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Full Length Research Paper

Optimization of medium composition for protease production by *Paecilomyces marquandii* in solid-state-fermentation using response surface methodology

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The present work was aimed to evaluate the optimization of medium composition for protease production by *Paecilomyces marquandii* in solid-state-fermentation using response surface methodology. Wheat bran was used as substrate for protease production. The variables tested were as follows: pH, tryptone and days of incubation. The proteolytic activity was measured by caseinolytic method. The Central Composite Design (CCD) was applied to determine the optimal concentration of the tested significant variables. A full factorial central composite experimental was used with six replicates at the central point. A total of 20 experiments were used to investigate the variables. The best fermentation conditions found after optimization were pH 5.0, tryptone 0.02 g/l and 5 days of incubation which yielded about 118 U/ml. The enzyme production of submerged fermentation (46 U/ml) was 2.57 times lower than the highest enzyme production of solid state fermentation (118 U/ml). The results indicate that pH, tryptone and days of incubation optimized the production of proteases by fungus *P. marquandii* in solid state fermentation. This is the first work about optimizing the production of proteases in solid state fermentation by the fungus *P. marquandii*.

Key words: Solid state fermentation, *Paecilomyces marquandii,* protease, optimization, response surface.

INTRODUCTION

Microbial protease represents about 60% of all the industrial enzyme's sales in the world due to their applications in several industrial sectors (Gupta et al., 2002). Furthermore, according to Godfrey and West (1996), the industrial use of enzymes is in full development and the estimates of trade in this sector in 2005 were around 1.7 to 2 billion dollars in the world. Beck et al. (1987) report that the fungus *Paecilomyces marquandii* produces proteases with interesting potential use commercial in the laundry industries, due to its low temperature for the optimum activity. However, industrial production of enzymes is still limited due to cost of substrates used in microorganism cultivation.

Filamentous fungi are exploited for the production of industrial enzymes due to their ability to grow on solid substrate and produce a wide range of extracellular enzymes. The great advantages offered by fungal enzymes are low material costs coupled with high and faster productivity and the ease with which the enzymes can be modified (Sharma et al., 2007). At present, due to high cost of substrates and mediums used, the overall cost of enzyme production is very high and therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view (Kammoun et al., 2008). Also solid-state fermentation (SSF) has certain advantages over the conventional submerged

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fermentation (SmF), like low production cost, saving of water and energy, less waste effluent problem and stability of the product due to less dilution in the medium (Pandey, 2003; Holker and Lenz, 2005).

According to Hajji et al. (2008), in biotechnological studies, the growth medium is generally optimized with the one-variable-at-a-time method, in which all variables but one is held at a constant level, and then the optimum level of the testing variable is determined. Although, especially due to the lack of interaction between factors involved, it is time consuming and incapable of detecting the true optimal. The response surface methodology is an empirical modeling technique that evaluates the relation between a group of controlled experimental factors and the results observed a selected criterion (Ambati and Ayyanna, 2001). The factorial design and the regression analysis help to validate the effective factors and to build blocks for studying their interactions, serving as well to select the optimum conditions of the variables for a chosen response (Sharma et al., 2007).

The present work was aimed to evaluate the optimization of medium composition for protease production by *P. marquandii* in solid-state-fermentation using response surface methodology.

MATERIALS AND METHODS

Microorganism and media

The isolate of the nematophagous fungus *P. marquandii* was obtained from a soil located in the Zona da Mata region, Viçosa, Minas Gerais, Brazil, 20°45'20"S, 42°52'40" W 20, 649m altitude. The isolate was kept in test tubes containing Potato Dextrose Agar (PDA), at 4°C, in the dark for 10 days. The inocula of spores (10^6 spores ml^{-1}) were prepared from 10-day-old colonies by flooding with 10 ml of sterile distilled water and scraping off the agar plates. The minimal medium for enzyme production was established in grams per liter: Glucose (1.0); KH_2PO_4 (1.5); K_2HPO_4 (1.0); $MgSO_4$ (0.2); $CaCl_2$ (0.2); NaCl (0.2) according to Gradisar et al. (2005), modified.

