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Full Length Research Paper

In vitro larvicidal action of *Paecilomyces marquandii* crude extract

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The aim of this present work is to produce *Paecilomyces marquandii* crude extract and its *in vitro* larvicidal action. Saccharose, glucose, coffee residue, soluble starch, insoluble starch, soybean meal and wheat bran were the carbon sources tested. $NaNO_3$, NH_4Cl, soybean meal, $(NH_4)_2SO_4$, yeast extract, casein peptone, tryptone and NH_4NO_3 were the nitrogen sources tested. Central composite design was applied to determine the optimal concentration of the tested significant variables. The best carbon sources were glucose and soybean meal, while the best nitrogen source observed was tryptone. The greatest value for proteolytic activity was 246.58 U/ml due to the concentration of tryptone and pH value. Results showed that the optimum pH was 8.0, while the optimum temperature was 60°C. The optimized extract obtained from *P. marquandii* exhibited *in vitro* larvicidal activity with 60.1% reduction. Tryptone and pH optimized the production of proteases by fungus *P. marquandii*.

Key words: Nematophagous fungi, *Paecilomyces marquandii,* crude extract, larvicidal action, surface response.

INTRODUCTION

Proteases represent one of the three largest groups of industrial enzymes; however, enzyme industrial production is still limited due to substrates costs used in microorganisms cultivation (Godfrey and West, 1996; Kashyap et al., 2001). Thus, the optimal design of the culture medium helps in understanding the interactions among the nutrients at varying concentrations and in calculating the optimal of each nutrient for a maximal enzyme production in less time and at lower cost. Multivariate experiments are designed to reduce the number of experiments and to produce more precise results than those obtainable by univariate experiments (Box and Draper, 1987; Khurana et al., 2007).

The crude extract can be used as *in vitro* biological control against nematodes of domestic animals (Braga et al., 2010). Among the nematophagous fungi that are capable of producing proteases, the genus *Paecilomyces* stands out. This genus produces proteases and has been used successfully in combating nematode parasites of plants, domestic animals and humans (Khan et al., 2004; Braga et al., 2008a, b; Soares et al., 2010).

Currently, the penetration of the cuticle of nematodes or their eggshells has been assumed to be the consequence of mechanical forces exerted by the fungi, in combination with cuticle-degrading enzymes (such as proteases) produced by fungi. Proteases have been shown to play a critical role during host infection, and therefore have attracted particular attention (Liang et al., 2011).

The aim of this present work is to produce *Paecilomyces marquandii* crude extract and its *in vitro* larvicidal action.

MATERIALS AND METHODS

Fungus

The nematophagous fungus *P. marquandii* was used. This isolate originates from the soil of Brazil. The fungus was maintained on 2% potato-dextrose-agar (2%PDA) medium at 4°C and transferred every 7 weeks. Petri dishes containing 2% PDA medium were

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incubated at 28°C for 10 days and maintained at 4°C before use. After ten days, spores produced by *P. marquandii* were scraped from the Petri dishes and ten milliliters of distilled water containing 0.1% Tween-80 were added. After this step, the spores were counted using a Neubauer counting chamber, adjusted to 10^6 spores ml^{-1} according to the modified technique of Kammoun et al. (2008).

Selection of the carbon and nitrogen sources

The initial screening of the carbon and nitrogen most significant sources for maximum protease production was performed by one-variable-per-time method. The carbon sources examined were as follows: saccharose, glucose, coffee residue, soluble starch, insoluble starch, soybean meal, wheat bran. The nitrogen sources tested were as follows: $NaNO_3$, NH_4Cl, soybean meal, $(NH_4)_2SO_4$, yeast extract, casein peptone, tryptone and NH_4NO_3.

The minimal medium for enzyme production was established in grams per liter: 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. To test carbon sources, wheat bran was used as nitrogen source. To test nitrogen sources, the best carbon source tested was used.

The flasks containing the growth media were autoclaved at 121°C for 15 min. After inoculating with the appropriate volume of the spore suspension, they were kept on a rotary shaker for six days at 28°C and 180 rpm. After fungus growth, the culture medium was filtered using Whatman filter paper No. 1 for performing the enzymatic assay.

Enzymatic assay

Proteolytic activity was measured as described by Soares et al. (2010).

Central composite design

Central Composite Design (CCD) was applied to determine the optimal concentration of the tested significant 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 five replicates at the central point. A total of 13 experiments were used to investigate the variables (Table 3). Design Expert 7 trial version was used to analyze the experimental data. The interaction between the variables and the response (enzymatic activity) were calculated by using the following second order polynomial equation:

$$Y = a_0 + \sum a_i x_i + \sum a_{ii} x_2 + \sum a_{ij} x_i x_j$$

Where Y represents response variable, a_0 is the interception coefficient, a_i coefficient of the linear effect, a_{ii} the coefficient of quadratic effect and a_{ij} the coefficient of interaction effect. x_i and x_j denotes the coded levels of variable X_i and X_j in experiments. X_i was coded as x_i according to the following equation:

$$xi = (\mathrm{Xi\text{-}X0})/\Delta Xi$$

Where x_i (dimensionless) is the coded value of the variable X_i, X_0 is the real value of X_i at the center point level, and the ΔX_i is the step change value. All the experiment was performed in triplicate.

