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ARTICLE

Concomitant production of delta-endotoxins and proteases of *Bacillus thuringiensis* subsp. *kurstaki* in a low-cost medium: effect of medium components

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ABSTRACT *Bacillus thuringiensis* is a bacterium, commonly used as a biological pesticide, and produces entomotoxic parasporal crystals (delta-endotoxins) and photolytic enzymes involved in several biological processes. The aim of present work was to enhance the production of delta-endotoxins by an isolated *Bacillus thuringiensis* subsp. *kurstaki* strain. The adopted approach was based on studying both delta-endotoxins and proteolytic activities production. Trials were carried out based on the Plackett-Burman experimental design. The statistical analysis revealed that the soybean meal was the main significant ingredient (confidence level= 99.5%) for both delta-endotoxins and proteolytic activities productions. Starch and $FeSO_4$ were considered as significant ingredients for only protease production, the confidence levels were 98.8% and 97% respectively. The study of the nutrients effects brought out that K_2HPO_4, $FeSO_4$ and starch, exhibit an opposite effect on the synthesis of delta-endotoxins and proteolytic activity production. On the other hand, $MnSO_4$, $MgSO_4$ and soybean meal are considered as the ingredients having dual positive effect on proteolytic activities and delta-endotoxin productions, whereas KH_2PO_4 had an inhibitory effect. The optimisation strategy using mathematical methods offers an efficient technique to optimize media leading to the increase delta-endotoxin production by *Bacillus thuringiensis* subsp. *kurstaki* strains by clustering of the effects of medium components.
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KEY WORDS

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Bacillus thuringiensis is an ubiquitous Gram-positive bacterium. The distinctive property of *B. thuringiensis* is its high specific entomopathogenicity due to production of insecticidal crystal toxins (Cry proteins called delta-endotoxins) that accumulate in the cell as crystalline inclusions during sporulation of the bacterium (Schnepf et al. 1998). The principal target pests of *B. thuringiensis* insecticides include lepidopterous, dipterous, and coleopterous species.

B. thuringiensis products are produced by fermentation (Bernhard and Utz 1993). Several proteolytic enzymes are synthesized by *Bacillus* species during growth and sporulation phases (Doi 1972). Indeed, proteins are required during sporulation (Yezza et al. 2006). Recently, we evidenced for the first time a relationship between delta-endotoxins and proteases production (Ennouri et al. 2013). Reducing proteolytic activities in the fermentation medium increased the accumulation of delta-endotoxins in the insecticidal crystal proteins. Moreover, productivity of microbial enzymes, such as proteases and delta-endotoxins, can be regulated by optimizing nutritional supplements of *B. thuringiensis* especially with the development of economic medium that requires the selection of complex carbon and nitrogen sources (Zouari et al. 1998). Nutritional supplements of *B. thuringiensis* can be optimized by statistical methods. The most cost-beneficial of these methods is the statistical design of experiments. Indeed, these techniques are frequently used to understand the effects of several variables and to describe, predict and improve the behaviour of any process in limited number of experiments. One of these models is the Plackett-Burman design that is proven to be an interesting tool to optimize complex variables (Badawy et al. 2000).

In the present study, seven nutritional components, including KH_2PO_4, K_2HPO_4, $MgSO_4$, $FeSO_4$, $MnSO_4$, starch and soybean meal, have been selected to identify the main factors affecting delta-endotoxin and protease production by *B. thuringiensis* strain, using Plackett-Burman design.

Since a Plackett-Burman design is useful to find out the important variables in a system and is suitable when more than five independent variables are to be investigated, this model was used to test the effects of seven factors (nutritional ingredients) at two levels. This type of design allows the evaluation of a large number of factors in a small number of experiments. In fact, only 12 experiences were generated

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compared to a typical experimental program with seven factors, each at two levels that requires 128 experiences due to nutritional component level combinations.

Materials and Methods

Microorganism and cultivation media

B. thuringiensis subsp. *kurstaki* S7, S3 and BUPM13 were wild type strains isolated and identified in our laboratory (LPIP, CBS, Tunisia). HD-1 strain was used as a reference in this study.

