Statistical optimization of medium components and physicochemical parameters to simultaneously enhance bacterial growth and esterase production by *Bacillus thuringiensis*

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Running title: Optimization of esterase production by *Bacillus thuringiensis*

Abstract

Bacillus thuringiensis is a genus extensively studied because of its high potential biotechnological application, principally in biocontrol techniques. However, the optimization of esterase production by this strain has been scarcely studied. The aim of this work was to select and optimize the physicochemical and nutritional parameters which significantly influence the growth and esterase production of *Bacillus thuringiensis*. To this purpose six nutritional factors and two physicochemical parameters were evaluated using a Plackett-Burman design. Significant variables were optimized using Box-Benhken design and through the desirability function to select the levels of the variables that simultaneously maximize microbial growth and esterase production. The optimum conditions when applying simultaneous optimization of the responses under study resulted to be: glucose concentration, 1 g/L; peptone concentration, 15 g/L and NaCl concentration, 3.25 g/L. Under these optimal conditions, it was possible to achieve a 2.5-log CFU/mL increase in bacterial growth and 113-fold increase in esterase productivity, compared to minimal medium without agitation.

Keywords

Esterase; medium optimization; Plackett-Burman design; Response surface methodology; microbial growth.

1. INTRODUCTION

The development and evolution of biotechnology over the past four decades expanded the field of industrial enzymes application, constituting as a consequence, a worldwide market of great importance (Kirk *et al.* 2002; Pandey *et al.* 2006). Among them, lipolytic enzymes are of growing interest because of their wide range of applications. Esterases (EC 3.1.1) represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds (Bornscheuer 2002; Costas *et al.* 2004). Bacteria produce different classes of lipolytic enzymes, among which carboxylesterases (esterases, EC 3.1.1.1) and triacylglycerol lipase (lipases, EC 3.1.1.3) are two of the most important. These enzymes can be differentiated by their substrate specificity (Arpigny and Jaeger 1999; Costas *et al.* 2004). Esterases hydrolyze water soluble or emulsified esters with short-chain carboxylic acids (less than or equal to ten carbon atoms), whereas lipases prefer long chain acyl-glycerides (greater than or equal to ten carbon atoms) (Jeon *et al.* 2009; Nardini and Dijkstra 1999).

Different uses of industrial esterases include organic chemical processing, detergent formulation, synthesis of biosurfactants, oleochemistry, dairy production, agrochemicals, paper manufacture, nutrition, cosmetics, and pharmaceutical processing; most of them related to its excellent stereospecificity, enantioselectivity and regioselectivity (Jaeger and Eggert 2002; Pandey *et al.* 1999; Sharma *et al.* 2001). The interest in these enzymes also resides in the fact that they do not require cofactors, are usually rather stable and are active even in organic solvents (Bornscheuer 2002). However, the large diversity of reactions and substrates handled by esterases in nature is still poorly explored. In part, this is caused by

69 their limited commercial availability (Bornscheuer 2002). Therefore, the identification of
70 new esterases with potential industrial applications is highly desirable and would be
71 invaluable to the growing industry of biocatalysts (Jeon *et al.* 2009).
72 *Bacillus thuringiensis* is the most important microorganism with entamopathogenic
73 activity. Bacterial toxicity is due to endotoxinproteins, which are synthesized during
74 sporulation and assembled into parasporal crystals that are toxic when ingested by larvae of
75 certain insect orders. Nowadays, *Bacillus thuringiensis* formulations account for 80-90% of
76 the biopesticides in the global market (Crickmore 2006; Moraga *et al.* 2004). Although the
77 ecology of *B. thuringiensis* is greatly unknown, the cosmopolitan nature of its distribution
78 is widely accepted, which may indicate a wide diversity of natural habitats and, as a
79 consequence, a large battery of hydrolytic enzymes. In spite of the potential
80 biotechnological use of these enzymes, this subject has been scarcely studied (Tenorio-
81 Sánchez *et al.* 2010).
82 For enzyme production at an industrial scale it is necessary, in the first instance, the
83 identification of physicochemical factors affecting enzyme production, and then the
84 optimization of the level of each one of these factors to be used to maximize enzyme yield
85 and activity. Nowadays there are different statistical methods available to evaluate a large
86 number of variables using a small number of assays, which minimize time invested and
87 reduce experimental costs (Agüero *et al.* 2011; Rajendran *et al.* 2008). One of these
88 methods is the Plackett-Burman design, which allows an efficient screening of several
89 variables to identify those that significantly affect the responses (Queiroga *et al.* 2013;
90 Rajendran *et al.* 2008). Then, response surface methodology (RSM) is applied to find
91 which levels of the selected factors maximize microbial growth and esterase production.

RSM is considered suitable for optimization of enzyme production because allows a simultaneous evaluation of the factors tested as well as the interaction between these factors (Singh *et al.* 2011; Vijayaraghavan and Vincent 2014).

The aim of this study was to screen and optimize the most important factors affecting bacterial growth and esterase production by a *Bacillus thuringiensis* strain. A Plackett-Burman statistical design was applied to select the variables that significantly influence each response, followed by RSM to optimize the levels of those variables that enhanced simultaneously esterase production and microorganism growth.

2. MATERIALS AND METHODS

2.1. Microorganism maintenance and inoculum preparation

The bacterial strain used in this work was isolated from defatted soy pellet and identified as *Bacillus thuringiensis* (Mazzucotelli *et al.* 2013).

Stock culture was maintained on soft Luria- Bertani (LB) agar media (1% w/v tryptone; 1% w/v NaCl; 0.5% w/v yeast extract; with 3.5 % w/v agar- agar; pH 7) at -18ºC. Before use, the strain was cultured in LB broth for 24 h at 37◦C. Approximately 0.1 mL of culture was transferred to 9.9 mL of LB broth at 2 consecutive 24 h intervals immediately before each experiment.

2.2. Esterase production and bacterial growth determination

Esterase activity was measured by the method described by Pera *et al.* (2006). Bacterial cells were pelleted by centrifugation at 10,000 rpm (4472g) for 15 min at 4ºC. The cell-free culture supernatant was regarded as the enzyme solution. Substrate, p-nitrophenyl butyrate (*p*NPB), was prepared at 1 mM in acetone.

The reaction mixture was prepared with: buffer mixture (phosphate buffer 100 mM, pH 7; arabic gum 0.1% w/v; Triton X-100 0.4% w/v), substrate (*p*-nitrophenyl butyrate 1 mM); and enzyme solution, in an 8:1:1 ratio, respectively.

A control sample was carried out incubating the reaction mixture without the enzyme solution. All samples were incubated at 37°C until the appearance of a yellowish coloration. Esterase activity was measured spectrophotometrically at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per minute (Pera *et al.* 2006). The molar absorptivity of *p*-nitrophenol under the assay conditions was found to be 0.00639 L/(µmol·cm). Esterase activity was expressed as U/L.

