

DEVELOPMENT OF A COST EFFECTIVE MEDIUM FOR PRODUCTION OF *BACILLUS THURINGIENSIS* BIOINSECTICIDE USING FOOD BARLEY

Mohammad Shojaaddini¹, Saeid Moharramipour^{1*}, Mahvash Khodabandeh², Ali Asghar Talebi¹

¹Department of Entomology, Faculty of Agriculture, Tarbiat Modares University, Tehran, P.O. Box 14115-336, Iran

²National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

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Abstract: Genetically manipulated food barley, *Hordeum vulgare* L. var. *valfajr*, was compared to commercial-grade starch as carbon sources in groundnut and soybean based media for supporting growth, sporulation and delta-endotoxin production by *Bacillus thuringiensis* Berliner *B. thuringiensis* kurstaki HD-1 and a promising newly isolated *B. thuringiensis* strain (referred to as BTA) were used. MgCl₂, CaCl₂, and MnCl₂ were used as trace-elements. Culture media were compared in shaken flasks and then in 5 l Fermentors. Biomass, delta-endotoxin levels and the number of spores as colony forming units (CFU) were evaluated. For each of the two strains, biomass and delta-endotoxin synthesis were not significantly different in soybean-based media after substitution starch by barley flour. There were significant differences between the two strains with respect to biomass and toxin production. Evaluated costs of media preparation showed that food barley is an economical alternative to commercial-grade starch in the production of BTA bioinsecticide.

Key words: food barley, culture media, *Bacillus thuringiensis*, bioinsecticide

INTRODUCTION

Over the past century, the insecticidal bacterium *Bacillus thuringiensis* Berliner proved to be an interesting minor pathogen of caterpillars, thus being an environmentally safe insecticide for controlling pests of major crops and vectors of human diseases. *B. thuringiensis* bioinsecticides are composed of mixture of spores and crystals harvested from production media. Spores and crystals of *B. thuringiensis* are readily produced by aerated liquid fermentation. They are easily harvested and, when properly formulated, have a long shelf life (Zouari *et al.* 2002; Prabakaran and Balaraman 2006; Jing-Wen *et al.* 2007). At present the cost of *B. thuringiensis* production using existing fermentation technology is high because of a high cost of the production medium. Less expensive media for culturing of *B. thuringiensis* will facilitate production of biopesticides by utilization of cost effective alternative agricultural products. Fermentation studies showed that large-scale production of *B. thuringiensis* is possible at a relatively competitive cost. Although progressive development of *B. thuringiensis* as a bioinsecticide is evident, key solutions may lead to further commercialization and development of this bacterium as an environmentally safe insecticide. A high cost of *B. thuringiensis* products is due to production being mostly located in developed countries where production costs are higher, and also expenses paid in transportation to other countries. Thus, local production could significantly reduce costs of pest control and help with the development and improvement

of local fermentation industries, besides the utilization of more economic agricultural products.

In order to reduce production costs, many raw materials and cheap byproducts were tested and used. Media containing fishmeal, and corn-steep liquor for production of *B. thuringiensis* were reported (Saalma 1983; Kumar *et al.* 2000). Media containing molasses, corn extract, and mineral salts (Dregval *et al.* 1999), corn steep liquor, glucose, and mineral salts (Luna 2004), glucose, corn extract, sodium humate, and mineral salts (Dregval *et al.* 2002), or potato, common sugar, and Bengal gram (*Cicer arietinum* L.) (Poopathi and Kumar 2003) were all used for growth and production of *B. thuringiensis*. Powders of edible leguminous seeds and mung bean *Vigna radiata* (L.) R. Wilczek were used as major sources of protein together with different combinations of soluble starch and/or sugarcane molasses as major carbohydrate sources for the production of delta-endotoxin (Mummigatti and Raghunathan 1990). Similarly, soybean flour, groundnut cake powder and, wheat bran extract (*Triticum aestivum* L.) were separately used in large-scale production of *B. thuringiensis* bioinsecticide (Prabakaran and Balaraman 2006).

