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Bioprocess Development for High Cell Mass and Endospore Production by *Bacillus thuringiensis* var *israelensis* in Semi-Industrial Scale

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The process for high cell mass and spore production by *Bacillus thuringiensis* var *israelensis* (*Bti*) on semi-industrial scale was developed. Cultivations were conducted in shake flask and bioreactor levels to develop a semi-industrial scale process for the production of *Bti* cells and spores. The composition of the most suitable cultivation medium was further optimized to enhance biomass and spore production and also to reduce the production time. Furthermore, cultivations were performed in 16-L stirred tank bioreactor to investigate the scalability of this process and to study the effect of different aeration rates. The optimization of the main three components of the cultivation medium, i.e. glucose, ammonium sulphate and phosphate, resulted in a significant increase in both cell growth and spore formation by about 32% and 119%, respectively. Scaling up the cultivation process from shake flask to 16-L bioreactor further improved the process. Maximal cell mass of 16.06 $g.L^{-1}$ concomitant with a spore production of 152×10^7 $spores.mL^{-1}$ was achieved in $1v.v^{-1}.min^{-1}$ aerated culture. In terms of cell growth and spore production, these results were 5- and 8.5-fold higher, respectively, compared with the initial un-optimized medium in shake flask culture. The results obtained in this study improved the process of *Bti* production in semi-industrial scale through the optimization of both the cultivation medium composition and the aeration rate in pilot scale stirred tank bioreactor.

Keywords: *Bacillus thuringiensis* var *israelensis*, Spore production, Pilot scale, Batch culture, Stirred tank bioreactor, Aeration.

Bacillus thuringiensis(Bt) based biopesticides are the major microbial pest control agents (MPCAs) widely used to prevent plant diseases caused by insects in many crops[1,2]. *Bt* based bioinsecticides were first commercialized in France in 1938 with limited success. However, based on the new development in microbial cell cultivation strategies and the possibility of conducting such cultivations in large industrial scale, these products showed extensive market growth since 1950s. Initially, *B. thuringiensis* var *kuristaki* (the first isolated strain for biological control) dominated the market and was used for biological control of lepidopteran pests in many agriculture crops. After the discovery of *B. thuringiensis* var *israelensis* (*Bti*) in 1976, the

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*Bt*business was further expanded based on the larvicidal activity of this bacterium against *Aedes aegyptii*. This type of mosquito acts as the main vector of many worldwide dangerous diseases such as malaria and dengue. In 2010, it was reported that more than 200 *Bt*-based biopesticides account of 53% of the world market of MPCAs with annual revenues between 120 and 140 million US dollars[3,4].Nowadays, *Bti* strains are sold worldwide under different trade names such as Bactimos®, Vectobac®, Aquabac®, and Teknar®. When the spores of this bacterium are applied to the field and consumed by mosquito larvae, they release toxins in the mosquito's gut leading to termination of feeding and consequently causing larval death. However, for field application, bacterial spores should be applied continuously to the environment since the *Bti* remain effective from 24 h to over one month based on the product formula and the environmental conditions. Thus, based on this huge market demand, studies on cell mass and spore production by *Bti*areof special industrial interest.

The nutritional requirements of different varieties of *Bt*strainsare known to be variable and highly dependent on the type of strain. An optimum culture medium for one variety may be unsuitable to the others, underlining the difficulty in defining the most suitable culture medium for each particular *Bt*strain[5].Most of industrial cultivation media are based on complex carbon and nitrogen sources. However, simple carbon sources yield the best results and carbohydrate concentration influences the production of protein crystals[6,7]. For nitrogen source, soybeanmeal is widely used in the cultivation of some *Bt* strains as a cheap source, and it was found to induce better spores and endotoxin production, irrespective of the carbon source used[5].Another study reported thatprotein rich media inhibit *Bt*sporulation while promoting cell growth[8]. Thus, it was recommended to be used in the stage of inoculum preparation. The authors concluded that, culture media with a high carbon to protein ratio arebetter for the sporulation and crystal formation. Furthermore, soybean powder was found to be a good and an economic nitrogen source for the large scale industrial production of *Bt*. It has been reported that the level of toxicity produced from soybean powder was comparable to thatobtained from conventional yeast nitrogen saltmedium[9]. Moreover, soybean powder permits high biomass production and spore formation in shorter fermentation periods. On the other hand, the optimum level of phosphate,added asK_2HPO_4, for the optimum production of d-endotoxin was found to be 1 $g.L^{-110}$. Additionally, this study showed that there is no link between cell growth and the production of d-endotoxin.On the other hand, higher phosphate concentrations (150mM) inhibited the production of protein crystals[11].It was also found that increasing the phosphate concentration in the medium increased the strength and potency of the produced d-endotoxin product[12]. Increasing the concentration of dipotassium phosphate, at least up to 0.2 M resulted in an increase in the yield and activity of d-endotoxin produced.

