontrolled pH bioreactor were 4.14 gL^{-1} at 36h and 3.7×10^6 spore mL^{-1}, respectively. The maximal cell dry mass and spore production, $Spore_{max}$ for controlled pH bioreactor were 3.36 gL^{-1} at 26 h and 3.23×10^6 spore mL^{-1}, respectively. In conclusion, batch cultivation in 16-L bioreactor with the new optimized production medium under uncontrolled pH condition increased of the cell dry mass and number of spores up to 23 % and 47 % , respectively.*

Keywords: *Bacillus thuringiensis var israelensis, Spore production, Shake flask, Batch culture, Bioreactor*

I. Introduction

Major concern around the worldwide against utilization of chemical pesticides which give harmful effects to the human and environment. Nowadays, increasingly utilizing of biological pesticides as a safe and alternative for controlling agricultural catastrophe and human disease vectors [1]. *Bacillus thuringiensis* var *israelensis* (Bti) is used as a natural agent for biological control. Bti as an anaerobic and gram positive sporulating bacterium can produced parasporal insecticidal protein (d-endotoxin) to against mosquito, black fly larvae and fungus gnats. Its famously used as a bioinsecticide to control mosquito populations around the world [2,3]. It was first discovered at Israel in year 1976 and can produce a toxic substance which is crystal that fatal when ingested by the larvae [3]. During the stage of spores formation, Bti can produce some delta-endotoxins which is responsible for the insecticidal activity [3]. Bti have a big potential effect to the three insect species such as Lepidoptera (Butterflies, Moths), Diptera (mosquitoes, biting flies), and Coleoptera (Beetles) [2,4]. Several studies have been conducted to the development of the culture conditions to improve the spores and or crystal protein production [4-9]. Thus, this bacterium became one of the main components for biological control in good agriculture practice and to promote the production of healthy and safe food [10]. Microbial productivity can be improved by doing research on the various parameters of nutritional, bioprocessing development and development of strain itself. Moreover, the nutritional requirement for *Bacillus thuriengiensis* requires economic production medium for large scale production. Furthermore, development of a cost effective medium for production high biomass and optimization cultivation parameters such as pH to grow in large scale bioreactors was successfully develop by several authors to reduce accumulation of byproducts and improving process yields towards reducing production costs [11-14]. At the cellular level, each stage of sporulation, germination and vegetative growth phase can be considered as multi- processes which is different in their nature and their special needs. As reported by Cromwick *et al.* [15], it is important to controlled pH at 6.5 for production of gamma-PGA by *Bacillus licheniformis*. In the present study, *Bacillus thuriengiensis* var. *israelensis* was used as the potential biological control microorganism. Bioprocess development for cultivations process in the new production media were conducted in submerged cultures to study the effects

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of controlled and uncontrolled pH on growth kinetics and spore production of *Bacillus thuriengiensis* var. *israelensis* in semi-industrial scale 16L-bioreactor. The objective is to determine the best pH conditions whether uncontrolled or controlled at pH 7 for the cell growth and sporulation of Bti. It is desirable to understand microbial fermentation processes in order to control them at their optimal conditions. This has been proved from the previous studied successfully developed a process for the production of Bti under different aeration rate by using 16-L bioreactor [16].

II. Materials and methods

1. Microorganisms and maintenance of culture

Bacillus thuringiensis var *israelensis* (WICC- B108) was obtained from Wellness Industries Culture Collection (WICC), Institute of Bioproduct Development, UTM, Malaysia. This strain source originally from American Type Culture Collection (ATCC, University Boulevard Manassas, VA, USA). This strain was delivered in frozen glycerol. Cells were first propagated on Luria Bertani (LB) agar medium was adjusted to 7.0 before sterilization of the following compositions ($g\ L^{-1}$), Peptone, 10.0,Yeast extract, 5.0, NaCl, 5.0 and agar 20.0, incubated at 37 °C for 24 hours. The colonies formed were harvested by 50% glycerol solution and aspirated to be placed in series of 2 ml sterile cryovials tubes. These tubes were then immediately frozen at 4.0 °C for one day followed by further storage as working cell bank at -80 °C ultra-deep freezer for further utilization.

