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Optimization of Bioinsecticides Overproduction by *Bacillus thuringiensis* subsp. *kurstaki* Using Linear Regression

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Abstract

A multiple linear regression analyses were performed to screen for the significant factors simultaneously influencing production of delta-endotoxin, proteolytic activities and spore formation by a *Bacillus thuringiensis kurstaki* strain. Investigated factors included: pH of the medium, available oxygen and inoculum size. It was observed that oxygen availability was the most influencing setting on both delta-endotoxins production and spores counts, followed by initial pH of the medium and inoculum size. On other hand, pH of medium was found to be the most significant parameter for proteolytic activity, followed by inoculum size and dissolved oxygen. Our results suggested that the first order with two-factor interaction model seemed to be more satisfactory than simple first order model for optimization of delta-endotoxin overproduction. The coefficients of determination (R^2) indicated a better adequacy of the second order models to justify the obtained data. Based on results, relationships between delta-endotoxins production, proteolytic activities and spores counts were established. Our results can help to balance delta-endotoxins production and its stability.

Key words: *Bacillus thuringiensis kurstaki*, delta-endotoxins, multiple linear regression, proteases, spores

Introduction

Bacillus thuringiensis is an aerobic, Gram-positive and spore-forming bacterium. *B. thuringiensis* grows under aerobic conditions with few nutrient requirements (Zouari *et al.*, 2002). After exhaustion of one or more nutrients, *B. thuringiensis* produces a spore and parasporal crystal proteins (also called delta-endotoxins) exhibiting insecticidal activity with high specificity to insects larvae belonging to Lepidoptera, Diptera, Coleoptera, Hymenoptera, Orthoptera orders (Feitelson, 1993). *B. thuringiensis* production, especially concerning the spore-crystal complex, depends principally on the composition of the culture medium components and fermentation conditions (Ghribi *et al.*, 2007a). Furthermore, some studies had shown that *B. thuringiensis* bioinsecticides production is achieved by significant levels of enzymes in the growth media (Zouari and Jaoua, 1999; Chen *et al*., 2004). The production and secretion of enzymes by microorganisms is induced by medium ingredients and other physical factors such as inoculum size and available oxygen (Celik and Calik, 2004; Singh *et al.*, 2004). Proteolytic activities are essential to assimilate proteins as nitrogen source, but they are involved in the stability of proteins produced by the bacterium as metabolites. In our previous work (unpublished), we showed that a strong relationship exists between accumulation of delta-endotoxins of *B. thuringiensis* and the available proteolytic activities in the medium. It is then necessary to establish a balanced system in which the synthesis and accumulation of delta-endotoxins in the fermentation broth is not counteracted by of proteolytic activity of *B. thuringiensis*.

There is a common interest to investigate such balance. It is still difficult to predict simultaneously yields of multiple bioproducts using a given set of cultivation parameters. Multiple linear regression is a statistical method used to analyse the relationship between one response variable (dependent variable) with two or more variables (independent variables). If the relation between the dependent and other independent variables could be found using multiple linear regression, a better control strategy could be sought.

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The aim of this study was to optimise external factors which play an important role in improving the production of *B. thuringiensis* delta-endotoxins and to find the relationship between the most important parameters which are the inoculum size, initial pH and available oxygen, then to predict delta-endotoxins, proteases concentrations and spores counts using multiple linear regression. The optimisation process was carried out by development of mathematic equations characterizing the experimental results as a function of the factor level.

Experimental

Materials and Methods

Microorganisms. The bacterial strain S7 was isolated and identified as *B. thuringiensis kurstaki* in our laboratory. It was shown that it exhibits promising potentials in pest caterpillars control (data unpublished). The strain was maintained by streak inoculating on Luria Broth (LB) nutrient plates (g/l): yeast extract 5, peptone 10, NaCl 5 and agar 15, incubated at 30°C for 24 h and stored at 4°C for further use.

