Optimization of medium components and physicochemical parameters to simultaneously enhance microbial growth and production of lypolitic enzymes by *Stenotrophomonas* sp.

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Synopsis

The optimization of lipase and esterase production and bacterial growth of a *Stenotrophomonas sp* strain was developed. For this purpose, the effect of five different medium components and three physicochemical parameters were evaluated using a Plackett-Burman statistical design. Among eight variables, stirring speed, pH and peptone concentration were found to be the most effective factors on the three responses under evaluation. An optimization study applying Box-Benhken response surface methodology was used to study the interactive effects of the three selected variables on lipase/esterase production and microorganism growth. Predicted models were found to be significant with high regression coefficients (90–99%). By using the desirability function approach, the optimum condition applying simultaneous optimization of the three responses under study resulted to be: stirring speed of 100 rpm, pH of 7.5 and a peptone concentration of 10 g/L, with a desirability value of 0.977. Under these optimal conditions, it is possible to achieve in the optimized medium a 15-fold increase in esterase productivity, a 117-fold increase in lipase production, and a 9-log CFU/ml increase in bacterial growth, compared to the basal medium without agitation.

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[1] **Keywords**
Lipase; Medium optimization; Plackett-Burman design; Response surface methodology; *Stenotrophomonas sp.*

[2] **Abbreviations**
Box-Benhken (BB); Bacterial Growth (BG); Basal Medium (BM); Esterase Production (EP); Luria- Bertani (LB); Lipase Production (LP); Plackett-Burman (PB); Response Surface Methodology (RSM); Sum of Squares (SS)

1.- Introduction

Over the last decades, lipolytic enzymes (such as lipases and esterases) have been attracting enormous attention due to their multifaceted properties, which find usage in a wide array of industrial applications, such as food ingredients and products, detergent, production of fine chemicals, pharmaceuticals and cosmetics, pulp and paper industry, textile and leather industries (1, 2). A large number of additional hydrolytic applications have been described for these enzymes including flavor development for dairy and bakery products, beverages, milk chocolate and sweets. This is due to the selective hydrolysis of fat triglycerides that releases free fatty acids that can act as either flavors or flavor precursors (3, 4). Nowadays, the treatment of waste and wastewater by lipases and lipolytic bacteria looks to become very important; this includes the breakdown of fat solids, the prevention or cleaning of fat films and the cleaning of fat-containing waste effluents (3, 5, 6). The effluents from different industries contain a high concentration (>100 mg/L) of lipids (fats and oils) (7). The high concentration of these compounds in wastewater generates problems of accumulation in piping and equipment, and usually also causes difficulties in biological treatment processes, because the lipids form a layer on the water surface and decrease the oxygen transfer rate in the aerobic process (3, 7). One of the ways to reduce the initial concentration of lipids is the application of enzymatic pretreatments, which can be applied prior or together with the biological treatment step. This would improve the biological degradation of fatty effluents, increasing efficiency and reducing the treatment time (8, 9).

Lipolytic enzymes are produced by many microorganisms, including bacteria, fungi, yeasts and actinomyces (10). Among them, the extracellular enzymes are of considerable commercial importance, as their bulk production is relatively easy (11). Bacteria produce different classes of lipolytic enzymes, being the two of utmost importance the carboxylesterases (esterases, EC 3.1.1.1), and the triacylglycerol lipase (lipases, EC 3.1.1.3),

which can be distinguished based on their substrate specificity (10, 12). Lipases hydrolyze the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol. The natural substrates of lipases are long-chain triacylglycerols (>10 carbon atoms) (1, 2, 12). Esterases hydrolyze 'simple' esters and usually only triglycerides composed by short chain fatty acids (<10 carbon atoms). Most lipases also act as esterases; however esterases rarely catalyze reactions with insoluble substrates, which, in turn, are specifically hydrolyzed by lipases (12).

One of the most novel sources of lipolytic enzymes is the microorganism *Stenotrophomonas* sp. This genus has been extensively studied, mainly *S. maltophila*, since the latter is associated to human disease in immunocompromised patients (13, 14, 15). However in recent years this genus has received special attention because of their biotechnological properties that are currently under study. Some *Stenotrophomonas* sp. have a high level of intrinsic resistance to heavy metals and antibiotics (16, 17) and also have promising applications in bioremediation and phytoremediation (18, 19, 20). Many *Stenotrophomonas* sp. can produce antimicrobial compounds that protect plants, as well as generate factors that can promote plant growth (20, 21). Some researchers have described the production of extracellular enzymes by *Stenotrophomonas* sp. including proteases, lipases, nucleases, chitinases, elastases, fibrinolysin and hyaluronidase (20, 22, 23, 24). However, the production of these enzymes as well as the factors affecting this process, are not fully understand and, at our best knowledge, the optimization of lipase and esterase production by *Stenotrophomonas* species has been scarcely studied.

The optimization of medium components is the primary step in the overproduction of extracellular enzymes. The use of the classical method that involves the change of "one-variable-at-a-time", while keeping others at a constant level, was found inefficient. This method is extremely time-consuming, and thus expensive when a large number of variables

are to be considered. Furthermore, this single dimensional task does not explain interaction effects among the variables on the fermentation process (25, 26, 27). Statistical tools that are currently available enable to evaluate a large number of physicochemical parameters and factors influencing the fermentation process with limited number of experiments, minimizing experimental efforts, reducing costs and obtaining information regarding the interaction between the studied variables (26, 28). One such approach is Plackett-Burman design that allows efficient screening of key variables, to identify the statistical significance of factors on the dependent variables for further optimization (25, 26). Once the critical components for enzyme production and bacterial growth are identified, the optimum level of each parameter for maximize the responses are selected through response surface methodology (RSM) (29). RSM is a well-known method applied in the optimization of medium constituents and other critical variables responsible for lipase production and bacterial growth (26, 30, 31), because can provide information about interactions among variables, and necessary data to design and optimize the process, even with multiple responses at the same time (27, 29).

