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# Development of a Cost Effective Bioprocess for Production of an Iranian Anti-Coleoptera *Bacillus thuringiensis* Strain

G. Salehi Jouzani[1*], S. Abbasalizadeh[1] M. F. Moradali[1], and H. Morsali[1]

## ABSTRACT

**One of the major obstacles of Bt production as a biopesticide is its expensive bioprocess and fermentation. Therefore, the objective of the present study was to optimize growth condition and develop a low-cost bioprocess for mass production of a native coleopteran-effective *Bacillus thuringiensis* (Bt) strain (KH4) based on agricultural wastes, at incubator and batch fermenter level. Preliminary experiments showed that the optimum pH and temperature for the strain were 6.5 and 30°C, respectively. The maximum growth and spore/crystal production were observed in the medium containing 2% molasses and 3% corn steep liquor as carbon and nitrogen sources, respectively. Different concentrations of the sea salt were used as a new cheap and available mineral source. Sea salt with final 0.003% w/w concentration showed the highest rate of growth for the strain. The experiments in Batch fermenter showed that volume of 2% bacterial inoculation in total volume of medium culture was the best concentration as preculture. It was shown that pH significantly decreased at the beginning of logarithmic phase, whereas it significantly increased at the end of the logarithmic phase. By increasing fermentation period, the oxygen demand was increased, and by increasing oxygen concentration up to 70%, the bacterial growth and the spores/crystal production was increased. Based on the results, the growth condition of the strain was optimized and a new cheap and available commercial fermentation medium was developed for mass production of the strain in the batch systems.**

**Keywords**: Agricultural waste, Bt, Economical growth media, Fermentation.

## INTRODUCTION

During the last decades, the insecticidal bacterium *Bacillus thuringiensis* (Bt) proved to be an environmentally safe insecticide for controlling pests of major crops and vectors of human diseases (Khojan *et al*., 2013; Salehi Jouzani *et al*., 2008a). Spores and crystals of *Bt* are readily produced by aerated liquid fermentation (Zouari *et al.,* 1999, 2002). Currently, the cost of *Bt* production using existing fermentation technology is high because of a high cost of the production medium (Prabakaran *et al.,* 2009). Finding available economic sources and optimization of a cost-effective process for production of *Bt* strains are known as one of the most important steps of biopesticide development at industrial scale (Amin *et al.,* 2008; Brar *et al.,* 2005a).

In recent years, several studies using different *Bt* strains and materials have been done to find economical and locally available media for the commercial production of *Bt* bioproducts (Amin *et al.,* 2008; Berbert-Molina *et al.,* 2008a; Brar *et al.,* 2005a, 2005b; Ouhib *et al.,* 2006; Rao *et al.,* 2007; Shojaaddini *et al.,* 2010). Polysaccharides and carbohydrates in the agricultural byproducts, such as molasses, grain powder byproducts or hydrolyzed starch, food industries wastewater (like whey, permeate and corn steep liquor), municipal and domestic wastewater (sewage sludge) and dextrose have been used as the

[1]Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Islamic Republic of Iran.
[*]Corresponding author; e-mail: gsalehi@abrii.ac.ir





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carbon source in the medium (Anderson and Jayaraman, 2003; Berbert-Molina *et al.,* 2008a; Brar *et al.,* 2005a, 2005b; Keshavarzi *et al.*, 2005). Also, some cheap byproducts, such as ammonium salts, amino acids or protein-rich substances of plant or animal origin, including soy and corn processing byproducts (corn steep), oil-free cotton seed flour, yeast extract and whey protein, fish meal, cottonseed meal, soybean, yeast extract and casein have been used as nitrogen source (Amin *et al.,* 2008). The minerals can be provided from mineral water, natural sea water and different salts (Ghribi *et al*., 2007b). In addition to medium contents growth conditions, including oxygen supply, pH, agitation rate, and temperature are very important in spore-crystal production which could be optimized based on the type of strain and medium contents (Brar *et al.,* 2005b, 2005c; Khodair *et al.,* 2008; Kraemer-Schafhalter and Moser, 1996; Liu and Bajpai, 1995; Wu *et al.,* 2002; Yezza *et al.,* 2005).

Previously, we isolated and characterized more than 100 different Iranian native *Bt* strains (Nazarian Amirani *et al.,* 2009; Salehi Jouzani *et al.,* 2008a, 2008b; Seifinejad *et al.,* 2008). The strain (KH4) that showed high entomotoxicity against *Xanthogaleruca luteola* Mull. (Elm Leaf Beetle) and *Leptinotarsa decemlineata* (Colorado Potato Beetle), and contains an interesting combination of different important coleopteran-active *cry* genes, including *cry34*, *cry35*, *cry8B*, *cry18*, *cry7A*, *cry28* and *cry8A* (Nazarian-Amirani *et al.,* 2009). The objective of current study was to develop cost-effective medium based on available agricultural waste sources and to optimize growth conditions for this native coleopteran effective strain of *Bt* at incubator and lab bioreactor scales.

## MATERIALS AND METHODS

### Strains and Cultures

The selected native *Bt* strain (KH4) was used in the present study (Nazarian Amirani *et al.,* 2009; Salehi Jouzani *et al.,* 2008a, 2008b; Seifinejad *et al.,* 2008). The strain was grown at 28ºC in nutrient broth (Difco) with vigorous shaking (120 rpm) or on nutrient broth agar composed of 5 g $L^{-1}$ meat peptone, 3 g $L^{-1}$ meat extract and, 12 g $L^{-1}$ agar, and then stored at 4°C. Primary spore and crystal production were performed using PGSM medium (Brownbridge and Margalit, 1986) at 28°C for 72 hours (Salehi Jouzani *et al*., 2008a, b).

### Temperature and pH Optimization

Two hundred mL of the sterile PGSM medium (Brownbridge and Margalit, 1986) in three replicates inoculated with a volume of 3 % of the isolates biomass to obtain pre-culture medium. Three temperatures including 25, 30 and 35°C were considered to find the optimum temperature of the strain. To optimize the pH condition, pH was adjusted in the range of 6.5, 7, and 7.5 using 1M HCl and 1M NaOH. Bacterial growth was evaluated on a shaker at 150 rpm for 16 hours of incubation.