Temperature greatly affects the bacterial growth, sporulation and protein crystal production. The cultivation of *Bt*is usually performed at a temperature around 30°C. On the other hand, the typical pH profile of culture is generally characterized by a decrease from the initial pH, which is usually set between 6.8-7.2. The pH decrease to 5.8 due to the release of acetate, then risesagain up to 7.5-8 as a results of acetate consumption[13].*Bt*is an aerobic microbe, and oxygen supply is one of the major factors influencing the performance and conditions of the bacterial growth. It has been found that the *Bt*bacterium fails to survive or sporulateat low oxygen conditions. Toxin synthesis is known to be dependent on sufficient aeration levels. In small scale shake flask level, the air/medium ratio of 19:1 yielded higher cell growth, spore count and 6-fold increase in toxin potency compared to the culture having air/medium ratio of 9:1[12]. It was also reported that cultivation under high dissolved oxygen (DO) promotes cell mass production but lower spore and toxin yields[14]. The cells produced under low aeration levels possess a greater capacity to synthesize toxin during sporulation.

Therefore, the present study was undertaken to optimize the process of microbial cell mass and spore production by *Bti* in semi-industrial scale. Firstly,the bioprocess was optimized in small scale by selecting the most suitable medium for cell growth and sporulation followed by simple medium optimization of different ingredients in shake flask level. Secondly, the

process was scaled up to a pilot scale 16-L stirred tank bioreactor to investigate the effect of aeration rate on the kinetics of cell growth and spore production.

MATERIALS AND METHODS

Microorganism

The bacterial strain used in this study was a standard strain of *Bacillus thruringienssi* var *israelensis* ATCC 700872,kindly provided by EntoGenex Sdn. Bhd. This strain was obtained originally from American Type Culture Collection (ATCC, Manassas, VA, USA). Once received, the strain was activated in Luria Bertani (LB) agar medium of the following composition ($g.L^{-1}$): Peptone 10.0; Yeast extract 5.0; NaCl 5.0, and Agar 20.0. The pH of medium was adjusted to 7.0 before sterilization. After 24 h cultivation at 30°C, the arisen colonies were harvested in 50% glycerol solution ($v.v^{-1}$). The harvested cell suspension was stored in 2 mLCryovials (NalgenNunc. Int., Rochester, NY, USA)and were stored at -80°C ultradeep freezer as master cell bank. To minimize intrapopulation and inoculums quality variations during this study, each experiment was started by revival of one glycerol vial in LB vegetative broth culture. The stored cells viability was periodically checked to ensure the cell stability during storage.

Inoculum preparation

Inoculum was prepared by inoculating 250 mL Erlenmeyer flasks containing 50 mL of LB liquid medium with 500 mLfrom cell suspension stored in glycerol culture. The inoculated flasks were incubated on a rotary shaker (Innova 4080 New Brunswick Scientific Co., NJ, USA) at 200 rpm and 30°C for 24 h. The grown cells were used to inoculate either 250 mL Erlenmeyer flasks or stirred tank bioreactor containing production medium with an inoculum concentration of 5% ($v.v^{-1}$) of inoculums density of 1.0 OD_{600}.

Culture media for cell growth and spore production

Based on the previously published data by other research groups, the suitability of eight different media was evaluated for cell growth and spore production by *B. thuriginentis*. Table 1 summarized composition of different media used in this study. For all media, the pH was adjusted to 7.0 before sterilization. The carbon source of each medium was sterilized separately and added to the fermentation medium before inoculation. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 30°C.

Bioreactor cultivations

Cultivations in stirred tank bioreactor were conducted using the optimized medium, previously selected from the medium screening step, and run under the same cultivation conditions as in shake flasks in terms of inoculum size, temperature and pH. The bioreactor used in this study was 16-L stirred tank bioreactor (BioEngineering, Wald, Switzerland) with a working volume of 8-L. The stirrer was equipped with two 6-blade Rushton turbine impellers ($d_{i\ (impeller\ diameter)}$ = 85 mm; $d_{t\ (tank\ diameter)}$=214 mm, $d_i d_t^{-1}$ = 0.397). The agitation speed was adjusted to 600 rpm and was kept constant during the cultivation time. Unless otherwise mentioned, aeration was performed using filtered sterile air supplied continuously to the bioreactor with a rate of 1.0 $v.v^{-1}.min^{-1}$. Foam was suppressed by the addition of silicon antifoam grade A (Sigma-Aldrich Inc., MO, USA). pH and DO were determined throughout the cultivation process using liquid filled pH electrode and DO polarographic electrodes (Ingold, Mittler-Toledo, Switzerland), respectively.