Thus, the aim of this work was to evaluate the effect of different medium components and physicochemical parameters on lipase and esterase production and microorganism growth using a *Stenotrophomonas* sp. strain. For this purpose, a Plackett-Burman statistical design was used to select the variables that significantly influence the lipase and biomass production. After that, a simultaneous optimization was carried out by applying response surface methodology to obtain medium conditions that enhanced lipase/esterase production and microorganism growth.

2. Materials and methods

2.1. Microorganism and culture maintenance

The bacterial strain used in this work was isolated from defatted soy pellet and identified as *Stenotrophomonas* sp. (24).

The isolated culture was maintained in 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. The strain was activated in two steps. First, a loop was inoculated in 10 ml LB broth and incubated at 37°C for 24-48 h; subsequently, 2 mL of active culture were centrifuged at 10,000 rpm for 3 min at 4°C. The obtained precipitate was added to 10 mL fresh LB broth and statically incubated at 37°C for 24-48 h.

Before the experiments, the strain was cultured in LB broth for 24 h at 37ºC. For each culture, 0.1 mL was transferred to 9.9 mL LB broth at two consecutive 24-h intervals.

2.2. Prescreening assay: effect of incubation time on bacterial growth and lipase/esterase production

To define the incubation time for experimental runs, growth kinetics of *Stenotrophomonas sp* was determined. Aliquots from *Stenotrophomonas sp* culture taken from the fresh culture were inoculated in 250 mL Erlenmeyer flask with 100 mL of basal medium (BM) composed by 1% w/v tryptone; 1% w/v NaCl; 0.5% w/v yeast extract. The initial cell concentration was approximately 5.10^7 $CFU.mL^{-1}$. The flasks were incubated at 35°C during 72 h on an orbital shaker at 100 rpm. Samples were taken at regular intervals for bacterial growth and lipase and esterase activities determination.

Bacterial growth (BG) was determined by serial dilution method on LB agar plates (32). The plates were incubated at 37ºC for 24-48 h and the numbers of colonies were determined. Microbial counts were expressed as log CFU/mL.

For lipase production (LP) and esterase production (EP), the cells were separated from the medium by centrifugation at 10,000 rpm for 15 min at 4ºC. The clarified supernatant was

used as source of enzyme. Lipase and esterase activity were measured spectrophotometrically at 405 nm with *p*-nitrophenyl palmitate (*p*NPP) and *p*-nitrophenyl butyrate (*p*NPB) as substrates at 37°C, respectively. A buffer mixture was prepared with phosphate buffer 100 mM (pH=7.0), arabic gum (0.1 % w/v) and Triton X-100 0.4 % (w/v) according to the method of Pera *et al.* (33). Substrates, p-nitrophenyl derivates, were prepared at a 1 mM concentration in acetone. The reaction mixture was prepared with buffer mixture, substrate and enzyme solution in 8:1:1 ratio, respectively. A blank was performed by incubating the reaction mixture without the enzyme solution, which is added at the time of measurement. The blank takes into account possible substrate degradation during incubation as well as the inherent color of the culture medium. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per minute (33). The molar absorptivity of *p*-nitrophenol under the assay conditions was found to be 0.00639 L/(µmol·cm). Lipase and esterase activities were expressed as U/L.

2.3. Factors affecting microbial growth and enzymes activities: Plackett-Burman design

A Plackett-Burman (PB) statistical design was performed in order to determine nutritional and physicochemical parameters (k_i) that significantly influence lipase and esterase production and bacterial growth. This design assumes that there are no interactions between different factors in the range of each variable under consideration and a linear approach is considered sufficient for screening:

$$Y = \beta_0 + \sum \beta_i X_i \quad ; \qquad i = 1,2,...,k \tag{1}$$

where Y is the estimated function for the responses (lipase production, esterase production or bacterial growth), X_i is a dimensionless coded value of x_i (independent variable), β_0 is the model intercept and β_i are the regression coefficients (26, 28).

In this work, eight factors were screened to evaluate the relative importance of them on LP and EP as well as on BG. The factors were five medium components: glucose (as carbon source), olive oil (as carbon source and inducer), Tween 80 (as carbon source and inducer), peptone (as organic nitrogen source), $CaCl_2$ (as inducer); and 3 operating conditions: temperature, pH and agitation. The factors were tested at two levels, high (+) and low (−): temperature (x_1; -1: 25; +1: 35ºC); pH (x_2; -1: 6; +1: 8); stirring speed (x_3; -1: 0; +1: 100 rpm); glucose (x_4; -1: 1; +1: 10 g/L); olive oil (x_5; -1: 0.5; +1: 10 g/L); Tween.80 (x_6; -1: 0.5; +1: 10 g/L); peptone (x_7; -1: 1; +1: 10 g/L); $CaCl_2$ (x_8; -1: 0.05; +1: 0.5 g/L). These variables and their levels were selected based on a preliminary literature review in order to determine the most important factor affecting LP, EP and BG. For the particular case of pH levels, these were fixed in function of a preliminary test in which the range of appreciable growth of the strain was determined (data not shown).

The screening was carried out through a 12-trials experimental run and the level of each factor within each run was determined by the PB matrix (Table 1). Each row in Table 1 represents a trial and each column represents an independent (assigned) or *dummy* (unassigned) variable. All experiments were carried out in triplicate and the average values of lipase production (Y1), esterase production (Y2) and bacterial growth (Y3) were taken as responses. *Dummy* variables were used to estimate experimental error in data analysis.