### Optimizing Cost-effective Media at Incubator Scale

### Optimizing of Carbon Source

To find the optimum economic carbon source for the *Bt* strain, three different carbohydrates, including hydrolyzed starch (hydrolyzed by HCl at pH: 2.0 and 121°C for 15 minutes), molasses (sucrose, Bx: 66.8, Pol: 42.2, Q: 63.5, pH: 4.9) and condensed milk ultra filtration permeate (Lactose: 14.7%, Protein: 17 %, BX: 15, pH: 6.4) were separately used. Glucose was used as the control. Yeast extract (2%) was used as nitrogen source. Minerals, including (NH4) 2SO4 (2 g $L^{-1}$), MgSO4.7H2O (0.3 g $L^{-1}$), CaCl2.2H2O (0.0205 g $L^{-1}$), MnSO4.H2O (0.015 g $L^{-1}$), CuSO4 (0.001 g $L^{-1}$), FeSO4 (0.01 g $L^{-1}$), KH2PO4 (0.78 g $L^{-1}$), K2HPO4 (1 g $L^{-1}$) were used as mineral sources.





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The waste waters used in the medium were diluted based on the amount of their sugar contents, till the amount of carbon reached 1.5 percent. The volume of 200 mL medium in three replicates was used for each Erlenmeyer flask. Then, pH of each medium was adjusted based on the optimum pH for the *Bt*-strain. Media were sterilized at 121°C and cooled at room temperature. Six mL pre-culture (3% volume) of the strain was added to the medium, and then cultured at 30±1°C in a shaker incubator (Jal Company, Tehran , Iran) with 200 rpm. To study growth kinetics of the bacteria, 10 mL of culture medium was harvested at definite intervals (2-4-6-8-12-16-20-24 hours after incubation). To achieve an appropriate level of bacterial concentration (OD≤ 1.2 at 600 nm), suspension containing the bacterial culture was diluted with Saline buffer, and the growth rate was measured via spectrophotometer at 600 nm (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). The quantity of spores (CFU) and total protein were measured after 72 hours according to Zouari *et al.* (2002).

### Optimization of Nitrogen Sources

Corn steep liquor, a byproduct of corn wet-milling, was used as nitrogen source for growth of the strain. Corn steep at 1, 2, and 3% of the media volume was added to the selected carbon source and mineral. Each treatment was repeated at three replicates. Evaluation of bacterial growth kinetic and spore and crystal production was performed as mentioned above. The sporulation time was investigated by Gram staining and microscopic observation.

### Sea Salt as an Alternative Source of Minerals

Sea salt (Sera marine salt, Sera company, Heinsberg, Germany) as an available and economic alternative minerals source was used. The salt was dissolved in water 30 g $L^{-1}$ (3%), and the performance of various dilutions, including two (0.3%), three (0.03%) and four (0.003%) times on growth and the spore and crystal production were evaluated comparing with the minerals commonly used in the *Bt* cultures. The selected carbon and nitrogen sources in previous experiments were used in the composition of the medium in these experiments. Bacterial growth kinetic study and quantification of spore and crystal production were performed according to the previous section.

### Fermenter Experiments

#### Culture Medium and Inoculum Preparation

The selected carbon and nitrogen sources in the previous experiments (experiments at Shaker incubator level) were mixed. The sea salt was separately dissolved at pH 2, and added to the mixture and the final pH was adjusted to 6.5. To prepare active pre-culture, 1 mL of culture of the strain (culture medium was the same) kept at -80°C was added to 5 mL of the selected culture medium, and was incubated for 10-12 hours at 30°C in a bio-shaker (model BR-3000LF, Japan) with 200 rpm. Then, 1.5 mL of active culture was added into 150 mL of culture medium, and incubated for 10-12 hours at 30°C in a bio-shaker (model BR-3000LF, Japan) with 200 rpm.

#### Fermentation Conditions

Fermentation process for the strain was performed in a two-liter batch Fermenter (BIOFLO 2000, New Brunswick Scientific, USA) by using 1.5 L volume selected culture medium. Each treatment was repeated three times. Fermentation was performed at optimum growth temperature, different pH values, percentages of inoculation (1-3%), and different amounts of oxygen (50, 70, and 90 % of saturation). To





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adjust oxygen concentration, automatic control was used to set the mixer speed in the range of 300-1,200 rpm and aeration rate 1VVM. Adjusting of pH was performed by automatic addition of 2 normal hydrochloric acid and NaOH solution. Sampling was performed in the sterile conditions at 2, 4, 6, 8, 12, 16, 20, 24, 48 and 72 hours after incubation. Finally, the samples (10 mL) were kept at -20°C for later analysis. The growth kinetic and the amount of spore and crystal for different factors were studied. Bacterial growth kinetics in the fermenter was performed with continuous and simultaneous measurement of optical density (OD) 660 medium by turbidity meter equipped with a fiber optic sensor (Turbidity transmitter, Trb 8300, Mettler-toledo), which was placed inside the vessel of the bioreactor. The quantity of spore and crystal production was performed as described in the previous sections.

### Spores and Crystals Characterization

To characterize spores and crystals produced during the experiments, one mL of culture was poured in 1.5 mL vials, the culture was centrifuged at 3,000 rpm for 5 minutes, and supernatant was discarded. Pellet was suspended in one mL 0.1M NaCl and again centrifuged at 3,000 rpm for 5 minutes. This mixture was finally suspended in 200 µL of distilled water and was kept at -20°C. Fifty µL of spore-crystal mixture were colored by Gram staining method (Liu and Bajpai, 1995; Liu and Tzeng, 2000.) and observed by Nikon microscope with magnification of 100.

## RESULTS

### Optimization temperature and pH

Temperature and pH optimization experiments at Erlen Mayer scales showed that at the pH: 6.5 (Figure 1-a) and 30°C (Figure 1-b), the maximum *Bt* biomass was achieved in PGSM medium.