Sample preparation and cell dry weight determination

Samples in the form of two flasks containing 50 mL each, or 25 mL of broth in case of bioreactor, were withdrawn at different time intervals during the cultivation in a centrifugation tube. Immediately after sampling, the optical density was measured by using spectrophotometer (DR/250, Hach Co., Loveland, CO., USA) at 600 nm after proper dilution. In medium containing soybean meal, the solid particles were allowed to settle first for 10 seconds and the supernatant was used for OD determination. The OD of the culture was converted to dry cell mass through a linear correlation standard curve previously performed for this strain. Based on the standard curve, one unit OD_{600} was equal to 0.28 $g.L^{-1}$.

Spore determination

Thespores were determined by the modified Schaaffer and Fulton method[22]. FiftymL of fresh fermentative broth was fixed on

microscopic slides and was stained by spore staining technique using malachite green and safranin. In this method, malachite green dye was added to the fixed microbial smear and exposed to steam for 20 min. After that time, the slide was washed using distilled water, dried and the counter stain (safranin) was added for 1 min, and the excess safranin stain was washed then using distilled water. After staining, the slide was heat dried. The dried slides were examined for the quantitative determination of green colored spores using phase contrast microscope (DM2500 P, Leica, Germany) equipped with digital color camera (DFC420C, Leica, Germany) and connected with image processing and analysis software (Leica QWin).

RESULTS AND DISCUSSION

Medium screening for cell growth and spore production

As reported by many authors, *Bt*strains exhibit different growth and sporulation kinetics, that have been found to be highly strain specific and depend on the cultivation medium used. Based on the previous published research, cell growth and spore production of *Bti* strain under study were investigated in eight different cultivation media as described before in materials and methods section. The inoculated flasks were incubated at 30°C on rotary shaker at 200 rpm for 48 h. Samples were taken after 24 and 48 h for pH, cell growth and spore formation analysis. As shown in Figure (1), medium 4 was the best for supporting cell growth as cell mass reached about 2 $g.L^{-1}$ after 24 h cultivation followed by media 5, 7 and 8. The other tested media did not support cell growth, where the biomass produced wasabout 0.5 $g.L^{-1}$ or less. After 48 h incubation, the maximal biomass of about 3.1 $g.L^{-1}$ was also achieved in medium 4 followed by media 7, 5 and 8.

On the other hand, after 24 h cultivation, the maximal spore production of about 8.5×10^6 $spores.mL^{-1}$ was also achieved in medium 4 followed by media 5 and 7 whichalso, to a lesser extent, supported spore formation of about 4×10^6 and 3.2×10^6 $spores.mL^{-1}$, respectively. However, the number of spores increased significantly after 48 h cultivation reaching about 15×10^7, 9.8×10^7 and 7.9×10^7 $spores.mL^{-1}$ for media 4, 3 and 5, respectively. Based on these results, it is obvious that medium 4 which is composed of ($g.L^{-1}$): glucose, 10; soybean flour, 30; KH_2PO_4, 5; K_2HPO_4,

Table 1. Different industrial media used for *B. thuringiensis*growth and spore production

Components (g/L)	Medium No							
	1[15]	2[16]	3[17]	4[18]	5[14]	6[19]	7[20]	8[21]
Glucose	-	5.0	1.0	10.0	10.0	15.0	-	5.0
Sucrose	-	-	-	-	-	-	1.0	7.0
Glycerol	-	-	-	-	-	5.0	-	-
Soybean flour	-	-	-	30.0	-	-	60.0	-
Yeast extract	5.0	2.0	3.0	-	5.0	2.0	-	2.0
Peptone	-	-	-	-	-	-	-	2.0
Hydrolyzed casein	4.5	-	-	-	-	-	-	-
$(NH_4)_2SO_4$	6.0	2.0	2.0	-	5.4	-	-	-
$(NH_4)_2PO_4$	-	-	-	-	2.0	-	-	-
K_2HPO_4	-	1.4	0.5	-	5.0	1.0	8.7	5.0
KH_2PO_4	-	1.4	-	0.78	5.0	1.0	-	5.0
$NaNH_4PO_4.4H_2O$	-	-	-	-	1.5	-	-	1.5
$MgSO_4.7H_2O$	0.61	0.2	0.3	0.05	0.3	0.04	0.05	-
$CaCl_2.2H_2O$	0.33	0.08	0.02	0.05	-	0.08	0.05	0.0102
$MnSO_4.H_2O$	0.006	0.05	0.015	0.03	0.01	0.001	0.03	-
$CuSO_4$	-	-	0.01	-	-	-	-	-
$FeSO_4$	-	-	0.01	0.01	0.01	-	0.01	-
$CaCO_3$	-	-	-	-	20.0	-	-	-
$MgCl_2$	-	-	-	-	-	-	-	0.0203
$MnCl_2$	-	-	-	-	-	-	-	0.001

Table 2. Kinetics of cell growth and spore production by *Bti* in shake flask and in bioreactor using different media and different cultivation conditions

Parameter	Shake flask cultivation (medium optimization)		Bioreactor batch cultivation (different aeration rates)		
	Un-optimized	Optimized	0.5 $v.v^{-1}.min^{-1}$	1.0 $v.v^{-1}.min^{-1}$	2.0 $v.v^{-1}.min^{-1}$
X_{max}	3.25	4.90	9.16	16.06	12.3
dx/dt [$g.L^{-1}.h^{-1}$]	0.077	0.160	0.458	0.689	1.025
m [h^{-1}]	0.047	0.065	0.100	0.091	0.166
Sp_{max} [$´10^7$ mL^{-1}]	17.8	39.1	83.0	152.0	116.0

X_{max}: maximal cell dry weight; dx/dt: growth rate;m: specific growth rate; Sp_{max}: maximal number of spores.