Each trial was conducted in erlenmeyer flasks (250 mL) containing 100 mL of BM added with medium components and conditions according to PB matrix (Table 1). Each flask, after autoclaving at 120°C for 15 minutes and cooling to room temperature (25°C), was inoculated with 1% (v/v) culture (cell concentration approximately: 5.10^7 $CFU.mL^{-1}$). The flasks were incubated for a period of 48 h with orbital shaking and after this period microbial growth and lipases activities were determined according to the previously detailed procedures.

The effect (E) of each independent variable (or factor, x_i) was determined from the following equation:

$$E(x_i) = M_i^+ - M_i^- \quad (2)$$

where M_i^+ and M_i^- are the means of response from the trials with factor x_i present at high and low levels, respectively (28, 34).

Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{eff} = \frac{\sum E_d^2}{n} \quad (3)$$

where V_{eff} is the variance of the effect, E_d is the effect for the dummy variable and n is the number of dummy variables.

The standard error (S.E.) of an effect is the square root of the variance of an effect and the significant level (p-value) of each effect is determined using the Student´s t-test:

$$t_{(x_i)} = \frac{E_{(x_i)}}{S.E.} \quad (4)$$

Data were analyzed using REG procedure of SAS software version 8.0 (SAS Inst. Inc., Cary, N.C., U.S.A. 1999). Results were evaluated using the following statistically parameters: sum of squares (SS), percentage contribution to the SS, t-value, p-value and confidence level. The factors were screened at a confidence level of 95% on the basis of their effects.

2.4. Optimization of the selected factors: Response Surface Methodology

2.4.1. Box-Benhken design

Once the three main factors were established through Plackett-Burman analysis, the level of each factor was optimized in order to maximize the response variables under study (LP, EP and BG) using response surface methodology (RSM) with a Box-Benhken (BB) design, with a total number of experimental combinations calculated as:

$N = k^2 + k + C_p$, where k is the number of independent variables and C_p is the replicate number of the central point (35).

For a 3-level-3-factor BB design with three replicates at the central point, a total of 15 experimental runs are needed (Table 3). Each variable was tested in three different coded levels: low (−1), middle (0) and high (+1), together with the response variable.

Each trial was conducted in an erlenmeyer flask (250 mL) containing 100 mL of BM added with different peptone concentration and physical parameters according to BB matrix (Table 3). Each flask was inoculated with 1% (v/v) culture (cell concentration approximately: 5.10^7 CFU mL^{-1}) and were incubated for a period of 48 h, at 25ºC. After this period microbial growth and lipase and esterase activities were determined according to previously detailed procedures.

In developing the regression equation the factors were coded according to:

$$X_i = \frac{x_i - x_{0,i}}{\Delta x_i} \qquad (5)$$

where X_i is the coded value of the i^{th} independent variable, x_i is the natural value of the i^{th} independent variable, $x_{0,i}$ is the natural value of the i^{th} independent variable at the center point and Δx_i is the steep change value.

All experiments were performed in a random order and were independently repeated three times.

Once responses (LP, EP and BG) were measured for each trial, an independent second-order polynomial model was fitted to each response:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (6)$$

where Y is the predicted response (LP or 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).

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.

2.4.2. Simultaneous optimization

When several response variables need to be optimized, the independent optimization of each one can lead to conflicting results, i.e., improving one response may have an opposite effect on another one. For these situations "desirability function" approach is one of the most widely used methods for simultaneously optimize several responses (36). The general approach is to first convert each response (y_n) into an individual desirability function (d_n). The desirability scale ranges from 0 to 1, where, if the response is at its goal or target, then d_n = 1, and if the response is outside an acceptable region, then d_n = 0. Each response is then standarized in desired functions d_n of the type.

$$d_n = h_n(y_n) \tag{7}$$

where *n* is the total number of responses in the measure.

Derringer and Suich (37) used the following modified desired function:

$$d_n = \begin{Bmatrix} 0 & if\ y_n \le y_n^{\min} \\ \left(\dfrac{y_n - y_n^{\min}}{y_n^{\max} - y_n^{\min}}\right)^r & if\ y_n^{\min} \le y_n \le y_n^{\max} \\ 1 & if\ y_n \ge y_n^{\max} \end{Bmatrix} \tag{8}$$

where $y_n^{\min}$ is the minimum acceptable value of y_n; $y_n^{\max}$ is the maximum value that is considered desirable and *r* is a positive constant. If *r* =1, the d_n increases linearly as y_n

increases; if r >1, the d_n changes more rapidly towards the y_n^{max} and if r <1, the d_n changes less rapidly towards the y_n^{max} .

The individual desirability functions from the considered responses are then combined to obtain the overall desirability D, defined as the geometric average of the individual desirability.

$$D = (d_1, d_2, ..., d_n)^{1/n} \quad (9)$$

where $0 \leq D \leq 1$, a high value of D indicates the more desirable and best functions of the system, which is considered as the optimal solutions of this system. The optimum values of factors are determined from the value of individual desired functions that maximizes D.

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- Prescreening assay: effect of incubation time on bacterial growth and lipase/esterase production

To establish the incubation time, the isolated culture of *Stenotrophomonas* sp. was maintained in LB broth and pH 7. Lipase and esterase activities were measured at different incubation periods in order to determine the lower incubation time in which the maximum enzyme production was observed. The time needed to achieve maximum lipase and esterase production was found to be 48 h. Therefore, all the cultures were incubated for 48 h for monitoring BG, LP an EP.

3.2- Placket-Burman design

From the PB design, the results of lipase and esterase productions and bacterial growth are presented 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.

Regarding BG, the statistical analysis of these results showed that agitation speed was the most and the only significant variable ($p<0.01$) affecting BG (Table 2). As and additional selective criteria, it is also useful to consider the contribution of each factor to the total SS. As it can be observed in Table 2, the major contributions to the SS for BG are due to agitation speed which contributes with 90.2 %, followed by pH (7.4 %) and peptone concentration (1.9%), giving these three factors a total contribution of 99.4 %.

