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Response Surface Analysis on the Effect of Temperature and pH on Growth and Proteolytic Activity of Thermophilic *Bacillus* sp.

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ABSTRACT

Proteolytic activity and cell biomass of thermophilic Bacillus *sp. strain were evaluated at various levels of initial pH and temperature by applying response surface methodology. The mineral medium containing yeast extract (0.01%) and starch (1%) was used throughout the experiment. The results of statistical analysis revealed the polynomial model with high coefficient of determination (R^2 = 0.8) for the biomass and total proteolytic activity of the strain studied. This model showed a satisfactory adjustment of the statistic model with the experimental data. The p values showed that the temperature and pH had significant effect on biomass and proteolytic activity ($P<0.05$) of strain tested. The highest proteolytic activity (2.333 U/ml/h) of the* Bacillus *sp. was predicted at 41^oC and pH 4.8. The high biomass values were observed at broad range of temperature and pH.*

Key words: *Bacillus*, proteolytic activity, response surface methodology

INTRODUCTION

Thermophilic bacteria of genus *Bacillus* sp. receive great interest due to their biotechnological potential in relation to the production of variety of extracellular enzymes that are used in industrial scale. The proteases are one of the most important industrial enzymes in the world, used in laundry detergents, in leather preparation and food industry (Rao et al., 1998). It is well known that the effect of environmental conditions, such as nutrients, medium composition, variations of pH and temperature have strong effect on the production of proteases (Anwar and Saleemuddin, 1998; Johnvesly and Naik, 2001; Nehete et al., 1985). Such parameters may be optimized by using the mathematical and statistical models that minimize the number of experiments, instead of the classical methods that involve changing one independent variable while unchanging all others at fixed level. The response surface methodology (RSM) that consists of factorial design and regression analysis, helps in evaluating the effective factors and building the models to study the interaction and select optimum conditions of variables for a desirable response (Beg et al., 2003; Myers and Montgomery, 2002). RSM was used to optimize the production of several extracellular enzymes

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such as xilanases (Bocchini et al., 2002), α-amylase (Júnior et al., 2007; Tanyildizi et al, 2005), proteases (Beg et al., 2003; Puri et al., 2002; Thys et al., 2006) and β-glucanase (Tang et al., 2004; Tari et al., 2006) produced by several species of *Bacillus* sp. and other bacteria.

The aim of this study was to optimize the temperature and pH for the extracellular proteolytic enzymes produced by *Bacillus* sp. with the help of full-factorial composite design using RSM.

MATERIAL AND METHODS

Bacterial strain and medium

The thermophilic strain of *Bacillus* sp. TR17 isolated from the semi arid pasture soil of Northeast of Brasil was used in this study. The minimum and maximum growth temperature of strain studied was 30 and 65ºC, respectively. For the strain identification, the following analyses were used: API 50CH test (Biomerieux), Automatized Identification System Biolog (MicroLog System, Release 4.2, 2001) and Probabilistic Identification of Bacteria (PIB) (Bryant, 1995). The strain showed low similarity with *Geobacillus thermoglucosidasius* (R - 0.45) in the Biolog analysis, and high similarity (R - 0.98) with Taxon 41 in the PIB analysis. The API 50CH pattern did not match with any standard strain of this test.

Protease assay and biomass determination

The minimal mineral medium (0.05% $CaCl_2 \cdot 2H_2O$, 0.02% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.01% NaCl) with addition of 0.01% of yeast extract and one of the following substrate: glucose, glycerol and starch (10 g l^{-1}) was used to determine the best carbon source for the proteases production. The strain was incubated in 30 ml of the medium at 40 ºC and pH 7.0 for 48 h, after that the cultures were centrifuged at 6000 rpm for 10 min (4ºC), and then cell free supernatant was used for the estimation of proteolytic activity using azocasein (Sigma) as a substrate. The reaction mixture containing 0.5 ml of cell-free supernatant, 0.5 ml of 0.025% azocasein in 0.2M phosphate buffer (pH 7.0), was incubated for 30 min at 40ºC. The reaction mixture with water instead of supernatant was used as a control. After incubation, the reaction was stopped by addition of 1 ml of 10% trichloroacetic acid, and after 30 min, the solution was centrifuged at 12,000 rpm for 15 min. The optical density of the solution was measured at 420 nm, and one unit of enzyme activity was defined as the quantity of enzyme that increased the absorbance of 1.0 in relation to blank. Total activity was expressed as unit/ml/h. Total protein content in the cell-free supernatant was determined by Lowry et al. (1951) with bovine serine albumin as a protein standard. The biomass of *Bacillus* sp. was determined by drying the cells for 24 h at 100ºC.