5; $MgSO_4.7H_2O$, 0.05; $MnSO_4.H_2O$, 0.03; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.05 and $(NH_4)_2SO_4$, 1.5 was the most suitable medium for cell growth and spore production. The superiority of this medium composition may be attributed to its balanced C:N ratio compared to other media. Another possible explanation is the use of soy flour in medium 4. The best two media, in terms of cell growth,were media 4 and 7, which include soybean flour as the main ingredient. Soybean powder is a good and economical nitrogen source for the large scale industrial production of *Bt*. Additionally, the level of spores and bacterial toxin production produced in soybean powder was reported as much as equal to those produced in the expensive widely used yeast extract rich medium[9].

Kinetics of cell growth and spore production in un-optimized medium

To obtain a better understanding of the kinetics of cell growth and spore formation by *Bti* using the selected highly productive medium, cultivation was carried out in shake flask batch culture for 48 hours. During that time samples were taken every 6 hours and were analyzed for pH, cell dry weight and spore formation. As shown in Figure (2), cells grew exponentially without any noticeable lag phase with a growth rate of 0.077 $g.L^{-1}.h^{-1}$. The cells reached a maximal cell mass of about 3.25 $g.L^{-1}$ after 42 h. On the other hand, spores were firstly produced in the culture medium after 12 h. At that time the number of cells bearing spores was about only 1.25×10^5 $spores.mL^{-1}$. The number of sporulated cells increased gradually by time and reached about 17.8×10^7 $spores.mL^{-1}$ after 42 h cultivation, where almost 100% of *Bacillus* cells were sporulated. Therefore, increasingthe cultivation time further up to 48 h didn't show any increase in the number of sporulated cells. It is also worthy to note that during the first 8 hours, the pH dropped from 7 to 5.5. Then, it increased gradually and reached about 7.93 after 42 h and kept more or less constant for the rest of the cultivation time. This pH trend exhibited in Figure (2) follows the trend of a typical *Bt* fermentation broth as reported before, where the pH starts at 6.8-7.2, decreases to 5.8 as acetate is released, then rises to 7.5-8.0 as the produced acetate is being consumed[13]

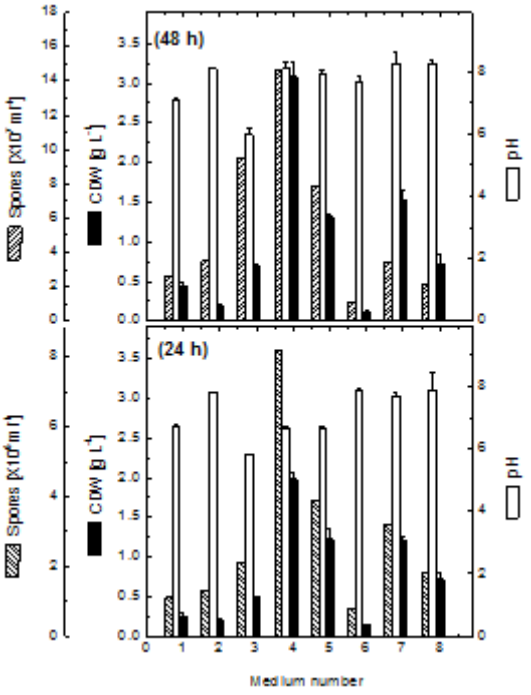

Fig. 1. Effectof different cultivation media on cell growth, spore production and pH of *B. thuringiensis* var *israelensis* in shake flask level

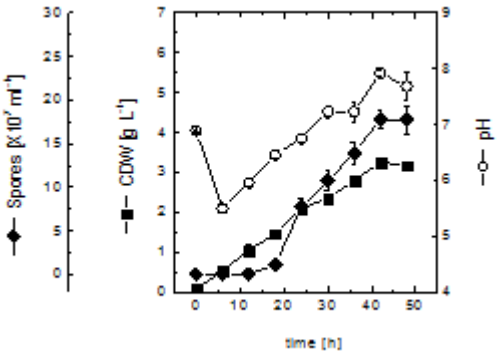

Fig. 2. Kinetics of cell growth and spore formation of *B. thuringiensis* var *israelensis*cultivated in un-optimized medium in shake flask fermentation at 30°C for 48 hours