Regarding LP, the most significant variable was, again, agitation speed ($p<0.01$) (Table 2). Considering the mainly contribution to the SS for LP, the most important factors were agitation speed (87.4 %), temperature (3.6 %) and peptone (3.3 %), giving the three factors a total contribution of 94.3 %.

When considering EP, in the same way as in previous responses, agitation was the most significant factor affecting EP (Table 2). Considering the mainly contribution to the SS for EP, it was associated to agitation speed (46.5%), pH (38%) and glucose concentration (6.8%), giving the three factors a total contribution of 91.2%.

3.3- Bacterial growth and enzymes production optimization

3.3.1. Response modeling

From Placket Burman analysis, the most effective factors with significant impact on the three responses and those that contribute most to the total SS were considered and applied for the optimization studies using Box-Benhken response surface methodology. In this way, stirring speed, pH and peptone were found to be the most effective factors and were selected for the optimization studies.

The Box-Behnken design matrix with the experimental results obtained for BG, LP and EP under conditions established by the 15-trial experimental is shown in Table 3. As can be observed, high variations in response variables were found as a function of factors under evaluation. The experimental data for each response were used to calculate the coefficients of the second order polynomial (Eq. 6) to obtain the significance of the coefficients 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 coefficient of variation are presented in Table 4. High correlation coefficient obtained for dependent variables (0.98, 0.90 and 0.99 for BG, EP and LP, respectively) indicates a close agreement between the experimental results and the theoretical values predicted by the models. Furthermore, relatively lower values obtained for the coefficient of variation indicates precision and reliability of the conducted experiments.

A summary of the analysis of variance (ANOVA) for the quadratic models is shown in Table 5. The ANOVA of the regression models indicates that the resultant three models were highly significant (p= 0.0005, 0.0405 and 0.0060 for BG, EP and LP, respectively) exhibiting no significant lack of fit. Hence, these models can be used to describe the effects of the selected independent variables (stirring speed, pH and peptone concentration) on BG, LP and EP.

Analysis of significance of each term of polynomial model obtained for BG indicated that only stirring speed (lineal and quadratic terms) resulted significant for this response. Thus, a simplified version of the polynomial equation for BG can be expressed as follows:

$$Y_1 = 15.11 + 2.17 \cdot X_1 + 1.80 \cdot X_1^2$$

where Y_1 is the BG (log UFC/L) and X_1 is the codified variable for stirring speed.

Canonical analysis shows that stationary point was a saddle point suggesting movement away from these points would cause an increased or decreased response, depending upon

movement direction. Figure 1 presents the response surface showing the combined effect of stirring speed with each of the other two variables on BG (maintaining the third one at its middle value). The significant effect of stirring speed could be observed in these graphs. The positive coefficient for this variable indicates that bacterial growth increases when stirring speed increase, reaching maximum BG at 100 rpm. On the other hand, bacterial growth was not influenced by pH level within the range tested nor peptone concentration in the culture medium. This fact could be observed from Figure 1 as BG did not change with these variables.

Analysis of significance of each term of polynomial model obtained for LP indicated that linear and quadratic terms of stirring speed and pH, quadratic term of peptone and the interaction between stirring speed and peptone resulted significant for this response. Thus, a simplified version of the polynomial equation for LP can be expressed as follows:

$$Y_2 = 1.57 + 2.56 \cdot X_1 + 0.3739.X_2 + 2.62 \cdot X_1^2 - 0.54 \cdot X_2{}^2 + 0.48 \cdot X_3^2 + 0.75 \cdot X_1 \cdot X_3$$

where Y_2 is the LP (U/L), X_1 is the codified variable for stirring speed, X_2 is the codified variable for pH and X_3 is the codified variable for peptone concentration.

Canonical analysis show that stationary point was a saddle point suggesting movement away from these points would cause an increased or decreased response, depending upon movement direction. Figure 2 represents the response surface showing the combined effect of two variables with the third one maintained at its middle value. As in BG, the positive coefficient and the quadratic effect for stirring speed indicated that LP increases when stirring speed increase, reaching maximum lipase production at 100 rpm.

Analysis of significance of the polynomial model obtained for EP indicated that stirring speed and peptone (lineal terms) resulted significant for this response (Table 4). Thus, a simplified version of the polynomial equation for EP can be expressed as follows:

$$Y_3 = 5.25 + 0.84 \cdot X_1 + 0.91 \cdot X_3$$

where Y_3 is the EP (U/L), X_1 is the codified variable for stirring speed and X_3 is the codified variable for peptone concentration.

Canonical analysis show that stationary point was a saddle point suggesting movement away from these points would cause an increased or decreased response, depending upon movement direction. Figure 3 represents EP as a function of stirring speed and peptone concentration, holding pH at its middle value, since this variable presented the least influence on the response. Again, the significant effect of stirring speed and peptone could be observed in these graphs. The positive coefficient for these variables indicated that EP increases when stirring speed and peptone concentration increase.

3.2.2. Optimization and validation

The optimum levels for bacterial growth, esterase and lipase production were determined using the desirability function approach. Second order polynomial models obtained in this study were used for each response in order to determine the specified optimum medium conditions that enhanced lipase and esterase production and bacterial growth. The simultaneous optimization was applied for selected ranges of stirring speed, pH and peptone concentration as 0-100 rpm, 6-8, and 0-10 g/L, respectively. The results of the simultaneous optimization indicated that the levels of the optimized conditions were X_1= 1 (100 rpm), X_2= 0.5 (7.5 pH), and X_3= 1 (10 g/L), with the predicted optimum responses of 19.17 log CFUmL^{-1} for BG, 7.78 UL^{-1} for EP and 8.15 UL^{-1} for LP. The general "goodness" score (r) for the simultaneous optimization was 0.977.

