PRODUCTION AND CHARACTERIZATION OF CELLULOLYTIC ENZYMES BY *ASPERGILLUS NIGER* AND *RHIZOPUS* SP. BY SOLID STATE FERMENTATION OF PRICKLY PEAR[1]

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ABSTRACT - Prickly palm cactus husk was used as a solid-state fermentation support substrate for the production of cellulolytic enzymes using *Aspergillus niger* and *Rhizopus sp.* A Box-Behnken design was used to evaluate the effects of water activity, fermentation time and temperature on endoglucanase and total cellulase production. Response Surface Methodology showed that optimum conditions for endoglucanase production were achieved at after 70.35 h of fermentation at 29.56°C and a water activity of 0.875 for *Aspergillus niger* and after 68.12 h at 30.41°C for *Rhizopus sp.* Optimum conditions for total cellulase production were achieved after 74.27 h of fermentation at 31.22°C for *Aspergillus niger* and after 72.48 h and 27.86°C for *Rhizopus sp.* Water activity had a significant effect on *Aspergillus niger* endoglucanase production only. In industrial applications, enzymatic characterization is important for optimizing variables such as temperature and pH. In this study we showed that endoglucanase and total cellulase had a high level of thermostability and pH stability in all the enzymatic extracts. Enzymatic deactivation kinetic experiments indicated that the enzymes remained active after the freezing of the crude extract. Based on the results, bioconversion of cactus is an excellent alternative for the production of thermostable enzymes.

Keywords: Filamentous fungi. Response surface methodology. Bioprocesses. Semi-arid region. Enzymatic characterization.

PRODUÇÃO E CARACTERIZAÇÃO DE ENZIMAS CELULOLÍTICAS POR *ASPERGILLUS* NIGER E *RHIZOPUS SP.* DURANTE A FERMENTAÇÃO EM ESTADO SÓLIDO DA PALMA FORRAGERIA

RESUMO - Palma forrageira foi usada como fonte de nutrientes para a produção de enzimas celulolíticas durante a fermentação em estado sólido com auxílio dos fungos filamentosos *Aspergillus niger* e *Rhizopus sp.* Os efeitos da ativiade de água, do tempo de fermentação e da temperatura sobre a produção enzimática foram determinados segundo o protocolo padrão de Delineamento Box-Behnken. A condição ideal para produção das enzimas endoglucanase pelo fungo *Aspergillus niger* foi 70,35 h de fermentação, atividade de água de 0,875 e temperatura de 29,56 ° C enquanto que para o fungo *Rhizopus sp.* foi 68,12 h e 30,41 ° C. Entretanto, a celulase total produzida pelo fungo *Rhizopus sp.* foi otimizada em 74,27 h de fermentação e temperatura de 31,22 ° C e para o fungo *Aspergillus niger* foi 72,48 h e 27,86 ° C. A atividade de água só tinha uma influência significativa sobre a endoglucanase para *Aspergillus niger*. A alta estabilidade dos extratos enzimáticos em diferentes tepenraturas e pH's foram confirmadas após caracterização enzimática, indicando viabilidade em aplicações industriais. Os ensaios de desativação cinética indicaram que essas enzimas permaneceram ativas após o congelamento do extrato bruto. Com base nos resultados, utilizar a palma forrageira com matéria prima em bioprocessos é uma alternativa excelente para a produção de enzimas termoestaveis.

Palavras-chave: Fungos Filamentosos. Metodologia de Superfície de Resposta. Bioprocessos. Região semi-árida. Caracterização Enzimática.

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INTRODUCTION

The *caatinga* is an exclusively Brazilian biome that covers around 850,000 km^2 and represents approximately 10% of the national territory. A large portion of this biome is located in the Brazilian arid and semi-arid regions, which account for 89.3% of Northeastern Brazil (OLIVEIRA et al., 2010).

Prickly pear cactus is a rustic, of Mexican origin that is adapted to dry conditions (NOBEL et al., 2002; NOBEL; ZUTTA, 2008). The prickly pear fodder is well adapted to the conditions of the Brazilian semi-arid region, enduring long periods of drought due to its physiological properties such as its efficient photosynthetic process (VASCONCELOS et al., 2007). In the Brazilian semi-arid region, the use of prickly pear as animal feed in diverse livestock production systems is due to its high level of total digestive nutrients, carbohydrates, ash, vitamins and iron. In addition, prickly pear can be used in landscaping, conservation and remediation of soils, and in the production of medicines, cosmetics and colorants (FERREIRA et al., 2003; CHIACCHIO et al., 2006).

Prickly pear contains a high amount of total digestible nutrients, and has higher levels of soluble carbohydrates, ash, vitamins, nitrogen and iron content. It also has a relatively high concentration of calcium (3%), potassium (2.5%) and phosphorus (0.15%) and low levels of dry matter (11%), crude protein (4%), neutral detergent fiber (26%) and acid detergent fiber (18%) (ARAUJO et al., 2005; DUBEUX et al., 2006; SANTOS et al., 2015).

In Brazil, the economic use of prickly pear in animal feed is limited to non-industrial production processes. However, because of its composition, this plant can be used as a raw material for the production of highly useful products such as bioethanol, enzymes and flavor compounds. Enzymes are proteins that act as catalysts to accelerate the speed of chemical reactions and are frequently commercially produced for use in a variety of industrial applications. Commercial enzymes are currently used in food, pharmaceutical, effluent-treatment, textile, paper and cellulase industries (SOCCOL et al., 2010).

The use of enzyme extracts in the industrial processes can increase economic efficiency both in the production process and in the commercialized products. However, this is limited by the high production cost of enzymes. Currently, synthetic culture media is used for enzyme production, which elevates the production costs. These costs may be reduced through the use of plant biomass as a source of microorganism culture. As such, the use of biomass as the raw material for the production of compounds with industrial applications through biological, thermal and/or chemical processes are becoming more popular with the advancements in technology (GALEMBECK et al., 2009; GHORAI et al., 2009; BELMESSIKH et al., 2013).

