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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

(Biochrom®, Cambridge, England). The denatured enzyme was used as a negative control.

2.8. Biochemical properties of the protease produced by Acremonium sp. L1-4B

The crude enzyme extract resulting from statistical validation test was submitted to a biochemical characterization study.

2.8.1. Temperature and optimal pH

To determine the optimal temperature of the protease, the enzyme extract was incubated in the range 10–80 °C. The optimal pH of the enzyme was determined using the buffers sodium citrate (pH 3.0; 4.0), sodium acetate (pH 5.0), sodium phosphate (pH 6.0; 7.0) and Tris–HCl (8.0; 9.0) at 50 mM. The results of optimal temperature and pH were expressed in relative activity (%).

2.8.2. Stability to temperature and pH

Thermal and pH stability were evaluated by incubating the enzyme extract for 180 min under the same conditions of temperature and pH described in the previous assay (Section 2.8.1). They were expressed as residual activity (%).

2.8.3. Effect of NaCl in the catalytic reaction

To determine the effects of NaCl on protease activity, the azocasein substrate was prepared with different concentrations of NaCl (0–3000 mM) and the results were expressed in relative activity (%).

2.8.4. Effect of surfactants and other substances in the enzymatic activity

The following components were evaluated: surfactant Triton X-100 (5%), Tween 20 (5%), Tween 80 (5%) and Polyethylene glycol 2000 (5%); chemical denaturant urea (5%), bleaching agent H_2O_2 (5%) and β-mercaptoethanol reducer (1 and 5 mM). The enzyme extract was previously incubated for 30 min at 26 ± 2 °C together with the measured substance. The relative activity was determined using the enzyme extract without addition of agents as a control.

2.8.5. Effect of protease inhibitors

The effect of different inhibitors such as iodoacetic acid (1 and 10 mM); phenylmethylsulfonyl fluoride (PMSF; 1 and 10 mM); pepstatin A (1 mM) and ethylenediaminetetraacetic (EDTA; 1 and 10 mM) were evaluated. The enzyme extract was incubated for 30 minutes with the inhibitor and then an aliquot was removed and subsequently subjected to detection of enzyme activity. The results were calculated relative to the control without inhibitor.

2.9. Determination of the molecular weight and zymography

The electrophoresis with polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) was performed according to the method described by Laemmli (1970), using a stacking gel at 4% and a resolution of 13.75%, GE Healthcare molecular weight standard (LMW Calibration Kit For SDS Electrophoresis, Amersham Place, England). The run proceeded at 4 °C, 284 V, 25 mA, 7 W for 81 min. The gel was stained in a solution containing *Comassie Brilliant Blue* R250 0.25% (w/v) for 120 min and destained in a solution containing 45% methanol, 45% water and 10% acetic acid for 24 h. Subsequently, it was subjected to GE Healthcare silver staining (Silver Staining Protein kit, protein, Uppsala, Sweden). The molecular weight was estimated using the LabImage 1D image analysis software (Loccus, Cotia, São Paulo, Brazil). The zymography was performed according to Egito et al. (2007), with modifications, using gelatin as substrate (0.2 mg/mL). The run was performed at 4 °C, 320 V, 50 mA, 16 W for 62 min. The gel was incubated in a solution of Triton X-100 at 2.5% for 20 min at 4 °C. Then, three washes were then made with water and incubated in a Tris–HCl buffer at 50 mM, pH 7.5, containing $CaCl_2$, 15 Mm, for 120 min at 37 °C. The gel was stained and destained with the same solutions used for the electrophoresis. However, the destaining occurred until a hydrolysis zone visible was visible.

3. Results and discussion

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

Data from 2^3 factorial design showed a variation of protease activity secreted by *Acremonium* sp. L1-4B from 0.07 to 191.32 U/mL, indicating the importance and the need to use experimental designs to achieve higher yields of enzyme production. The difference between the lowest and highest (tests 6 and 3, respectively) showed an increase of approximately 2.733 times of the response variable, so it can be foreseen that the compositional parameters of the assay 3 may possibly provide better stimulus conditions for the expression and consequently the production of protease by *Acremonium* sp. L1-4B (Table 2).

The results as assessed by analysis of variance (ANOVA) (Table 3), revealed that the regression was statistically significant and the lack of fit not was statistically significant. The model adjustment was measured by the coefficient of determination (R^2) which had a value of 0.9333 and R^2_{Adj}=0. 8334. Indicating that 93.33% of the total variation in residual activity was explained by the adjusted model.