Bacterial growth (BG) was determined by a serial dilution method on LB agar plates (Benson 2002). The plates were incubated at 30ºC for 24-48 h and the numbers of colonies were determined. Microbial counts were expressed as log CFU/mL.

2.3. Experimental design and data analysis

2.3.1. Medium components and physicochemical parameters

In this study, eight nutritional and physicochemical factors were evaluated to determine which one significantly affected BG and EP. The selected factors were six medium components: glucose (as carbon source), olive oil (as carbon source and esterase inducer),

Tween 80 (as carbon source and esterase inducer), peptone (as organic nitrogen source), $(NH_4)_2SO_4$ (as inorganic nitrogen source), NaCl (for isotonic balance); and 2 operating conditions: pH and agitation rate.

Both the factors as their levels were selected based on a preliminary literature review, analyzing which are the main nutritional components and physicochemical parameters that could affect BG and EP. To select the pH levels, a preliminary test was conducted in which the range of appreciable growth of the strain was evaluated (data not shown). Each factor was examined at two levels: -1 as the low level and +1 as the high level. The levels selected for each factor were: agitation speed (x_1; -1: 0; +1: 120 rpm); pH (x_2; -1: 6; +1: 9); glucose (x_3; -1: 1; +1: 10 g/L); olive oil (x_4; -1: 0; +1: 10 g/L); Tween 80 (x_5; -1: 0; +1: 10 g/L); peptone (x_6; -1: 1; +1: 15 g/L); $(NH_4)_2SO_4$ (x_7; -1: 0; +1: 10); NaCl (x_8; -1: 1; +1: 10 g/L).

To determine the shortest incubation time at which the maximum esterase production by *Bacillus thuringiensis* was achieved, a preliminary assay was performed. For this purpose, 1 mL of fresh culture of the strain was transferred into a 250-mL Erlenmeyer flask containing 100 mL of fresh culture medium (1% w/v tryptone; 1% w/v NaCl; 0.5% w/v yeast extract) and incubated on an orbital shaker at 100 rpm, 30°C for 120 h. Samples were taken at regular time intervals, in which bacterial growth and esterase activity were determined.

2.3.2. Selection of significant variables by Plackett–Burman design

The Plackett–Burman experimental design was used to evaluate the relative importance of various nutrients and physicochemical parameters for esterase production and bacterial growth by *B. thuringiensis* in submerged fermentation. Table 1 represents the Plackett–

160 Burman experimental design for 12 trials with two levels for each variable and the
161 corresponding esterase production and bacterial growth. Variables *X1* to *X8* represent the
162 medium components and physicochemical parameters and d_1 to d_3 represent the *dummy*
163 variables/unassigned variables.

164 Each trial was conducted in Erlenmeyer flasks (100 mL) containing 30 mL of culture
165 medium, with the components and conditions outlined by the PB matrix (Table 1). Each
166 flask was inoculated with 1% (v/v) culture (cell concentration approximately: 5.10^5
167 CFU/mL). The flasks were incubated for a period of 96 h with orbital shaking and after this
168 period BG and EP were determined.

169 The Plackett–Burman design assumes that there are no interactions between the different
170 media constituents, x_i, in the range of variables under consideration. A linear approach is
171 considered to be sufficient for screening:

172 $$Y = \beta_0 + \sum \beta_i X_i \quad ; \qquad i = 1,2,...,k \qquad (1)$$

173 where Y is the estimated target function for the responses (esterase production or bacterial
174 growth), β_0 is the model intercept, β_i are the regression coefficients and X_i is a
175 dimensionless coded value of x_i (independent variable).

176 In this fractional factorial design the main effect of each independent variable (*E*) may be
177 calculated as the difference between the average of measurements made at the high level
178 (+1) of the factor and the average of measurements made at the low level (-1). A large *E*
179 value obtained for a factor, either positive or negative, indicates that the factor has a large
180 impact on response; while a coefficient close to zero means that the factor has little or no
181 effect (Agüero *et al.* 2011; Levin *et al.* 2005). *Dummy* variables were used to estimate
182 experimental error in data analysis. The significance of each variable was determined via

183 Student's *t*-test. All experiments were carried out in triplicate and the average values of
184 esterase production (Y1) and bacterial growth (Y2) were taken as responses.
185 Data were analyzed using REG procedure of SAS software version 8.0 (SAS Inst. Inc.,
186 Cary, N.C., U.S.A. 1999. Based on a regression analysis of the variables, a confidential
187 level of 95% ($p<0.05$) for each factor was considered to have a significant effect on a
188 response. Experimental error and standard error were calculated as described in
189 Mazzucotelli *et al.* (2015).
190
191 *2.3.3. Optimization of selected components by Response Surface Methodology*
192 The next step in the formulation of the culture medium and incubation conditions was to
193 determine the optimum levels of the significant variables for EP and BG. For this purpose,
194 response surface methodology was used to optimize the screened components using Box–
195 Behnken (BB) design.
196 To evaluated 3 factor at 3 levels (low, −1; middle, 0; high, +1), with three replicates at the
197 central point, the BB design needs a total of fifteen combinations or experimental runs
198 (Table 3) (Aslan and Cebeci 2007).
199 Each test was carried out in a 100 mL Erlenmeyer flask with 30 mL of culture medium
200 comprised by the same factors used in the Placket-Burman design, keeping all them at its
201 average level, except for the factors to be optimized, which were added to the
202 concentrations determined by the BB matrix (Table 3). Each flask was inoculated with 1%
203 (v/v) of active culture (approximate initial cell concentration: 5.10^5 CFU/mL) and
204 incubated for a period of 96 h, at 30°C, with intermediate level of agitation (60 rpm). After

this period, microbial growth and esterase activity were determined. Table 3 shows BB matrix with the experimental results for both responses.

Once responses (EP and BG) were measured for each trial, the behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (2)$$

where Y is the predicted response (EP, or BG), β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient for the interaction effect, and X_i is a dimensionless coded value of x_i (independent variable).

All experiments were performed in a random order and were independently repeated three times. Data were analyzed using SAS software (version 9.0, North Carolina, U.S.A). The goodness of the fit model was evaluated by the lack of fit, the determination coefficient (R^2) and the analysis of variance (ANOVA). Statistical testing of the model was done by the Fisher's statistical test. The robustness of the model was assessed by the determination coefficient (R^2), correlation coefficient (R) or F-test.

2.3.4. Simultaneous optimization: Desirability function

The "desirability function" approach is one of the most widely used methods when several responses variables need to be simultaneously optimized. This method resolves the conflict often found in independent optimization, when improving one response may have an opposite effect on another one (Derringer 1994). The advantages of using desirability functions include the following: (1) responses that have different scaling can be compared, (2) the transformation of different responses to one measurement is simple and quick, and (3) both qualitative and quantitative responses can be used (Derringer and Suich 1980).The

general approach is to first convert each response (y_n) into an individual desirability function (d_n). The scale of the desirability function ranges between d_n=0, which suggests that the response is completely unacceptable, and d_n = 1, which suggests that the response is exactly the target value (Mazucotelli *et al.* 2015).