In order to test the reliability of the models in predicting optimum responses and in accordance with the optimization results obtained from RSM with the desirability function, verification experiments were carried out at the optimum levels. The results indicated that the mean experimental values were 18.52 log CFU/mL for BG, 7.53 U/L for EP and 8.19 U/L for

LP at the selected optimum conditions of stirring speed, pH and peptone concentration. The predicted results matched well with the experimental results obtained using optimum conditions ($p<0.01$) which validated the RSM models with a good correlation.

4. Discussion

The wide usage of lipases on wastewater treatment has persuaded researchers to obtain lipases exhibiting various properties. The use of lipases improves the biological degradation of fatty effluents, increasing efficiency and reducing the treatment time (8, 9). The large scale production of lipases requires not only the screening of potential lipase producers, but also the optimization of the chemical and physical components of the culture media (38).

From the results obtained through the PB design, the most effective factors with significant impact on lipase and esterase production and bacterial growth and those that contribute most to the total SS were: stirring speed, pH and peptone. Then, these three factors were considered and applied for the optimization studies using Box-Behnken response surface methodology.

For the three studied responses (BG, LP and EP), the most significant variable was agitation speed. Different mechanisms could explain the effect of stirring speed on microbial growth under aerobic submerged fermentations. Among them, agitation allows oxygen supply to culture media improving the O_2 mass transfer to the growing microbial cells (39). Also, a continuous agitation enables a homogenous temperature and adequate mixing of culture components. But it is important to consider that beyond certain values, turbulent flows may cause disruption of cells or damage of cells membranes due to shear forces, and the formation of stagnant eddies that may negatively affect mass transfer for both oxygen and substrate (26, 29, 39, 40). Similar to the effect observed in BG, agitation speed also presented a positive effect on LP and EP, indicating that moving from the low level (0 rpm) to the high level (100

rpm) increases lipase/esterase production. In the present work, the increased in LP and EP caused by the agitation speed can be directly related to the increase in the viable cells number in the culture and/or the stirring can be correlated to an increment in lipase secretion or in the enzyme activity. Many researchers have investigated this dependence (38, 41, 42).

In the same way, diverse researchers have reported microbial strains with different optimum agitation speed for lipase/esterase production. Ebrahimpour *et al.* (43) reported a *Geobacillus* sp. strain that presented the optimum lipase production at stationary condition. Khoramnia *et al.* (44) have found a *Staphylococcus xylosus* strain whose optimum agitation speed for lipase production was 60 rpm. Several authors have studied the behavior of other strains and found the optimum at higher stirring speed. Veerapagu *et al.* (45) reported for *Pseudomonas gessardii* strain an optimal agitation speed of 160 rpm for lipase production. Iftikhar *et al.* (38) studied the production of extracellular lipase by *Rhizopus oligosporus* and obtained the maximum enzyme production at 250 rpm. Lower stirring speeds seemed to limit oxygen levels, along with the lacking of homogeneous suspension of the fermentation medium, affecting microorganism growth. Higher stirring speeds resulted in mechanical and/or oxidative stress, excessive foaming, disruption and physiological disturbance of the cells (38). Elibol and Özer (42) investigated lipase production by *Rhizopus arrhizus* at four different agitation speeds, i.e. 75, 100, 150 and 200 rpm, and found that maximum lipolytic activity was manifested at 150 rpm (42).

From the PB results, another factor that contributed significantly to the SS for *Stenotrophomonas* sp. growth and enzyme production was pH. It is known that each microorganism has a pH range in which its growth is possible and usually has a well-defined optimum pH (46). While the optimum growth range was established at the prescreening (pH 6 to 8), important differences were found within the pH range tested for BG. The optimum pH range is generally narrow enough so that small changes in pH can have large effects on

the growth rate of the microorganism, because it can denature or alter the structure and function of an enzyme (46). The effect of pH on microbial growth and enzymes production was positive meaning that all responses were enhanced by slightly alkaline pH. The same behavior was found by others authors (47, 48, 49). The 3D structure of lipases and esterases is based on an α/β-hydrolase fold and its active site comprises a Ser-His-Asp catalytic triad and an oxyanion hole. In order to function, enzymes require that the catalytic residues have the appropriate protonation state in the active pH range (50, 51). The direct result of a pH change is a modification in the equilibrium concentrations of the protonated and deprotonated forms of the residues. Thus, pH is of key importance for enzyme activity. Most lipases show optimum activity at pH levels above 7, which is consistent with the ionization properties of histidine (pKa: 6.5) (50, 51). From the BB results, the regression analysis of the data showed that LP was significantly affected by pH. Results revealed that LP production increased with alkaline pH. Many researchers have reported different bacteria strains that also showed its optimum pH for lipase/esterase production within the alkaline range. Sangeetha *et al.* (52) isolated a *Bacillus pumilus* SG2 which presented an optimum pH for lipase production at 9.0. In the same way, Kumar *et al.* (53) evaluated the production of lipase from a *Bacillus coagulans* BTS-3 strain, and this microorganism showed maximum activity at pH 8.5. Immanuel *et al.* (47) investigated the production of extracellular lipase of a *Serratia rubidaea* strain and observed that pH range optimum for maximum lipase production were 7-8. Also, other researchers have reported lipolytic bacterial strains with optimal pH for enzyme production at neutral pH (49, 54) or slightly acidic pH (55, 56).