### Carbon Source Optimization

The maximum growth and spore ($5.2\times10^9$ $mL^{-1}$) and crystal production (750 mg $L^{-1}$) was observed in the medium containing sucrose (molasses), and surprisingly showed lower growth and spore/crystal production in glucose and hydrolyzed starch, respectively (Figures 2 and 3). The minimum growth and spore ($2.3\times10^9$ $mL^{-1}$) and crystal production (170 mg $L^{-1}$) of the strain was occurred in UF permeates, which shows that the strain could not use lactose as carbon source (Figures

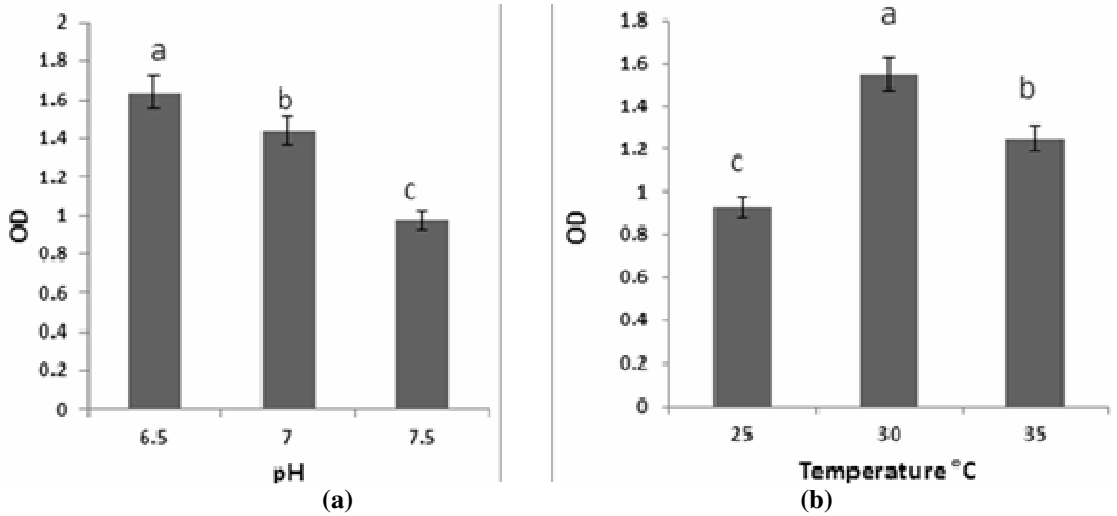


**Figure 1.** KH4 growth at different pH (a) and temperatures (b). Optical density (OD).





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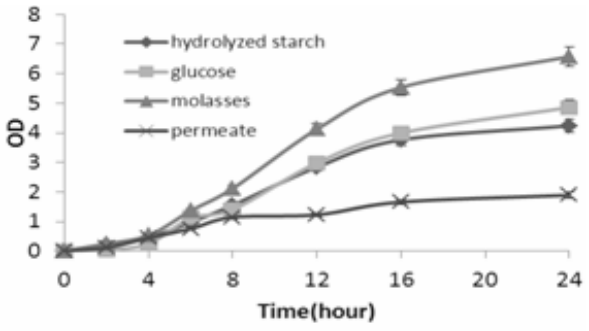
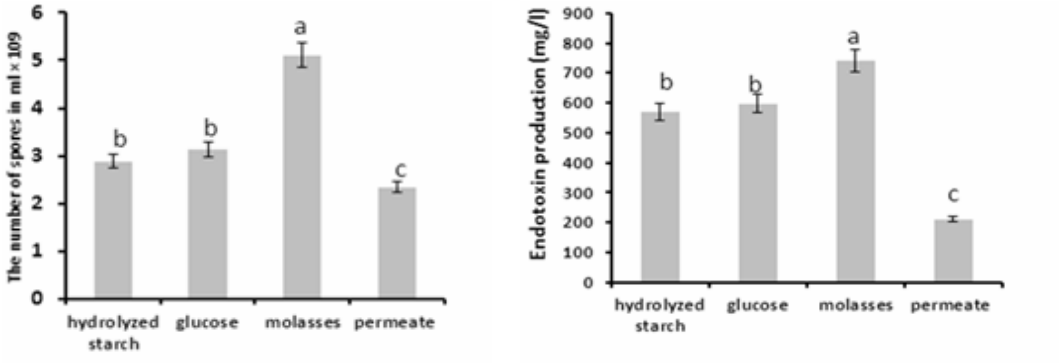



**Figure 2.** Growth kinetics of KH4 in different carbon sources.




(a) (b)

**Figure 3.** KH4 spores (a) and endotoxin (b) production in different carbon sources.

2 and 3). The highest production of spores and δ-endotoxin were quite consistent with growth kinetics and the best carbon source was molasses.

The maximum spore and crystal production was observed when volume of molasses in the medium was about 2%. It was shown that by increasing concentration of carbon source, spore and δ-endotoxin production was declined (Figure 4).

## Nitrogen Source Optimization

Kinetics of bacterial growth in the medium containing corn steep showed that the strain was well able to use corn steep as a nitrogen source, and surprisingly, the strain growth was higher than that of yeast extract (Figure 5). The reason for this may be that corn steep is rich in nitrogen sources such as soluble amino acids and proteins (Liggett and Koffler, 1948).

It was shown that the strain had similar spores and δ-endotoxin production in the media containing corn steep and yeast extract, and therefore, significant difference was not observed ($P \leq 0.05$, Table 1). Also, according to the influence of concentration of nitrogen sources on growth and production of crystals and spores, the concentration of corn steep was evaluated on the mentioned factors (Figure 6). The optimum concentration of corn steep was 3 percent.

## Mineral Resource Optimization Using Sea Salt

Molasses (2% sucrose) and corn steep (3%) were used as carbon and nitrogen sources, respectively. To evaluate the sea salt efficiency on the strain growth, growth kinetics was measured in different sea salt concentrations (Figure 7). It was observed that 4-fold dilution of sea salt showed the highest rate of KH4 growth. Surprisingly, the strain showed different growth in sea salt in comparison to standard mineral salts (Figure





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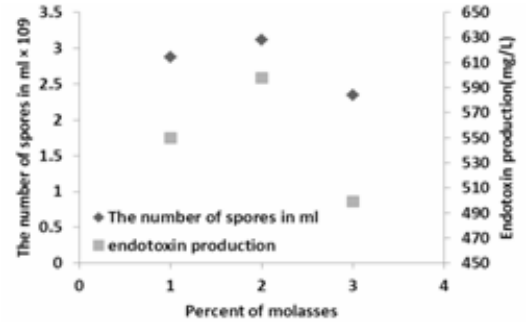