Barley (*Hordeum vulgare* L.) grain is used in malt production and as a source of food for humans and animals. Hull-less or naked barley is a genetically improved variety that allows easier removal of a hull. It was investigated as whole grain for several potential new applications (Helm and Francisco 2004). In Iran, barley is cultivated on 1.83 million hectares of land under diverse and vari-

*Corresponding address:
moharami@modares.ac.ir

able agro-climatic conditions. It ranks second among the cultivated crops in terms of hectares and is produced to approximately three million tones per year. Barley is primarily produced and used as animal feed in Iran, it is also consumed as malt, food in the form of "barley soup" and ma-ol shaer (non-alcoholic beer) (Grando and Macpherson 2008). Food barley can play an important role in the sustainability of agricultural production systems in Iran. The goal of the present work was to develop fermentation media using food barley rather than commercial-grade starch for low-cost production of bioinsecticide by a newly isolated *B. thuringiensis* strain with suitable characteristics for the control of pest caterpillars.

MATERIALS AND METHODS

B. thuringiensis strain

A new *B. thuringiensis* aizawai strain, having certain promising characteristics in controlling pest caterpillars (data not published) was used. It was recently isolated by Shojaaddini from a wild population of Indian meal moth, *Plodia interpunctella* Hübner. *B. thuringiensis* kurstaki HD-1, kindly provided by Dr. J-F Cherles (Pasteur Institute, Paris, France), was used as reference strain. The bacterial strains were grown on nutrient agar medium (Merck KGaA, Darmstadt, Germany) composed of 5 g/l meat peptone, 3 g/l meat extract and, 12 g/l agar and then stored at 4°C.

Media preparation

Hull-less grains of food barley (*Hordeum vulgare* var. *valfajr*, facultative type) and commercial soybean cake (*Glycine max*), containing 46% proteins, were obtained from Sobhan food-industry, Tehran, Iran. Commercial-grade starch, (Nemooneh Co., Karaj, Tehran) and groundnut (*Arachis hypogea* L.) were purchased from the Iranian market. All materials were finely grinded and all media were prepared at 2.5% (w/v). Medium "A" consisted of soybean flour and starch, "B" consisted of soybean flour and food barley flour, "C" consisted of groundnut powder and starch and, "D" consisted of groundnut powder and food barley flour. Ingredients of media "B" and "C" were soaked in distilled water for 1 h. The ingredients of all media were dissolved in distilled water and filtered through a plastic strainer (mesh size 100 µm) to remove all the insoluble solid particles. To each liter of the above media, 10 ml of stock salt solution (20.3 g MgCl₂, 10.2 g CaCl₂, and 1.0 g MnCl₂/l of distilled water) was added, according to the method by Prabakaran and Balaraman (2006). The pH of the medium was adjusted to 7.0 prior to inoculation.

Bioinsecticide production in shaken flasks

Inocula were prepared by transferring cells from nutrient agar media into 10 ml of nutrient broth liquid media (Merck KGaA, Darmstadt, Germany) containing 5 g/l meat peptone and 3 g/l meat extract. After 12 h of incubation at 30°C in a shaker-incubator at 200 rpm, optical densities were determined and equally adjusted for all four different media. Culture broths were then used to inoculate 1 l erlenmeyer flasks, containing 250 ml of the above

four culture media. These flasks were then incubated for 72 h at 30°C in a shaker-incubator at 200 rpm.

Seed culture preparation

The seed culture was produced using shaken flasks. First-stage seed culture was prepared by inoculating 10 ml of nutrient broth in a test tube with one loop-full of strain and incubating the tube in a shaker-incubator at 30°C, 200 rpm for a period of 10 h. Second-stage seed culture was prepared by transferring 10 ml of first stage seed into 190 ml of the same medium in a 1 l erlenmeyer flask and incubating in a shaker-incubator at 30°C, 200 rpm for a period of 10 h.