On the other hand, it was found that all independent variables and their interactions are statistically significant ($p < 0.05$) or marginally significant ($p < 0.10$) (Table 4). The interaction refers to the combined or synergistic action of two or more factors, or the interaction of two or more factors is related to the fact that the effect of the level of one of them depends on the level of the other.

It was found that the factors temperature and yeast extract (both statistically significant) have negative effects, indicating that, when increasing the temperature from 15 to 25 °C and the concentration of yeast extract from 0.5% to 1.5%, there was a decrease in the production of protease. As the pH was marginally significant factor and their interactions were statistically significant, it was determined to continue in the direction of the interaction effect of greater magnitude (Table 4).

In a work by Badoei-Dalfard and Karami (2013), the authors found that temperature, pH, $MgSO_4$ and NaCl were the variables that most influenced the production of protease by *Bacillus* sp., using the Plackett–Burman design, since it was statistically significant ($p < 0.05$), that is, they affected the production of protease,

Table 2
Matrix of the 2^3 fractional design with three center points associated with response variable total activity of proteases produced by *Acremonium* sp. L1-4B.

Run	Temperature (ºC)	pH	Yeast extract (%)	Proteases activity (U/mL)
1	15.0	4.0	0.5	189.75 ± 0.00
2	25.0	4.0	0.5	15.22 ± 0.01
3	15.0	8.0	0.5	191.32 ± 0.00
4	25.0	8.0	0.5	35.42 ± 0.00
5	15.0	4.0	1.5	135.30 ± 0.02
6	25.0	4.0	1.5	0.07 ± 0.00
7	15.0	8.0	1.5	10.57 ± 0.00
8	25.0	8.0	1.5	5.77 ± 0.01
9	20.0	6.0	1.0	53.47 ± 0.00
10	20.0	6.0	1.0	33.75 ± 0.00
11	20.0	6.0	1.0	48.15 ± 0.03

Table 3
Analysis of variance (ANOVA) for the production of protease by *Acremonium* sp. L1-4B according to the 2^3 factional design with three center points.

Source	Sum of squares	Degrees of freedom	Mean square	*F-Value*	*p-Value*
Regression	48435.98	6	8072.66	9.34	0.0243*
Residual	3457.29	4	864.32		
Lack of Fit	3249.11	2	1624.55	15.61	0.060
Pure error	208.18	2	104.09		
Total	51893.26	10			

* Statistically significant. Confidence level 95%.

Table 4
Estimate of the effects of variables in production of protease by *Acremonium* sp. L1-4B according to the 2^3 factional design with three center points.

Variables	Effects	*t*	*p*-Value
(1) Temperature	−117.615	−16.3032	0.003 s
(2) pH	−24.315	−3.3704	0.077 ms
(3) Yeast extract	−70.000	−9.7030	0.010 s
1 by 2	37.265	5.1655	0.035 s
1 by 3	47.600	6.5981	0.022 s
2 by 3	−35.200	−4.8792	0.039 s

ms: Marginally significant. s: Statistically significant. Confidence level 95%.

showing positive effects. In the production of alkaline protease by *Marinobacter* sp. GA CAS9, the authors reported that the enzyme production was strongly influenced by NaCl concentration, meat extract, $CuSO_4$, incubation temperature and initial pH values, obtaining values between 298.15 and 487.24 U/mL using the same statistical method, since the remaining parameters (lactose, $CuSO_4$ and incubation time) were identical for these two activity extremes. However, the pH had a positive effect, and temperature and concentration of NaCl had negative effects (Kumar et al., 2014). It was shown that protease secretion in the studies described above was strongly influenced by physical–chemical parameters in the cultivation employing different microorganisms, thus highlighting the importance of using statistical tools for the modeling of the experimental design.

3.2. Optimization of protease production using Central Composite Design

A new experimental matrix was made to increase the production of protease by *Acremonium* sp. L1-4B, all variables were evaluated in five levels: temperature (13, 14, 15, 16 and 17 °C), pH (6.5, 7.0, 7.5, 8.0 and 8.5) and concentration of yeast extract (0.0, 0.2, 0.4, 0.6 and 0.8% w/w). The levels of these factors have been modeled and the results of the predicted are presented in Table 5.