Inocula preparation method. Inocula were prepared as follows: one isolated colony was dispensed in 3 ml of LB medium and incubated overnight at 30°C. 0.5 ml were used to inoculate 250 ml shake flasks containing 50 ml LB medium. After 6 h of incubation at 30°C in 200 rev/min in a rotary shaker (New Brunswick Scientific, Edison, NJ, USA), the culture broth was used to inoculate the media. The $O.D._{600}$ was determined using a SmartSpec™ 3000 UV-visible spectrophotometer (Bio-Rad Laboratories).

Cultures conditions. *B. thuringiensis* strain was grown in complex medium for delta-endotoxin production. Economic complex medium (Ghribi *et al.*, 2007b) contained (g/l): soybean flour, 25; starch, 30; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.3; $MnSO_4$, 0.1; $FeSO_4$, 0.1. The initial pH was adjusted and $CaCO_3$ (20 g/l) were added for maintaining pH value. All flasks are sterilized at 121°C for 20 min. Shake flask cultures were hold in shake flasks containing 25 ml of medium and incubated at 30°C on a rotary shaker at 200 rev/min for 72 h.

Quantification of delta-endotoxin production. Crystal proteins were solubilized before protein concentration assay. Crystal-spore pellets were washed twice with 1 M NaCl and twice with bi-distilled water. Samples were incubated in 0.05 M NaOH (pH 12.5) for 2 h at 30°C in a rotary shaker (200 rev/min). Obtained fraction was collected by centrifugation with 13000 × g for 10 min. The supernatant, containing the alkali-soluble insecticidal crystal proteins was used to estimate delta-endotoxin concentration by Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as a protein standard. Samples were measured after 10 min at 595 nm. The values are the average of three measures of two separate runs.

Proteolytic activity assay. Protease activity was determined according to modified Kunitz method (Kunitz, 1946). Supernatant of culture medium was diluted with Tris-HCl buffer pH 7.0. Proteolytic activity was assayed by incubating 1 ml diluted enzyme solution with 1 ml casein (1% w/v) for 20 min at 60°C. The reaction was stopped by adding 3 ml of 5% trichloroacetic acid (TCA). The mixture was incubated for 30 min to precipitate the total non-hydrolysed proteins and peptides. Blanks were prepared with inactivated enzymes containing supernatants. After the incubation, samples and blanks were centrifuged at 13000 × g, for 15 min. The absorbance of the samples was measured at 280 nm. One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1 µg of tyrosin within 1 min at 60°C. The presented values are the average of three measures of two separate runs.

Viable spore count. Viable spore counts from the bacterial culture were measured by colony counting after heating the culture sample at 80°C for 10 min in order to destroy vegetative cells, The LB plates were incubated at 30°C for 24 h. Colonies were counted and statistically analysed (and expressed as c.f.u. ml^{-1}). The values are the mean of three determinations of two separate runs.

Effect of aeration, inoculum volume and initial pH on *B. thuringiensis* production. The effect of aeration on *B. thuringiensis* subsp. *kurstaki* was determined by growing the strain S7 into various shake flask volumes (250 ml, 500 ml and 1000 ml) and constant medium volume of 25 ml. Consequently, the quotient, corresponding to the volume of air in the flask compared to culture volume, was equal to 9, 19 and 39. The initial pH was adjusted to 6.5, 7.0 or 7.5 in order to investigate the effect of various initial pH on *B. thuringiensis* bioinsecticides production. The optical density at 600 nm of the inoculum prepared in LB medium, was measured and calculated volumes were used to inoculate the cultures with inoculum sizes of 0.100, 0.150 and 0.200, corresponding to 1.35×10^8, 2×10^8 and 2.7×10^8 cells/ml respectively.

Linear Regression model. The purpose of the model is the recovery of specific information about the culture conditions influenced by several variables observed simultaneously and the estimation of the dependent variable from the other observed independent variables. The multiple regression analyses were conducted to establish prediction equations (Minitab Inc., State College, PA).