Fermentation procedure

Two media were selected for the purpose of bioinsecticide production in the fermentor. Actively growing second stage seed cultures were used to inoculate the fermentors at 10% (v/v) and optical density (OD) of 0.4. The fermentor used in this study (Tryton, Pierre Guerin, France) had a working volume of 5 l and controlled the fermentation variables automatically. The medium (2 l) was sterilized *in situ* and the fermentation process was then started in batch mode with the following conditions: temperature 30°C, stirrer speed 250 rpm, pH controller was connected to 2N NaOH and the pH was never allowed to drop below 6.8. Moreover, rise in pH above 7.2 was controlled with HCl 20% (v/v). Dissolved oxygen (DO) was maintained between 20 and 40% saturation by controlling the air flow that was set at 1 l/min. Silicone at 0.98 g/ml (Merck KGaA, Darmstadt, Germany) was used as antifoam agent. Fermentation was terminated after the completion of the spore crystal complex. Samples were withdrawn at 2 h intervals and used for delta-endotoxin determination. The fermentation was carried out twice for each medium.

Cell mass

After fermentation was completed, two samples (45 ml) were taken from each fermentor and then centrifuged at 8 000 rpm for 15 min. The supernatants were discarded and the cell pellets were lyophilized. Dry weight was calculated and expressed in grams per liter (g/l).

Spore count

The two culture samples taken from each fermentor were heat treated at 80°C for 15 min, serially diluted, and plated onto NA plates. Plates were then incubated at 37°C for 20 h and the developed *B. thuringiensis* colonies were counted and expressed in CFU (colony forming units) per ml.

Delta-endotoxins determination

Delta-endotoxin concentration was determined in a solubilized crystal preparation from each culture medium as described by Prabakaran and Balaraman (2006). In summary, 1 ml of culture medium was centrifuged for 10 min at 10 000 g and the resulting pellet was washed twice with NaCl (1 m/l) and twice with distilled water. The pellet was then suspended in 1 ml of NaOH (50 mmol/l, pH 12.5) in order to solubilize delta-endotoxin crystals. After

2 h of incubation at 37°C, total proteins in the supernatant was measured by using the Bio-Rad Protein Assay Kit (München, Germany), in accordance with the method by Bradford (1976). The values presented are the averages of the results of two determinations of two separate experiments for each culture condition. The toxin synthesis yield was calculated as the ratio of delta-endotoxin (mg/l) relative to CFU (spores/l).

Analysis of protein profile

Purified crystal preparations from each fermentor were incubated with trypsin (Sigma) for 5 h at 30°C. Active toxins were solubilized at 100°C in sample buffer: Tris-HCl (62.5 mM, pH 6.8), SDS (2%), glycerol (10%), mercaptoethanol (5%) and bromophenol blue (0.1%), before being loaded on the gel. SDS-PAGE was performed using the standard discontinuous buffer system of Laemmli (1970) using 10% polyacrylamide gel to confirm the presence of toxin bands of molecular weight of 57–68 kDa for these two Lepidoptera specific strains.

Statistical analysis

All data obtained from the determination of cell mass, delta-endotoxin production and CFU counts were the mean of two separate samples for each culture media.

Data were statistically analyzed by SPSS software (Version 9.0.0) using the one-way ANOVA test to compare the means.

RESULTS

Bioinsecticide production in shaken flask. Comparison of the two strains

According to the pooled data obtained from all four culture media, biomasses produced by the two strains were significantly different ($F = 201.33$, $p < 0.01$) and HD1 produced 1.5 fold more mean dry weight than the BTA strain (12.52 ± 0.22 g/l and 8.20 ± 0.21 g/l by HD1 and BTA, respectively). Delta-endotoxin synthesis yield by the two strains, was significantly different ($F = 6.049$, $p < 0.05$) and HD1 synthesized approximately 3.7 fold more toxin than BTA. Results of spore count produced by the two strains were significantly different ($F = 11.36$, $p < 0.01$) and the highest mean spore count of 357.18×10^6 CFU/ml was observed in the BTA strain that was approximately 4.8 fold higher than for HD1 strain (74.67×10^6 CFU/ml) (Table 1).