**Figure 4.** The effect of carbon sources concentration on spore and endotoxin production of KH4.

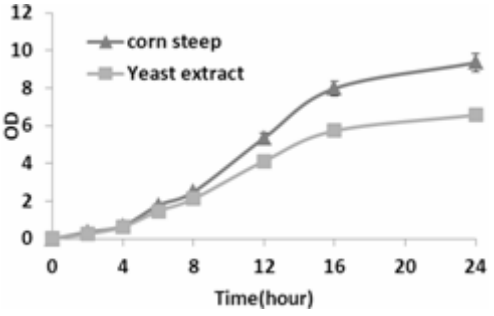


**Figure 5.** Comparing KH4 growth kinetics in corn steep and yeast extract as nitrogen sources.

**Table 1.** Spore and δ-endotoxin production by KH4 in the medium containing corn steep as a nitrogen source.

| Nitrogen Source | Spores $mL^{-1}$ | δ-Endotoxin (mg $L^{-1}$) |
|---|---|---|
| Corn steep liquor | $5.3\times10^{9}\pm0.2$ | 750 ± 25 |
| Yeast extract | $5.0\times10^{9}\pm0.2$ | 710± 20 |

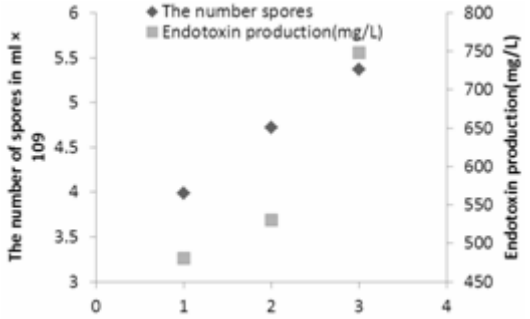


**Figure 6**. The effect of corn steep on KH4 spores (a) and endotoxin (b) production.

7). The amount of δ-endotoxin and spore production of KH4 in medium containing sea salt and mineral salts was not significantly different (P≤ 0.05, Table 2).

## Optimization of Preculture Volume and pH at Fermenter Conditions

Three different volumes of bacterial inoculations, including 1, 2 and 3 % of fermenter medium were used to evaluate the effect of preculture volume on growth kinetics. It was found that by increasing percentage of inoculation, lag phase decreased and growth rate was increased (P≤ 0.05, Figure 8). By increasing the volume of preculture from 1 to 2%, the quantity of spore and crystals were significantly increased, whereas, increasing from 2 to 3% did not show significant effect on spore and crystal production (P≤ 0.05, Figure 9). Therefore, the amount of 2% was selected as preculture volume.

To optimize pH in fermenter conditions, it was automatically regulated in the range of 6.5, 7 and 7.5, and its impact on growth kinetics of the strain was investigated. The optimum pH for KH4 strain was 6.5, respectively (Figure 10). It was shown that the maximum growth kinetics and also spore and crystal production were observed at the mentioned optimum pH for the strain (Figure 11). These results were





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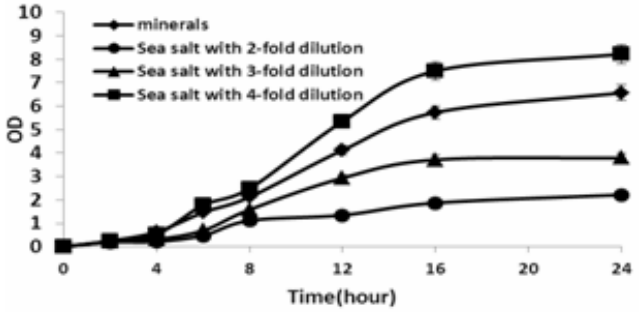


**Figure 7**. KH4 growth kinetics in sea salt as a mineral source.

**Table 2.** Spore and δ-endotoxin production by KH4 in the medium containing sea salt as mineral source.

| Mineral source | Spores $mL^{-1}$ | δ-Endotoxin (mg $L^{-1}$) |
|---|---|---|
| Sea salt | 5.2×109±0.2 | 740 ± 30 |
| Minerals | 5.0×109±0.2 | 720± 25 |

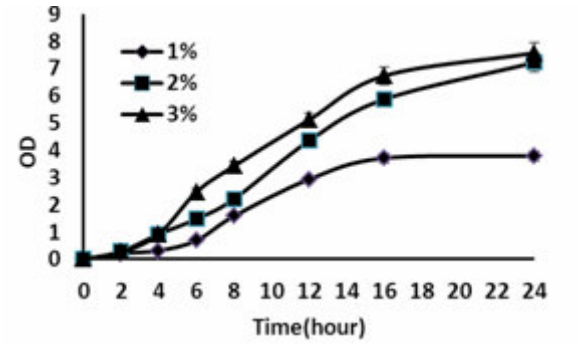


**Figure 8.** The effect of preculture volumes on growth kinetics of KH4 at fermenter conditions.

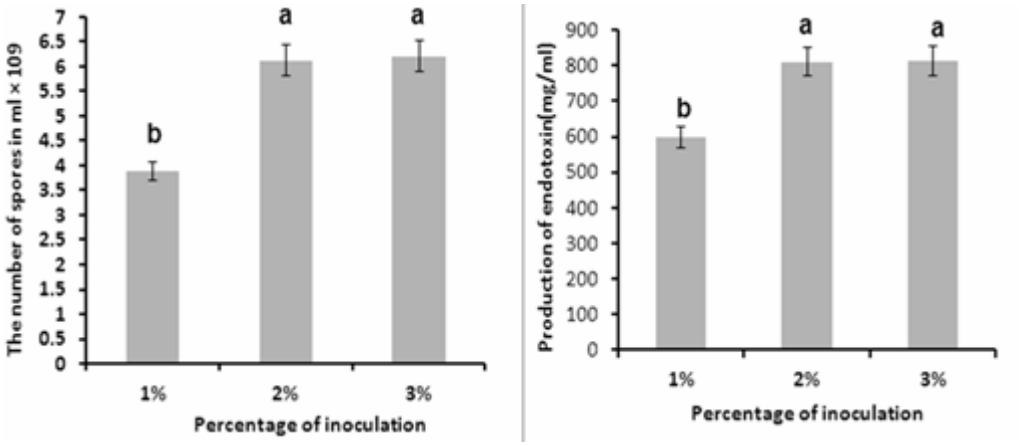


**Figure 9.** The effect of preculture volumes on spore (a) and endotoxin (b) production in KH4 after 72 hours incubation.

consistent with the results of the incubator experiments. (Figure 1). Furthermore, monitoring of trend of pH change during different growth phases showed a significant pH decrease and increase at the beginning and end of logarithmic phase, respectively.