2. Inoculum preparation

For each experiment, 1 cryogenic vial from working cell bank (containing 1 ml) was taken and used to inoculate 250 ml Erlenmeyer flask containing 50 ml LB medium. The inoculated flasks were incubated on rotary shaker (Innova 4080, New Brunswick Scientific Co., New Jersey, USA.) at 200 rpm and 37 °C for 24 hours. The propagated cells were then either inoculated into the shake flask or bioreactor containing cell production medium

3. Medium for cell mass cultivation

The medium chosen for biomass and spore production was optimized in shake flask study. The compositions of the production media were as follows [gL^{-1}]: glucose, 10; yeast extract, 30; KH_2PO_4, 5; K_2HPO_4, 5; $MgSO_4$. $7H_20$, 0.005; $MnSO_4.H_2O$, 0.03; $FeSO_4$, 0.01; $CaCl_2.7\ H_2O$, 0.05; NaH_2PO_4, 1.5; $NH_4H_2PO_4$, 1.5. pH of medium was adjusted to 7.0 before sterilization. Medium used for spread plate was LB medium added with $15gL^{-1}$ of agar. The compositions of the fermentation medium in the 16-liter bioreactor (BioEngineering, Wald, Switzerland) were the same as in case of the flask study.

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4. Growth kinetics in submerged shake flask cultivation and bioreactor cultivations and process condition

For shake flask task, cultivations were carried out using 250 ml Erlenmeyer flask containing 50 ml LB liquid medium. After 24 hours incubation period, the culture was then used to inoculate the production medium. Later, the inoculated flasks were incubated at 37 °C on rotary shaker at 200rpm for 24 hours. With regards of bioreactor experiments, cultivations were executed in 16-L pilot scale in situ sterilizable bioreactor with working volume of 6-L. The stirrer of the bioreactor was equipped with two four bladded rushton turbines. The temperature and agitation speed were adjusted based on the required conditions (for pH, aeration and fed batch studies). For pH, under controlled conditions, the pH was maintained at 7 throughout the study. In contrast for uncontrolled pH conditions whereby pH was not controlled throughout the course of experiment. pH was measured on-line using in situ sterilizable pH electrode (Mittler Toledo, Switzerland) and controlled at a constant value of pH 7, hence pursued via the addition of either 4 M NaOH and 2 M HCl solutions, connected to acid/base controller equipped with peristaltic pump. Meanwhile for uncontrolled pH, it was not controlled throughout the course of experiment. Aeration was performed by sterile air and adjusted to rate of 1.0 vv-1min-1 using an integrated thermal mass flow controller. Dissolved oxygen concentration was first adjusted to 100% saturation before inoculation and measured using polarigraphic DO electrode (Mittler Toledo, Switzerland). Antifoam agent (Silicon antifoam, Sigma, USA) was added to suppress foaming, when necessary.

5.0 Analytical methods

5.1 Sample preparation and Cell dry weight determination

Samples in the form of 2 flask of 50 ml broth for each shake flask or 30 ml in case of bioreactor cultivation were taken at different time interval during cultivations. For immediate biomass determination, one ml of samples were aspirated and added to 9 ml distilled water, which is done in universal bottles. Dilution repeated until 1000 dilution. The optical density was measured by using spectrophotometer (DR/250, Hach C0., Loveland, CO., USA). Absorbance was taken for each dilution and its replicate at 600 nm. Dilution that portrays conformity in results is taken as results and cell dry weight is calculated based on the standard curve, one unit OD_{600} was equal to $0.3\ g\ L^{-1}$.

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5.2 Spore Determination- Malachite Green Staining

Using an inoculation loop, distilled water is placed on the slide, and then loop is flamed. After it is allowed to cool, some samples are taken up aseptically and mixed well with water. Samples are mixed well to avoid clumping of cells and to ease observation under microscope. Slide is then heat fixed and placed on water bath (beaker containing water placed on hot plate). Malachite green is smeared all over the smear. After 5 minutes, excess stain is removed and allowed to cool with stain for 3 minutes. Slide is rinse thoroughly to remove stain and is immediately covered with Safranine for 2 minutes. Stain is rinsed and blotted dry before observing under the microscope completed with digital color camera (DM2500 P, Leica, Germany) and connected with image processing and analysis software (Leica Qwin, Germany).

5.3 Spore Determination- Spore count.

From samples obtained, dilution is done from 10^{-1} to 10^{-6}. However, dilutions 10^{-3} to 10^{-6} are taken and placed in water bath at 80 °C for 10 minutes. Using sterile pipette tips, 0.1 ml is aspirated from each dilution and is withdrawn into respective Petri dish. Spreader is used and is spread evenly on the surface. Petri dish is incubated at 30 °C for 24 hours.