Simultaneous optimization, desirability functional analysis and 3D plots of the responses were performed using Statistica software (version 7.0, Stat Soft Inc., Tulsa, USA).

3. RESULTS

3.1. Screening of significant variables using Plackett–Burman design

The shortest incubation period to achieve the maximum esterase production by *Bacillus thuringiensis* was determined in a preliminary assay. It was found at 96 h of culture incubation, and no significant differences were found with longer incubation times. Therefore, for further assays all the cultures were incubated for 96 h for monitoring BG and EP.

A total of eight variables were analyzed with regard to their effect on esterase production and bacterial growth using a Plackett–Burman design. The design matrix selected for the screening of significant variables and their corresponding responses are shown in Table 1. The statistical analysis of these data (effect, parameter estimate, SS, contribution to SS, t- and p-values) is presented in Table 2.

Factors evidencing p-values of less than 0.05 were considered to have significant effects on the response, and were selected for further optimization studies. Glucose, with a probability value of 0.0235 was determined to be the most significant factor for BG, followed by NaCl

(0.0293), peptone (0.037) and pH (0.0416) (Table 2). The lower the probability value, the more significant are the factors on bacterial growth of *B. thuringiensis*. Two of the four significant variables screened, glucose and NaCl, exerted a negative effect on the response, therefore an increase in concentration of both components was coupled with a decrease in strain growth. The other two significant variables, peptone and pH, exerted positive effect, thus BG is favored by the addition of peptone to the culture medium and incubating under neutral-alkaline pH conditions. All remaining variables were irrelevant on BG and therefore, were removed from the following analysis.

From the statistical analysis of the results, it was found that only the concentration of peptone significantly influenced EP by *B. thuringiensis* (p=0.0468) (Table 2). Peptone presented a positive effect on EP, indicating that an increase in peptone concentration in the culture medium provides an increase in the production of this enzyme.

3.2. Optimization of significant variables using response surface methodology

Based on Placket Burman results, the factors with most significant impact on the two responses (glucose, peptone and NaCl concentration) were considered for the optimization studies using Box-Benhken response surface methodology. The design matrix and the corresponding results of RSM experiments to determine the effects of the selected factors are shown in Table 3.

The experimental data for each response (BG and EP) were statistically analyzed to obtain the significance of the model. A summary of the analysis of variance (ANOVA) for the quadratic models is shown in Table 4.

For BG, the p-value for the model (0.0122) and the non-significant lack of fit indicate that the obtained experimental data has a good fit with the model. Similar results were obtained for EP, with a p-value of 0.0316 and non-significant lack of fit. Therefore, the predicted models can be used to describe the effects of the selected independent variables (glucose, peptone and NaCl concentration) on BG and EP by *B. thuringiensis*.

The experimental data for each response were used to calculate the coefficients of the second order polynomial (Eq. 2) in order to obtain the significance of the models. The significance of each coefficient was determined by student's t-test and p-values. The regression coefficients for the second order polynomial equations and results for the linear, quadratic and interaction terms, as well as the correlation coefficients (R^2) and the coefficients of variation, are presented in Table 5. The coefficient R^2 was calculated as 0.94 for BG and 0.91 for EP, indicating good agreement between the experimental and the predicted values.

Analysis of significance of each term of the obtained polynomial models indicated that only linear terms of peptone and glucose concentration were significant for BG, and linear terms of peptone, NaCl and glucose concentration for EP. In both responses, quadratic terms and interaction between variables were not significant.

The responses, BG (log CFU/mL) (Y1) and EP (U/L) (Y2) can be expressed in terms of the following simplified regression equations:

$$Y_1 = 8.51 - 0.84 \cdot X_1 + 0.75 \cdot X_2$$

$$Y_2 = 6.93 - 3.41 \cdot X_1 + 5.43 \cdot X_2 - 3.55 \cdot X_3$$

where X_1 is the codified variable for glucose concentration, X_2 for peptone concentration and X_3, for NaCl concentration.

296 To aid visualization, the response surfaces for BG and EP are shown in Figures 1 and 2.
297 Figure 1 presents the response surface showing the combined effect of peptone and glucose
298 concentration on BG, holding NaCl at its middle value, since this variable presented the
299 least influence on the response. The significant and positive effect of peptone concentration
300 can be observed in Figure 1, where bacterial growth is favored by the addition of peptone to
301 the culture medium. It can be observed that the maximum value of BG is reached at the
302 upper limit of the range of peptone concentration (15 g/L). With respect to glucose
303 concentration, the effect of this variable on BG was significant but with a negative effect
304 meaning that a reduction in BG is observed with an increase of glucose concentration in the
305 culture medium. On the other hand, addition of NaCl did not significantly influence the
306 growth of *Bacillus thuringiensis*. This effect can be seen when plotting BG *vs*. NaCl (figure
307 not shown), where the response (BG) is almost constant in the range of NaCl concentration
308 tested.
309 The relationship between EP and the three independent variables is depicted in Figure 2 (A-
310 C), maintaining in each case, the third variable in a middle level. It can be observed from
311 these figures the significant effect of peptone concentration on EP. As the effect is positive,
312 *i.e.* there is an increase in the EP with an increase of peptone concentration in the culture
313 medium, reaching the maximum production at the upper limit of peptone tested (15 g/L).
314 Regarding the other two factors effect, although both glucose and NaCl concentration were
315 significant on EP, the variation levels of these factors within the range tested showed less
316 impact on the response than the observed for the same variations in the concentration of
317 peptone. This can be seen in Figure 2, where the slope of the graphs, that represents the

change in PE, is significantly smaller for variations in NaCl and glucose concentration than the observed for variation of peptone.

Optimum levels for the three selected factors (peptone, glucose and NaCl concentration) were determined to simultaneously obtain maximum bacterial growth and esterase production. Second-order polynomial models obtained in this study were utilized in order to determine the specified optimum conditions. By applying the method of desirability function, the optimal concentration of each component in the culture medium was: glucose, 1 g/L; peptone, 15 g/L and NaCl, 3.25 g/L. At these conditions, the predicted optimum responses were 10.44 Log CFU/mL for BG and 22.296 U/L for EP. The general "goodness" score (r) for the simultaneous optimization was 0.999. Verification experiments were carried out at the optimum levels to test the reliability of the models in predicting optimum responses. The results indicated that the predicted and experimental results matched properly ($p<0.05$), therefore the RSM models were validate with a good correlation.