The acrystalliferous strain HD-1cryB, was obtained by plasmid curing from the wild strain HD-1. The *B. thuringiensis* strains were streaked on LB (Luria Bertani) plates, incubated for 24 hours at 30 ± 0.1°C and then preserved at 4°C for future use. LB medium with the following composition (g l^{-1}) was used for the preparation of the pre-inoculum and inoculum: peptone, 10.0; yeast extract, 5.0; NaCl, 5.0. For fermentation medium, a complex economic medium (Ghribi et al. 2007) was used containing the following components (g l^{-1}): starch, 25; soybean meal, 20; $MgSO_4$, 0.3; $MnSO_4$, 0.02; $FeSO_4$, 0.02; K_2HPO_4, 1; KH_2PO_4, 1. $CaCO_3$, (20 g l^{-1}) were added for keeping pH stability. All media used in this study were adjusted to pH 7.0 ± 0.01 before autoclaving.

Culture conditions

For pre-inoculum preparation, a loopful of *B. thuringiensis* grown on Luria Bertani (LB) plate was used to inoculate a 3 ml of sterilised LB medium and incubated in a rotary shaker (New Brunswick incubator shaker model INNOVA 44®, USA), at 30°C and 200 rpm overnight. For inoculum preparation, 250 ml erlenmeyer flasks containing 50 ml LB medium were inoculated with 1% (v/v) of the pre-inoculum and incubated in a rotary shaker at 30°C and 200 rpm for 6 hours. The volume of culture inoculum was determined on the basis of a final absorbance of approximately 0.15 measured at 600 nm. The 250 ml flasks containing 20 ml of complex economic medium were incubated with estimated inoculum volume. In such media, to obtain the same initial bacterial concentrations, the volume of inoculum was calculated based on optical density (OD) measured before inoculation. Samples taken periodically from the incubated cultures were subjected to microscopical examination. When 90% (or more) of the *B. thuringiensis* cells had lysed, releasing the spores and crystals, the fermentation process was considered as finished.

Determination of delta-endotoxins

One ml of collected samples at the end of fermentation was centrifuged at 13,000×g for 10 minutes at a temperature of 4°C. The supernatants were discarded. The pellets were washed twice with 1 ml of 1 M NaCl solution and twice with 1 ml of distilled autoclaved water. The crystal proteins in the pellet were dissolved in one ml of 50 mM NaOH (pH 12.5) for 2 h at 30°C with vigorous shaking. The suspension was centrifuged at 13000×g for 10 minutes at 4°C and the pellet was discarded. The supernatant, containing the alkali-soluble insecticidal crystal proteins was used to define the delta-endotoxin concentration by Bradford method using bovine serum albumin as standard protein. Delta-endotoxins concentration was measured spectrophotometrically at 595 nm using a SmartSpec 3000® UV–visible spectrophotometer (Bio-Rad Laboratories Inc.). The negative control (the acrystalliferous strain HD-1cryB) was included in each experiment and each cultural condition. Indeed, we considered the possible contribution of dissolved proteins from spore coat, cell debris and particulate or insoluble materials in the protein levels measured after treating the pellets by NaOH. Toxin contents were calculated as the result of subtracting the total proteins measured with the HD-1cryB strain from the total proteins measured with the toxin-producing strains. The obtained values were the mean of three values of two separate experiments.

Determination of proteolytic activity

Protease activity (PA) was determined according the modified method of Kunitz. The culture solution was centrifuged at 13,000×g for 15 minutes at a temperature of 4°C. The supernatant was used for the assay of proteolytic activity: to one ml Tris-HCl casein solution (1% w/v in 100 mM Tris-HCl buffer pH = 7) one ml of the diluted sample was added. The reactions were carried out at 60°C for 20 min and then stopped by the addition of 3 ml of 5% trichloroacetic acid (TCA,w/v). The mixture was centrifugated at 13000×g for 20 minutes and a total of 1 ml supernatant was carefully removed to measure peptide content. The protease activity in the supernatant was measured spectrophotometrically at 280 nm. One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1 µg tyrosin within 1 minute at 60°C. The presented values are the mean of three values of two separate experiments.

Plackett-Burman Design

This study was aimed to screening the important medium components with their main effects and not the interaction effects between different medium ingredients and therefore, Plackett-Burman design was employed. Seven components were selected and each variables were represented at two levels, high concentration (+) and low concentration (-) as shown in Table 1. Each column contains the same number of positive and negative signs. Hence, each row form a trial run and each column form an assigned variable. The effect of each variable was determined by:

$$E(x_i) = (\sum C^+_i - \sum C_i^-) / N$$

Where $E(x_i)$ is the content effect of the studied variable, $\sum C_i^+$ and $\sum C_i^-$ are the sums of the obtained values at high and

Table 1. Assigned concentrations of variables at different levels in Plackett-Burman design.