5.4 Total Protein Determination (Lowry Method)

The protein content of the crude enzyme source was determined by the method of Lowry [17] using bovine serum albumin as standard. 0.5 ml of sample and 2.5 ml Lowry C reagent were added and left for 10 minutes under room temperature. Later, 0.5 ml of diluted Folin reagent was added and mixed immediately. After 20 minutes, developed colour was measured using Spectrophotometer (DR/250, Hach C0., Loveland, CO., USA) at 750 nm.

5.5 Residual Glucose Determination (3,5-dinitrosalicylic acid, DNS Method)

1000µl of DNS solution was mixed to diluted samples, consisting of 100 µl samples diluted to 900 µl distilled water. The samples with replicates are place in water bath at 95 °C. After 15 minutes, samples were taken out and placed in container with tap water at room temperature. 8 ml of distilled water were added into each tube containing samples and vortex vigorously. Absorbance was neared at 540 nm using spectrophotometer. Glucose concentration was determined using the pre-prepared standard curve.

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III. Results and discussion

1. Cell growth kinetics in optimized media

B. thuringiensis var *israelensis* cultivation was executed using an optimized cultivation media and conditions in shake flask level to identify the cell dry weight, sporulation, pH, total protein and residual glucose. As displayed in Figure 1, initially, cells grew exponentially with growth rate of 0.313 $gL^{-1}h^{-1}$ and reached 3.20 g L^{-1} at hour 8. The specific growth rate is 0.250 h^{-1}. The robust exponential growth without significant lag phase was attributed by the presence of both glucose and nitrogen source in cultivating media [18]. However, it was reported that increasing the concentration of both carbon and nitrogen sources has resulted increased of the cell mass. As mentioned in Figure 1, Bti growth exponentially almost 8 hours and then the cells have entered the stationary phase. The highest cell dry weight of 4.62 g L^{-1} was obtained after 36 hours cultivation before entering the death phase.

The sporulation starts to increase after 12 hours and exhibit a steady increased until 52 hours of cultivation. This is because at the beginning of the fermentation process was characterized by a rapid period of adaptation of the cells to the culture medium resulting in a shorter lag phase [19]. As mentioned in Figure 1, the spores were rapidly produced after 52 hours cultivation which is the death phase of the vegetative cells [20] . This is due to the formed of intracellular spores are liberated from the lysed cells [18]. More cells that contain the spores lyse, more spores are liberated to the medium and hence the steep rise after 52 hours. $Spore_{max}$ is 3.29×10^6 Spore mL^{-1}. During the cell growth phase, the glucose concentration decreased progressively in culture with the rate of 0.141 g $L^{-1}h^{-1}$. Carbon source which is available in glucose and in yeast extract is consumed for cell growth, accumulation of vegetative cells and synthesis of spores [20].

For the first 4 hours, pH of the medium decreased from pH 7.59 to pH 6.48. This is because during the glucose utilization, organic acid such as acetic acid is produced significantly thus lowering the pH. Then, the increased of pH may be associated with sporulation process and cell lysis [8]. In another report, more metabolic acids were found to be secreted into the media during the exponential growth phase [21]. These acids were utilized by vegetative cells as a carbon source after glucose was depleted during stationary phase [21]. In Figure 1, pH level rises after 4 hours and reached up to 8.7 at 72 hours. Total protein also decreases with time. The ammonia compound present in yeast extract is consumed for propagation of cells and delta endotoxin products of bacillus [8].

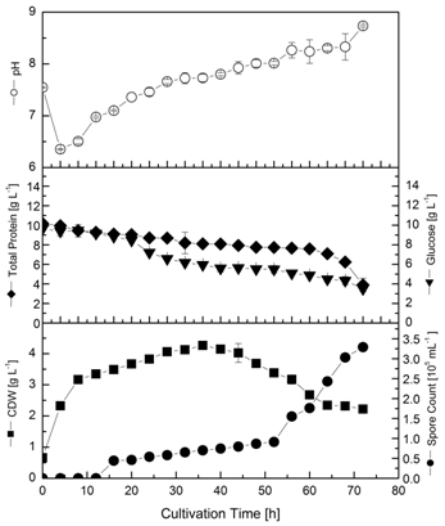

Figure 1. Cell growth, glucose consumption, spore production and change in pH during Bti cultivation in shake flask culture