Regarding peptone concentration, while not significant effect on microbial growth was observed from the PB analysis, both the production of lipases and esterases were favored with an increase in peptone concentration. Different authors reported the optimization of culture conditions for lipase/esterase production, and many of them found that peptone

resulted as a significant (34, 57, 58, 59). In all the cases mentioned above, an increase in peptone concentration provided an increase in lipase production. Peptone is used by the bacteria as a nitrogen source. For cells, nitrogen is one of its main components, and their use is essential for protein synthesis and nucleic acids. Thus providing a good source of nitrogen is very important not only for increasing the biomass but also for the synthesis of metabolites of interest such as enzymes (46). The type of nitrogen source in the medium influences the production levels of lipases. Generally, a high yield of lipase is observed when organic nitrogen sources are used (60). Many other researchers have studied the effect of the addition of peptone to the culture media and most authors have found peptone concentration as a significant factor on the lipases/esterases production. Organic nitrogen sources favored the lipase production by *Stenotrophomonas maltophilia* (57), *Bacillus* sp. (61), *Staphylococcus* sp. (54) between others. Maybe this effect is due to the complex nature of these compounds that provide other components to the culture medium, as vitamins, growth factors, and surfactant, besides the nitrogen (57). The optimal peptone concentration for the production of both enzymes depends on the microorganism under study. Rajendran *et al.* (26) evaluated the lipase production by a *Candida rugosa* strain by RSM and the maximum production of enzyme was found at the maximum peptone concentration assayed (6 g/L). Shukla *et al.* (30) studied the lipase production by a *Rhizopus oryzae* strain using RSM and found that the maximum enzyme concentration was obtained at a peptone concentration of 0.51 g/L. Ananthi *et al*. (62) performed the optimization of lipase production by *Bacillus cereus* strain, with a one-factor method and found that the optimum concentration of peptone was 20 g/L.

4. Conclusion

Stenotrophomonas sp. is one of the most novel sources of lipolytic enzymes. In spite of being a well-known microorganism because of its biotechnological properties, the production of

extracellular enzymes for this bacterium has been scarcely studied. In the present work the simultaneous optimization of bacterial growth and lipase production by this strain was carried out using statistical methods that allowed on one hand, to reduce experimental efforts, and secondly, to evaluate the interaction of various factors on the studied responses.

The large scale production of lipases and esterases allows evaluating its application in different industrial processes, in which the pretreatment of fatty wastewaters seems to be one of the most promising applications. For this purpose, it is not only necessary the screening of potential enzymes producers, but also the optimization of medium components and physicochemical parameters of the culture media. The Plackett-Burman design was applied to determine the factors that exhibited a significant influence on lipase/esterase production and bacterial growth by *Stenotrophomonas* sp. Thereafter, using Box–Behnken response surface methodology, the three factors that resulted significant between assayed variables (stirring speed, peptone concentration and pH) were optimized. By applying the desirability function approach, optimal levels of the three parameters were found to be 100 rpm, 10 g/L and 7.5 (for stirring speed, peptone concentration and pH, respectively) achieving a lipase production of 8.19 (U/L), 7.53 (U/L) for esterase production and 18.52 (log UFC/mL) for bacterial growth. Under these optimal conditions, it is possible to obtain a 15-fold increase in esterase productivity, a 117-fold increase in lipase production, and a 9-log CFU/ml increase in bacterial growth, in the optimized medium compared to the basal medium without agitation.

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

Run	Variables											LP	EP	BG
	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	2.6405	2.5496	15.8369
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.1398	0.8842	13.2373
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	2.8923	5.6276	19.6680
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.9351	0.6112	17.0078
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	0.1887	1.4081	10.5925
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	2.5728	3.5168	18.9132
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	3.3027	4.4621	17.9674
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	3.2879	1.0329	16.5396
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	0.0147	0.2876	10.7777
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.0909	0.2579	11.0469
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	0.0477	1.2701	11.6211
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.0704	0.5004	9.6139

[a] X_i are the independent variables (factors): temperature (X_1), pH (X_2), stirring speed (X_3), glucose concentration (X_4), olive oil (X_5), Tween 80 (X_6), peptone (X_7) and $CaCl_2$ concentration (X_8).

[b] d_i are the *dummy* variables.

Table 2: Statistical parameters for microbial growth, lipase production and esterase production

Response	Coded Variable	Variable	Effect	Parameter estimate	SS	Contribution to total SS (%)	p-value	Confidence level
BG	X_1	Temperature	0.074	-0.659	0.017	0.012	0.920	7.97
	X_2	pH	1.863	1.988	10.410	7.388	0.073	92.73
	X_3	Stirring speed	3.985	1.775	127.033	90.152	0.002	99.75
	X_4	Glucose	-0.096	-0.839	0.028	0.020	0.897	10.29
	X_5	Olive oil	0.376	0.309	0.423	0.300	0.622	37.83
	X_6	Tween.80	0.308	-0.266	0.284	0.202	0.684	31.62
	X_7	Peptone	-0.936	-0.074	2.626	1.864	0.265	73.46
	X_8	$CaCl_2$	0.171	-0.554	0.088	0.062	0.818	18.15
LP	X_1	Temperature	-0.508	-0.254	0.774	3.571	0.245	75.51
	X_2	pH	0.351	0.175	0.369	1.703	0.393	60.72
	X_3	Stirring speed	2.141	1.257	18.948	87.407	0.006	99.43
	X_4	Glucose	-0.074	-0.037	0.016	0.076	0.646	35.35
	X_5	Olive oil	0.318	0.159	0.304	1.404	0.433	56.75
	X_6	Tween.80	-0.376	-0.188	0.424	1.956	0.364	63.59
	X_7	Peptone	0.489	0.244	0.717	3.308	0.259	74.09
	X_8	$CaCl_2$	0.204	0.102	0.125	0.575	0.603	39.67
EP	X_1	Temperature	-0.659	-0.329	1.302	4.172	0.374	62.55
	X_2	pH	1.989	0.994	11.859	37.992	0.052	94.84
	X_3	Stirring speed	1.775	1.099	14.502	46.460	0.040	95.97
	X_4	Glucose	-0.839	-0.420	2.114	6.772	0.277	72.32
	X_5	Olive oil	0.309	0.154	0.286	0.917	0.659	34.10
	X_6	Tween.80	-0.266	-0.133	0.213	0.681	0.702	29.76
	X_7	Peptone	-0.074	-0.0373	0.017	0.053	0.914	8.63
	X_8	$CaCl_2$	-0.554	-0.277	0.922	2.953	0.446	55.43

Table 3. Box- Behnken design matrix and mean values of lipase and esterase production and microbial growth under different experimental conditions after 48 hs of incubation.