The effects of variables were modeled and are presented in Table 6. Temperature, pH and yeast extract in their quadratic terms, had a negative effect, indicating a region of maximum protease production values at the center point or near it. An increase in pH (from 6.5 to 8.0) and in yeast extract concentration (0–0.8) determined a higher protease production averaged 3.08 and 148.80 U/mL, respectively. In linear term, the effect pH was not significant, the effect temperature was marginally significant ($p < 0.10$), and the only effect statistically significant ($p < 0.05$) was yeast extract. This is the variable with the greatest influence on enzyme production in the experimental field studied. In this way, Kammoun et al. (2008) reported that the use of organic nitrogen sources may have a significant effect on the production of enzymes, since it can have a natural and mineral percentage of carbon in its composition.

Table 5
Experimental matrix, results obtained in the CCD in the production of proteases by *Acremonium* sp. L1-4B and activity provided for in the model.

Run	Temperature (°C)	pH	Yeast extract (%)	Proteases activity (U/mL)	
				Experimental	Predicted
1	16.0	8.0	0.6	401.70 ± 4.24	386.84
2	16.0	8.0	0.2	373.20 ± 0.85	363.02
3	14.0	7.0	0.6	440.85 ± 1.06	433.06
4	16.0	7.0	0.2	369.75 ± 1.06	349.47
5	15.0	7.5	0.4	451.50 ± 4.67	440.21
6	15.0	7.5	0.4	431.25 ± 8.70	440.21
7	15.0	7.5	0.4	423.00 ± 2.97	440.21
8	15.0	6.5	0.4	394.05 ± 7.85	399.94
9	15.0	7.5	0.0	304.05 ± 3.18	330.00
10	16.0	7.0	0.6	374.85 ± 1.06	381.55
11	13.0	7.5	0.4	380.25 ± 6.15	391.36
12	15.0	7.5	0.8	422.85 ± 1.91	422.29
13	15.0	7.5	0.4	459.45 ± 5.73	440.21
14	14.0	8.0	0.2	381.45 ± 8.70	356.78
15	14.0	7.0	0.2	350.25 ± 5.30	347.13
16	17.0	7.5	0.4	339.00 ± 3.39	412.50
17	15.0	8.5	0.4	393.00 ± 6.36	412.50
18	14.0	8.0	0.6	432.15 ± 1.91	434.46

Table 6
Estimate of the effects of variables in production of protease by *Acremonium* sp. L1-4B according to CCD design.

Variables	Effects	*T*	*p*-Value
Temperature (ºC) (L)	−22.64	−2.46	0.091 ms
Temperature (ºC) (Q)	−48.00	−5.014	0.015 s
pH (L)	7.47	0.81	0.476
pH (Q)	−24.03	−2.51	0.087 ms
Yeast extract (%) (L)	54.87	5.96	0.009 s
Yeast extract (%) (Q)	−45.30	−4.73	0.018 s
Temperature (ºC) × pH	1.95	0.16	0.881
Temperature (ºC) × Yeast extract (%)	−26.93	−2.24	0.111
pH × Yeast extract (%)	−4.13	−0.34	0.754

ms: Marginally significant. s: Statistically significant. Confidence level 95%.

A multiple regression analysis based on the least squares method was performed using STATISTICA software Version 8.0. According to Eq. (2), the enzymatic activity followed a second order polynomial model with 10 coefficients.

Enzymatic activity

$$\begin{aligned}(\text{U/mL}) = &- 7941.71\ (\pm 2170.094) + 721.02{\cdot}T\ (\pm 170.102)\\ &- 24.00{\cdot}T^2\ (\pm 4.786) + 707.42{\cdot}\text{pH}\ (\pm 340.205)\\ &- 48.06{\cdot}\text{pH}^2\ (\pm 19.146) + 1754.53{\cdot}\text{YE}\ (\pm 645.914)\\ &- 566.22{\cdot}\text{YE}^2\ (\pm 119.659) + 1.95{\cdot}\text{T}{\cdot}\text{pH}\ (\pm 12.037)\\ &- 67.31{\cdot}\text{T}{\cdot}\text{YE}\ (\pm 30.093) - 20.62\ \text{pH}{\cdot}\text{YE}\ (\pm 60.186)\end{aligned} \quad (2)$$

where T is the temperature (°C), YE is yeast extract concentration (%) and pH is hydrogenic potential.

The Analysis of Variance (ANOVA) for the response surface model represented in the Eq. (2), is presented in Table 7.

The Fisher-based test (F test) indicated that the adjusted equation 2 was statistically significant (Fcal > Ftab) and the lack of fit showed a good concordance (Fcal < Ftab) between the predicted response model and the experimental values. The quality of the model adjustment was measured by the coefficient of determination (R^2) 0.8709 and $R^2_{Adj} = 0.7255$. This indicated that the adjusted model explained 87.09% of the total variation in the residual activity.