Results and Discussion

Multiple linear Regression analysis. Regression analysis demonstrates the statistical relationship between one or more parameters and the response variable to forecast new observations. The output is expressed as a simple linear model such as $Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \varepsilon$, where Y is the dependent (response) variable. The variables to be predicted are $\beta_0, \beta_1, \beta_2$ and β_3, are empirical constants and x_1, x_2 and x_3 are the parameters influencing the studied response and with the error term ε.

Regression results define the statistical significance, direction and size of the relationship between a parameter and the response. Sign of each coefficient suggests the direction of the relationship. The coefficients describe the change in the response for one unit of change in the predictor while considering other parameters in the model constant. The significance of coefficients was estimated by student's t-test and p-values. The higher the level of the t-value and the lower the p-value, the more significant is the coefficient (Khuri and Cornell, 1987).

Three multiple linear correlations were tested in which, delta-endotoxins concentration (Y_1), proteases concentration (Y_2) and spore counts (Y_3) are the dependent variables. The applicable range of all the parameters of the regression model is the following: optical density of inoculum varied from 0.10 to 0.20, available oxygen quotient, varied from 9 to 39, and the initial pH medium varied from 6.5 to 7.5.

Results of delta-endotoxins productions, proteolytic activity and spores counts depending on culture parameters (pH, available oxygen and inoculum volume) are presented in Table I. Delta-endotoxin concentrations ranged from 2597 mg/l to 3464.68 mg/l, enzymatic activity values varied from 434.09 U/ml to 954.55 U/ml whereas spores counts ranged from 70×10^7 c.f.u./ml to 150×10^7 c.fu./ml according the experimental conditions. Regression coefficients and corresponding t-values for the model were obtained which are shown in Table II. The regression model proposed for estimation of delta-endotoxin production is as follows:

$$Y_1\ (\text{mg/l}) = 4465 + 13.4\ x_1 - 326\ x_2 + 3080\ x_3 \quad \text{(Eq. 1)}$$

Where (x_1) is the available oxygen ratio, (x_2) is initial pH medium and (x_3) is the optical density at 600 nm of inoculum (OD_{600}). The analysis of multiparametric function, such as the example shown in Table II, indicates its adequacy, with a satisfactory R^2 (72.5%). Analysis of the p-values indicates the level of significance of each effect. Effects with p-value < 0.05 are highly significant. p-value < 0.1 shows a moderate significant effect and p-value < 0.2 suggests that the effect can be considered (Moita *et al.*, 2005). The p-values for all variables are less than 5%. The p-values indicate that x_1 was the most significant (p = 0.001), followed by x_2 (p = 0.005) and x_3 (p = 0.018) in multiple linear correlations.

Trials and results for proteolytic activities of *B. thuringiensis* are showed in Table I. The regression coefficients and t-values for the model were obtained (Table II).

Table I
Study of the effects of oxygen, initial pH of medium and optical density (OD) of inoculum at 600 nm on delta-endotoxin productions, proteolytic activity and spores counts.