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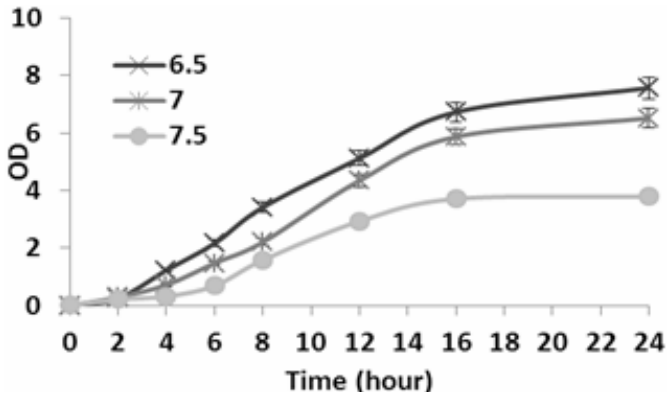
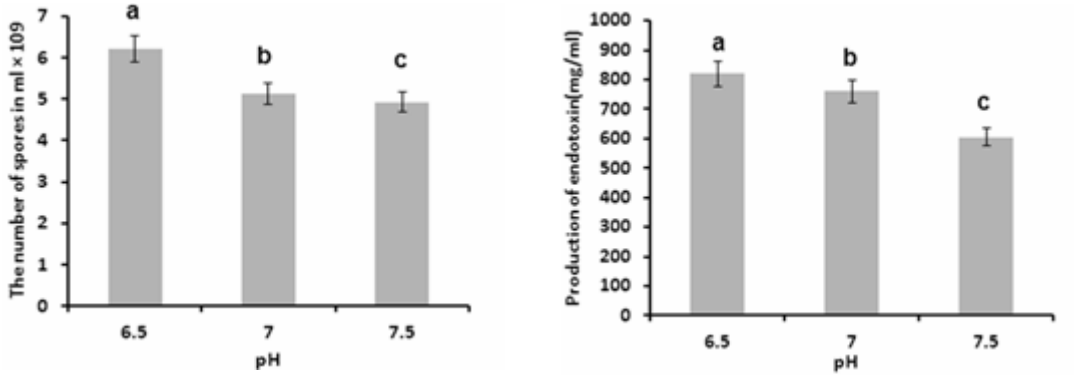



**Figure 10.** The effect of different pH levels on KH4 growth kinetics at fermenter conditions.




(a) (b)

**Figure 11.** The effect of pH on spore (a) and endotoxin (b) production by KH4 after 72 hours.

### Optimization of Oxygen Concentration

To optimize the oxygen concentration, the percentage of dissolved oxygen during the process was controlled by adjusting stirring speed and keeping the intensity of aeration constant during fermentation. Oxygen consumption pattern showed that by increasing fermentation period, the oxygen demand was increased, and during 4, 8 and 12 hours after inoculation, the concentration of dissolved oxygen was 50, 70, and 90%, respectively (Figure 12). By increasing oxygen concentration, the bacterial growth and the spores and crystal production was increased (Figure 13). The results showed that the effect of oxygen depended on the type of culture medium and strain, and also the level of oxygen consumption during the production of spores and δ-endotoxin was reduced. According to the results, oxygen concentration of 70% was recommended for the studied strain.

## DISCUSSION

Bioprocess engineering, including optimization of growth conditions and also finding economic and locally available media is known as one of the most important stages for the commercial production of *Bt* bioproducts (Brar *et al.,* 2005a, 2005b; Ouhib *et al.,* 2006; Shojaaddini *et al.,* 2010). Therefore, the present study was devoted to the design of a low cost and effective bioprocess for mass production of a native selected strain (KH4). The temperature and pH optimization experiments at incubator and bioreactor scales showed that the optimum temperature and pH for the strain was in the range of other previously isolated and introduced strains of *Bt* (Amin *et al.,*





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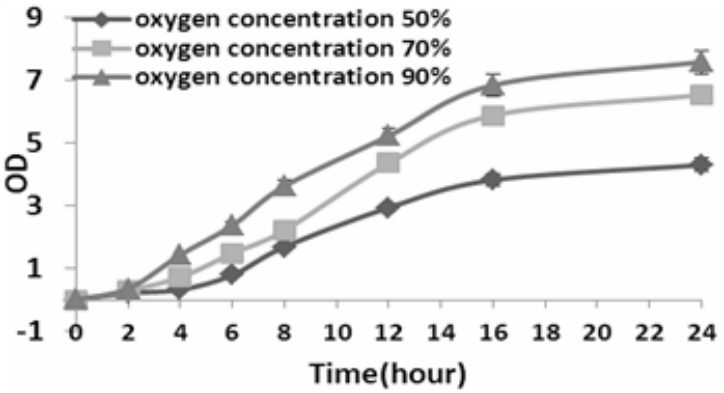
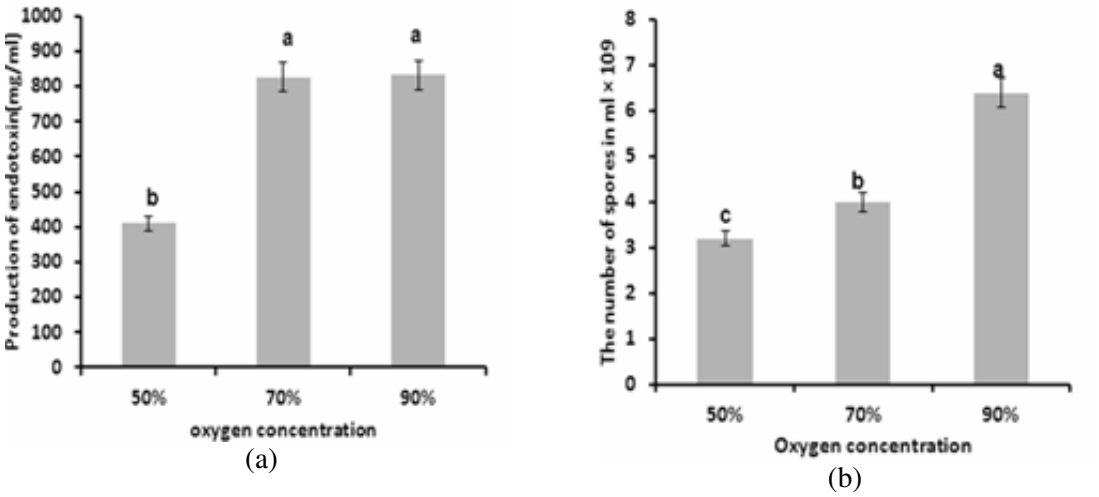



**Figure 12.** The effect of oxygen concentration on KH4 growth kinetics at fermenter conditions.




(a) (b)