Optimized values are calculated by its partial differentiation with respect to temperature, pH and yeast extract. Response

Table 7
Analysis of variance (ANOVA) for the data presented in Table 5.

Source of variation	Sum of squares	Degrees of freedom	Mean square	*Fcal*	*Ftab*	*p*-Value
Regression	29313.71	9	3257.08	6.94	3.39	0.005*
Residual	3754.88	8	469.36			
Lack of fit	2885.52	5	577.10	1.99	9.01	0.302
Pure error	869.35	3	289.78			
Total	29073.51	17				

* Statistically significant. *Fcal*: F calculated; *Ftab*: F tabled. Confidence level 95%.

surface graphics Fig. 1(A, B, and C) were drawn to determine optimal levels of independent variables according to Eq. (2). Each response surface shows the effect of two factors, while the third was fixed at its central point.

The relative effect of pH and temperature on the production of protease is shown in Fig. 1(A). The change in temperature from 13 °C to 14 °C resulted in an increase of enzyme production averaged 20.93 U/mL. There was a loss in enzyme activity (Fig. 1(B)) with the decrease in the concentration of yeast extract at higher temperature levels, fixating at 16 °C. When decreasing the concentration of the yeast extract from 0.6% to 0.2%, a loss in enzyme production averaged 34.32 U/mL was found. However, with a lower temperature (14 °C) and an increase from 0.2% to 0.6% of yeast extract concentrations, the protease activity increased from 365.85 to 436.50 U/mL. This indicates the presence of a negative interaction effect between these two variables in the production of protease (Tables 5 and 6).

In Fig. 1(C), it is possible to observe that, with a lower yeast extract concentration (0.0–0.2%), enzyme production is reduced, regardless of the tested pH. However, protease is secreted to the maximum between 0.4% and 0.7% of yeast extract, and at pH 7.2–8.0. The use of the response surface methodology in this study allowed guiding safely and efficiently the parameters that directly influence the production of proteases, which only reinforces that reported by Mnif et al. (2012), according to which one of the devices used to provide more bioprocessing economy is the use of response surface methodology for optimization of medium components.

3.3. Validation

The results demonstrated a high degree of similarity between simulated and experimental data. The model was validated using the independent variables temperature (14 °C), pH (7.54) and yeast