Experimental design

After selection of best medium, the next stage was the determination of the optimal levels of two variables, initial pH (5.0-11.0) and temperature (30-70ºC), on protease production and bacterial growth. For this purpose, the response surface approach by using a set of experimental design (central composite design with five coded levels: -√2, -1, 0, 1, √2) was performed. For two factors, the design was made up of a full 2^3 factorial design with its eight points augmented with three replications of the centre points (both factors at level 0). The range and levels of experimental variables tested are presented in Table 1. The central values (zero level) chosen for the experimental design were pH 8.0 and temperature 50 ºC. The set of 11 experiments that consisted of eight unique combinations and three replications in central point were carried out (Table 3).

The culture was grown in mineral medium that contained 0.01% yeast extract and 1% of starch, at pH and temperature designed for each experimental set for 48 h. The biomass (mg of cells/ml), total activity (U/ml/hour) and protein content (mg/ml) were determined as described above. Results were analyzed by the Experimental Design Module of the Statistica software 6.0. The effect of each factor was evaluated by ANOVA (95% and 99%).

Table 1 - Experimental range and levels of the independent variables used.

Variables	Symbol coded	Range and levels				
		$-\alpha$ ($-\sqrt{2}$)	-1	0	1	$+\alpha$ ($\sqrt{2}$)
Temperature	X_1	30.0	35.9	50.0	64.1	70.0
pH	X_2	5.0	5.9	8.0	10.1	11.0

The model permitted the evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the dependent variable. The response surface contour plots were drawn to illustrate the main and interactive effects of the independent variables on protease production. The optimum values of the selected variables were obtained by solving the regression equation and by analyzing the response surface contour plot.

RESULTS AND DISCUSSION

The biomass of *Bacillus* sp. varied from 9.0 to 15.0 mg/ml on different media, and its proteolytic activity was the highest in the mineral medium containing yeast extract and starch (40°C, pH 7.0) (Table 2). This medium was selected for further study on the proteolytic activity and biomass of *Bacillus* sp. that were analyzed as shown in Table 3.

Table 2 - Effect of substrate on total proteolytic activity and biomass of *Bacillus* sp. strain.

Strain	Yeast extract	Yeast extract + Glucose	Yeast extract + Starch	Yeast extract + Glycerol
Total activity (U/ml)	0.764 ± 0.003*	1.031± 0.006	1.909± 0.011	0.819± 0.005
Biomass (mg/ml)	15.00± 0.120	9.00± 0.098	12.00± 0.100	15.00 ± 0.092

* Standard deviation

Table 3 - Experimental design used in response surface methodology studies showing observed extracellular proteins production, biomass and proteolytic activity of thermophilic *Bacillus* sp. strain.

Experiments	Temperature (°C)		pH		Total proteins mg/ml	Biomass (mg/ml)	Total activity (U/ml/h)
	Coded values	True values	Coded values	True values			
1	+1	64.1	+1	10.1	0.310	23.00	0.936
2	-1	35.9	+1	10.1	0.996	10.00	0.980
3	+1	64.1	-1	5.9	0.331	18.00	0.920
4	-1	35.9	-1	5.9	1.061	15.00	1.012
.5	+√2	70.0	0	8.0	0.813	9.00	0.484
6	-√2	30.0	0	8.0	0.885	14.00	0.612
7	0	50.0	+√2	11.0	0.526	25.00	1.230
.8	0	50.0	-√2	5.0	0.374	41.00	2.648
9	0	50.0	0	8.0	0.584	35.00	1.620
10	0	50.0	0	8.0	0.856	36.00	1.760
11	0	50.0	0	8.0	0.694	38.00	1.840

The growth of bacterial strain and protease production occurred in all the conditions tested (Table 3). According to the ANOVA results shown in Table 4, polynomial model obtained presented a high determination coefficient (R^2=0.85), showing good adjustment of the model to the experimental data. As indicated by P-values, temperature showed significant quadratic effect and pH had linear effect on the proteolytic activity ($p<0,05$). Temperature showed significant quadratic effect, and pH also linear effect, on the biomass ($p<0,05$) (Table 4).

On the basis of experimental data showed in Table 3, the following equations of the polynomial model for predicting the optimal point for total activity (TA) and biomass (B) were calculated:

$TA = 5.185 – 0.001x^2_1 – 0.777x_2 + 0.013x_1. x_2$

$B= 94.799 – 0.025x^2_1 – 15.478x_2 – 0.093\ x^2_2 + 0.312x_1. x_2$

where x_1 is temperature, and x_2 is pH.
Figs. 1 and 2 show the plots of response surface for the total proteolytic activity (U/ml/h) and biomass (mg/ml) of *Bacillus* sp., respectively.