2. Comparison between the effects of controlled & uncontrolled pH for kinetic of cell growth and spore production in batch cultivations using 16-L stirred tank bioreactor

This experiment was carried out to determine the effect of pH control and uncontrolled on the kinetics of cell growth and spore production with regards to glucose consumption. Figure 2 and Figure 3 present the data of cell cultivation under uncontrolled and controlled pH condition, respectively, during the 48 hour cultivation. In both cultures, the dissolved oxygen level was started with 100% and was not controlled during cell cultivation process. As shown, cell grew exponentially in both cultivations with different rates without any significant lag phase. However, uncontrolled pH condition was more favourable for cell mass production which is 4.14 g L^{-1} at 36 hour compared to controlled pH bioreactor which is 3.36 g L^{-1} at 26 hour. These differences in growth rate and cell yield are linked to the rate of glucose consumption, which also reflects the cell physiology status activity. For uncontrolled pH culture, glucose concentration decreased gradually with consumption rate of 0.156 g $L^{-1}h^{-1}$ and 0.173 g $L^{-1}h^{-1}$ for controlled pH cultivation. Dissolved oxygen (DO) level falls rapidly when cell grows in the exponential phase.

For uncontrolled pH bioreactor, at 0 hour to 4 hour pH were maintained at pH 7.0 as a standard cultivating condition for the Bti. Since thuringiensin is a mixed growth associated product in the cultivation of Bti, showing variation of pH value in the exponential growth and stationary phases. The pH reading was decreased because glucose utilization and the liberation of organic acids at stationary phase [2]. Then, pH was increased at death phase associated with sporulation process and cell lysis. The cell lysis results in liberation of cytoplasmic constituents and contributes for the pH elevation and indication of the presence of protein compounds of cellular origin [2]. Total protein analysis readings were reducing progressively with time. The ammonia compound present in yeast extract is consumed for propagation of cells. The protein was also consumed to produce delta endotoxin of bacillus [22].

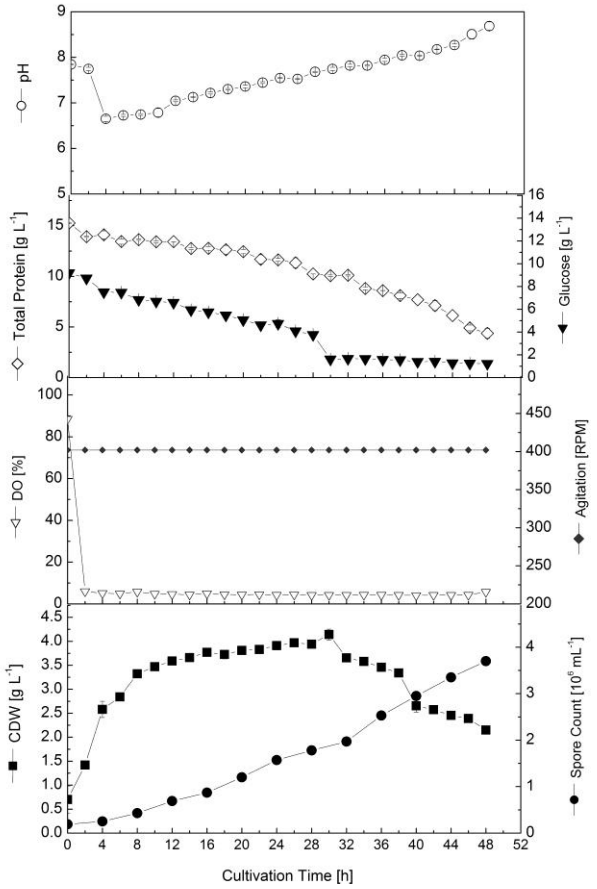

Figure 2. Cell growth, glucose consumption, spore production and change in pH during Bti cultivation in batch bioreactor under uncontrolled pH.