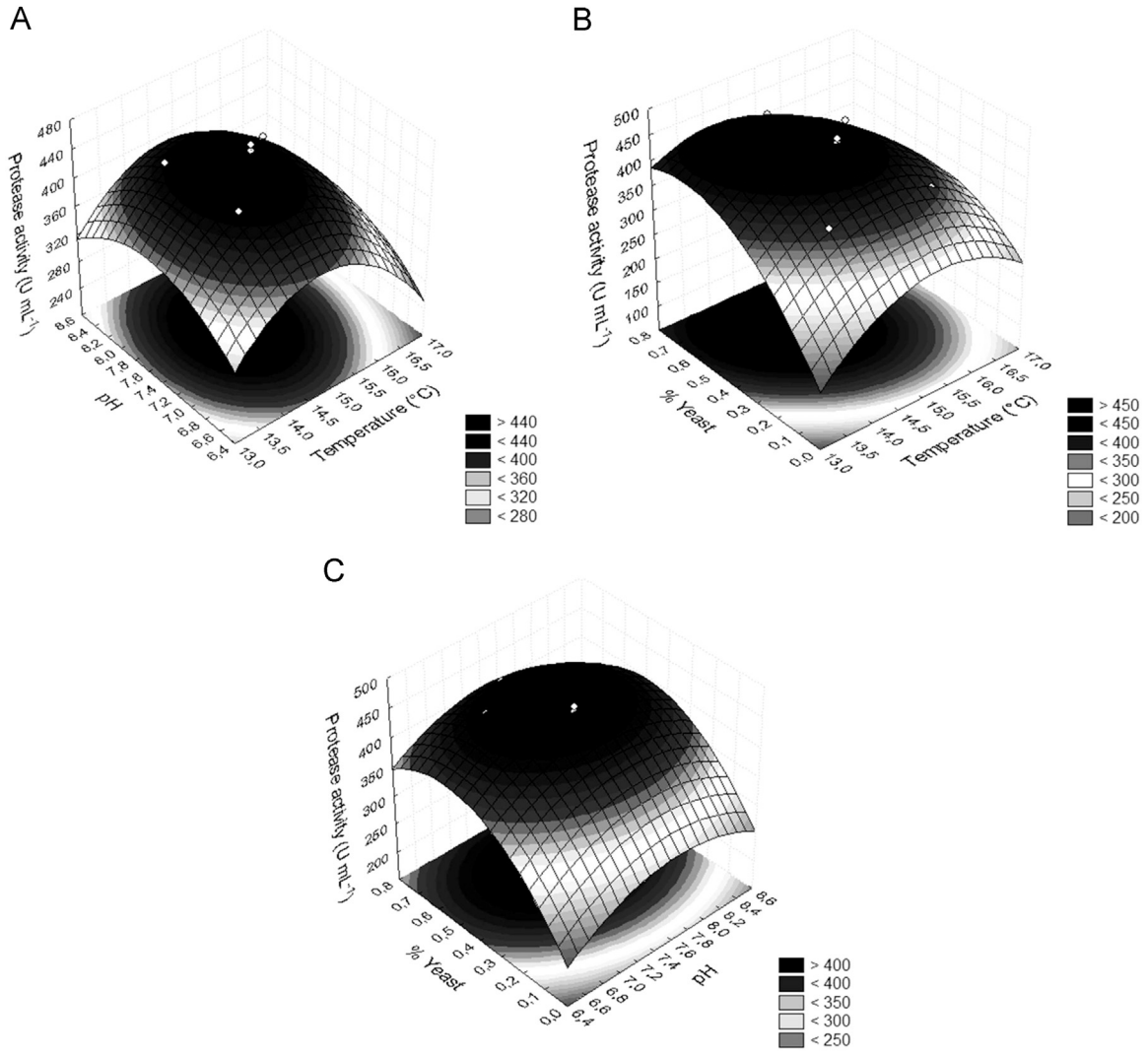

Fig. 1. Response surface and contour curve for protease production by *Acremonium* sp. L1-4B, interaction of temperature and pH (A), in function of temperature and yeast extract (B), and yeast extract and pH (C).

extract (0.55%). The proximity of the predicted response of protease activity (445.48 U/mL) and the experimental response (447.65 ± 2.6 U/mL) defined the validation of the model. In this study, two statistical methods were used. It was thus possible to increase enzyme activity by 2.35 times (134.94%) in optimal conditions when compared to the best conditions obtained in the factorial design. Shabbiri et al. (2012), using *Central Composite Design*, there was a 2.0 times increase in the production of protease by *Brevibacterium linens* DSM 20158. Meena et al. (2013), using Taguchi methodology with optimized conditions, the alkaline protease production by *Pseudomonas aeruginosa* increased by 1.29 time (28.8%) In contrast, during the production of protease by *Exiguobacterium* sp. CFR26M after optimal conditions obtained in the CCD, the authors obtained a 6.30 times increase (Kumar and Suresh, 2014).

3.4. Biochemical properties of the protease produced by Acremonium sp. L1-4B

3.4.1. Temperature and optimal pH

The enzyme exhibited a higher catalytic activity at 50 °C. However, it showed a relative activity above 20% at 10 °C and about 17% at 80 °C (Fig. 2(A)). The optimal protease was at pH 8.0, showing a higher relative activity at 87% in the pH range 6.0–9.0 (Fig. 2(B)). However, the biocatalysis was influenced negatively with pH 3.0 and 4.0, showing, respectively, 1% and 8.5% of relative activity under these conditions. The enzyme showed activation characteristics suggesting the high flexibility of the biomolecule, predisposed to catalyze at low and high temperatures, and a greater efficiency in the neutral and alkaline pH range. Similar results were found by Irwin et al. (2001), evaluating a protease secreted by marine psychrophilic strain PA-43, which showed a better activity between 55 and 60 °C and pH 8.3. However, the protease of isolated bacteria *Pseudoalteromonas* sp. NJ276 in Antarctica showed its optimal value with a temperature of 30 °C and pH 8.0. Although microorganisms come from cold environments, distinct characteristics may possibly be related to the genetic variability of the strains and their physiological adaptation (Wang et al., 2008). However, when evaluating the protease of a mesophile fungal, Abidia et al. (2011) found that the enzyme secreted by *Botrytis cinerea* showed similar characteristics to those found in this study at an optimal temperature of 50 °C and pH 8.0. Also studying mesophiles, Savitha et al. (2011), observed that proteases produced by *Graphium putredinis* demonstrated their optimal value at 50 °C at pH 7.0. On the other hand, the protein secreted by *Trichoderma harzianum* showed a higher catalytic activity at 60 °C and pH 8.0. According to Shankar et al. (2011), pH affects the ionization of amino acids, which form the primary and secondary structures of the enzyme, and the temperature directly influences the speed of the enzymatic reaction. This statement is in agreement with the results found in this study, in which the temperature inferred directly in enzyme activation, and the strong acidification caused inactivation of the protein; possibly, the structure of its surface was altered, causing a lack of interaction between the catalytic site of the enzyme and its substrate.