Variable (g l^{-1})	Symbol	-1	+1
KH_2PO_4	X1	0.5	1.5
K_2HPO_4	X2	0.5	1.5
Mg SO_4	X3	0.1	0.5
$FeSO_4$	X4	0	0.02
$MnSO_4$	X5	0	0.02
Starch	X6	25	35
Soybean meal	X7	20	30

Table 2. Screening of *B. thuringiensis* strains for delta-endotoxin and proteolytic enzyme production, using complex medium into 250 shake flasks.

Strain reference	Delta-endotoxins (mg l^{-1})	Proteolytic activity (IU)
HD-1	2178 ± 41	231 ± 46
S7	2384 ± 33	275 ± 25
S3	1968 ± 74	710 ± 31
BUPM13	1270 ± 50	1352 ± 24

low levels respectively for delta-endotoxin and protease productions from the trials where the measured variable (x_i) was presented at high and low concentrations; and N is the number of runs. The significance level (p-value) of concentration effect was estimated using student's *t*-test. The influence of all ingredients on delta-endotoxin and protease productions was evaluated. The significance effect of components medium was evaluated using Pareto charts analysis. These charts contain bars and a line graph. Individual value effects are represented in descending order by bars, and the line represent the cumulative total. The component (bars) exceeding the line have significant effect. Plackett-Burman design and analysis of the results were done using Minitab 15 software.

Results

Delta-endotoxin production by B. thuringiensis strains

In order to select one *B. thuringiensis* strain for the study of the correlation between delta-endotoxin and proteolytic activities, four strains (HD-1, S7, S3 and BUPM13) were cultured using the complex medium, mainly composed of starch (25 g l^{-1}) and soybean meal (20 g l^{-1}) (Table 2). Delta-endotoxin concentrations were ranged between 1270 mg l^{-1} for strain BUPM13 and 2384 mg l^{-1} for strain S7. *B. thuringiensis* BUPM13 strain showed the lowest production of delta-endotoxin with 41.68% less compared to HD-1 which is considered as a *B. thuringiensis* subsp. *kurstaki* reference strain. *B. thuringiensis* strain S7 showed the highest yield with almost 9.5% higher than HD-1. However, *B. thuringiensis* BUPM13 showed the highest yield of proteases with 485% more compared to reference strain HD-1. Proteases activities were varied between 231 IU for strain HD-1 and 1352 IU for strain BUPM13. In order to improve delta-endotoxin production for further large scale applications, S7 was selected for the improvement investigations.

Effects of nutritional components on delta-endotoxin production

A Plackett-Burman design was employed to evaluate the main effect of the medium components for delta-endotoxin production by *B .thuringiensis* subsp. *kurstaki* S7. The design matrix with 12 different runs is presented in Table 3. The results of delta-endotoxins production using the experimental design showed a wide variation from 1863 mg l^{-1} (run n°6) to 4397 mg l^{-1} (run n°2).

The data on delta-endotoxins production level given in Table 3 was subjected to statistical evaluation through multiple linear regression analysis, student's t-test for p-value and confidence level. Estimated t-value, p-value and confidence level giving the effect of variables on delta-endotoxin pro-

Table 3. Plackett-Burman design randomized runs and the responses.

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Delta-endotoxins production (mg l^{-1})	Proteases concentrations (IU)
1	+1	+1	-1	+1	-1	-1	-1	2203 ± 98	146 ± 10
2	-1	+1	+1	+1	-1	+1	+1	4397 ± 85	538 ± 24
3	-1	-1	+1	+1	+1	-1	+1	3042 ± 102	943 ± 15
4	+1	+1	-1	+1	+1	-1	+1	2817 ± 91	791 ± 29
5	+1	-1	+1	+1	-1	+1	-1	2476 ± 110	180 ± 18
6	-1	-1	-1	-1	-1	-1	-1	1863 ± 88	452 ± 12
7	+1	+1	+1	-1	+1	+1	-1	1926 ± 105	250 ± 22
8	+1	-1	+1	-1	-1	-1	+1	2780 ± 117	575 ± 11
9	+1	-1	-1	-1	+1	+1	+1	3615 ± 104	473 ± 16
10	-1	+1	+1	-1	+1	-1	-1	2173 ± 81	578 ± 20
11	-1	-1	-1	+1	+1	+1	-1	2186 ± 90	272 ± 23
12	-1	+1	-1	-1	-1	+1	+1	3711 ± 108	350 ± 27

Table 4. Linear multiple regression analysis of Plackett-Burman design (delta-endotoxins).