For both bioreactors, the sporulation starts to rise after 4 hours of cultivation. Uncontrolled pH bioreactor produced more spores which was 3.7×10^6 Spores mL^{-1} when compared with controlled pH bioreactor only 3.23×10^6 Spores mL^{-1}. Spores are usually rapidly produced during the stationary phase [23]. This is correlated with studies by Tzheng [24] where the pH was maintained at a constant value during the whole experiment gave a significant increase of the vegetative cells concentration and sporulation. Throughout the cell growth phase, the glucose concentration decreased progressively with the rate of 0.156 $gL^{-1}h^{-1}$ for uncontrolled pH bioreactor and 0.173 g L^{-1} h^{-1} for controlled pH bioreactor. As mentioned before, carbon source which is available in glucose and in yeast extract is consumed for cell growth, accumulation of vegetative cells and synthesis of spores [23].

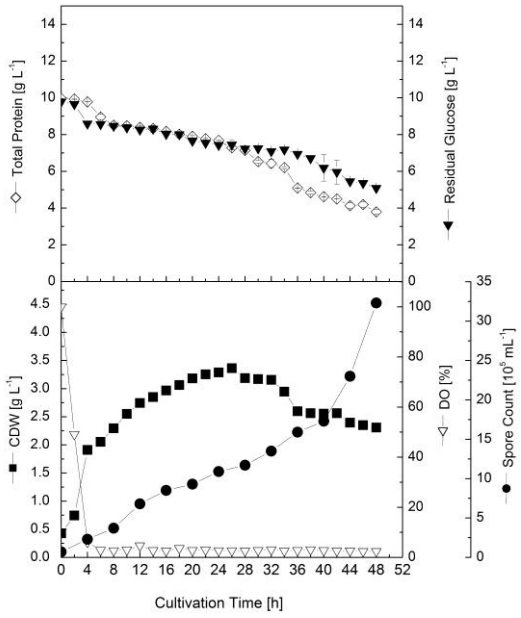

Figure 3. Cell growth, glucose consumption, spore production and change in pH during Bti cultivation in batch bioreactor at Controlled pH.

In summary, table 1 presents the differences between different growth parameters in shake flask and bioreactor cultivations under controlled and uncontrolled pH condition. As shown, biomass was not increased by transferring the process from shake to bioreactor level. However, significant increase in specific growth rate concomitant with glucose consumption rate was observed in bioreactor culture of controlled pH. Improvement of cell growth rate and glucose consumption in bioreactor is mainly attributed to the better mixing which improve cell physiological status in addition to controlling the pH of culture as observed in many other processes involving aerobic microorganisms [25,26].

Table 1. Kinetic parameters of cell growth, glucose consumption and spore production during different modes of submerged cultivations of Bti in shake flask and in bioreactor

Parameter	Shake Flask	Bioreactor	
		Uncontrolled pH	Controlled pH
X_{max} [g L^{-1}]	4.623	4.14	3.363
dx/dt [g $L^{-1}h^{-1}$]	0.313	0.328	0.370
μ [h^{-1}]	0.250	0.250	0.499
$-Q_{glucose}$ [g $L^{-1}h^{-1}$]	0.141	0.156	0.173
$Spore_{max}$[Spore mL^{-1}]	3.290×10^{6}	3.7×10^{6}	3.23×10^{6}
Q_{Spore} [Spore/mL/h]	1.088×10^{4}	1.08×10^{5}	1.88×10^{5}
$Y_{P/X}$ [Spore/g]	7.120×10^{2}	8.937×10^{2}	9.60×10^{2}

X_{max}: maximal cell dry weight; dx/dt : growth rate; μ: specific growth rate; $-Q_{gluc}$: glucose consumption rate; $Spore_{max}$: maximal spore production; Q_{spore}: Spore production rate.

IV. Conclusion

In summary, kinetic data for all experiments conducted in this study are summarized in Table 1was mentioned above. On comparing these results together, as a conclusion on scaling up the process from shake flask to bioreactor level, uncontrolled pH bioreactor produced more cell which is 4.14 g L^{-1} compared to controlled pH bioreactor, 3.36 g L^{-1}. For spore production, uncontrolled pH bioreactor produces more spores which is 3.7×10^{6} Spore mL^{-1} than controlled pH bioreactor which is 3.23×10^{6} Spore mL^{-1}. Shake flask experiment is for determination of the optimum conditions for the fermentation process. Therefore, uncontrolled pH bioreactor produced more spores and high cell growth rate result. Further study process optimization in bioreactor will continue with uncontrolled pH bioreactor since it produced more spore and high cell growth rate. It will reduce the cost of acid and base utilization and also save operating and maintenance cost of pH controller.

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