**Figure 13**. The effects of oxygen concentration on KH4 spore (a) and endotoxin (b) production.

2008; Anderson and Jayaraman, 2003; Berbert-Molina *et al.,* 2008b; Brar *et al.,* 2005a). The strain showed different responses to different carbon sources, and its maximum and minimum growth and spore/crystal production were observed in the medium containing sucrose (molasses) and UF permeates, respectively. These results indicated that the strain was capable to produce hydrolytic enzymes with high activity, and could use various carbon sources, including monosaccharide, disaccharide and polysaccharide for the growth and proliferation (Avignone-Rossa *et al.,* 1992; Avignone-Rossa and Mignone, 1995). Review of similar studies indicate that, in the early stages of many optimization tests, glucose and yeast extract have been used as carbon and nitrogen sources, respectively. But, mass production seeks the use of cheaper sources, and extensive studies have been done to develop alternative sources (Amin *et al.,* 2008). For example, Ghribi *et al* (2007a, 2007b) used soybean and starch instead of the two mentioned sources, and showed that 15 g $L^{-1}$ starch and 30 g $L^{-1}$ soybeans could produce significant amounts of live cells, spores, and protein crystals.

Δ-endotoxin and spore production in the strain was influenced by the concentration of carbon source, and the maximum efficiency was observed when volume of molasses in the medium was about 2%. It was shown that by increasing concentration of carbon source, spore and δ-endotoxin production declined. Other studies showed that this effect varies according to different carbon sources. For example, high concentration of glucose and gruel caused limitations in δ-





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endotoxin production, but, when Ghribi *et al.* (2007a) used starch as a carbon source; high concentrations of it caused no limitation in the production of delta δ-endotoxin. Berbert-Molina *et al.* (2008a; 2008b) showed that the concentration of carbon source (glucose) highly affected the growth kinetics and spore and crystal production rate, and glucose concentration more than 75 g $L^{-1}$ inhibited the growth and spore and crystal production. Also, the various sources of carbon such as starch, glucose, glycerol, lactose, maltose, sucrose, flour, millet flour, sugar and carbohydrates from urban and food industry waste waters have been used in other researches for optimization of producing *Bt*, spores, toxin, and evaluation of production of various enzymes (Adjalle *et al.,* 2007; Amin *et al.,* 2008; Avignone-Rossa *et al.,* 1992; Khodair *et al.,* 2008; Kraemer-Schafhalter and Moser, 1996; Ouhib *et al.,* 2006; Rao *et al.,* 2007). Chang *et al.* (2008) used starch processing wastewater (SPW) as a new medium for production of *Bt*. They claimed that SPW was a cheap and highly available in China and, therefore, they introduced this material as new source. Shojaaddini *et al.* (2010) used food barley flour as carbon source for production of two *Bt* strains, and showed effective mass production of the studied strains. The food barley flour is available in Iran, but it is expensive in comparison to wastes used in the current study.

Kinetics of bacterial growth in medium containing corn steep showed that the strain was well able to use corn steep as a nitrogen source, and surprisingly, the strain growth was higher than that of yeast extract. This may be because corn steep is rich in nitrogen sources such as soluble amino acids and proteins (Liggett and Koffler, 1948). Liu and Bajpai (1995) reported similar results, and introduced corn steep as a suitable source for nitrogen. Prabakaran and Hoti (2008) showed that the presence of amino nitrogen in the composition of nitrogen source increase the spore and crystal production and also toxicity against pests. Our primary analysis showed that corn steep contained between 1-3% amino nitrogen which may increase the spore and crystal production in the studied strains. Valicente and Mourao (2008) achieved the highest amount of spores and protein crystals using 1% corn sugar as carbon source, and 3% soybean meal as nitrogen source. Peptone, soybean meal, and soy protein have been evaluated as nitrogen sources in production of *Bt* (Rao *et al.,* 2007), but soybean is not widely cultivated in Iran and, therefore, corn steep seems to be a more appropriate resource.

The results of the present study showed that sea salt can be used as an alternative mineral source, because the rate of growth and δ-endotoxin/spore production in the media containing 0.003% of sea salt was similar to the medium containing the commonly used and expensive mineral salts. Ghribi *et al.* (2007b) have also shown similar results and high performance of sea salt in bacterial growth and toxin production.

The experiments at bioreactor scale confirmed the results of the experiments at incubator level on optimum temperature and pH. It was shown that the optimum preculture concentration was 2%, and by increasing percentage of inoculation, lag phase decreased and growth rate increased. These results were expected because, generally, increasing the number of starter cells in preculture, cause shorter lag phase and faster achieving maximum biomass. Several reports have emphasized the important impact of environmental factors such as percentage of inoculum in the production of spores and crystals (Kraemer-Schafhalter and Moser, 1996; Yezza *et al.,* 2005).

Oxygen consumption pattern showed that by increasing fermentation period the oxygen demand was increased, and by increasing oxygen concentration, the bacterial growth and the spores and crystal production was increased. These results were in accordance with those of previous studies (Avignone-Rossa and Mignone, 1995; Huang *et al.,* 2007; Kraemer-Schafhalter and Moser, 1996). Ghribi *et al.*





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(2007a, 2007b) which showed that the rate of oxygen saturation during the process had important effect on cell growth and final production of spore and crystal, and depending on the content of the medium, the optimum amount of oxygen was different. Thus, the optimum aeration rate during the process was 60 and 70% for the media containing glucose and starch as carbon source, respectively. Also, Wu *et al.* (2002) showed that the change in the rate of stirring and aeration had a very strong effect on the amount of exotoxin production in the *Bt* bacterium in fermenter conditions. The results of the present study also show that the effect of oxygen depended on the type of culture medium and strain, and oxygen consumption during the production of spores and δ-endotoxin was reduced.

In conclusion, in the present study, the growth condition of the *Bt* strain, including temperature, pH, oxygen demand, and preculture volume, was optimized and a new commercial fermentation medium containing cheap and available carbon, nitrogen, and mineral sources was developed for mass production of the *Bt* strain. These results will promote the commercialization of this coleopteran-effective *Bt* strain.