Volume	pH	OD inoculum	Delta-endotoxins production (mg/l)	Proteolytic activity (U/ml)	Spores counts (10^7/ml)
39	7.5	0.1	2597.01 ± 85.7	434.09 ± 20.5	100 ± 5
19	7	0.15	2711.93 ± 61.3	556.82 ± 13.7	120 ± 7
9	7.5	0.15	2748.1 ±87.7	625 ± 23.9	140 ± 5
9	6.5	0.15	2783.47 ± 44.36	534.09 ± 22	130 ± 4
9	7.5	0.2	2783.47 ± 86.6	954.55 ± 27.4	146 ± 6
19	7.5	0.2	2788.97 ± 43.34	727.27 ± 17.8	147 ± 2
19	7.5	0.1	2789.59 ± 62.74	545.45 ± 19.18	100 ± 5
9	7	0.15	2803.83 ± 43.85	572.73 ± 26	150 ± 7
19	6.5	0.1	2858.82 ± 60.6	472.73 ± 21.9	94 ± 5
39	7.5	0.15	2908.7 ± 61.3	615 ± 11	89 ± 6
19	6.5	0.15	3029.19 ± 91.9	440.91 ± 27.27	90 ± 5
39	7	0.1	3031.18 ± 43.85	545.45 ± 16.36	70 ± 7
19	6.5	0.2	3244.83 ± 85.7	436.36 ± 20	90 ± 3
39	7	0.2	3289.78 ± 62	545.45 ± 23.18	95 ± 5
39	7	0.15	3415.19 ± 89.8	477.27 ± 20.45	97 ± 5
39	6.5	0.15	3464.68 ± 94.11	488.64 ± 15	96 ± 4

Table II
Regression results of delta-endotoxins and proteases concentrations, and colonies forming units (c.f.u./ml)

Term	Delta endotoxins			Proteases			Colonies forming units (c.f.u./ml)		
	Coefficient	Standard effect (t)	p-value	Coefficient	Standard effect (t)	p-value	Coefficient	Standard effect (t)	p-value
Constant	4464.8	6.47	0.000	–851.5	–2.20	0.048	–38.33	–0.60	0.562
x_1	13.390	4.11	0.001	–3.240	–1.77	0.102	–1.3626	–4.49	0.001
x_2	–326.43	–3.46	0.005	181.66	3.43	0.005	21.794	2.48	0.029
x_3	3080	2.73	0.018	1421.8	2.25	0.044	182.8	1.74	0.108
	S = 155.757 R^2 = 72.5% R^2 (adjusted) = 65.6%			S = 87.4089 R^2 = 64.5% R^2 (adjusted) = 55.7%			S = 14.5134 R^2 = 73.5% R^2 (adjusted) = 66.9%		

The regression model proposed for determination of protease concentration is shown in Equation (2):

$$Y_2\,(\text{U/ml}) = -851 - 3.24\,x_1 + 182\,x_2 + 1422\,x_3 \quad \text{(Eq. 2)}$$

The regression results in Table II reveal that the correlation yields a R^2 value of 0.645, which means that the proposed model can explain 64.5% variation in the response. The p-values reveal that x_2 ($p = 0.005$) was highly significant, followed by x_3 ($p = 0.044$), while x_1 showed a moderate significant effect ($p = 0.102 \geq 0.1$). It can be seen that the available air has a negative effect and pH of the medium and inoculum size have positive effect, which means that an increase in the inoculum size and/or pH of the medium and a decrease of the available oxygen lead to improve enzymatic activity. Table II shows the variables effects on spore counts. The regression model for determination of spore counts is presented in Equation (3):

$$Y_3\,(10^7/\text{ml}) = -38.3 - 1.36\,x_1 + 21.8\,x_2 + 183\,x_3 \quad \text{(Eq. 3)}$$

In the multiple linear correlation (Table II), the correlation yields the smallest R^2 of 73.5%. Additionally, p-value for the slopes of two parameters (x_1 and x_2) are less than 5%, these p-values indicate that the parameters are significant variables in this correlation. The p-value of inoculum (x_3) indicates the relative significance volume in variation of spores counts ($p = 0.108 \geq 0.1$).

Data analysis shows that oxygen was found to enhance the delta-endotoxins contents. However, it has an inhibitory effect on both proteolytic activity and spores counts. Foda *et al.* (1985) noted the failure of the organism to sporulate under low aeration levels. In other study, Maldonado-Blanco *et al.* (2003) obtained the most toxic *B. thuringiensis* bioinsecticide producted at high aeration rates, in spite of inhibition of sporulation.