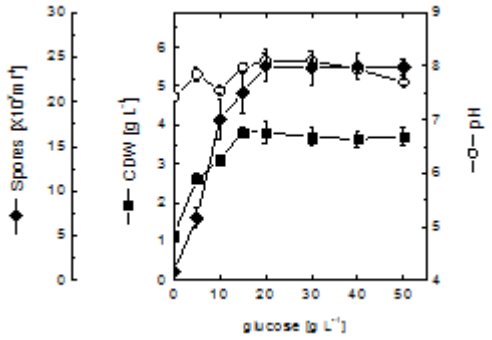

Fig. 3. Effectof different initial glucose concentrations on cell growth, spore production and pH of *B. thuringiensis* var *israelensis*. Cultivations were conducted for 48 hours in shake flasks at 200 rpm and 30°C

Effect of glucose concentration on cell growth and spore formation of Bti

Carbon source is a very important component in *Btcultivation*, since it acts asa source of energy and cell biomaterial synthesis.In previous studiesit has been reported that simple carbon sources yield better results for cell growth, spore formation and toxin production. Moreover, the concentration of carbon source also showed a significant effect on the yield of spore production[5]. Therefore, the effect of glucose concentration on growth and spore formation of the strain understudy was carried out by cultivating the cells in mediumcontaining different glucose concentrations rangingfrom 0.0to 50 g.L^{-1}. The results showed in Figure (3) demonstrates that increasing the initial glucose concentration from 10 g.L^{-1} (the initial concentration of glucose in medium) to 15 g.L^{-1} increased cell growth from 3.1 g.L^{-1} to 3.8 g.L^{-1}. Further increase in glucose concentration didn't show any increase in biomass production. The effect of glucose concentration on cell growth and spore production was also studied by other authors. Upon using initial glucose concentrations higher than 50 g.L^{-1}, a significant decrease in both cell growth and spore production by *Bt*var. *galleriae*was observed[23].On the other hand Scherrer*etal.*[24]reported a significant reduction in both cell count and spore formation when the glucose concentration in the culture only

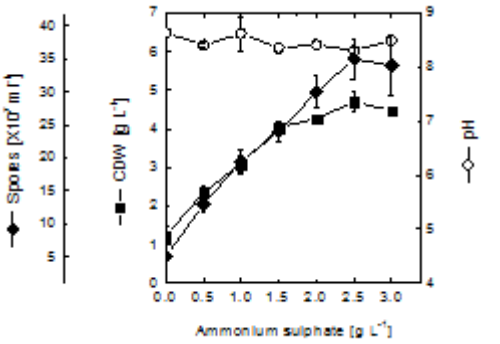

Fig. 4. Effectof different initial ammonium sulphate concentrations on cell growth, spore production and pH of *B. thuringiensis* var *israelensis*. Cultivations were conducted for 48 hours in shake flasks at 200 rpm and 30°C

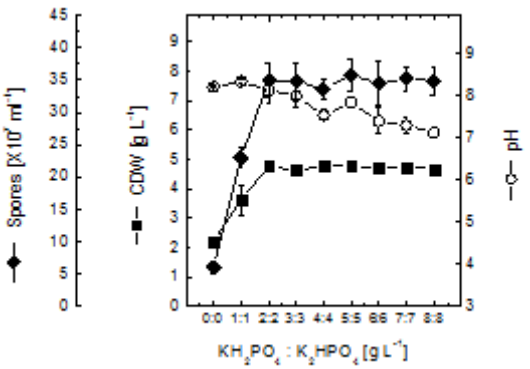

Fig. 5. Effectof different initial phosphate concentrations on cell growth, spore production and pH of *B. thuringiensis*var*israelensis*. Cultivations were conducted for 48 hours in shake flasks at 200 rpm and 30°C.

exceeds 8 g.L^{-1}. This limitation was explained due to the increased acid production in culture medium, which exhibited a negative effect on bacterial growth.

In the present study, it was clearly observed that the final pH of the medium is generally lower for medium containing high glucose concentration. This based on fact that high glucose concentration supports organic acid biosynthesis in most of *Bt* strains[6]. The results of spore formation showed also that, the number of spores in culture was as low as 1×10^7 spores.mL^{-1} in medium without glucose. Significant increase in spore formation was observed when glucose was added to the culture medium. The maximal number of spores produced in the culture was about 24×10^7 spores.mL^{-1},when the culture was supplemented with 20 g.L^{-1} glucose. Further increase in glucose concentration did not show any effect on both cell growth and spore production. In conclusion, increasing the glucose concentration in the medium from 10 g.L^{-1} (the initial medium) to 20 g.L^{-1} resulted in a significant increase in both cell growth and number of spores by about 22% and 33%, respectively.