Run	Variables			Experimental Responses		
	Stirring speed $X_1(x_1)$	pH $X_2(x_2)$	Peptone $X_3(x_3)$	BG	EP	LP
1	-1(0 rpm)	-1(6)	0(5 g/L)	14.982	3.655	0.843
2	-1(0 rpm)	1(8)	0(5 g/L)	14.596	4.582	1.157
3	1(100 rpm)	-1(6)	0(5 g/L)	18.805	5.815	5.919
4	1(100 rpm)	1(8)	0(5 g/L)	19.257	6.969	6.693
5	0(50 rpm)	-1(6)	-1(0 g/L)	15.571	3.159	0.676
6	0(50 rpm)	-1(6)	1(10 g/L)	15.014	4.264	1.394
7	0(50 rpm)	1(8)	-1(0g/L)	15.374	4.320	2.353
8	0(50 rpm)	1(8)	1(10 g/L)	15.118	5.281	1.622
9	-1(0 rpm)	0(7)	-1(0g/L)	14.137	4.025	2.472
10	1(100 rpm)	0(7)	-1(0g/L)	19.187	3.919	5.911
11	-1(0 rpm)	0(7)	1(10 g/L)	15.563	5.425	1.933
12	1(100 rpm)	0(7)	1(10 g/L)	19.382	7.727	8.367
13	0(50 rpm)	0(7)	0(5 g/L)	15.116	5.428	1.561
14	0(50 rpm)	0(7)	0(5 g/L)	15.207	5.238	1.579
15	0(50 rpm)	0(7)	0(5 g/L)	14.991	5.069	1.562

Table 4. Regression coefficients (from coded data) and R^2 of the response surface models

Coefficients	Responses		
	BG	**EP**	**LP**
β_0 (intercept)	15.1050***	5.2451***	1.5675***
β_1 (stirring speed)	2.1690***	0.8429**	2.5605***
β_2 (pH)	-0.0033	0.5323	0.3739*
β_3 (peptone)	0.1011	0.9091***	0.2379
β_{12} (speed x pH)	0.2095	0.0569	0.1151
β_{13} (speed x peptone)	-0.3078	0.6019	0.7484**
β_{23} (pH x peptone)	0.0754	-0.0361	-0.3622
β_{11} (speed x speed)	1.8014***	0.5144	2.6226***
β_{22} (pH x pH)	0.0036	-0.5039	-0.5368**
β_{33} (peptone x peptone)	0.1609	-0.4850	0.4808**
Coefficient of Variation	2.4546	12.6404	11.9750
R^2	0.9846	0.9051	0.9928

* Significant at 0.05 level.
** Significant at 0.01 level.
*** Significant at 0.001 level.

Table 5. Results of the ANOVA for regression equation for lipase and esterase production and bacterial growth

Responses	Source	DF	SS	MS	F value	Pr>F
BG	Linear	3	37.724		79.98	0.0001
	Quadratic	3	12.054		25.56	0.0018
	Cross-product	3	0.577		1.22	0.3922
	Total model	9	50.356		35.59	0.0005
	Lack of fit	3	0.763	0.254	21.64	0.0545
	Pure error	2	0.023	0.012		
EP	Linear	3	14.564		12.19	0.0098
	Quadratic	3	2.955		2.47	0.1765
	Cross-product	3	1.467		1.23	0.3911
	Total model	9	18.986		5.30	0.0405
	Lack of fit	3	1.926	0.642	19.92	0.0682
	Pure error	2	0.064	0.032		
LP	Linear	3	54.020		145.64	0.0001
	Quadratic	3	27.833		75.04	0.0001
	Cross-product	3	2.818		7.60	0.0261
	Total model	9	9.299		32.97	0.0060
	Lack of fit	3	0.156	0.052	4.74	0.0802
	Pure error	2	0.088	0.003		

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

Figure 1. Response surface plot a) effect of pH and stirring speed on bacterial growth b) effect of peptone concentration and stirring speed on bacterial growth.