Variables	Estimate	t statistic	p-value	Confidence %
Constant	2766.3	25.25	0.000	
KH_2PO_4	-129.6	-1.18	0.302	69.8
K_2HPO_4	105.3	0.96	0.391	61.9
$MgSO_4$	33.2	0.30	0.777	23.3
$MnSO_4$	87.9	0.80	0.467	53.3
$FeSO_4$	-139.2	-1.27	0.273	72.7
Starch	286.1	2.61	0.059	94.1
Soybean meal	627.9	5.73	0.005 a	99.5

a: significant at $p < 0.05$

duction are shown in Table 4. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect. In this work, variables with confidence levels greater than 95% were considered as significant. On the basis of the calculated p-values at 95% confidence level ($\alpha = 0.05$), soybean meal (confidence level of 99.5%), is identified as the significant medium component on delta-endotoxin production. Thus, it seems that only the supply of soybean meal in the medium was required to delta-endotoxin production by *B. thuringiensis* S7.

Based on the Plackett-Burman design, the effect of independent variables on delta-endotoxin production is given by the first order linear model. The regression coefficient of the model ($R^2 = 91.73\%$) validates that the model is well fitted with the experimental results. The p-value in the ANOVA test presented in Table 5 was 0.047 (less than 0.05). Thus, this could confirm the validity of the proposed design concerning delta-endotoxin production. The mean absolute error of 379.477 was the average value of the residuals. The main effect of each medium component on delta-endotoxin production is summarized in Figure 1.

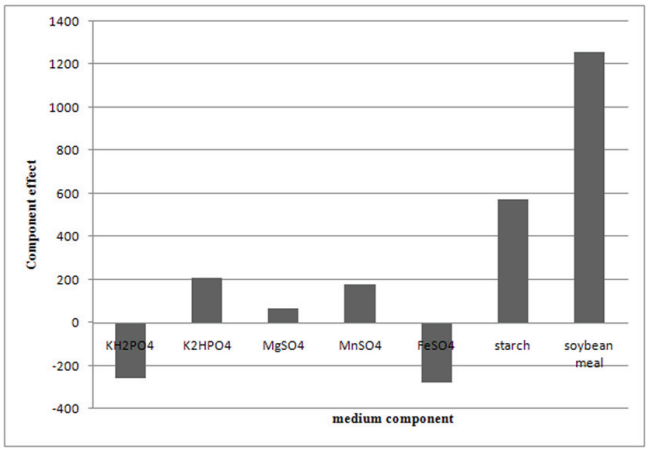

Figure 1. Main effect on delta-endotoxins amount of the media constituents after randomisation using Plackett-Burman design.

Table 5. ANOVA test for delta-endotoxin response.

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-value
Model	6385623	7	912232	6.33	0.047
Residual error	576011	4	144003		
Total	6961634	11			

$R^2 = 91.73\%$; R^2 (adjusted) = 77.25%; Mean absolute error = 379.477

The highest delta-endotoxins production was achieved in association with $MgSO_4$ (+66.3), $MnSO_4$ (+175.8), followed by K_2HPO_4 (+210.5), starch (+572.2) and soybean meal (+1255.7), but restrained by $FeSO_4$ (-278.5) and KH_2PO_4 (-259.2) (Fig. 1).

Analysis of the Pareto chart of medium components (Fig. 2) denoted that the soybean meal significantly affected the delta-endotoxin production. Pareto charts are helpful because they can be employed to identify those factors that have the best cumulative effect on the output, and therefore screen out the less significant factors in an analysis.