Effect of ammonium sulphate concentration on cell growth and spore formation of Bti

Based on the results of the previous experiment, glucose was added tocultivation medium in a concentration of 20 g.L^{-1}. In this experiment, different concentrations of ammonium sulphate ranging between 0.0 and 3.0 g.L^{-1} were added to the culture medium to investigate the effect of this inorganic nitrogen source. In this medium, organic nitrogen (soybean meal) and inorganic (ammonium sulphate) sourceswere used. This was based on the fact that inorganic nitrogen source alone is not suitable to support *Bti* growth,

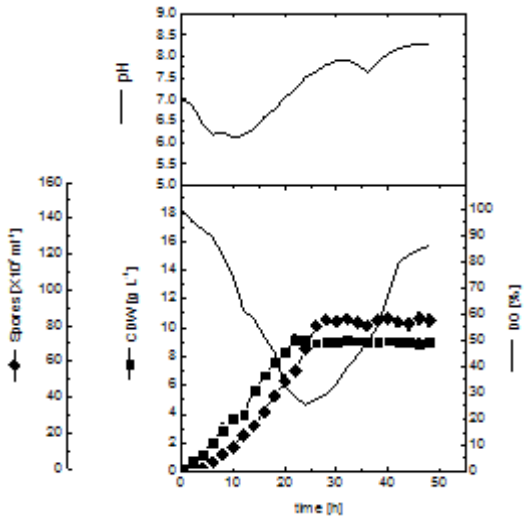

Fig. 7. Kinetics of cell growth and spore formation during batch cultivation of *Bti* in 16-L stirred tank bioreactor at an aeration rate of 0.5 v.v^{-1}.min^{-1}.

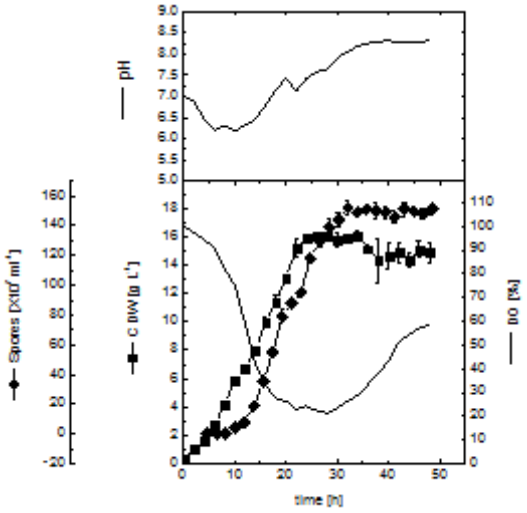

Fig. 8. Kinetics of cell growth and spore formation during batch cultivation of *Bti* in 16-L stirred tank bioreactor at an aeration rate of 1.0 v.v^{-1}.min^{-1}

since the bacteria required some amino acids and peptides essential for their growth, and they should be added to the culture medium in the form of complex organic nitrogen sources such as yeast extract and soybean[25].Thus, it was also reported by some authors that the combination of organic and inorganic nitrogen sources yielded the highest spore number concomitant and d-endotoxin[26]. According to the results of this study, maximal cell growth of about 4.7 g.L^{-1} was produced in culture supplemented with 2.5 g.L^{-1} ammonium sulphate. Concomitant to this increase in cell mass production, a significant increase in spore formation in the culture was also observed. The maximal number of spores of 35×10^7 spores.mL^{-1} was also obtained using this concentration.

Further increase in ammonium sulphate concentration above 2.5 g.L^{-1}didn't show any

significant effect on either cell growth or spore formation. On the other hand, it has been also reported that ammonium sulphate at a concentration of 4.7 concentration of 4.7 $g.L^{-1}$was optimal for supporting cell growth[21]. The addition of both inorganic and organic nitrogen in a mixture is necessary. In spite of the fact that amino nitrogen supports cell growth and spores production, it has been reported that organic nitrogen sources above certain limits inhibit the sporulation, and accordingly organic nitrogen rich medium is more recommended for the preparation of inoculum[8].