Solid-state fermentation

Wheat bran from a local market located in the Zona da Mata region, Viçosa, Minas Gerais, Brazil, was used as substrate for protease production. Fermentations were carried out in erlenmeyers flasks (250 mL) with 10 g of wheat bran, supplemented with 50 ml of the minimal medium, in addition of tryptone in concentrations defined by the experimental design. Moisture was adjusted to 50% (v/w) using 5 ml of liquid minimal medium and 10 g of wheat bran, each flask was covered with hydrophobic cotton and autoclaved at 121°C for 15 min. After cooling, each flask was inoculated with 2mL of the suspension previously prepared and incubated in a rotary shaker at 28°C and 180 rpm at different times defined by the experimental design.

Submerged fermentation

Fermentations were carried out in erlenmeyers flasks (250 mL) with 10 g of wheat bran, supplemented with the minimal medium, with final volume of 50 ml. After cooling, each flask was inoculated with 2 mL of the suspension previously prepared and incubated in a rotary shaker at 28°C and 180 rpm for 6 days.

Central composite design

The Central Composite Design (CCD) was applied to determine the optimal concentration of three independent variables. The effect of these significant variables in the enzymatic activity was studied in 5 experimental levels as follows: -α, -1, 0, 1, + α, where $\alpha = 2^{n/4}$, n is equal to number of variables and 0 corresponds to the central point. A full factorial central composite experimental was used with six replicates at the central point. A total of 20 experiments were used to investigate the variables. Table 1 shows the analyzed factors and respective high and low levels. The interaction between the variables and the response (enzymatic activity) were calculated using the following second order polynomial equation:

$$Y = b_o + \Sigma b_i x_i + \Sigma b_{ij} x_i x_j + \Sigma b_{ii} x^2_i \quad i = 1, 2, 3, \ldots\ldots\ldots, k$$

where y is the measured response, b_0 is the intercept term, b_i, b_{ij}, and b_{ii} are the measures of the effects of variables x_i, $x_i x_j$, and x_{2i}, respectively. The variable $x_i x_j$ represents the first-order interaction between x_i and x_j ($i<j$). The variable Xi was coded as *xi* according to the following equation:

$$x_i = (X_i - X_{oi}) / \Delta X_i \quad i = 1,2,3\ldots\ldots\ldots\ldots,k$$

where xi is a dimensionless value of an independent variable, X_i is the real value of an independent variable, X_{0i} is the real value of the independent variable at the center point, and ΔX_i is the step change. Minitab Release 15 software was used for regression and graphical analyses of the data obtained. The statistical significance of the regression coefficients was 95%.

Extraction of the enzyme

For isolation of protease produced under SSF, the flasks containing the fermented matter was mixed with distilled water (1:5, w/v) by stirring on a magnetic stirrer for 30 min at room temperature (25°C). The slurry was then squeezed through cheesecloth followed by centrifuging the whole content at 10,000 × *g* for 10 min at 4°C to remove the insoluble matters. The clear supernatant was used for the protease assay (Mukherjee et al., 2008). For isolation of protease produced under SmF, after fungus growth, the culture medium was filtered using Whatman filter paper No. 1 for performing the enzymatic assay.

Enzymatic assay

The protease activity was measured as described by Joo and Chang (2005) modified. The volumes of solutions used in this method were as follows: 50 µl crude extract, 450 µl of Tris-HCl 100 mM pH 7.0 and 500 µl of 1% casein pH 8.0. The reaction medium was incubated for 15 min at 43ºC and the reaction interrupted by adding 1 ml of 10% trichloroacetic acid. After 10 min, the reaction medium was centrifuged at 10,000 x g for 5 min, the supernatant was collected and the absorbance was determined spectrophotometrically at 280 nm. A standard curve of tyrosine was constructed by varying the concentrations of tyrosine. One unit of protease was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the test conditions used.

Table 1. High and low levels (-1 and +1) with the three selected variables: pH (A), tryptone (B) and days of incubation (C) in the Response Surface Methodology experimental design.

