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Extracellular serine proteases by *Acremonium* sp. L1-4B isolated from Antarctica: Overproduction using cactus pear extract with response surface methodology

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ABSTRACT

Acremonium sp. L1-4B isolated from lichen in Antarctica was used to produce extracellular proteases through submerged fermentation using cactus pear extract (*Opuntia ficus-indica* Mill.). A 2^3 factorial design was applied to optimize the protease production using three independent variables, namely temperature, pH and concentration of yeast extract, was also used a Central Composite Design (CCD) under Response Surface Methodology (RSM). All variables and interactions analyzed in the factorial design were significant or marginally significant, a Central Composite Design was developed, and the Response Surface Methodology towards the highest point it was established. The experimental model was validated under 14 °C, pH 7.54, and 0.55% yeast extract, showing a protease activity of 447.65 ± 2.6 U/mL by a prediction model of 445.48 U/mL. The enzyme showed a molecular weight of 59 kDa; it was inhibited in the presence of PMSF (serine protease); it presented optimal conditions at pH 8.0 and 50 °C; it remained stable at pH in the 3.0–9.0 range and between 10 and 40 °C; it showed a tolerance to 3000 mM NaCl as well as to surfactants, hydrogen peroxide and urea at 5%. This paper presents a proposal for an economically attractive production methodology using cactus pear as a primary source of carbon. In addition, the protease secreted by *Acremonium* sp. L1-4B presented a combination of biochemical characteristics that grants a promising variability of biotechnological applications.

1. Introduction

Extremophile microorganisms are molecularly adapted to the development and spread in hostile environments. They are commonly subjected to various stress conditions such as high or low temperatures, extreme pH, different salt concentrations, high levels of radiation and pressure, and water and nutrient restrictions (Gomes and Steiner, 2004).

Enzymes synthesized by psychrophilic microorganisms have developed a variety of structural features that grants them a high degree of flexibility, a low activation enthalpy and a high specific activity at low temperatures, so that these biocatalysts have attracted great attention for applications that seek to reduce energy consumption (Joshi and Satyanarayana, 2013; Siddiqui and Cavicchioli, 2006).

Secretion of enzymes by microorganisms is generally affected by physical–chemical and nutritional conditions. The evaluation

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process of the components of the environment and the optimization of parameters such as pH and temperature is a crucial step for enzyme production in commercial practice. In order to analyze the influence of these elements, the response surface methodology it has been commonly used (Fleuri and Sato, 2008; Joshi and Satyanarayana, 2013).

The use of agricultural and agro-industrial waste products in bioprocesses has shown a relevant productive viability in obtaining enzymes with an industrial and an economic interest. Castro and Sato (2013) suggest a combination of residues in the fermentation process for obtaining synergistic enzymes such as protease and α-amylase. Maciel et al. (2011) cactus used as substrate in solid fermentation to produce pectinolytic enzymes with *Aspergillus niger*. In submerged fermentation, Gomes et al. (2014) used a medium consisting of cactus pear, bark of cassava and corn steep liquor, obtaining a significant production of cellulases, xylanases and phytases from *Aspergillus tamarii*.

The cactus pear (*Opuntia ficus-indica* Mill.) is a widely cultivated cactus in northeastern Brazil. It has a moderate nutritional value and is largely used as forage for ruminant herds and less significantly as human food (Bezerra et al., 2012; Oliveira et al., 2011). The genus *Opuntia* is known as a producer of mucilage, a complex carbohydrate with excellent water absorption capacity (Sáenz et al., 2004).

Aiming to establish parameters for the production of proteases through a filamentous fungus isolated in Antarctica, experimental models were employed in order to enable the use of cactus pear as a major substrate in the fermentation process, and thus detect the biochemical characteristics of the enzyme obtained in the study.

2. Materials and methods

2.1. Microorganism and inoculum

In this study, the filamentous fungus *Acremonium* sp. L1-4B isolated from a lichen sample on the Antarctic continent was used. It is preserved in the research collection associated with the Brazilian Collection of Microorganisms of Environment and Industry (CBMAI). The line was reactivated in agar Potato Dextrose Agar at 15 °C for 360 h. The inoculum was standardized in a NaCl solution (0.3%) and Tween 80 (0.1%) containing 10^6 conidia per mL.