Effect of phosphate concentration on cell growth and spore formation of Bti

Phosphate ions are vital for the growth, sporulation and toxin production by*Bt*. In the production medium of this study, inorganic phosphate source was used in a form of equal concentration of KH_2PO_4 and K_2HPO_4. In this experiment, the phosphate concentration was changed while keeping the ratio between both phosphate sources equal as in the initial medium. After 48 h cultivation in shake flask culture, cell growth, number of spores and pH of culture were determined (Fig. 5). As shown, the addition of inorganic phosphate source to this medium was important for both cell growth and spore production. The maximal cell growth of about 4.9 $g.L^{-1}$ concomitant with the highest number of spores produced in culture reaching 35×10^7 $spores.mL^{-1}$ was obtained in culture supplemented with equal balanced concentrations of KH_2PO_4 and $K_2HPO_4$of 2.0 $g.L^{-1}$ each. Further increase in inorganic phosphate concentration neither increased cell growth nor spore production. However, it is worthy to note that, there was a direct relation between the final pH of culture and the initial phosphate concentration. Increasing the phosphate concentration decreased in the final pH in all concentrations applied. Thus, it can be concluded that increasing the phosphate concentration promotes acid production in the culture medium. The results of low sporulation in culture without phosphate were in agreement with those obtained by Iegen*et al.*[11], who reported that phosphate limitation in the culture can lead to low cell growth and spore formation. In another study, it was observed that the concentration of inorganic phosphate in the culture added as dipotassium hydrogen phosphate (K_2HPO_4), optimal for cell growth and spore formation, was 1.0 $g.L^{-110}$. In conclusion, the phosphate source in this culture should be reduced from 5.0 $g.L^{-1}$ for each inorganic phosphate source understudy to only 2.0 $g.L^{-1}$ each. Further increase in the phosphate concentration had no effect on cell growth or spore production,however, it may switch the pathway toward more acid production.

Kinetics of cell growth and spore production in optimized medium

Based on the results of the previous experiments, new medium formula was developed for producing the biological control agent by *Bti*. This medium composed of ($g.L^{-1}$): glucose, 20; soybean flour, 30; KH_2PO_4, 2; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.05; $MnSO_4.H_2O$, 0.03; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.05 and $(NH_4)_2SO_4$, 2.5. Figure (6) demonstrates the kinetics of cell growth and spore production when *Bti*cells were cultivated for 48 h. As shown, cells grew exponentially without any significant lag phase with a growth rate of 0.16 $g^{-1}.L^{-1}.h^{-1}$ reaching a maximal biomass of about 4.9 $g.L^{-1}$ after 30 hours. This value of biomass produced in this medium was almost 47% higher than the biomass concentration obtained in the initial medium (before optimization) under the same cultivation conditions. In terms of spore production, the maximal number of spores of 39.1×10^7 $spores.mL^{-1}$ was obtained also after 30 h. This value was about 119% higher than those

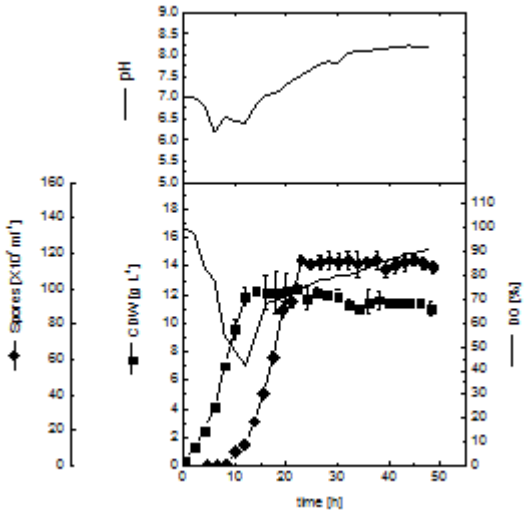

Fig. 9. Kinetics of cell growth and spore formation during batch cultivation of *Bti* in 16-L stirred tank bioreactor at an aeration rate of 2.0 $v.v^{-1}.min^{-1}$

values obtained in the initial medium before optimization. Moreover, the maximal concentration of spores was achieved after only 30 hours whereas the maximal spore production in the un-optimized medium was recorded after 42 hours (Figure 1). Thus, we can conclude that,the new medium composition is highly supportive for both biomass and spore production for this *Bti*strain. Moreover, this medium decreased the spore production phase by 12 hours in addition to saving in part the cost of medium components since the inorganic phosphate in the medium was reduced from 5.0to 2.0 $g.L^{-1}$ only. Accordingly, this optimized medium is more suitable for large scale production of *Bti*.

Kinetics of cell growth and spore production during batch cultivation in 16-L stirred tank bioreactor under different aeration rates

After successful optimization of the cultivation medium in small scale, cultivations were conducted in 16-L stirred tank bioreactor. This step was important to investigate the scalability of this process and to investigate the effect of aeration on the kinetics of cell growth and spore production. It is well known that *Bt*is a highly aerobic microorganism, and oxygen supply is one of the major factors influencing the performance of this type of bacteria. Thus, the effect of aeration on the kinetics of cell growth and spore production by *Bti* was investigated in 16-L stirred tank bioreactor. In this experiment, three different bioreactors were run in parallel to investigate the aeration effect by setting the aeration rate in these three bioreactors at 0.5, 1.0 and 2.0 $v.v^{-1}.min^{-1}$. For this purpose, air was supplied to the bioreactors continuouslyand controlled by an integrated on-line mass flow controller (Bronkhorst High-Tech B.V., Ruurio, The Netherlands). All other cultivation parameters in terms of temperature, agitation, inoculum age and size were constant for all bioreactors. Figures (7, 8 and 9) demonstrate the cultivation profile of *Bti* in batch culture in 16-L bioreactor under different aeration rates of 0.5, 1.0 and 2.0 $v.v^{-1}.min^{-1}$, respectively.