4. DISCUSSION

Bacillus thuringiensis is a genus extensively studied due to its high potential for biotechnological applications in biocontrol techniques, mainly for the wide variety of extracellular hydrolytic enzymes produced, such as proteases, amylases, esterases, chitinases and nucleases (Agrahari 2011; Mazzucotelli *et al.* 2013; Öztürk *et al.* 2008; Tenorio-Sánchez *et al.* 2010). Despite the great biotechnological potential of *B.*

thuringiensis, the production of the enzymes as well as the factors affecting this process have not been extensively studied (Tenorio-Sánchez *et al.* 2010).

Increased productivity of enzymes during the fermentation process is of great importance, since lower costs of production could promote new industrial applications. The productivity of esterases is affected by different factors, such as agitation speed, pH, medium composition and presence of inducers, among others (Kishan *et al.* 2013). In this study, the most important factors affecting bacterial growth and esterase production by a *Bacillus thuringiensis* strain were screened and optimized through the use of several statistical tools.

Figures 1 and 2 show the effect of the three most significant factors (peptone, glucose and NaCl concentration) on bacterial growth and esterase production. In both figures, it can be observed that peptone concentration influenced linearly and positively on both responses (*i.e.* an increase in peptone concentration in the culture medium caused a lineal increase in microbial growth and enzyme production in the tested range).

Peptone is used by bacteria as a nitrogen source, which is then utilized for synthesis of proteins and nucleic acids. Therefore, the incorporation of a good source of nitrogen in the culture medium is essential for increasing biomass and for enzymes production (Madigan *et al.* 2003). Among nitrogen sources, peptone is often reported due to its positive incidence on bacterial growth and esterase production. This behavior could be associated to the complex structure of peptone, which not only contains a mixture of peptides and amino acids, derived from the hydrolysis of meat proteins, it also provides other components, such as vitamins, growth factors and surfactants (Hasan-Beikdashti *et al.* 2012). Amezaga *et al.* (1995) studied the role of peptone on *Listeria monocytogenes* growth, and found two main functions: firstly as a nutritional supplement for protein synthesis, and secondly as a source

363 of amino acids and peptides that can generate a mechanism that maintains cell turgidity.
364 Mukesh Kumar *et al.* (2012) studied a *Bacillus* sp. strain whose preferential nitrogen source
365 for cell growth was peptone, versus other organic and inorganic nitrogen sources.
366 In a previous work, Mazzucotelli *et al.* (2015) reported the optimization of culture
367 conditions for a *Stenotrophomonas* sp. strain, and it was found that peptone concentration
368 was a non-significant factor for bacterial growth, but it was significant for esterase/lipase
369 production. Both bacterial growth and enzyme production by that particular
370 *Stenotrophomonas* strain showed to be more affected by changes in agitation speed than by
371 the levels of nutritional factors. In this study, for *Bacillus thuringiensis* strain, only
372 nutritional factors affected BG and EP, while peptone concentration was the most
373 significant factor on both responses. Many others researchers have studied the effect of
374 adding peptone to the culture medium and most of them have found that the peptone
375 concentration is a significant factor on the production of lipases/esterases (Hasan-
376 Beikdashti *et al.* 2012; Rajendran *et al.* 2008; Salihu *et al.* 2011a; Salihu *et al.* 2011b).
377 Optimal concentration of peptone for producing lipases/esterases depends on the
378 microorganism under study.
379 While some strains found their optimum at low peptone concentrations (Rajendran *et al.*
380 2008; Shukla *et al.* 2007) other strains show an increase in response with higher peptone
381 concentrations in the culture medium (Ananthi *et al.* 2013; Ren *et al.* 2006; Sooch and
382 Kauldhar 2013). Sooch and Kauldhar (2013) and Ren *et al.* (2006) reported that once the
383 maximum production was reached (at 30 g/L and 15.4 g/L, respectively) higher peptone
384 concentrations inhibited the synthesis of lipases. The inhibition of lipases/esterases
385 production by peptone can be attributed to the complex nature of this nitrogen source, in

386 which certain constituents can cause toxic effects when found at high concentrations
387 (Sooch and Kauldhar 2013).
388 Glucose concentration showed a negative and linear effect for both responses (growth and
389 esterase production) for the tested strain. In most microorganisms, glucose is the most
390 readily metabolizable carbohydrate and provides a quick energy supply (Madigan *et al.*
391 2003). Therefore, it was expected that the addition of glucose to the culture medium
392 increased the growth rate and biomass formation of this strain. However, from the analysis
393 of the results it can be observed that increasing glucose concentration had an inhibitory
394 effect on growth. The *Crabtree* effect describes the phenomenon by which the growth
395 under aerobic conditions of some types of yeasts, such as *Saccharomyces cerevisiae*, is
396 inhibited or suppressed by glucose. This is a phenomenon present in glucose sensitive
397 microorganisms, which will adopt different catabolic pathways according to the
398 concentration of this substrate (Al-mhanna 2010). In the particular case of *S. cerevisiae*, the
399 suppressing effect exerted by glucose on respiration is due to the fact that this carbohydrate
400 represses the synthesis of, or directly inactivates, respiratory enzymes (van den Brink
401 2009). This phenomenon has also been detected in facultative anaerobic bacteria (as is the
402 case of *B. thuringiensis*), though it was found that the regulatory mechanism for glucose
403 utilization was different to that found for yeasts (Al-mhanna 2010). It has been reported in
404 *Bacillus thuringiensis* (Anderson and Jayaraman 2003) and *Serratia* (Liu 2014), that the
405 *Crabtree* effect appears when glucose concentration in the culture medium was 27.85 g/L
406 and 20 g/L, respectively. Moreover, the inhibitory effect on the production of
407 lipases/esterases caused by the addition of glucose to the culture medium is well
408 documented for many bacteria, molds and yeasts.

409 Glucose has a catabolite repression effect on the expression of these enzymes (Gupta *et al.*
410 2004; Hasan-Beikdashti *et al.* 2012; Korbekandi *et al.* 2008; Łukaszewicz *et al.* 2013).
411 Regulatory pathways involved in lipase induction and regulation at the gene level are
412 poorly understood. In most of reported cases, when glucose is the sole carbon source,
413 extracellular lipase activity is detected only after depletion of this substrate in the culture
414 broth. Fickers *et al.* (2005) studied the catabolic repression effect of glucose on lipase
415 production in *Yarrowia lipolytica* through different mutant strains, and concluded that the
416 presence of glucose in the culture medium cause the over expression of the HXK1 gen,
417 responsible for encoding hexokinase enzyme. Hexokinase catalyzes the initial reactions of
418 the intracellular metabolism of hexoses (such as glucose and fructose) through their
419 phosphorylation in the glycolytic pathway. When the kinase activity increases, it could be
420 observed a reduction of lipase production, due to HXK1 gen which is involved in the
421 downregulation of the LIP2 gene, encoding the extracellular lipase (Fickers *et al.* 2005).
422 NaCl is known for maintaining isotonicity between the environment and the bacterial
423 cytoplasm. While most microorganisms have mechanisms that allow them to cope with
424 saline stress, incubation in a hypotonic or hypertonic medium, causes a decrease in cell
425 viability (Madigan *et al.* 2003; Tortora *et al.* 2007). With respect to the effect of NaCl
426 concentration on growth of *B. thuringiensis*, RSM analysis showed that none of the terms
427 were significant. Therefore, using NaCl in the culture medium in a range of 0.1 to 1% (w/v)
428 would ensure the normal growth of this strain. Similar results were reported by Faille *et al.*
429 (1999) while studying the effect of culture medium components on recovery of cell
430 viability in *B. thuringiensis*. They found that with NaCl concentrations between 0.2 and
431 0.8% (w/v) the greatest counts were obtained, however these counts decreased significantly