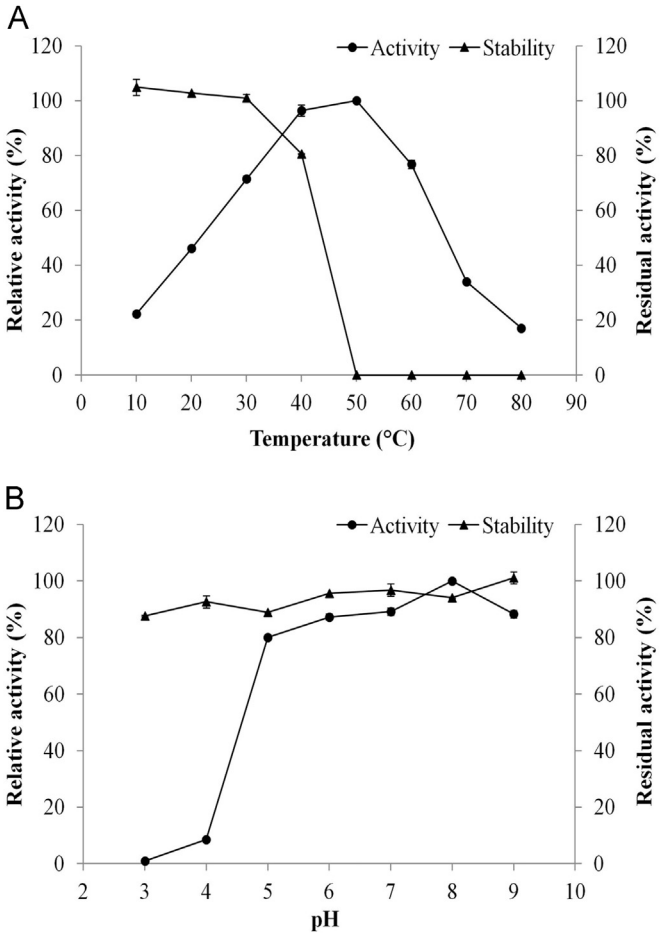

Fig. 2. Effects of temperature (A) and, pH (B) on activity and stability (180 minutes) of proteases produced by *Acremonium* sp. L1-4B.

3.4.2. Stability to temperature and pH

The protease secreted by the psychrophile *Acremonium* sp. L1-4B showed to be thermally stable for 180 min at 10–40 °C temperature range, showing thermolability at temperatures above 50 °C (Fig. 2(A)). However, when contrasted with different pH ranges, the enzyme showed stability and a residual activity above 87% at all tested pH ranges at 180 min of testing (Fig. 2(B)). Wang et al. (2008) found that the protease of psychrophile *Pseudoalteromonas* sp. NJ276 remained stable at 30 °C for 50 min; when incubated at 50 °C, the enzyme lost 55% of its activity after 20 min and it was completely inactivated in 30 min. However, it demonstrated stability in the pH range 7.0–9.0 for 60 min. Vázquez et al. (2008) observed that the enzyme from the Antarctic bacterium *Pseudoalteromonas* sp. P96-47 showed thermostability for 60 min when below 30 °C. However, it showed inactivation at 60 °C in the same time interval. It also showed a greater stability in neutral to alkaline pH ranges, maintaining more than 80% of the activity at pH 6–10 after 180 min of incubation at 4 °C. On the other hand, proteases from the mesophile *Bacillus subtilis* AP-MSU6, investigated by Maruthiah et al. (2013), remained stable at temperatures of 40 and 150 °C for 50 and 75 min, respectively. The enzymes showed stability at pH 9.0 for 180 minutes and at pH 10.0 for 90 min. The results found in the studies described above only confirm that there is a strong interrelation between the original habitat of the microorganism and the characteristics of its biomolecules. According to Siddiqui and Cavicchioli (2006), proteins from psychrophiles exhibit more flexibility and thermolability than those secreted by its counterparts. The high flexibility of the molecule is accompanied by low stiffness, giving to the protein a lower enthalpy of activation but a greater thermal lability. However, the thermolability of the protease secreted by *Acremonium* sp. L1-4B, evaluated in this study, can also be considered as qualitative for the development of certain industrial processes. This is because, according to Vázquez et al. (2008), the thermal lability arising from microbial protease may also be a characteristic sought after in some biotechnological procedures, such as in the food industry, since they can be selectively inactivated in food cooking. In addition, the activity of these biomolecules can be exploited, for example, in the production of food hydrolysates, for it generates