Evaluation of factors affecting protease production

The corresponding response, proteolytic activity is shown in Table 3, according to the experimental design matrix of variables. Proteolytic activities, obtained by using the experimental design, ranged from 146 IU (run n°1) to 943 IU (run n°3). Estimated t-value, p-value and confidence level giving the effect of variables on protease production are shown in Table 6. In this study, variables with confidence levels greater than 95% were considered as significant. On the basis of the calculated p-values at 95% confidence level ($\alpha = 0.05$), soybean meal (confidence level = 99.5%), starch (confidence level = 98.8%) and $FeSO_4$ (confidence level =

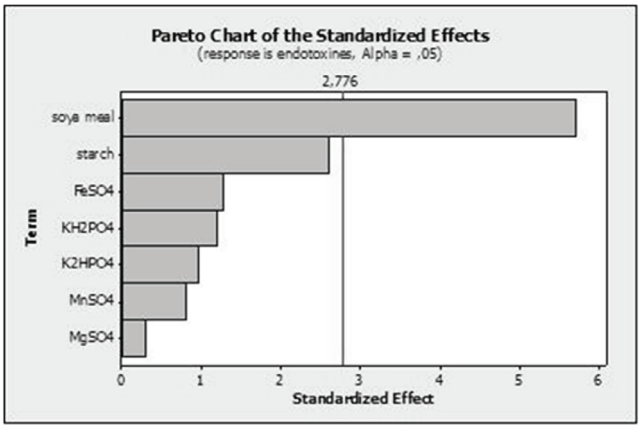

Figure 2. Effect of medium components on delta-endotoxins production.

Table 6. Linear multiple regression analysis of Plackett-Burman design (proteases).

Variables	Estimate	t statistic	p-value	Confidence %
Constant	462.7	17.10	0.000	
KH_2PO_4	-59.8	-2.21	0.091	90.9
K_2HPO_4	-20.3	-0.75	0.496	50.4
$MgSO_4$	48.3	1.78	0.149	85.1
$MnSO_4$	16.1	0.59	0.584	41.6
$FeSO_4$	88.9	3.29	0.030 a	97.0
Starch	-118.4	-4.37	0.012 a	98.8
Soybean meal	149.3	5.52	0.005 a	99.5

a: significant at $p < 0.05$

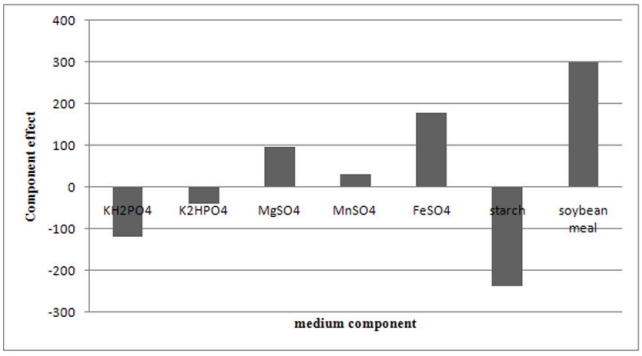

Figure 3. Main effect on proteases amount of the media constituents after randomisation using Plackett Burman design.

Table 7. ANOVA test for protease response.

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-value
Model	609652	7	87093	9.91	0.021
Residual error	35156	4	8789		
Total	644808	11			

$R^2 = 94.55\%$; R^2 (adjusted) = 85.01%; Mean absolute error = 93.75.

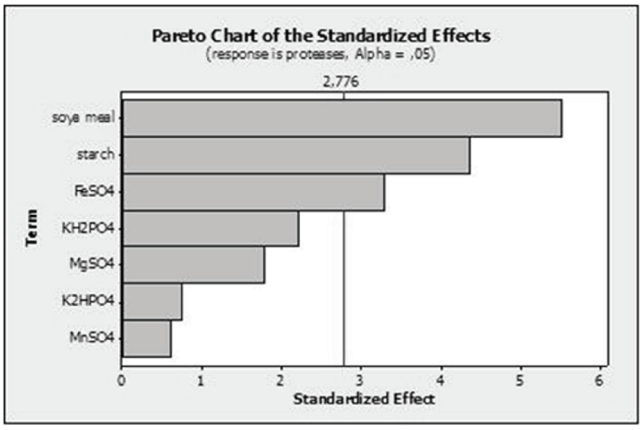

Figure 4. Effect of medium components on proteases production.

97%) were identified as the most significant medium components on bacterial proteolytic enzymes production. Based on the Plackett-Burman design, the effect of independent variables on protease production is given by the first order linear model. It was found that p-value of three variables was less than 0.05. This indicated that they were significant factors on the protease production. So the presence of soybean meal, starch and $FeSO_4$ in the medium is required for proteolytic activity production by *B. thuringiensis* subsp. *kurstaki* S7. The regression coefficient of the model ($R^2 = 94.55\%$) validates that the model is well fitted with experimental results.