In vitro larvicidal activity

Positive faeces for nematode gastrointestinal were collected from the rectum of horses (*Equus caballus*). Coprocultures were then carried out and third stage larvae (L_3) were obtained after seven days, identified and quantified in an optical microscope (10 x objectives). Baermann readings showed that 100% of the detected L_3 were cyathostomin (Bevilaqua et al., 1993). Cyathostomin L_3 was washed thoroughly with a 10 mM PBS (pH 7.0, sterile), and suspension containing ~50 nematodes (20 µl) was transferred to a sterile tube. The crude extract (150 µl) was added to the cyathostomin L_3 (treated group) and boiled for 10 min before being added to the cyathostomin L_3 as control. Six replicates were performed for each group. After incubating the mixture at 26°C for 24 h, the numbers of cyathostomin L_3 were counted under a dissecting microscope (Braga et al., 2011a). Data were examined by analysis of variance (ANOVA) at significance level of 1% probability. The Tukey test (1% probability level) was used to assess predatory efficiency of L_3 compared with the control (Ayres et al., 2003). Reduction percentage of mean larva number was calculated with the following equation:

$$\text{Reduction (\%)} = (X_T\text{-}X_C) \times 100/X_T$$

$X_{T=}$Mean of Treated group
$X_{C=}$ Mean of Control group

RESULTS

Determination of optimal carbon and nitrogen sources

In the present work, the preliminary study for the optimization of protease production of the fungus *P. marquandii* was the screening of the most significant carbon and nitrogen sources, performed by the one-variable-per-time method. The selected carbon sources were added separately to the liquid medium containing wheat bran. However, according to the presented results in Tables 1 and 2, the best carbon sources were glucose and soybean meal and the best nitrogen source was tryptone.

Central composite design

Based on the screening preliminary study of the most significant carbon and nitrogen sources for the protease production, pH and tryptone were selected to perform the Response Surface Methodology (RSM). 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:

$$Y = -1091.07 + 326.21X_1 - 30.10X_2$$

Where Y is the enzymatic activity, and X_1 and X_2 are the coded levels of tryptone and pH, respectively. Results of the statistical significance of the regression model verified by the F test, and ANOVA are shown in Table 4. The

Table 1. Effect of different carbon sources (10 g/l) supplementation on *Paecilomyces marquandii* protease production in liquid media. Culture conditions: pH 6.0, 28°C, 180 rpm, wheat bran as nitrogen source (10 g/l).

Carbon sources	Proteolytic activity (U/ml)
Saccharose	27.6
Glucose	46.5
Coffee residue	9.47
Soluble starch	16.9
Insoluble starch	29.6
Soybean bran	46.0
Control (wheat bran)	35.9

Table 2. Effect of different nitrogen sources (10 g/l) supplementation on *Paecilomyces marquandii* protease production in liquid media. Culture conditions: pH 6.0, 28°C, 180 rpm, glucose as carbon source (10 g/l).

Nitrogen sources	Proteolytic activity (U/ml)
$NaNO_3$	13.3
NH_4Cl	2.14
Soybean bran	3.12
$(NH_4)_2SO_4$	1.64
Yeast extract	24.3
Tryptone	73.6
NH_4NO_3	1.97
Casein peptone	34.8
Control (wheat bran)	35.9

Table 3. Experimental design used for the establishment of response surface methodology using two variables (pH and tryptone) each one with 5 levels, with the values of proteolytic activity demonstrated.

Run order	pH	Tryptone (g/l)	Proteolytic activity (U/ml)
1	4.0	3.0	135.53
2	7.0	3.0	101.19
3	4.0	5.0	154.89
4	7.0	5.0	133.33
5	3.38	4.0	60.27
6	7.62	4.0	130.41
7	5.5	2.59	133.33
8	5.5	5.41	246.58
9	5.5	4.0	230.50
10	5.5	4.0	226.48
11	5.5	4.0	225.02
12	5.5	4.0	224.29
13	5.5	4.0	219.18

pH: -1 (4.0); 1 (7.0); 0 (5.5); -1.41 (3.38); 1.41 (7.62); Tryptone: -1 (3.0 g/l); 1 (5.0 g/l); 0 (4.0 g/l); - 1.41 (2.59 g/l); 1.41 (5.41 g/l).

central point was repeated five times to the error estimation. The regression coefficients and analysis of variance presented in Table 4 indicated a high significance of the model (R^2=0.89). The response

Table 4. Analysis of variance for the response equation developed in the protease production by *Paecilomyces marquandii*.

Source	SS	DF	MS	*F*-value	*P* > *F*
Model	38737.6	5	7747.5	11.28	0.003
pH	234.28	1	234.28	0.34	0.5776
Triptone	5599.98	1	5599.98	8.15	0.0245
pH*Triptone	40.83	1	40.83	0.059	0.8144
pH*pH	31912.14	1	31912.14	46.44	0.0002
Triptone*Triptone	2901.45	1	2901.45	4.22	0.0790
Error	4809.9	7	687.1		
Total	43547.4	12			

R^2: 0.89 R^2 (adj): 0.811 SS= Sum of Squares; DF = Degrees of Freedom; MS = Mean of Squares.