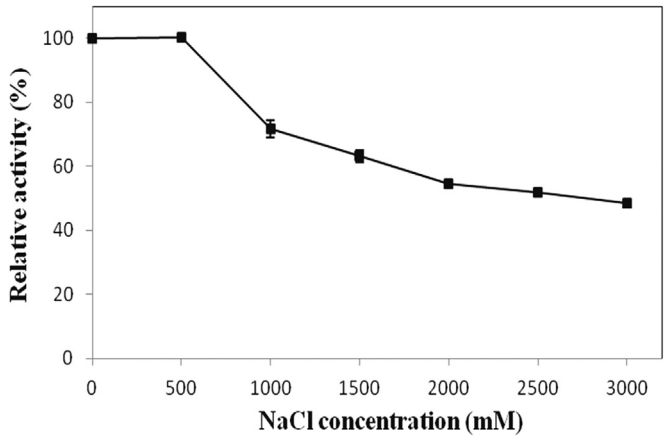

Fig. 3. Effect of NaCl concentration on catalytic activity of proteases produced by *Acremonium* sp. L1-4B.

less bitterness in food than when animal proteins are used.

3.4.3. *Effect of NaCl in the catalytic reaction*

The enzyme showed tolerance against NaCl concentrations ranging from 500 to 3000 mM. The relative activity of the enzyme in 500 mM of NaCl remained at 100%; with 1000 mM NaCl, it decreased to 72%. In summary, the progressive increase of salt concentration provided the decrease in enzyme activity. However, the relative activity was 48% when the catalyst occurred in the presence of 3000 mM NaCl (Fig. 3). Similar results were found in a study on proteases from the mesophile *Bacillus subtilis* AP-MSU 6, where Maruthiah et al. (2013) observed that there was a catalytic activity at concentrations from 500 mM to 2500 mM of NaCl, showing 74.47% of relative activity at a concentration of 1000 mM NaCl and approximately 40% in 2500 mM. Annamalai et al. (2014), suggest that enzymes with a high tolerance to salt are indispensable tools in biotechnological processes dependent of high salinity or osmotic pressure.

3.4.4. *Effect of surfactants and other substances in the enzymatic activity*

The effects of surfactants, bleaching agents, denaturants and enzyme reducing are shown in Table 8. With the addition of Tween 20 and Tween 80 in the reaction mixture, there was a slight increase of 1% and 12%, respectively, in the enzymatic activity in comparison with the control. However, the addition of Triton X-100 and PEG led to a catalytic decrease of 7% and 4%, respectively. H_2O_2 enhanced the enzyme activity by 34%. In the same way, there was an increase of 4% under the influence of urea. The reducing agent β-mercaptoethanol tested at concentrations of 1 and 5 mM showed a decrease in catalysis of 4% and 7%. Similar results obtained by Wang et al. (2008) were observed with active protease in cold temperatures, in which Tween 80 increased catalysis by 11%. However, the addition of Triton X-100 and H_2O_2 showed, respectively, a decrease of 31.5% and 11.4%. The protease of the thermophile evaluated by Zanphorlin et al. (2011) was negatively influenced in the presence of Triton X-100 and Tween 80, showing a decrease of 11.4% and 80%, respectively. However, the relative activity was potentiated in the addition of Tween 20 at 17.2%.

3.4.5. *Effect of protease inhibitors*

The enzyme extract was evaluated in comparison with protease inhibitors EDTA, iodoacetic acid, PMSF and pepstatin A. The enzyme secreted by *Acremonium* sp. L1-4B suffered inhibition in the presence of PMSF, showing at the end an activity of 6 and 3% at concentrations of 1 and 10 mM, respectively (Table 8). Anitha and Palanivelu (2013), also observed the inhibition by PMSF in studies with protease secreted by *Aspergillus parasiticus*. When subjected to the concentration of 0.1 mM PMSF, it showed a relative activity of around 2%. However, the 1 mM concentration did not show catalytic properties. Likewise, Zanphorlin et al. (2011), found a complete enzyme inhibition produced by thermophile *Myceliophthora* sp. under the influence of PMSF (5 mM). Cavello et al. (2013) found that the protease secreted by *Purpureocillium lilacinum* LPS#876 showed a 1.8% relative activity in the presence of 1.0 mM PMSF. Enzyme inhibition by PMSF suggests the inclusion of these enzymes in the group of serine proteases.