Analysis of variance (ANOVA) was applied to test the significant and adequacy of the model. The p-value in the ANOVA test (Table 7) was 0.021 (less than 0.05). This result indicated that the regression model was significant. The R-squared statistic indicates that the model as fitted explains 94.55% of the variability in protease amount. The mean absolute error of 93.75 was the average value of the residuals. The main effect of each medium component involved in protease production is summarized in Figure 3.

Indeed, soybean meal recorded the highest score (+298.7) for producing proteases followed by $FeSO_4$ (+177.8), $MgSO_4$ (+96.6) and $MnSO_4$ (+32.2). Nevertheless, proteases were not considerably affected by the presence of K_2HPO_4, KH_2PO_4 and starch as indicated by a negative value of the main effect. In fact, proteases amount was enhanced at low K_2HPO_4 (- 40.5), KH_2PO_4 (-119.7) and starch (-236.7). The positive value of the main effect of soybean meal, $FeSO_4$, $MgSO_4$ and $MnSO_4$ indicated that their high levels improved proteolytic activity in the complex medium.

As shown in Figure 4, production of proteolytic activity was designed using Pareto chart. It seems that soybean meal with starch and $FeSO_4$ are the most important factors at 95% confidence level on proteolytic activity production by *B. thuringiensis* subsp. *kurstaki* S7.

Discussion

According to Table 2, the high-producer strains of delta-endotoxins seemed to be low producers of proteases. In fact, Ennouri et al. (2013) reported that a negative correlation existed between delta-endotoxins and proteases productions.

The result indicated that K_2HPO_4 was one of the main factors affecting delta-endotoxins production, which agree with El Bendary (1999) reports. Ozkan et al. (2003) concluded that an efficient synthesis of crystal proteins by *B. thuringiensis israelensis* HD500 needed important concentrations of K_2HPO_4. The presence of Mn^{2+}, Mg^{2+} and Fe^{2+} in culture medium could promote growth and crystal formation (Morris et al. 1996). Therefore, a limitation of Fe^{2+} may cause the

accumulation of NADH which will result in feedback control of TCA cycle (Saksinchai et al. 2001). However, Ozkan et al. (2003) reported that Fe^{2+} negatively influenced toxin biosynthesis by *B. thuringiensis israelensis* HD500. Consequently, the requirement for minerals varies with strains as well as the nature of the basal medium. This result may demonstrate the effect of mineral salts on delta-endotoxin production in the complex medium.

The media used for industrial production of *B. thuringiensis* are composed of complex nitrogen and carbon sources. Production of *B. thuringiensis* has been found to vary drastically in media derived from various nutrient sources. Prabakaran et al. (2008) used locally available raw materials such as soybean flour, groundnut cake powder and wheat bran extract to improve the yield of cell mass and sporulation of *B. thuringiensis israelensis*. Whey and molasses, which can be used as low-cost and available substrates at an industrial scale, were potential carbon substrates for delta-endotoxin production (Içgen et al. 2002). In the gruel and fish meal medium, the production of bioinsecticides varied a lot, depending on *B. thuringiensis* strain. Diptera-specific strains produced less delta-endotoxins (1246-1998 mg l^{-1}) than lepidoptera-specific ones (3060-3301 mg l^{-1}) (Zouari et al. 2002). Some reports were interested in the optimization of Cry4Ba and Cry11Aa toxins production of *B. thuringiensis israelensis* HD500 (Tokcaer et al. 2006). The optimized toxins productions were 28.9 mg l^{-1} for Cry4Ba and 69.2 mg l^{-1} for Cry11Aa, using sucrose and yeast extract as carbon and nitrogen sources. Similarly, *B. thuringiensis israelensis* delta-endotoxins production was 415 mg l^{-1} through optimization of cultural conditions (Avignone Rossa et al. 1992), which were 10.59 folds lower than S7 delta-endotoxins production. So, considering the high production of bioinsecticides based on *B. thuringiensis* S7 is a promising strain for biotechnological applications.