The effect of initial pH of medium on delta-endotoxins production was also examined. The results indicate that it exhibits a supressive effect at high levels, on delta-endotoxins production but improves protease activity and spores counts. In fact, Alves *et al.* (1997) stated that the culture medium should be brought to neutral pH at harvest because high pH is optimal for *Bacillus thuringiensis* proteases and potentially damaging to the crystals. Furthermore, Ejiofor and Okafor (1989) concluded that low pH can restrain growth, sporulation and crystal formation of *B. thuringiensis*. Likewise, the volume of inoculum used for the production of crystal-spore complex has no significant effect on delta-endotoxins formation. High initial cell concentration in the production medium may result in a rapid consumption of oxygen and other nutrients. During fermentation, *B. thuringiensis* produces different types of proteases which can be supplied for the hydrolysis of protein components in the medium to amino acids that can be used for the microbial growth of *B. thuringiensis* and synthesis of useful metabolites (Tyagi *et al*., 2002). Besides, proteolytic activity kept on increasing until the end of fermentation process and could be due to the lysis of sporulated cells and release of intracellular proteases into the medium (Zouari and Jaoua, 1999). In addition, we conclude that it is recommended to use small amount of inoculum to limit the proteolytic action of secreted enzymes on crystals. Similarly, this explains previous results reported by Zouari *et al.* (1998).

The study of parameters involved in delta-endotoxins concentrations, protease concentrations and spores counts showed that sporulation phenomenon is strongly related with production of *B. thuringiensis* proteolytic enzymes. In fact, the sporulation stage in *B. thuringiensis* is marked by the synthesis of many types of proteins, including proteases (Bibilos and Andrews, 1988). Sporulation process is characterized by protein turnover as there is development of cortex and spore coats interfered by intracellular proteases.

Normal probability plot of residuals. Figure 1 shows the normal probability plot of the residuals for delta-endotoxins (a), proteases (b) and spores counts, expressed by colonies forming units (c). It can be seen