Table 8
Effects of surfactants, bleaching agent, chemical denaturant and inhibitors on proteases activity.

Chemical	Concentration	Relative activity (%)*
None		100
Surfactants		
Tween 20	5% (v/v)	101 ± 2.4
Tween 80	5% (v/v)	112 ± 1.0
Triton X-100	5% (v/v)	93 ± 5.7
PEG 2000	5% (v/v)	96 ± 4.6
Bleaching agent		
H_2O_2	5% (v/v)	134 ± 3.3
Desnaturant agent		
Urea	5% (w/v)	104 ± 2.1
Reducing agent		
β-mercaptoethanol	1 mM	96 ± 2.7
β-mercaptoethanol	5 mM	93 ± 1.3
Inhibitor		
EDTA	1 mM	83 ± 0.9
EDTA	10 mM	83 ± 1.8
PMSF	1 mM	6 ± 0.3
PMSF	10 mM	3 ± 0.3
Iodoacetic acid	1 mM	103 ± 1.1
Iodoacetic acid	10 mM	100.7 ± 0.5
Pepstatin A	0.1 mM	100.5 ± 2.3

* Data are show as relative activity (%) ± SD.

3.5. *Determination of the molecular weight and zymography*

The evaluation of the electrophoretic profile of proteins present in the crude extract secreted by psychrophile *Acremonium* sp. L1-4B enabled to detect the presence of distinct bands. However, the protease possibly has molecular weight of 59 kDa visualized through of zymography containing gelatin as substrate (Fig. 4). Different molecular weights were found in protease of psychrophilic microorganisms such as *Pseudoalteromonas* sp. NJ276, which had 28 kDa (Wang et al., 2008); *Flavobacterium psychrophilum*, 62 kDa (Secades et al., 2003); *Pedobacter cryoconitis*, 27 kDa (Margesin et al., 2008); and *Pseudomonas* sp., 45 kDa (Vazquez et al., 2004).

4. Conclusion

The 2^3 factorial design, associated with central composite design under response surface methodology proved to be a set of indispensable tools for establish a dynamic and efficient protease production method, through of the fungus *Acremonium* sp. L1-4B and cactus pear (*Opuntia ficus-indica* Mill.). The serine protease obtained in the study showed stability in all tested pH ranges, tolerance to different NaCl concentrations, surfactants, bleaching agents and reducing agents, as well as to chemical denaturant, thus exhibiting characteristics that suggest a great variety of applications in biotechnology processes.

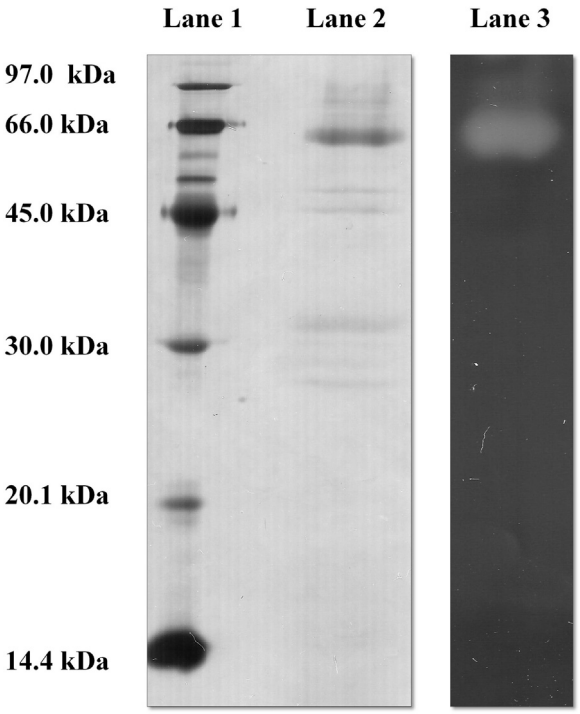

Fig. 4. SDS-PAGE and zymogram analysis of the protease from *Acremonium* sp. L1-4B. Lane 1: molecular weight of marker standard (14.4 kDa – α-Lactalbumin, 20.1 kDa – trypsin inhibitor, 30.0 kDa – carbonic anhydrase, 45.0 kDa – ovalbumin, 66.0 kDa – bovine serum albumin, 97.0 kDa – phosphorylase B); lane 2: crude enzyme extract; lane 3: zymography.

Acknowledgments

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