Comparison of four different media

Each of the two strains, using four different culture media showed no significant difference in producing

Table 1. Production of bioinsecticide by BTA and HD1 strains of *B. thuringiensis* using four different media in shaken flasks after 72 h

Strain	Media	CFU ¹ [10 ⁶ /ml]	Toxin synthesis yield ² [mg/10 ⁶ spore]	Dry biomass [g/l]±SE
BTA	A	733.400±33.5	0.1519±0.012	7.59±0.04
	B	261.340±23.7	0.5717±0.22	7.91±0.04
	C	250.600±19.1	0.7222±0.024	7.95±0.48
	D	183.340±23.3	0.6445±0.040	8.88±0.36
	Mean	357.18	0.552	8.20
HD1	A	113.340±12.6	1.0457±0.031	13.02±0.24
	B	78.000±8.4	1.4695±0.093	12.15±0.95
	C	22.730±2.5	4.5119±1.653	12.65±0.26
	D	84.610±7.7	0.7214±0.043	12.88±0.18
	Mean	74.67	1.937	12.52

¹ colony forming units

² toxin synthesis yield was calculated as the ratio of delta-endotoxin [mg/l] over CFU [spores/l]

Table 2. Comparative costs for production of one kg of dry biomass by the BTA strain using four different media

Media	Media components	Quantity used in media	Cost of quantity used [US \$] ¹	Total cost for production of 1 kg dry biomass [US \$]	
				shake flask	fermentor
Medium A	soybean	12.8 [g/l]	0.0151	11.18	8.62
	starch	12.2 [g/l]	0.0459		
	salt solution	0.315 [ml/l]	0.024		
Medium B	soybean	12.8 [g/l]	0.0151	5.93	6.18
	barley	12.2 [g/l]	0.0078		
	salt solution	0.315 [ml/l]	0.024		
Medium C	groundnut	12.8 [g/l]	0.0413	13.98	Nt
	starch	12.8 [g/l]	0.0459		
	salt solution	0.315 [ml/l]	0.024		
Medium D	groundnut	12.8 [g/l]	0.0413	8.23	NT
	barley	12.2 [g/l]	0.0078		
	salt solution	0.315 [ml/l]	0.024		

¹ all prices were calculated according to the prices of Iran domestic market on January, 2009, NT – not tested

Table 3. Production of bioinsecticide by BTA and HD1 strains of *B. thuringiensis* using two different media, in 5L fermentor

Strain	Media	CFU ¹ [10 ⁶ /ml]	Toxin synthesis yield ² [mg/10 ⁸ spore]	Dry biomass [g/l]±SE
BTA	A	212.01±12.7	1.51±0.06	9.85±0.34
	B	181.32±10.4	1.77±0.04	7.58±0.63
HD1	A	15.60±1.7	9.39±0.32	8.67±0.05
	B	31.40±2.2	9.58±0.65	10.57±0.04

¹ colony forming units; ² toxin synthesis yield was calculated as the ratio of delta-endotoxin [mg/l] by CFU [spores/l]

dry biomass (BTA: $F = 0.159$, $p = 0.922$; HD1: $F = 0.01$, $p = 0.970$). Also using these culture media had no significant effect on delta-endotoxin production by HD1 and BTA strains (BTA: $F = 2.63$, $p = 0.095$; HD1: $F = 3.54$, $p = 0.028$). Different spore counts were obtained for four used culture media ($F = 19.4$, $p < 0.01$) they were the highest for BTA strain (733.4×10^6 CFU/ml) in medium A and the lowest (183.34×10^6 CFU/ml) in medium D. Similarly, HD1 produced different amounts of spores ($F = 16.6$, $p < 0.01$) in different culture media so that the highest spore count was observed in medium A (113.34×10^6 CFU/ml) and the lowest in medium C (22.73×10^6 CFU/ml) (Table 1).