towards higher concentrations of NaCl. On the other hand, studying the effect of metallic ions on enzymatic activity is complex, probably because results are related to changes in the solubility of the ionized fatty acid at the interface, and in the catalytic properties of the enzyme itself (Kaiser *et al.* 2006). Therefore, NaCl concentration in the medium may have different effects on the production of enzymes, depending on the type of microorganism and the enzyme secreted.

In this work, it was observed that the optimum level of NaCl that maximized both responses (BG and EP) for *B. thuringiensis* was 3.25 g/L. Kumar and Valsa (2007) studied a strain of *B. coagulans* whose maximum lipase production was achieved in the presence of 5 g/L of NaCl. Huang *et al.* (2004) studied a strain of *Geotrichum marinum*, which showed an increase in lipase activity at concentrations of Na^+ 6 mM (0.35 g/L NaCl), but noted a decrease in lipase activity when the concentration exceeded 500 mM (29.2 g/L NaCl). Ju *et al.* (2012) studied the esterase production by a *Pseudomonas* sp. strain through RSM and found that the optimal NaCl concentration was 0.71 g/L.

5. CONCLUSION

Bacillus thuringiensis is a widely studied strain in the field of biotechnology because of its high insecticidal power. Although this strain has been used in recent years for biocontrol and it has been reported that shows various hydrolytic capacities, the production of extracellular hydrolytic enzymes of *B. thuringiensis* have not been extensively studied.

Taking into account the importance of these enzymes for several industries, a screening procedure was developed to identify those factors more relevant to maximize microbial

455 growth and enzyme production by *Bacillus thuringiensis*, followed by the optimization of
456 the medium components and physicochemical parameters. For this purpose different
457 statistical tools were used, which enabled to evaluate a high number of factors with limited
458 number of experiments, minimizing experimental efforts and reducing costs. Plackett-
459 Burman design allowed identification of the factors that significantly influence bacterial
460 growth and esterase production by this strain. Thereafter, using Box–Behnken response
461 surface methodology, the three factors that were significant between assayed variables
462 (peptone, glucose and NaCl concentrations) were optimized. The desirability function
463 approach was applied to simultaneously maximize both responses. Optimal levels of the
464 three parameters were found to be 1 g/L for glucose concentration, 15 g/L for peptone
465 concentration and 3.25 g/L for NaCl concentration. With these optimum levels, it was
466 possible to achieve 113-fold increase in esterase production (23.052 U/L) and a 2.5-log
467 CFU/mL increase in bacterial growth (10.2 log CFU/mL), in the optimized medium
468 compared to the minimal medium without agitation.
469

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471

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6. REFERENCES

Agrahari, S. 2011. Production of enzymes and degradation of feathers by soil microbes. Synopsis. Jaypee Institute of Information Technology.

Agüero, M.V., Kotlar, C.E., Roura, S.I. 2011. METHODS: Simultaneous optimization of biomass and protease biosynthesis by a local isolated *Pseudomonas* sp.—Selection of main medium components and operating conditions. Ind. Biotechnol. **7**(1): 53-62.

Al-mhanna, M.N.M. 2010. Observation of *Crabtree* effect and diauxic behaviour of yeast by using absorption. Chem. Eng. Trans. **21**: 1465-1470.

Amezaga, M.R., Davidson, I., McLaggan, D., Verheul, A., Abee, T., and Booth, I.R. 1995. The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. Microbiology. **141**: 41-49.

Ananthi, S., Immanuel, G., and Palavesam I. 2013. Optimization of lipase production by *Bacillus cereus* strain MSU AS through submerged fermentation. Plant. Sciences Feed. **3**(2): 31-39.

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. Q. **17**(3): 225-231.

Arpigny, J.L., and Jaeger, K.E. 1999. Bacterial lipolytic enzymes: classification and properties. Biochem. J. **343**: 177-183.

Aslan, N., and Cebeci, Y. 2007. Application of Box–Behnken design and response surface methodology for modeling of some Turkish coals. Fuel. **86**: 90-97.

Benson, H.J. 2002. Bacterial Population Counts. Pp. 87. In: Microbiological applications. 8th Edition. New York. McGraw Hill.

Bornscheuer, U.T. 2002. Microbial carboxyl esterases: classification, properties and application in biocatalysis. FEMS microbiology reviews. **26**(1): 73-81.

Costas, M., Deive, F.J., and Longo, M.A. 2004. Lipolytic activity in submerged cultures of *Issatchenkia orientalis*. Process Biochem. **39**: 2109-2114.

506 Crickmore, N. 2006. Beyond the sporepast and future developments of *Bacillus*
507 *thuringiensis* as a biopesticide. J. App. Microbiol. **101**: 616-619.
508 Derringer, G. 1994. A balancing act: optimizing a product's properties. Qual. Prog. **27**: 51-
509 58.
510 Derringer, G., and Suich, R. 1980. Simultaneous optimization of several response
511 variables. J. Qual. Technol. **12**: 214-219.
512 Faille, C., Fontaine, F., and Membre, J. 1999. Factors influencing recovery of heat-injured
513 *Bacillus thuringiensis* spores statistical approach. J. Food Sci. **64**(2): 363-366.
514 Fickers, P., Nicaud, J. M., Destain, J., and Thonart, P. 2005. Involvement of hexokinase
515 Hxk1 in glucose catabolite repression of LIP2 encoding extracellular lipase in the
516 yeast Yarrowia lipolytica. Curr. Microbiol. **50**(3), 133-137.
517 Gupta, N., Mehra, G., and Gupta, R. 2004. A glycerol-inducible thermostable lipase from
518 *Bacillus* sp.: medium optimization by a Plackett-Burman design and by response
519 surface methodology. Can. J. Microbiol. **50**(5): 361-368.
520 Hasan-Beikdashti, M., Forootanfar, H., Safiarian, M.S., Ameri, A., Ghahremani, M.H.,
521 Khoshayand, M.R., and Faramarzi, M.A. 2012. Optimization of culture conditions
522 for production of lipase by a newly isolated bacterium *Stenotrophomonas*
523 *maltophilia*. J. Taiwan Inst. Chem. E. **43**: 670-677.
524 Huang, Y., Locy, R., and Weete, J.D. 2004. Purification and characterization of an
525 extracellular lipase from *Geotrichum marinum*. Lipids. **39**(3): 251-258.
526 Jaeger, K.E., and Eggert, T. 2002. Lipases for biotechnology. Curr. Opin. Biotechnol. **13**:
527 390-397.
528 Jeon, J.H., Kim, J.T., Kang, S.G., Lee, J.H., and Kim, S.J. 2009. Characterization and its
529 potential application of two esterases derived from the arctic sediment
530 metagenome. Mar. Biotechnol. **11**(3): 307-316.
531 Ju, X., Yu, H.L., Pan, J., and Xu, J.H. 2012. Improved production of *Pseudomonas* sp.
532 ECU1011 acetyl esterase by medium design and fed-batch fermentation. Bioprocess
533 Biosyst. Eng. **35**(3): 323-331.