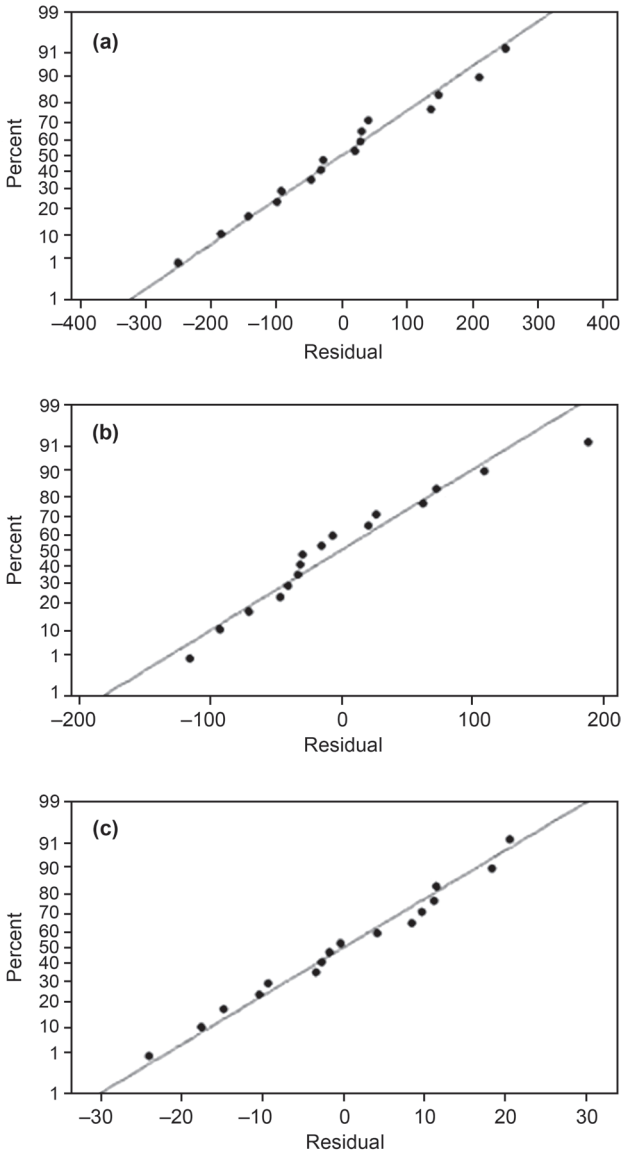

Fig. 1. Normal probability plot for the production of delta-endotoxins (a), proteases (b) and spores counts (c).

that for all the responses, the points fall close to the straight line. Hence, the data from the essays come from a normally distributed set. Normal probability plots are a graphical method for verifying the normality of the residuals. As seen in the figure, the normality assumption was reasonably satisfied as the points in the plot form straight line. All of the plots show that model is satisfactory to illustrate the final bacterial products (crystal proteins, proteases and spores) by multiple linear regression.

Development of regression model equation. In order to study the combined effects of the studied factors, experiments were performed for various combinations of the parameters using statistical methods. The model for multiple linear regression given n observations is: $Y = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \ldots \beta_p x_{ip} + \varepsilon_i$, where Y is the dependent variable, β_0 is the intercept, β_1, β_2 and β_p are the regression coefficient of each independent variable included in the regression model and ε_i is the random error term.

The different coefficients of the regression function, the t and p-values for delta-endotoxins, proteases and spores counts are presented in Table III. The polynomial equations for delta-endotoxins (Eq. 4), proteases (Eq. 5) and spores counts (Eq. 6) in terms of coded factors are given as following:

$$Y_4\ (\text{mg/l}) = -1140 + 157\ x_1 + 525\ x_2 + 17195\ x_3 - 22.0\ x_1x_2 + 77.1\ x_1x_3 - 2361\ x_2x_3 \quad \text{(Eq. 4)}$$

$$Y_5\ (\text{U/ml}) = 2212 + 31.30\ x_1 - 285\ x_2 - 21844x_3 - 3.40\ x_1x_2 - 63.90\ x_1x_3 + 3486\ x_2x_3 \quad \text{(Eq. 5)}$$

$$Y_6\ (10^7/\text{ml}) = 230 - 0.16\ x_1 - 16.3\ x_2 - 1770\ x_3 - 0.154\ x_1x_2 - 0.3\ x_1x_3 + 275\ x_2x_3 \quad \text{(Eq. 6)}$$

It is observed in Table III that low p-value (p = 0.013) of (x_1) confirms highly significance of this factor for delta-endotoxins production. The effect of (x_1x_2)

Table III
Estimated regression coefficients for delta-endotoxins concentrations, proteases and colonies forming units (c.f.u./ml) in coded units.

Term	Delta endotoxins			Proteases			Colonies forming units (c.f.u./ml)		
	Coefficient	Standard effect (t)	p-value	Coefficient	Standard effect (t)	p-value	Coefficient	Standard effect (t)	p-value
Constant	–1140	–0.41	0.692	2212	1.58	0.148	230.5	0.63	0.546
x_1	156.97	3.07	0.013	31.30	1.22	0.254	–0.158	–0.02	0.982
x_2	524.6	1.33	0.216	–285.1	–1.44	0.184	–16.32	–0.31	0.761
x_3	17195	1.11	0.295	–21844	–2.81	0.020	–1770	–0.87	0.408
x_1x_2	–22.036	–3.16	0.012	–3.398	–0.97	0.357	–0.1536	–0.17	0.871
x_1x_3	77.13	0.94	0.371	–63.90	–1.55	0.155	–0.33	–0.03	0.976
x_2x_3	–2361	–1.10	0.301	3486	3.23	0.010	275.3	0.97	0.357
	S = 119.954 R^2 = 87.7% R^2 (adjusted) = 79.6%			S = 60.22 R^2 = 87.4% R^2 (adjusted) = 78.9%			S = 15.8087 R^2 = 76.4% R^2 (adjusted) = 60.7%		