2.2. Production of proteases

The production of proteases occurred with cactus pear being used as the main carbon source. The cladodes of the cactus pear were obtained in the municipality of Garanhuns-PE, Northeastern Brazil, subjected to a cleaning process with sodium hypochlorite at 2% for 20 seconds, then with two washings with distilled water. The processing of cladodes was made in a processor and the obtained cactus pear extract was diluted in the ratio 1:5 (v/v) with H_2O deionized and stored at −20 °C. The carbon-nitrogen ratio of the validated medium (Section 2.5) was equal to 5.54 g (g carbon/g nitrogen). All production tests were conducted in Erlenmeyer flasks with a 125 mL capacity containing 25 mL of culture medium consisting of cactus pear extract associated with different yeast extract concentrations, pH and temperatures as described in the experimental design (Sections 2.3 and 2.4). The fermentation in a cooled incubator (model TE-422, TECNAL, Piracicaba, Brazil) lasted 96 h under orbital shaking at 120 rpm.

2.3. Evaluation of influence of different factors on enzyme production using 2^3 factorial design

A 2^3 factorial design was used with three central points, totaling 11 trials, in order to identify factors or independent variables that significantly influence enzyme production. In design, yeast extract concentration, temperature and pH of secretion of proteases was evaluated.

Table 1
Actual and coded values for optimization of production of proteases by *Acremonium* sp. L1-4B, using Central Composit Design.

Independent variables	Unit	Levels				
		−α*	−1	0	+1	+ α
Temperature	ºC	13.0	14.0	15.0	16.0	17.0
pH		6.5	7.0	7.5	8.0	8.5
Yeast extract	%	0.0	0.2	0.4	0.6	0.8

* 1.682.

2.4. Optimizing of production through Central Composite Design

Central Composite Design was employed to determine the best conditions for enzyme production. The design contained three variables, six axial points and four central points, totaling 18 assays. All variables were studied in five levels (−α, −1, 0, 1, α). In order to predict the production of the enzyme under the conditions of significant variables, the Response Surface Methodology was applied (Table 1).

The system behavior was explained by quadratic equation (Eq. (1)).

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{13}AC+\beta_{23}BC+\varepsilon \quad (1)$$

where Y is the experimental response, β_0 is the intercept, β_1, β_2 and β_3 are the linear coefficients, β_{11}, β_{22} and β_{33} are the quadratic coefficients, β_{12}, β_{13} and β_{23} are coupling coefficients and A, B, C, A^2, B^2, C^2, AB, AC and BC are independent variables and ε the experimental error.

2.5. Validation of the statistical model

To validate the composition of the medium, three additional experiments were conducted under conditions predicted for enzyme production. Protease activity in the enzyme extract was used as a dependent variable in the comparative study.

2.6. Statistical analysis

The results obtained in 2^3 factorial and Central Composite Design were processed in the Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA), to indicate the effects statistically significant ($p < 0.05$) or marginally significant ($p < 0.1$), and model adjustment to experimental data. All experiments were performed at random.

2.7. Protease activity

The protease activity was determined using azocasein (Sigma Aldrich, St. Louis, MO, USA) as a substrate according to the method described by Charney and Tomarelli (1947), with modifications. The reaction mixture containing 0.5 mL of azocasein at 0.5% (w/v) in a 50 mM sodium acetate buffer, pH 5.0 and 0.5 mL enzyme extract was incubated at 37 °C for 40 min. Then, 0.5 ml of trichloroacetic acid at 10% was added and centrifuged at 4000*g* for 10 min at 4 °C (model MIKRO 200 R, Andreas Hettich GmbH & Co. KG, Germany). An aliquot of 0.5 mL of the supernatant was added to 0.5 mL of potassium hydroxide at 500 mM. A protease unit was defined as the amount of enzyme capable of producing an increase of 0.001 in absorbance per minute of reaction at the wavelength of 430 nm, in UV–Visible spectrophotometer, model Libra S22