534 Kaiser, P., Raina, C., Parshad, R., Johri, S., Verma, V., Andrabi, K.I., and Qazi, G.N. 2006.
535 A novel esterase from *Bacillus subtilis* (RRL 1789): Purification and
536 characterization of the enzyme. Protein Express. Purif. **45**(2): 262-268.
537 Kirk, O., Borchert, T.V., and Fuglsang, C.C. 2002. Industrial enzyme applications. Curr.
538 Opin. Biotech. **13**(4): 345-351.
539 Kishan, G., Gopalakannan, P., Muthukumaran, C., Thirumalai Muthukumaresan, K.,
540 Dharmendira Kumar, M., and Tamilarasan, K. 2013. Statistical optimization of
541 critical medium components for lipase production from *Yarrowia lipolytica* (MTCC
542 35). J. Genet. Eng. Biotechn. **11**(2): 111-116.
543 Korbekandi, H., Abedi, D., Pourhossein, M., Motovali, M., Hejazi, M., Narimousaei, M.,
544 and Kabiri, M. 2008. Optimisation of *Candida rugosa* lipase esterase activity.
545 Biotechnol. **7**: 112-117.
546 Kumar, M.P., and Valsa, A.K. 2007. Optimization of culture media and cultural conditions
547 for the production of extracellular lipase by *Bacillus coagulans*. Indian J.
548 Biotechnol. **6**(1): 114-117.
549 Levin, L., Forchiassin, F., Viale, A. (2005). Ligninolytic enzyme production and dye
550 decolorization by Trametes trogii: application of the Plackett–Burman experimental
551 design to evaluate nutritional requirements. Process Biochem. **40**(3): 1381-1387.
552 Liu, Q. 2014. The preliminary study of the regulatory factors in Serratia Marcescens
553 producing prodigiosin. China. Thesis Magister. From:
554 http://www.globethesis.com/?t=2234330398484168.
555 Łukaszewicz, M., Jabłoński, S., and Krasowska, A. 2013. Characterisation of alkaline
556 lipase from an arctic yeast strain *Rhodosporidium babjevae* BD19. Eur Sci. J. 441-
557 449.
558 Madigan, M.T., Martinko, J.M., Parker, J., and Sánchez Pérez, M. 2003. Biología de los
559 microorganismos: Brock. 10th ed. Pearson, Préntice Hall, pp. 156-160.
560 Mazzucotelli, C.A., Ponce, A.G., Kotlar, C.E., and Moreira, M.R. 2013. Isolation and
561 characterization of bacterial strains with a hydrolytic profile with potential use in
562 bioconversion of agroindustrial by-products and wastes. Cienc. Tecnol. Aliment.
563 Campinas. **33**(2): 295-303.

564 Mazzucotelli, C.A., Agüero, M.V., Moreira, M.D.R., and Ansorena, M.R. 2015.
565 Optimization of medium components and physicochemical parameters to
566 simultaneously enhance microbial growth and production of lypolitic enzymes by
567 Stenotrophomonas sp. Biotechnol. Appl. Bioc. DOI: 10.1002/bab.1378.

568 Moraga, E., Tovar, E., Garcia, P., and Alvarez, C. 2004. Isolation, geographical diversity
569 and insecticidal activity of *Bacillus thuringiensis* from soil in Spain. Microbiol. Res.
570 **159**: 59-71.

571 Mukesh Kumar, D.J., Rejitha, R., Devika, S., Balakumaran, M.D., Immaculate, A., and
572 Kalaichelvan, P.T. 2012. Production, optimization and purification of lipase from
573 *Bacillus* sp. MPTK 912 isolated from oil mill effluent. Adv. Appl. Sci. Res. **3**(2):
574 930-938.

575 Nardini, M., and Dijkstra, B.W. 1999. α/β Hydrolase fold enzymes: the family keeps
576 growing. Curr. Opin. Struct. Biol. **9**: 732-737.

577 Öztürk, F., Açık, L., Ayvaz, A., Bozdoğan, B., and Suludere, Z. 2008. Isolation and
578 characterization of native *Bacillus thuringiensis* strains from soil and testing the
579 bioactivity of isolates against *Ephestia kuehniella zeller* (Lepidoptera: Pyralidae)
580 Larvae. Turk. J. Biochem. **33**(4): 202-208.

581 Pandey, A., Webb, C., Soccol, C.R., and Larroche, C. 2006. Enzyme Technology. Asiatech
582 Publisher Inc. Springer Science, USA.

583 Pandey, A., Benjamin, S., Soccol, C.R., Nigam, P., Krieger, N., and Soccol, U.T. 1999. The
584 realm of microbial lipases in biotechnology. Biotechnol. Appl. Biochem. **29**: 119-
585 1131.

586 Pera, L.M., Romero, C.M., Baigori, M.D., and Castro, G.R. 2006. Catalytic Properties of
587 lipase extracts from *Aspergillus niger*. Food Technol. Biotechnol. **44**(2): 247-252.

588 Queiroga, A.C., Pintado, M.E., and Malcata, F.X. 2013. Medium factors affecting
589 extracellular protease activity by *Bacillus* sp. HTS 102-A novel wild strain isolated
590 from Portuguese merino wool. Nat. Sci. **5**(6): 44.

591 Rajendran, A., Palanisamy, A., and Thangavelu, V. 2008. Evaluation of medium
592 components by Plackett-Burman statistical design for lipase production by *Cándida*
593 *rugosa* and kinetic modeling. Chin. J. Biotech. **24**(3): 436-444.

Ren, X.D., Yu, D.W., Han, S.P., and Yan, F. 2006. Statistical optimization of medium components for improved product ion of recombinant hyperthermophilic esterase. Chem. Res. Chin. Univ. **22**(2): 134-138.

Salihu, A., Alam, Z., AbdulKarim, I., and Salleh, H.M. 2011a. Optimization of lipase production by *Candida cylindracea* in palm oil mill effluent based medium using statistical experimental design. J. Mol. Catal. B-enzym. **69**: 66-73.