Variables	Variable code	Low level (-1)	High level (+1)
pH	A	4.0	7.0
Tryptone (g/l)	B	3.0	5.0
Days of incubation	C	3	7

Table 2. Experimental design used for the establishment of Response Surface Methodology using three variables (pH, tryptone and days of incubation) each one with 5 levels, with the values of proteolytic activity demonstrated.

Run Order	pH	Tryptone	Days	Activity (U/ml)
1	-1	-1	-1	13.0
2	1	-1	-1	9.53
3	-1	1	-1	14.3
4	1	1	-1	9.04
5	-1	-1	1	78.9
6	1	-1	1	51.0
7	-1	1	1	27.0
8	1	1	1	75.6
9	-1.68	0	0	0.00
10	1.68	0	0	114
11	0	-1.68	0	40.8
12	0	1.68	0	58.5
13	0	0	-1.68	0.00
14	0	0	1.68	101
15	0	0	0	70.0
16	0	0	0	72.7
17	0	0	0	109
18	0	0	0	92.7
19	0	0	0	94.1
20	0	0	0	118

pH: -1 (4.0); 1 (7.0); 0 (5.5); -1.68 (2.98); 1.68 (8.02). Tryptone: -1 (3.0 g/l); 1 (5.0 g/l); 0 (4.0 g/l); -1.68 (2.32 g/l); 1.68 (5.68 g/l). Days: -1 (3); 1 (7); 0 (5); -1.68 (1.64); 1.68 (8.36).

RESULTS

Table 2 presents the matrix of the complete factorial design with the correspondent responses in terms of enzyme activity. The results show that the highest enzyme production (118 U/ml) was obtained at central point conditions (pH 5.0, tryptone 4.0 g/l and 5 days of incubation). Three variables, pH, tryptone and days of incubation were selected to perform the Response Surface Methodology (RSM). A total of 20 experiments with different combinations of the selected parameters were performed. The function for the final response that provides the proteolytic activity after the removal of related terms to non-significant variables (p> 0.05) is the following:

$$Y = -513{,}050 + 58{,}6437X_3 - 7{,}04842X_1^2 - 736646X_2^2 - 4{,}53139\,X_3^2$$

where Y is the response value, that is, the enzymatic activity, and X_1, X_2 and X_3 are the coded levels of pH, tryptone concentration and days of incubation respectively.

The results of the statistical significance of the regression model verified by the F test, and ANOVA are shown in Table 3. The central point was repeated six times to the error estimation. The regression coefficients and analysis of variance presented in Table 3 indicated the significance of the model (R^2 = 0.77). In Figure 1, the results of three-dimensional response surface (3-D), plotted according to the model equation of protease production by the fungus *P. marquandii* are presented. The results indicated that the linear term of days of incubation showed significant effect (p> F: 0.005) on the protease production by *P. marquandii*. Besides, the quadratic effect of pH, tryptone and days of incubation also had significant effect, with the following values of p> F: (0.048) pH; (0.026) tryptone and (0.028) days of incubation. On the other hand, the linear effects of pH and tryptone, the interactive effect between pH and days of incubation, pH and tryptone and tryptone and days of incubation showed no significance (p> 0.05) in this work. The result of submerged fermentation (SmF) shows that the enzyme production was 46 U/ml.

DISCUSSION

The regression coefficients and analysis of variance indicated the significance of the model (R^2 = 0.77). Furthermore, the results shown indicate that the linear term of days of incubation and quadratic term of pH, tryptone concentration and days of incubation had a significant effect (p<0.001) in the production of protease by fungus *P. marquandii*. However, the linear effect of pH and tryptone concentration, and interactive effect between all studied variables didn't demonstrate significance (p>0.05) in this work. It may be noted that extremely basic or acidic pHs inhibit the production of protease. Besides, the results of this study also agree with Anbu et al. (2009) who reported that an exacerbated

Table 3. Analysis of variance for the response equation developed in the protease production by *Paecilomyces marquandii* in solid-state-fermentation.