The region of high proteolytic activity (TA) of *Bacillus* sp. was identified with temperature values lower than 53°C and pH lower than 6.0 (TA > 2.042 U/ml/h) (Fig. 1). The model predicted a maximum response for proteolytic activity of 2.333 U/ml/h at the point which was pH 4.8 and temperature 41°C.

Table 4 - Determination coefficient of polynomial model, p value and the interaction of temperature and pH for the variables tested. Values marked with asterisk are significant ($p<0.05$).

Variable/Effect	Biomass (mg/ml)	Total activity (U/ml/h)
R^{2*}	0.846	0.831
Value p - Linear		
Temperature	0.175	0.420
pH	0.034*	0.023*
Value p - Quadratic		
Temperature	0.002*	0.004*
pH	0.040*	0.583
Interaction		
Temperature X pH	0.082	0.849

R^{2*} - Determination coefficient

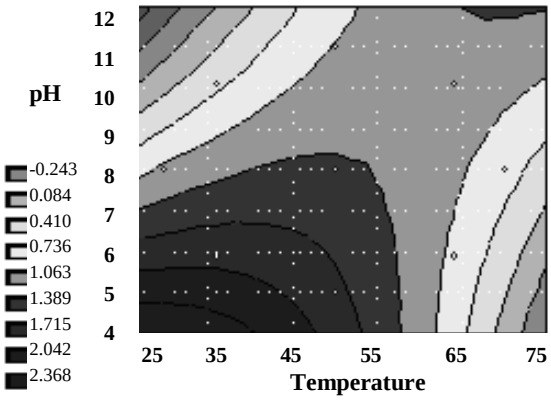

Figure 1 - Response surface curves for total proteolytic activity (U/ml/h) of *Bacillus* sp. as a function of temperature and pH.

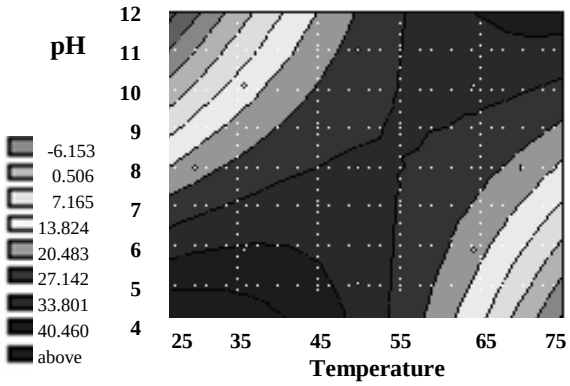

Figure 2 - Response surface curves for *Bacillus* sp. biomass (mg/ml) as a function of temperature and pH.

The acid proteases produced by the thermophilic *Bacillus* spp. were characterized by Murao et al. (1988) and Toogood et al. (1995). Kim (2002) characterized the acid protease produced by *B. subtilis* JM-3, and reported that optimal pH and temperature were 5.5 and 60°C, respectively. The high values of biomass on the level 33.8 mg/ml were observed in large range of temperature and pH (Fig. 2). For the temperature <42°C the maximum biomass was achieved at pH<5.0 (B>40.46 mg/ml).

Different applications of proteases require specific optimal pH for better enzyme activity. For example, the use of enzymes in the detergent industry requires alkaline pH, while the acid proteases are widely used in cheese-making, baking and meat tenderization.

Various studies have reported the optimization of alkaline proteases production by *Bacillus* spp. using RSM (Adinarayana and Ellaiah, 2002; Beg et al., 2003; Ellaiah and Adinarayana, 2001; Puri et al., 2002). Wu and Hang (2000) studied the acid proteases using the response surface methodology.

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RESUMO

Atividade proteolítica total e biomassa de uma linhagem de *Bacillus* sp. termofílico foram analisados em vários níveis de pH inicial e temperatura utilizando a metodologia de superfície de resposta. O meio mineral com extrato de levedura (0.01%) e amido (1%) foi utilizado no experimento. Os resultados de análise estatística da metodologia de resposta de superfície definiram um modelo polinomial para a biomassa e atividade proteolítica da linhagem de *Bacillus* sp. com alto coeficiente de determinação (R^2 = 0.8), mostrando um ajuste satisfatório do modelo estatístico obtido com os dados experimentais. Os valores de p mostraram que a temperatura e pH tiveram efeito significante em biomassa e atividade total (P <0.05) da linhagem testada. A atividade proteolítica mais alta (2.368 U/ml/h) da linhagem de *Bacilus* sp. foi prevista pelas curvas de superfície de resposta em temperatura a 41°C e pH igual a 4.8. Os valores de biomassa altos foram previstos para ampla faixa de temperatura e pH.

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