Salihu, A., Alam, Z., AbdulKarim, I., and Salleh, H. M. 2011b. Evaluation of nutritional components by Plackett- Burman design for *Penicillium citrinum* lipase production using palm oil mill effluent. Afr. J. Biotechnol. **10**(81): 18704-18708.

Sharma, R., Chisti, Y., and Banerjee, U.C. 2001. Production, purification, characterization, and applications of lipases. Biotechnol. Adv. **19**: 627-662.

Shukla, P., Garai, D., Zafar, M., Gupta, K., and Shrivastava, S. 2007. Process parameters optimization for lipase production by *Rhizopus oryzae* KG-10 under submerged fermentation using response surface methodology. J. Appl. Sci. Environ. Sanit. **2**(3): 93-103.

Singh, S.K., Singh, S.K., Tripathi, V.R., Khare, S.K., and Garg, S.K. 2011. Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic *Pseudomonas putida* SKG-1 isolate. Microb. Cell Fact. **10**: 114.

Sooch, B.S., and Kauldhar, B.S. 2013. Influence of multiple bioprocess parameters on production of lipase from *Pseudomonas sp*. BWS-5. Braz. Arch. Biol. Technol. **56**(5): 711-721.

Tenorio-Sánchez, S.A., Rojas-Avelizapa, N.G., Ibarra, J.E., Rojas Avelizapa, L.I., and Cruz-Camarillo, R. 2010. Characterization of a *Bacillus thuringiensis* strain isolated from a highly polychlorinated biphenyls contaminated soil. Tecnólog@. **3**(3).

Tortora, G.J., Funke, B.R., Christine, L., and Case, C.L. 2007. Introducción a la microbiología. 9ª edición. Ed Médica Panamericana SA. Buenos Aires. Pp 163-165.

621 Van den Brink, J. 2009. Energetic limits to metabolic flexibility: responses of
622 *Saccharomyces cerevisiae* to glucose–galactose transitions. Microbiology. **155**:
623 1340-1350.
624 Vijayaraghavan, P., and Vincent, S.G.P. 2014. Medium optimization for the production of
625 fibrinolytic enzyme by *Paenibacillus* sp. IND8 using response surface
626 methodology. The Scientific World J. **3**: 60.
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633 **Figures Legends**

634

635 **Figure 1-** Response surface plot - Effect of peptone and glucose concentration on BG

636

637 **Figure 2** - Response surface plot - a) effect of peptone and NaCl concentration on PE; b)

638 peptone and glucose concentration on PE; c) NaCl and glucose concentration on PE.

639

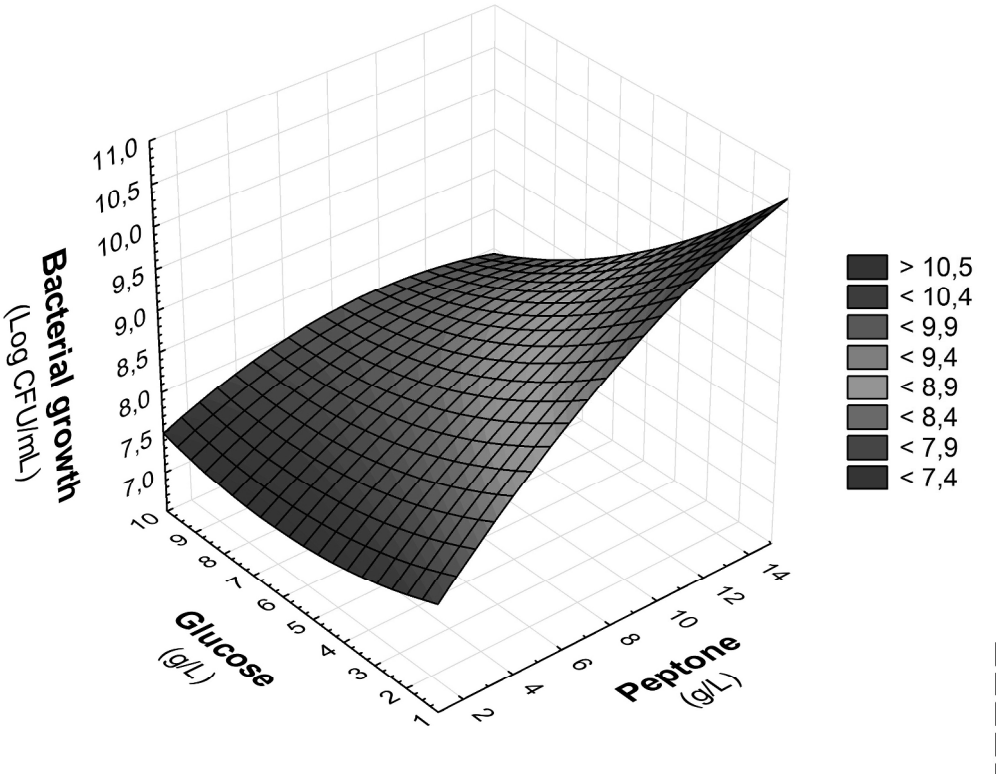

Figure 1- Response surface plot - Effect of peptone and glucose concentration on BG
267x206mm (300 x 300 DPI)

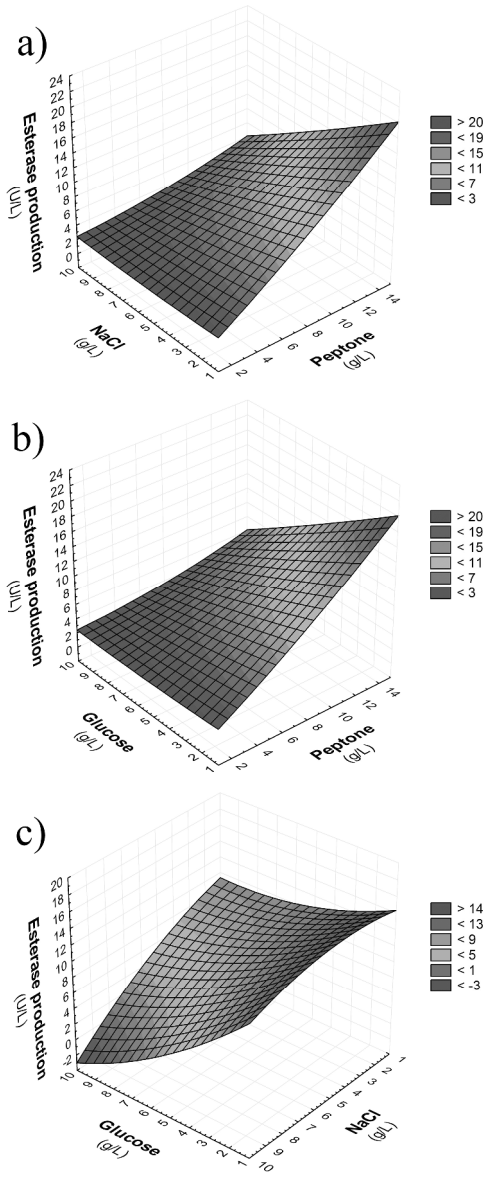

Figure 2 - Response surface plot - a) effect of peptone and NaCl concentration on PE; b) peptone and glucose concentration on PE; c) NaCl and glucose concentration on PE.
267x628mm (300 x 300 DPI)