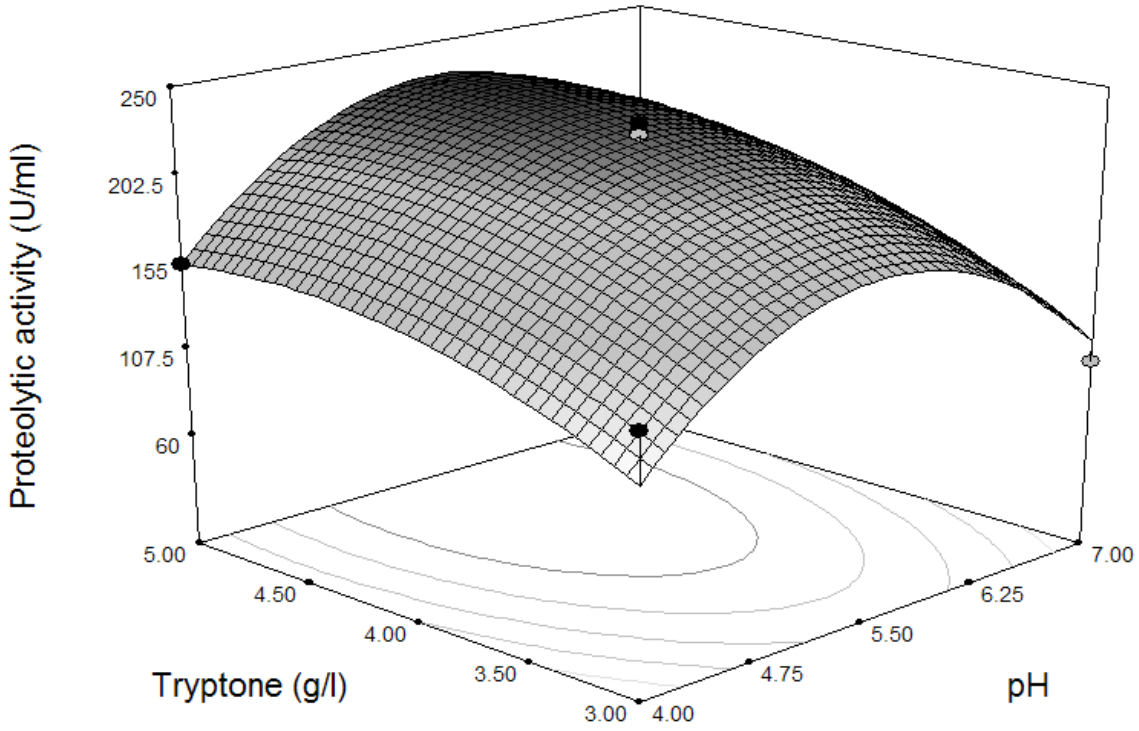

Figure 1. Response surface curve of protease production by the fungus *Paecilomyces marquandii*.

surface in Figure 1 shown was based on the final model, varying levels of two factors in their experimental range.

In vitro larvicidal activity

The enzymatic extract exhibited larvicidal activity in the tubes of the treated group after 24 h of interaction. There were differences ($p<0.01$) between the number of L_3 in the treated group tubes and in the control group, with 60.1% reduction of L_3 in the treated group in comparison with the control.

DISCUSSION

In this work, there was no difference ($p<0.01$) between the proteolytic activity between glucose and soybean meal. Howover, some studies have mentioned that the use of glucose as carbon source has been successfully performed on protease production by nematophagous fungi (Meyer and Wiebe, 2003; Braga et al., 2011b).

Results for the selected nitrogen sources are in accordance to Anbu et al. (2009) and Chellappan et al. (2006) that describe that the optimal concentration of tryptone has an important role in the production of

protease.

The linear term of tryptone and quadratic term of pH had a significant effect ($p<0.001$) on the production of protease by fungus *P. marquandii*. However, the linear effect of pH, the quadratic effect of tryptone and interactive effect between pH and tryptone did not demonstrate significance ($p>0.05$) in this work.

In the present study the three dimensional response surface showed an elliptical shape. However, it may be noted that an interaction between the variables studied was not observed, in spite of the chart elliptical. In this study, the optimized enzymatic extract of *P. marquandii* was effective in the destruction of cyathostomin L_3, suggesting proteolytic action. The nematophagous fungus *P. marquandii* produces proteases in solid-state-fermentation (Soares et al., 2010), but this is the first report of optimization for protease production by *P. marquandii* in liquid medium. Moreover, Khan et al. (2004) demonstrated that a serine protease secreted by fungus of genus *Paecilomyces* was effective in reducing the hatching of eggs and the destruction of *Meloydogine javanica* juvenile hatched. Thus, the results indicate that tryptone and pH optimized the production of proteases by fungus *P. marquandii* and its crude extract showed larvicidal action.

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