Source	SS	DF	MS	F-value	P > F
Model	24298.2	9	2699.8	3.77	0.025
Error	7162.7	10	1069.9		
Total	31460.9	19			

R^2 = 0. 77. SS = Sum of Squares; DF = Degrees of Freedom; MS = Mean of Squares.

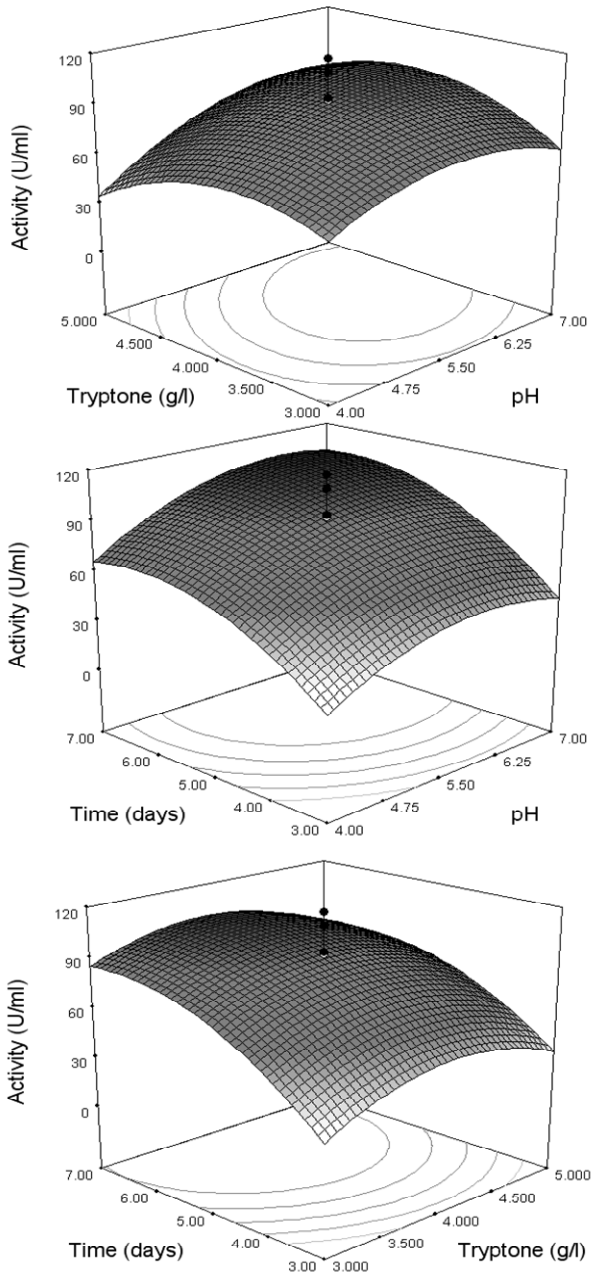

Figure 1. Response surface curve of protease production by the fungus *Paecilomyces marquandii* in solid state fermentation.

increase in the concentration of tryptone could lead to a decrease in protease production by fungi. In the present study the three-dimensional response surface (3-D) showed an elliptical shape suggesting that the operating conditions for the optimization have been well defined (Dutta et al., 2004). However, in the present study the interaction between the variables studied was not observed, in spite of the chart elliptical.

The enzyme production of submerged fermentation (46 U/ml) was 2.57 times lower than the highest enzyme production of solid state fermentation (118 U/ml). This result agrees with Sandhyaa et al. (2005), who reported that the enzyme production of submerged fermentation was 3.5 times lower than the protease production by *Aspergillus oryzae* in solid state fermentation, clearly demonstrating the superiority of SSF over SmF. It is interesting to note that, although a number of substrates have been tried in solid-state fermentation, wheat bran has been the preferred choice (Vishwanatha et al., 2010). In the present work, wheat bran seems to present a great nutritional potential for growth of *P. marquandii* and production of protease. Gradisar et al. (2005) realized a study with the *P. marquandii* keratinase, a new application of this species. However, this is the first work about optimizing the production of proteases in solid state fermentation by the fungus *P. marquandii*.

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