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## REFERENCES

1. Adjalle, K. D., Brar, S. K., Verma, M., Tyagi, R. D., Valero, J. R. and Surampalli, R. Y. 2007. Ultrafiltration Recovery of Entomotoxicity from Supernatant of *Bacillus thuringiensis* Fermented Wastewater and Wastewater Sludge. *Process. Biochem.,* **42:** 1302-1311.
2. Amin, G., Alotaibi, S., Narmen, A. Y. and Saleh, W. D. 2008. Optimization of a Fermentation Process for Bioinsecticide Production by *Bacillus thuringiensis. World J. Microbiol. Biotechnol.,* **24:** 2465-2471.
3. Anderson, R. K. I. and Jayaraman, K. 2003. Influence of Carbon and Nitrogen Sources on the Growth and Sporulation of *Bacillus thuringiensis var. galleriae* for Biopesticide Production. *Chem. Biochem. Eng.,* **17:** 225-232.
4. Avignone-Rossa, C., Arcas, J. and Mignone, C. 1992. *Bacillus thuringiensis* Growth, Sporulation and δ-Endotoxin Production in Oxygen Limited and Non-limited Cultures. *World J. Microbiol. Biotechnol.*, **8:** 301-304.
5. Avignone-Rossa, C. and Mignone C. F. 1995. *Bacillus thuringiensis* Growth and Toxicity. *Mol. Biotechnol.*, **4:** 55-71.
6. Berbert-Molina, M. A., Prata, A. M. R., Pessanha, L. G. and Silveira, M. M. 2008a. Kinetics of *Bacillus thuringiensis var. israelensis* Growth on High Glucose Concentrations. *J. Ind. Microbiol. Biotechnol.* 35:1397–1404
7. Berbert-Molina, M. A., Prata, A. M. R., Pessanha, L. G. and Silveira, M. M. 2008b. Kinetics of *Bacillus thuringiensis* var. *israelensis* Growth on High Glucose Concentrations. *J. Ind. Microbiol. Biotechnol.*, **35:** 1397-1404.
8. Brar, S. K., Verma, M., Barnabéa, S. and Tyagi, R. D. 2005a. Impact of Tween 80 during *Bacillus thuringiensis* Fermentation of Wastewater Sludges. *Process. Biochem.,* **40:** 2695-2705.
9. Brar, S. K., Verma, M., Tyagi, R. D., Valéro, J. R. and Surampalli, R. Y. 2005b. Starch Industry Wastewater-based Stable *Bacillus thuringiensis* Liquid Formulations. *J. Econom. Entomol.,* **98:** 1890-1898.
10. Brar, S. K., Verma, M., Tyagi, R. D., Valéro, J. R. and Surampalli, R.Y. 2005c. Sludge Based *Bacillus thuringiensis* Biopesticides: Viscosity Impacts. *Water Res.,* **39:** 3001-3011.
11. Brownbridge, M. and Margalit, J. 1986. New *Bacillus thuringiensis* Strains Isolated in Israel Are Highly Toxic to Mosquito Larvae. *J. Invertebr. Pathol.* **48:** 216–222.
12. Chang, M., Zhou, S. G., Lu, N. and Ni, J. R. 2008. Starch Processing Wastewater as a New Medium for Production of *Bacillus*





[ DOR: 20.1001.1.16807073.2015.17.5.13.2 ]

*thuringiensis*. *World J. Microbiol. Biotechnol.,* **24:** 441–447.
13. Ghribi, D., Zouari, N., Trabelsi, H. and Jaoua, S. 2007a. Improvement of *Bacillus thuringiensis* δ-Endotoxin Production by Overcome of Carbon Catabolite Repression through Adequate Control of Aeration. *Enzyme Microbial. Technol.*, **40:** 614–622.
14. Ghribi, D., Zouari, N., Trigui, W. and Jaoua, S. 2007b. Use of Sea Water as Salts Source in Starch and Soya Bean-based Media, for the Production of *Bacillus thuringiensis* Bioinsecticides. *Process. Biochem.*, **42:** 374-378.
15. Huang, K., Badger, M., Haney, K. and Evans, S. L. 2007. Large Scale Production of *Bacillus thuringiensis* PS149B1 Insecticidal Proteins Cry34Ab1 and Cry35Ab1 from *Pseudomonas fluorescens*. *Protein Expr. Purif.,* **53:** 325-330.
16. Keshavarzi, M., Salimi, H., Mirzanamadi, F. 2005. Biochemical and Physical Requirements of *Bacillus thuringiensis* subsp. Kurstaki for High Biomass Yield Production. *J. Agric. Sci. Technol.* **7**: 41-47.
17. Khojan, S., Keshavarzi, M., Zargari, K., Abdolahi, H. and Rouzbeh, F. 2013. Presence of Multiple *Cry* Genes in *Bacillus thuringiensis* Isolated from Dead Cotton Bollworm *Heliothis armigera*. Production. *J. Agric. Sci. Technol.,* **15:** 1285-1295.
18. Khodair, T. A., Abdelhafez, A. A. M., Sakr, H. M. and Ibrahim M. M. M. 2008. Improvement of *Bacillus thuringiensis* Bioinsecticide Production by Fed-batch Culture on Low Cost Effective Medium. *Res. J. Agri. Biol. Sci.,* **4:** 923-935.
19. Kraemer-Schafhalter, A. and Moser, A. 1996. Kinetic Study of *Bacillus thuringiensis* var. *israelensis* in Lab-scale Batch Process, *Bioproc. Biosys. Eng*. 14(3): 139-144.
20. Liggett, R. W. and Koffler, H. 1948. Corn Steep Liquor in Microbiology. *Microbiol. Mol. Biol. Rev.*, **12:** 297.
21. Liu, W. M. and Bajpai, R. K. 1995. A Modified Growth Medium for *Bacillus thuringiensis*. *Biotechnol. Progress*., **11:** 589-591.
22. Liu, B. L. and Tzeng, Y. M. 2000. Characterization Study of the Sporulation Kinetics of *Bacillus thuringiensis. Biotechnol. Bioeng.,* **68:** 11-17.
23. Nazarian Amirani, A., Jahangiri, R., Salehi Jouzani, Gh., Seifinejad, A., Soheilivand, S., Bagheri, O., Keshavarzi, M. and Alamisaeid, K. 2009. Coleopteran-specific and Putative Novel *Cry* Genes in Iranian Native *Bacillus thuringiensis* Collection. *J. Inverteb. Pathol.,* **102:** 101–109.
24. Ouhib, O., Clavel, T. and Schmitt, P. 2006. The Production of *Bacillus cereus* Enterotoxins is Influenced by Carbohydrate and Growth Rate. *Curr. Microbiol.*, **53:** 222-226.
25. Prabakaran, G. and Hoti, S. L. 2008. Influence of Amino Nitrogen in the Culture Medium Enhances the Production of δ-Endotoxin and Biomass of *B. thuringiensis var. israelensis* for the Large-scale Production of the Mosquito Control Agent. *J. Ind. Microbiol. Biotechnol.*, **35:** 961–965.
26. Prabakaran, G., Hoti, S. L. and Paily, K. P. 2009. Development of Cost-effective Medium for the Large-scale Production of a Mosquito Pupicidal Metabolite from *Pseudomonas fluorescens* Migula. *Biol. Control*., **48:** 264-266.
27. Rao, Y. K., Tsay, K. J., Wu, W. S. and Tzeng, Y. M. 2007. Medium Optimization of Carbon and Nitrogen Sources for the Production of Spores from *Bacillus amyloliquefaciens* B128 Using Response Surface Methodology, *Process. Biochem.,* **42:** 535-541.
28. Salehi Jouzani, G., Pourjan Abad, A., Seifinejad, A., Marzban, R., Kariman, K. and Maleki, B. 2008a. Distribution and Diversity of Dipteran-specific *Cry* and *Cyt* genes in Native *Bacillus thuringiensis* Strains Obtained from Different Ecosystems of Iran. *J. Ind. Microbiol. Biotechnol.,* **35:** 83-94.
29. Salehi Jouzani, G., Seifinejad, A., Saeedizadeh, A., Nazarian, A., Yousefloo, M., Soheilivand, S., Mousivand, M., Jahangiri, R., Yazdani, M., Maali Amiri, R. and Akbari, S. 2008b. Molecular Detection of Nematicidal Crystalliferous *Bacillus thuringiensis* Strains of Iran and Evaluation of Their Toxicity on Free Living and Plant Parasitic .ematodes. *Can. J. Microbiol.,* **54:** 812–822.
30. Seifinejad, A., Salehi Jouzani, G., Hosseinzadeh, A. and Abdmishani, C. 2008. Characterization of Lepidoptera-active *Cry* and *Vip* Genes in Iranian *Bacillus thuringiensis* Strain Collection. *J. Biol. Control.*, **44:** 216–226.