Bioinsecticide production in fermentor

Comparison of the two strains

After bioinsecticide was produced by the two strains in four different media in shaken flasks, the media preparation cost was calculated for each medium and according to a relatively lower cost of production of 1 kg dry biomass (Table 2), two soybean based media were selected for mass production in the fermentor stage. Pooled data of the two culture media (A and B) showed that biomass in HD1 and BTA strains were not significantly different ($F = 0.035$, $p > 0.05$). Delta-endotoxin synthesis yield was significantly different for the two strains ($F = 9.73$, $p < 0.05$) on two different culture media (Fig. 1). HD1 showed approximately 5.8 fold higher toxin synthesis yield in average. Spore counts did not show significant difference between strains ($F = 298$, $p < 0.01$) and maximum spore count of 212.01×10^6 CFU/ml was observed for the BTA strain in medium A (Table 3).

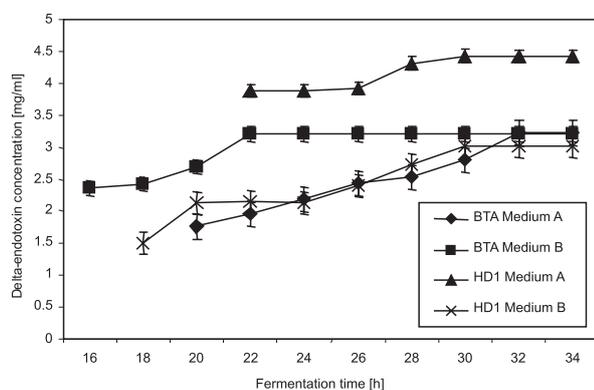


Fig. 1. Delta-endotoxin production by BTA and HD1 strains using two different media in 5 l fermentor

Comparison of two different media

For each of the two strains, biomass produced in different media, delta-endotoxin synthesis yields and also CFU counts were not significantly different ($F = 0.99$, $p > 0.05$; $F = 1.36$, $p > 0.05$; $F = 0.01$, $p > 0.05$, respectively).

Analysis of protein profile

In all four culture media used for bioinsecticide production, both in flasks or fermentors, when starch was substituted by barley, both strains were successful in delta-endotoxin synthesis (Fig. 2) and SDS-PAGE of trypsin-incubated solubilized delta-endotoxins showed toxin bands of about 57–68 kDa in both soybean-based culture media produced by the two strains (Fig. 2).

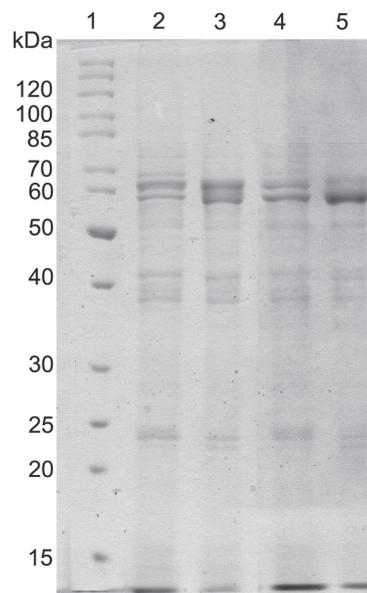


Fig. 2. SDS-PAGE of trypsin-activated *B. thuringiensis* protoxins from HD1 and BTA strain bioinsecticides, produced in 5l fermentors. Lanes: 1. Molecular mass, 2. BTA in medium A, 3. HD1 in medium A, 4. BTA in medium B, 5. HD1 in medium B

DISCUSSION

In this study, soybean or groundnut were used as nitrogen source, and starch or hull-less barley, as carbon source in ratios commonly used in most industrial scale productions of *B. thuringiensis* bioinsecticides. According to the results obtained this cultivar of barley could perfectly substitute starch in the soybean based media

Table 4. Comparative costs for production of one kg of dry biomass by the HD1 strain using four different media