It can be clearly observed that, in all cultures studied, the concentration of both biomass and spores were higher in bioreactor cultures compared to shake flask ones. In case of 0.5 $v.v^{-1}.min^{-1}$ aerated culture, cells grew exponentially without entering a lag phase with a growth rate of 0.458 $g.L^{-1}.h^{-1}$ and reached their maximal cell mass (9.16 $g.L^{-1}$) after 22 h. During this active growth phase the DO in culture dropped significantly and reached 25% saturation after 24 h. As cells entered the lag phase, the DO increased again in the culture reaching about 85% saturation at the end of cultivation time. During the exponential growth phase, the pH of culture dropped from 7.0 to about 6.0 after 10 h and increased gradually thereafter. On the other hand, spore formation proceeded parallel to cell growth and the maximal number of sporulating cells in the culture reached about 83×10^{7}spores.mL^{-1} after 28 h. This value of spores formed was almost double of that obtained in shake flask culture.

In the culture run at an aeration rate of 1.0 $v.v^{-1}.min^{-1}$, cell grew exponentially with a growth rate of 0.689 $g.L^{-1}.h^{-1}$, without any significant lag phase and reachedtheir maximal concentration of 16.06 $g.L^{-1}$ after 20 h. This value was about 75% than the biomass concentration obtained in 0.5 $v.v^{-1}.min^{-1}$aerated culture. During the growth phase, the DO also decreased in the culture and reached its minimal value of about 20% saturation at 30 h. After that time, the DO increased again in the culture and reached about 60% at the end of the cultivation time. Parallel to the DO drop, the pH also decreased in the culture during the exponential growth phase and reached about 6.0 after 10 h. After that time, the pH increased gradually up to 8.5 at the end of the cultivation. Spore formation in this culture started with cell growth and the maximal number of the spores (152×10^{7}spores.mL^{-1}) was obtained after 30 h. This concentration was about 83% higher than the concentrations obtained in 0.5 $v.v^{-1}.min^{-1}$aerated culture (83×10^{7}spores.mL^{-1}).

In the third culture, with the highest aeration rate of 2.0 $v.v^{-1}.min^{-1}$, cell growth rate was the highest among all cultures investigated. In this culture, the growth rate of the cells was about 1.025 $g.L^{-1}.h^{-1}$and the maximal cell mass of 12.3 $g.L^{-1}$ was reached after only 10 h. On the other hand, during the first 10 hours of the cultivation, the DO dropped significantly reaching about 40% saturation and increased gradually thereafter as cells entered the stationary phase. For spore production, after a lag phase of about 8 h, the number of spores increased gradually in the culture and reached its maximal value of 116×10^{7}spores.mL^{-1}after 20 h. This value was about 31% less than the numbers of spores

obtained in 1.0 $v.v^{-1}.min^{-1}$ aerated culture, but still higher by about 40% than the number of spores produced in 5.0 $v.v^{-1}.min^{-1}$ aerated culture. Thus, it can be concluded that aeration showed a positive effect on cell growth and spore formation of the *Bti* strain under study since all bioreactor cultures exhibited higher growth and spore production compared to shake flask cultures. On the other hand, a possible explanation for the less growth and spore formation in highly aerated culture could be that higher aeration can lead to the appearance of bacteriophage which preys on the bacteria, and thus subsequently reducing the yield of spores and toxin formation[27]. Moreover, It has been also reported that aeration rate and stirrer speed influence the production of the d-endotoxin[28].The authors reported that the most potent toxin was formed using a stirrer speed and an aeration rate of 300 rpmand 1 $v.v^{-1}.min^{-1}$, or 500 rpm and 0.6 $v.v^{-1}.min^{-1}$, respectively. The higher growth rate obtained in the 2.0 $v.v^{-1}.min^{-1}$ aerated culture may be due to the fact that higher DO in the culture promotes more biomass formation as previously reported[13]. The higher spore production in the low aerated cultures may be attributed to the DO pattern of these cultures compared to highly agitated culture since the optimal DO for spore production and toxin formation was reported to be of about 25% saturation[17].

CONCLUSION

Table (2) summarizes the kinetics of cell growth and spore production forthe cells cultivated in shake flask and in bioreactor levels under different conditions. As shown, the optimization of the cultivation medium resulted in a significant increase in both cell growth and spore formation by about 32% and 119%, respectively. Further improvement in this process was achieved by scaling up from shake flask to 16-L stirred tank bioreactor. Maximal cell growth of 16.06 $g.L^{-1}$ concomitant with the highest spore production of 152×10^{7}spores.mL^{-1} was achieved culture aerated at 1.0 $v.v^{-1}.min^{-1}$. These values were 5- and 8.5-folds higher in terms of cell growth and spore production, respectively, compared to the initial medium used in shake flask culture.

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