[ DOR: 20.1001.1.16807073.2015.17.5.13.2 ]

31. Shojaaddini, M., Moharramipour, S., Khodabandeh, M. and Talebi, A. A. 2010. Development of a Cost Effective Medium for Production of *Bacillus thuringiensis* Bioinsecticide Using Food Barley. *J. Plant Protec. Res.,* **50:** 9-14.
32. Valicente, F. H. and Mourao, A. H. C. 2008. Use of By-products Rich in Carbon and Nitrogen as a Nutrient Source to Produce *Bacillus thuringiensis* (Berliner)-based Biopesticide. *Neotrop. Entomol.*, **37:** 702-708.
33. Wu, W. T., Hsu, Y. L., Ko, Y. F. and Yao, L. L. 2002. Effect of Shear Stress on Cultivation of *Bacillus thuringiensis* for Thuringiensin Production. *Appl. Microbiol. Biotechnol.*, **58:** 175–177
34. Yezza, A., Tyagi, R. D., Valero, J. R. and Surampalli, R. Y. 2005. Production of *Bacillus thuringiensis*-based Biopesticides in Batch and Fed Batch Cultures Using Wastewater Sludge as a Raw Material. *J. Chem. Technol. Biotechnol.*, **80:** 502–510
35. Zouari, N. and Jaoua, S. 1999. The Effect of Complex Carbon and Nitrogen, Salt, Tween-80 and Acetate on Delta-endotoxin Production by a *Bacillus thuringiensis* subsp Kurstaki. *J. Ind. Microbiol. Biotechnol.,* **23:** 497–502.
36. Zouari, N., Achour, O. and Jaoua S. 2002. Production of Delta-endotoxin by *Bacillus thuringiensis* subsp Kurstaki and Overcoming of Catabolite Repression by Using Highly Concentrated Gruel and Fish Meal Media in 2- and 20-dm 3 Fermenters. *J. Chem. Technol. Biotechnol.*, **77:** 877-882.

## بهینه سازی فرایند تولید ارزان یک سویه بومی باکتری *Bacillus thuringiensis* موثر بر آفات سخت بالپوش

**غ. صالحی جوزانی، س. عباسعلی‌زاده، م. ف. مرادعلی، ح. مرسلی**

### چکیده

یکی از معضلات اصلی تولید باکتری بی تی به عنوان یک سم بیولوژیک، بالا بودن هزینه فرایند تولید و فرمنتاسیون این باکتری می باشد. لذا هدف از اجرای تحقیق حاضر بهینه سازی شرایط رشد و توسعه روش ارزان قیمت مبتنی بر پسماندهای کشاورزی برای تولید یک سویه بومی موثر بر آفات سخت بالپوش (KH4) در شرایط انکوباتور و فرمانتور بود. آزمایشات اولیه نشان داد که اسیدیته مطلوب برای سویه مذکور ۶/۵ و دمای مناسب آن ۳۰ درجه سلسیوس بود. آزمایشات در شرایط انکوباتور نشان داد که بهترین رشد باکتری و تولید اسپور/کریستال در محیط کشت حاوی ملاس ۲٪ (به عنوان منبع کربن) و همچنین آب استیپ ذرت (Corn steep) ۳٪ (به عنوان منبع ازت) بدست می آید. همچنین غلظت های مختلف نمک دریا به عنوان منبع ارزان قیمت و قابل دسترس جایگزین نمک های معدنی استفاده شد. بالاترین نرخ رشد باکتری با غلظت ۰/۰۰۳٪ نمک دریا بدست آمد. آزمایشات در فرمانتور ناپیوسته نشان داد که با استفاده از تلقیح اولیه ۲٪ باکتری بهترین نتیجه بدست می آید. نتایج نشان دادکه در شروع فاز لگاریتمی میزان اسیدیته به شدت کاهش می یابد، درحالیکه این میزان در پایان فاز مذکور افزایش یافت. با افزایش دوره فرمنتاسیون، میزان نیاز اکسیژن بطور معنی داری افزایش یافت.





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ضمنا با افزایش غلظت اکسیژن تا ۷۰٪ اشباع، میزان رشد و تولید اسپور/کریستال افزایش یافت. بر اساس نتایج بدست آمده، شرایط رشد و همچنین محیط کشت اقتصادی و قابل دسترس برای تولید انبوه سویه مذکور در شرایط تولید ناپیوسته مطلوب سازی شد.



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