In this study, bacteria were cultured in low-cost medium containing soybean meal as protein source and starch as carbohydrate source. Likewise, Iudina et al. (1993) concluded that the change of carbon source led to variations in the rate of endotoxin synthesis and crystal form when starch and corn flour were used as nutrient sources in fermentation of strain HD-1. Soybean flour is considered among the most inexpensive sources of nitrogen in applied microbiology. Vora and Shethna (1999) reported that growth and toxin production yields by *B. thuringiensis* subsp. *kurstaki* were enhanced when defatted soybean meal and groundnut seed meal extracts were used. Thus, starch and soybean meal are among the most adequate sources of carbon and nitrogen used in *B. thuringiensis* fermentation.

In some experiments summarized in Table 3, delta-endotoxin production increased with a moderate production of proteases. High delta-endotoxin production could be due to increased availability of nutrients and improved availability of nutrients as well as nutrient assimilation resulting in reasonable protease production. This is justified by the fact that *B. thuringiensis* proteases are produced under nitrogen rich conditions. In fact, protease activity is improved when *B. thuringiensis* grown in a nitrogen-rich medium, due to enhanced availability of nutrients from complex media. Besides, it's known that complex proteins induce higher levels of protease activity in the medium (Zouari and Jaoua 1999). In this case, the higher protease production in soybean meal rich media could be due to the fact that it was a nitrogen rich source for induction of proteases.

Proteolytic enzymes production by *Bacillus* is highly influenced by media components as carbon, nitrogen, presence of easily metabolisable sugars (Gupta et al. 2002) and metal ions (Varela et al. 1996). Li and Yousten (1975) concluded that proteolytic activity produced by *B. thuringiensis* subsp. *kurstaki* was dependent upon supplementation with Mn^{2+}. When a supplement of Mg^{2+} and K^{+} salts was added to the culture medium, a drastic increase in protease production was noted (Ellaiah et al. 2002; Nadeem et al. 2007). It is also known that carbon, nitrogen and sulphate sources contribute to alkaline protease production from *Bacillus* sp. (Chauhan and Gupta 2004).

With S7 strain, $MgSO_4$, $MnSO_4$ and soybean meal improve simultaneously delta-endotoxin production and proteolytic activity. In the same way, KH_2PO_4 has a retroactive impact on twice delta-endotoxins and proteases. $FeSO_4$ had a significant effect on both delta-endotoxins and protease production. At a high level, it promotes protease activity and has an inhibitory effect on delta-endotoxin production. However, starch and K_2HPO_4 have a positive effect on delta-endotoxin yield and an inhibitory action on proteolytic enzymes production, which is a very interesting finding for further formulation of the produced bioinsecticides. This means that the accumulation of proteolytic activity in the culture medium affects negatively the production of delta-endotoxins during fermentation. A negative aspect of the presence of proteolytic activity in Gram positive bacteria is their contribution to the overall degradation of proteins. *B. thuringiensis* requires hydrolysing complex proteins in order to satisfy its nutritional needs and synthesise different types of proteases from sporulation stage (Chu et al. 1992). Soybean meal, $MgSO_4$ and $MnSO_4$ positively affected delta-endotoxin production and proteolytic activity. Out of the three significant variables identified, KH_2PO_4, seems to negatively affect both toxin and protease production. On the contrary, K_2HPO_4, starch and $FeSO_4$ have an opposite effect by increasing delta-endotoxins production and decreasing proteolytic activity.

The secreted proteases from microorganisms are divided into two categories: intracellular and extracellular. Intracellular proteases are essential for metabolic processes, such as cell growth, protein turnover and differentiation, which mean that intracellular proteases were mainly involved in the synthesis of delta-endotoxins. In this case, the bacterium

needs nutrients for protoxin activation through intracellular proteases and thus for delta-endotoxins production (Reddy and Venkateswerlu 2002). This fact may explain that some medium components were needed for both delta-endotoxins and protease production. However, extracellular enzymes are needed for digesting nutrients and enable the cell to absorb hydrolyzed products in order to conclude microbial growth stages (Padmaja et al. 2008). That may elucidate the significance of antagonist effects of the nutrients on delta-endotoxins and proteolytic activity.

In this study, Plackett–Burman design was successfully used to screen the main factors affecting delta-endotoxins and protease production from seven factors. An improvement of almost 85% in delta-endotoxins produced by *B. thuringiensis* subsp. *kurstaki* S7 over the basal medium was reached upon optimization. Our finding revealed that a careful balance in nutrient compounds should be established to promote delta-endotoxins production and to minimize proteolytic enzyme activity. Further optimization by response surface methodology should be done to define the optimal values of the selected variables.

Acknowledgments

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