Media	Media components	Quantity used in media	Cost of quantity used [US \$] ¹	Total cost for production of 1 kg dry biomass [US \$]	
				shake flask	fermentor
Medium A	soybean	12.8 [g/l]	0.0151	6.53	9.80
	starch	12.2 [g/l]	0.0459		
	salt solution	0.315 [ml/l]	0.024		
Medium B	soybean	12.8 [g/l]	0.0151	3.86	4.43
	barley	12.2 [g/l]	0.0078		
	salt solution	0.315 [ml/l]	0.024		
Medium C	groundnut	12.8 [g/l]	0.0413	8.80	NT
	starch	12.2 [g/l]	0.0459		
	salt solution	0.315 [ml/l]	0.024		
Medium D	groundnut	12.8 [g/l]	0.0413	5.67	NT
	barley	12.2 [g/l]	0.0078		
	salt solution	0.315 [ml/l]	0.024		

¹ all prices were calculated according to the prices of Iran domestic market on August, 2008
NT – not tested

and the cost of media for producing one kg dry biomass would be lower by 28.3% and 57.8% during the production of BTA and HD1 bioinsecticides, respectively (Tables 2, 4). Using the “B” medium BTA reached a maximum toxin production after cultivation for only 22 h (Fig. 1), but HD1 reached the same level after 30 h. Using starch allows improvement of *B. thuringiensis* delta-endotoxin production mainly by the HD1 cultures (Ghribi *et al.* 2007). Such an improvement was of a great interest whilst calculating the cost of bioinsecticide production, and this study showed that interestingly, commercial-grade starch can be substituted by barley flour which has 57–63% starch in its chemical composition (Grausgruber *et al.* 2004). Moreover, hull-less barley usually has a higher total protein content (12.5–15.9%) than hulled barley and other cereals (Linko *et al.* 1989; Helm and Francisco 2004) and such proteins can be used as the second nitrogen source for the bulk production of *B. thuringiensis*. The other medium (A), with a relatively more expensive ingredient (commercial-grade starch), requires a longer cultivation period especially during BTA production, and thus has higher overall production costs. The toxin production yield of *B. thuringiensis* strains produced in our proposed medium (the soybean-based medium B) was comparable to the levels obtained using other media. We concluded that the use of food barley in production of *B. thuringiensis* has many advantages. It is available nearly throughout the world with relatively competitive costs; permits faster sporulation, comparable biomass production, and shorter fermentation time and is also easy to prepare and to store. Such a medium composition can provide very useful substrates for production of the *B. thuringiensis* bioinsecticide, particularly in Iran and countries that have high levels of barley production.

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POLISH SUMMARY

OPRACOWANIE OPŁACALNEJ POŻYWKI DO PRODUKCJI BIOINSEKTYCYDU *BACILLUS THURINGIENSIS* PRZY UŻYCIU SPOŻYWCZEGO JĘCZMIENIA

Porównywano zmieniony genetycznie jęczmień, *Hordeum vulgare* L. odmiana *valfajr*, ze skrobią handlowej klasy używanej jako źródło węgla oraz pożywki sojowe wykorzystywane do hodowli uzyskania zarodnikowania *B. thuringiensis* Berliner i do produkcji delta-endotoksyny.

Do badań użyto obiecujący, nowo izolowany szczep tej bakterii (BTA). Do pożywki w ilościach śladowych dodano następujące związki: $MgCl_2$, $CaCl_2$ i $MnCl_2$. Pożywki porównywano na wytrząsarce, a następnie przy użyciu sprzętu do fermentacji. Oceniana biomasa i wytwarzanie delta-endotoksyny, po zastąpieniu w pożywkach sojowych skrobi przez mąkę jęczmienną, nie różniły się istotnie. Stwierdzono natomiast różnice pomiędzy dwoma badanymi szczepami pod względem biomasy i produkcji toksyny. Porównanie kosztu przygotowania pożywek wykazało, że jęczmień spożywczy jest ekonomiczną alternatywą do produkcji bioinsektycydu BTA, w stosunku do skrobi handlowej jakości.