interaction has high influence on delta-endotoxin production ($p = 0.012$). In this case, the p-value ($p \leq 0.05$) in both responses for regression model equation implies that the polynomial model fitted well with the experimental results. However, (x_2), (x_3) and all interaction terms (except x_1x_2) were insignificant to the delta-endotoxins production. Furthermore, according to results of Table III, low p-values of (x_3), confirm highly significant effect of this factor for protease activity in the culture broth ($p = 0.020 < 0.05$). Likewise, the effects of x_2x_3 (p-value of 0.010) can be considered as highly significant. It was found that x_1, x_2 and all interaction terms (except x_2x_3) were insignificant to this response. On other hand, the regression analysis of spores counts results shown in Table III reveals that all terms are insignificant because of their high p-values ($p > 0.05$).

Similarly, variables effects and variable interactions on delta-endotoxins productions, proteolytic activity and spore counts of *B. thuringiensis* were analysed. It can be seen that (x_1) has active effect, while (x_1x_2) interaction denoted inhibitory effect on twice delta-endotoxins and proteases productions. All the other parameters have opposite effect on delta-endotoxins and proteases production. Finally, most parameters have similar negative effects on both protease activities and spores counts, except positive effect of (x_1) on proteolytic activity. In the same way, only (x_2x_3) has positive effect on proteolytic activity and spores counts. That suggests more associated relationships between proteolytic enzymes secretions and spores forming compared to delta-endotoxins concentrations. In fact, Freese and Heinze (1984) showed that high protease excretion by some *Bacillus* species starts during the sporulation phase. The relationship implying delta-endotoxins concentrations and proteolytic activities may be explained by the "disrupted action" of proteases on delta-endotoxins.

Additionally, R^2 values of 87.7%, 87.4% and 76.4% for delta-endotoxin concentration, proteolytic activity and spores counts, respectively, express high correlations between the observed and predicted values. Besides, an enhancement of coefficient of determination values in developed regression models confirms more accurate representative models of the experimental data, considering parameters interactions, compared to simple regression models.

According to the coefficients of determination values, the responses showed that the linear terms of the medium pH, initial inoculum volume, available oxygen and the interactions, have notable effects on delta-endotoxins, proteases production and spores formation. It implies that interaction effects of studied parameters were evident on production of delta-endotoxins, proteolytic enzymes secretion and spores counts by *B. thuringiensis kurstaki*. Moreover, this study proves that first order with two factor interactions model could be used in order to exhibit the possible relationships existing between the studied parameters and the effects on the responses. These methods are helpful for improving the accuracy of analytical predictive models in case of *B. thuringiensis kurstaki* studies.

In this work, multiple linear regression were used to test the relative importance of culture settings (initial pH, available oxygen and inoculum volume) on different microbial final products (delta-endotoxins, proteases and spores). Simple linear regression results suggested that the studied parameters have significant influence on bioinsecticides production. Furthermore, available oxygen and inoculum size showed a moderate significant effect on proteolytic activity and spores counts respectively. All other parameters showed high significant effect on twice proteolytic activity and spores counts. The study of multiple linear regressions of proposed models, including interactions parameters, reveals that negative interaction between available oxygen and initial pH must be considered for delta-endotoxins production. In addition, interaction between inoculum and initial pH showed a positive significant effect on proteolytic activity. The regression relations may be used to forecast concentrations of various *B. thuringiensis* products during fermentation. The regression equations presented in this study are specific for *B. thuringiensis* strain. It is important to note that these statements are suitable within the lower and upper limits of the factors: initial pH (6.5–7.5), optical density at 600 nm of inoculum size (0.10–0.20) and available oxygen ratio (oxygen volume /culture volume) (9–39).

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