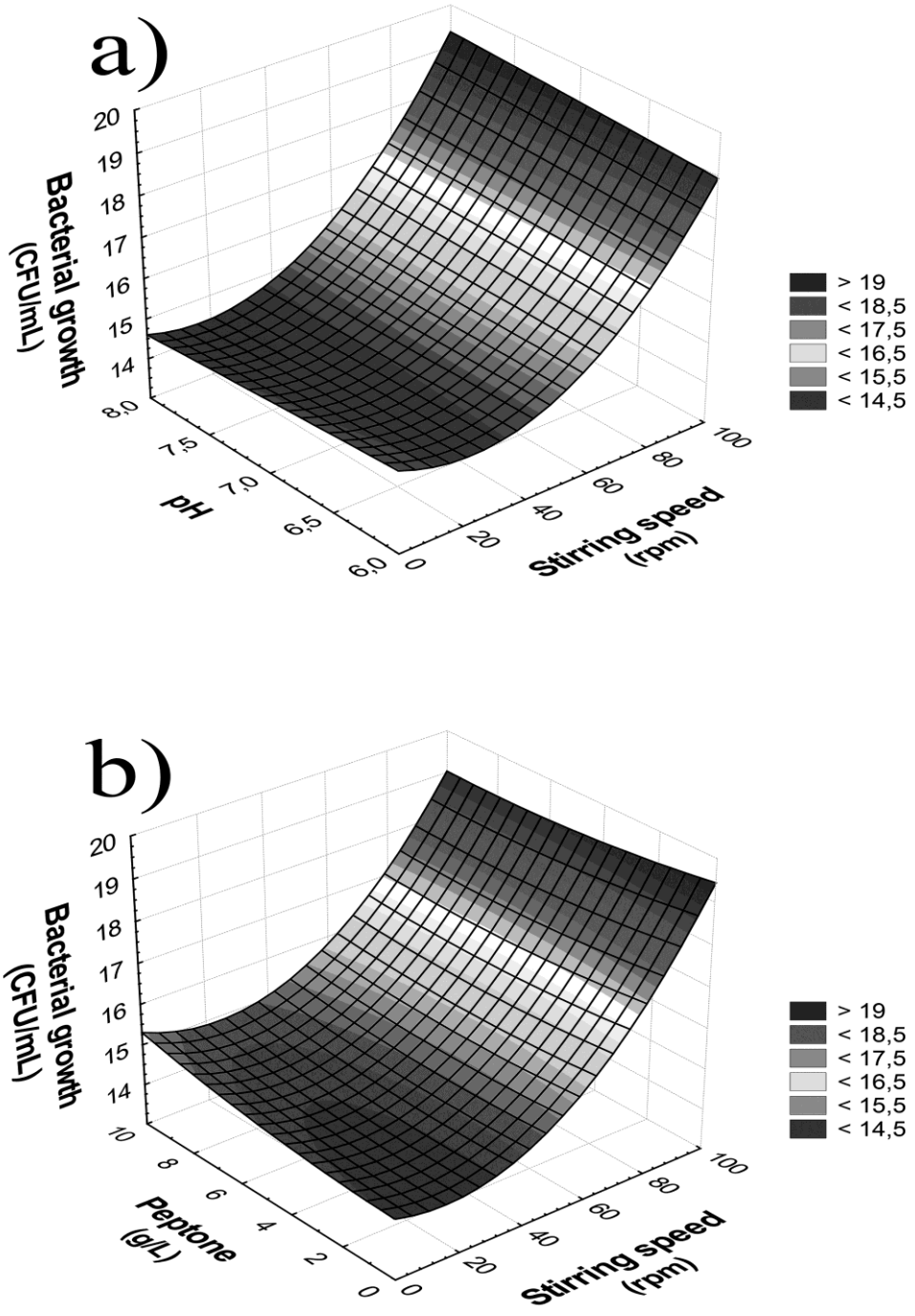

Figure 2. Response surface plot a) effect of pH and stirring speed on lipase production b) effect of peptone concentration and stirring speed on lipase production c) effect of peptone concentration and pH on lipase production.

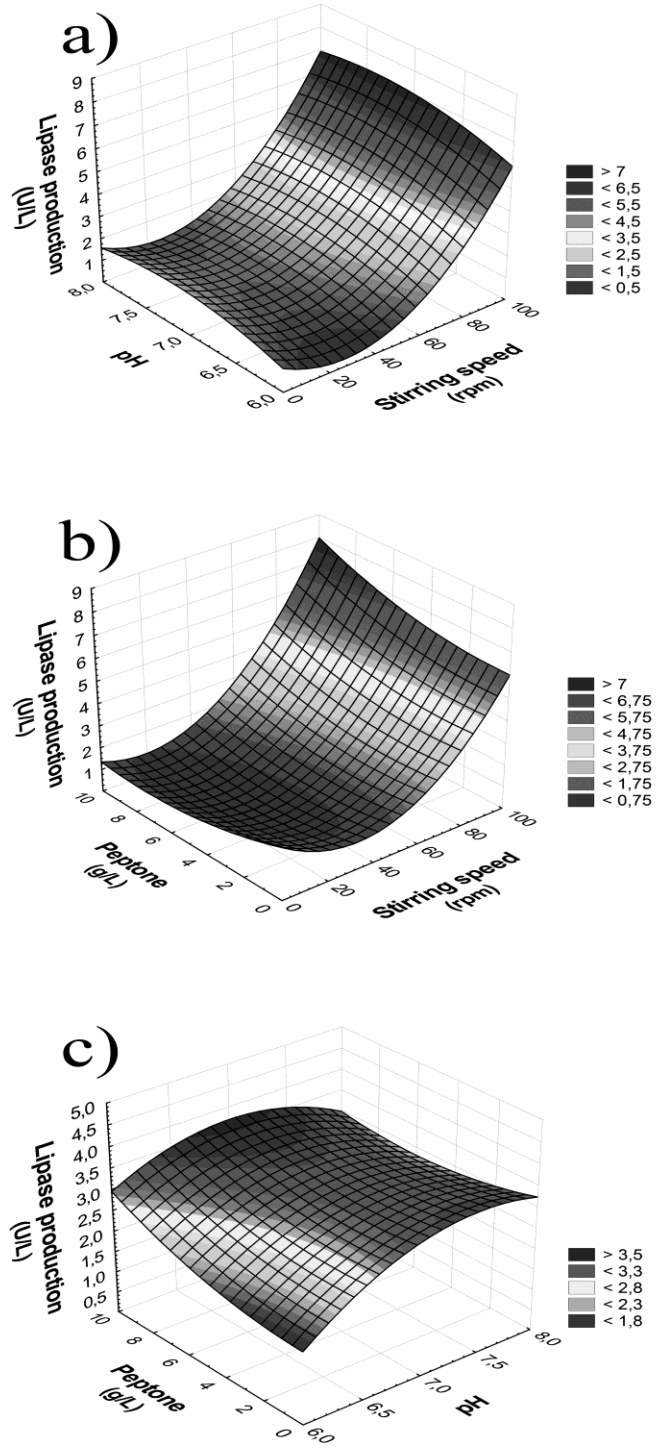

Figure 3. Response surface plot: effect of peptone concentration and stirring speed on esterase production.