Tables

Table 1 *- Plackett-Burman design matrix with coded values of independent variables, together with responses obtained for esterase production (EP) and bacterial growth (BG) for each run.*

Run	Variables											EP (U/L)	BG (Log CFU/mL)
	X_1[a]	X_2	X_3	X_4	X_5	X_6	X_7	X_8	d_1[b]	d_2	d_3		
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.205	5.50
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.602	8.71
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.791	7.89
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	2.822	8.15
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	5.613	8.72
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	10.340	7.68
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	4.500	8.54
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.040	3.60
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	0.283	7.87
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	24.500	9.48
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	9.220	8.38
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.204	7.70

[a] *X_i are the independent variables (factors): stirring speed (X_1), pH (X_2), glucose concentration (X_3), olive oil (X_4), Tween 80 (X_5), peptone concentration (X_6), $(NH_4)_2SO_4$ concentration (X_7) and NaCl concentration (X_8).*

[b] *d_i are the dummy variables.*

Table 2- *Statistical parameters for microbial growth and esterase production*

Response	Code	Variable	Effect	Estimated parameter	SS	SS contribution (%)	p-value	Confidence level
BG	X_1	Stirring speed	0.709	0.355	1.512	5.584	0.1511	84.89
	X_2	pH	1.271	0.635	4.839	17.872	0.0416	95.84
	X_3	Glucose	-1.582	-0.79167	7.521	27.777	0.0235	97.65
	X_4	Olive Oil	-0.172	-0.08667	0.090	0.332	0.6717	32.83
	X_5	Tween 80	-0.291	-0.145	0.252	0.931	0.4908	50.92
	X_6	Peptone	1.330	0.665	5.307	19.600	0.037	96.3
	X_7	$(NH_4)_2SO_4$	-0.630	-0.315	1.190	4.395	0.1875	81.25
	X_8	NaCl	-1.457	-0.72833	6.365	23.508	0.0293	97.07
EP	X_1	Stirring speed	6.152	3.17583	121.031	26.007	0.0752	92.48
	X_2	pH	2.754	-0.6075	4.429	0.951	0.6438	35.62
	X_3	Glucose	-5.873	-0.8875	9.452	2.031	0.5086	49.14
	X_4	Olive Oil	-3.922	-1.5925	30.433	6.539	0.2719	72.81
	X_5	Tween 80	2.691	1.74583	36.575	7.860	0.2374	76.26
	X_6	Peptone	5.116	3.87583	180.265	38.735	0.0468	95.32
	X_7	$(NH_4)_2SO_4$	3.528	1.39583	23.380	5.024	0.3241	67.59
	X_8	NaCl	-0.705	-2.2325	59.808	12.852	0.1563	84.37

Table 3- *Box- Behnken design matrix and mean values of esterase production and microbial growth under different experimental conditions after 96 h of incubation.*

Run	Variables			Experimental Responses	
	Glucose $X_1(x_1)$	**Peptone** $X_2(x_2)$	**NaCl** $X_3(x_3)$	**BG** (Log CFU/mL)	**EP** (U/L)
1	-1(1g/L)	-1(1 g/L)	0(5.5 g/L)	8.42	1.224
2	-1(1 g/L)	1(15 g/L)	0(5.5 g/L)	10.44	22.355
3	1(10 g/L)	-1(1 g/L)	0(5.5 g/L)	7.68	0.024
4	1(10 g/L)	1(15 g/L)	0(5.5 g/L)	7.89	5.962
5	0(5.5 g/L)	-1(1 g/L)	-1(1 g/L)	7.46	5.632
6	0(5.5 g/L)	-1(1 g/L)	1(10 g/L)	7.02	1.691
7	0(5.5 g/L)	1(15 g/L)	-1(1 g/L)	8.99	20.671
8	0(5.5 g/L)	1(15 g/L)	1(10 g/L)	9.25	2.999
9	-1(1 g/L)	0(8 g/L)	-1(1 g/L)	9.37	8.774
10	1(10 g/L)	0(8 g/L)	-1(1 g/L)	7.90	9.177
11	-1(1 g/L)	0(8 g/L)	1(10 g/L)	10.10	10.622
12	1(10 g/L)	0(8 g/L)	1(10 g/L)	8.12	0.512
13	0(5.5 g/L)	0(8 g/L)	0(5.5 g/L)	8.93	5.980
14	0(5.5 g/L)	0(8 g/L)	0(5.5 g/L)	8.35	6.523
15	0(5.5 g/L)	0(8 g/L)	0(5.5 g/L)	8.24	8.276

Table 4- *Results of the ANOVA for regression equation for esterase production and bacterial growth*

Response	Source	DF	SS	MS	F value	Pr >F
BG	Linear	3	10.237		23.36	0.023
	Quadratic	3	0.982		2.24	0.2014
	Cross-product	3	1.006		2.30	0.1950
	Total model	9	12.226		9.30	0.0122
	Lack of fit	3	0.455	0.152	1.10	0.5075
	Pure error	2	0.275	0.137		
EP	Linear	3	429.81		13.67	0.0076
	Quadratic	3	1.202		0.04	0.9888
	Cross-product	3	132.47		4.21	0.0777
	Total model	9	563.49		5.98	0.0316
	Lack of fit	3	49.509	16.503	11.46	0.0813
	Pure error	2	2.880	1.440		

DF, degrees of freedom; SS, sum of squares; MS, mean square.

Table 5- *Regression coefficients (from coded data) and R^2 of the response surface models*

Coefficient	Responses	
	BG	**EP**
β_0 (intercept)	8.5067***	6.9263*
β_1 (Glucose)	-0.8425**	-3.4125*
β_2 (Peptone)	0.7487**	5.4270**
β_3 (NaCl)	0.0962	-3.5537*
β_{11} (Glucose x Glucose)	0.3967	-0.0060
β_{12} (Glucose x Peptone)	-0.4525	-3.7982
β_{22} (Peptone x Peptone)	-0.2958	0.4709
β_{13} (NaCl x Glucose)	-0.1275	-2.6282
β_{23} (NaCl x Peptone)	0.175	-3.4327
β_{33} (NaCl x NaCl)	-0.031	0.3509
Coefficient of Variation	4.4730	43.97
R^2	0.9436	0.9149

* *Significant at 0.05 level.*

** *Significant at 0.01 level.*

*** *Significant at 0.001 level.*