Therefore, optimizing the production of enzyme extracts using renewable natural resources such as culture media for the growth of microorganisms may reduce production costs (PELIZER et al., 2007; AMIN et al., 2013). In this sense, it is necessary to study the application of prickly pear as a culture medium for the growth of microorganisms and optimize enzyme extract production (SANTANA et al., 2012).

The objective of this study was to investigate the use of prickly pear cv. Miúda (*Nopalea cochenillifera (L.) Salm Dyck*) as the main raw material for the production of the enzymes endoglucanase (CMCase) and cellulase by solid-state fermentation using *Rhizopus sp.* and *Aspergillus niger*. Box-Behnken Design (BBD) and Response Surface Methodology (RSM) were used to determine the optimal conditions for the production of these enzymes. Thus, the results from this study will help transform a low-commercial-value biomass into a product with many commercial applications.

MATERIAL AND METHODS

Materials

Base cuts of prickly pear (*Nopalea cochenillifera* (L.) *Salm Dyck*), were collected from the Field for Agricultural Studies from the State University of Southwest Bahia (UESB) located in Itapetinga, Bahia, Brazil, in January 2012. The base cuts consisted of the oldest pads, which contain a higher percentage of fibers relative to newer pads. After the pads were washed in running water, they were dried in an oven at 65ºC for 24 hours and ground in a Wiley mill to an approximate granulometry of 2 mm. The final water content of the sample after drying was 2% (m/m), determined with an infrared moisture analyzer (SHI-MOC-63U; Shimadzu, Kyoto, Japan). The chemical characterization of the prickly pear sample is shown in Table 1. Values are presented as mean ± SD from 6 replicates.

Microorganisms and culture conditions

The fungi *Aspergillus niger* and *Rhizopus sp.* were provided by the Laboratory of Agro-Industrial Waste Use (LABRA-UESB), where all the experiments were conducted. Microorganisms were inoculated in potato dextrose agar medium (Himedia, Mumbai, India) and incubated for 7 days at 35ºC in a temperature-controlled incubator (SL 222; Solab, Piracicaba, Brazil). The spores were collected using 0.01% (w/v) Tween 80 (Vetec, Duque de Caxias, Brazil) and counted with a Neubauer chamber and a binocular microscope (L1000; Bioval, São Paulo, Brazil).

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Table 1. Chemical characterization (g kg^{-1} of dry weight) of the cactus pear (*Nopalea cochenillifera*). Values are presented as mean ± SD from 6 replicates.

Nutrient	Composition (g kg^{-1} of dry weight)
neutral detergent soluble fiber [a]	654.90 ± 0.55
acid detergent soluble fiber [a]	386.00 ± 0.55
non-fibrous carbohydrate [b]	157.16 ± 0.55
total carbohydrate [b]	811.36 ± 0.55
lignin [c]	66.74 ± 0.55
cellulose [c]	319.26 ± 0.55
hemicellulose [c]	268.90 ± 0.55
mineral material [c]	103.02 ± 0.55
neutral-insoluble ashes [d]	73.32 ± 0.85
acid-insoluble ashes [d]	66.14 ± 0.75
ether extract [c]	32.98 ± 1.05
total protein [c]	52.64 ± 1.25
non-proteic nitrogen [d]	7.25 ± 0.75
neutral detergent non-soluble nitrogen [d]	2.22 ± 0.55
acid detergent non-soluble nitrogen [d]	1.56 ± 0.55

Methodologies: [a] Van Soest et al. (1991); [b] Sniffen et al. (1992); [c] AOAC (1995); [d] Silva & Queiroz (2002).

Solid state fermentation

Fermentation reactions were performed in an Erlenmeyer flask containing 10 g of residue. A spore suspension consisting of 10^8 spores per gram substrate was prepared. Afterwards, sterile water was added to the medium to adjust the moisture content. The cultures were performed according to the experimental design described below.

Experimental design

A Box-Benhnken Design with 18 runs including 4 repetitions in the central point was implemented to evaluate the influence of fermentation temperature, water content and fermentation time on the production of enzyme extracts. Coded and real values of variables are shown in Table 2.

Table 2. Coded levels and real values for each factor under study in solid state fermentation.

	Factorial design in coded values			Factorial design in real values		
Run	(X_1, h)	(X_2, a_w)	(X_3, ºC)	(X_1, h)	(X_2, a_w)	(X_3, ºC)
1	-1	-1	-1	24	0.786	25
2	-1	-1	+1	24	0.786	35
3	+1	-1	-1	120	0.786	25
4	+1	-1	+1	120	0.786	35
5	-1	+1	-1	24	0.903	25
6	-1	+1	+1	24	0.903	35
7	+1	+1	-1	120	0.903	25
8	+1	+1	+1	120	0.903	35
9	0	-1	0	72	0.786	30
10	0	+1	0	72	0.903	30
11	-1	0	0	24	0.865	30
12	+1	0	0	120	0.865	30
13	0	0	-1	72	0.865	25
14	0	0	+1	72	0.865	35
15	0	0	0	72	0.865	30
16	0	0	0	72	0.865	30
17	0	0	0	72	0.865	30
18	0	0	0	72	0.865	30

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Enzymatic extraction

After the fermentation process, enzymes were mechanically extracted using 5 mL of 50 mM sodium citrate buffer (pH 4.8) for each 1 g of residue. The resulting enzyme extract was collected and centrifuged at 100 *g* (CT-6000R; Cientec, Piracicaba, Brazil) for 10 min at 4ºC.

Determination of endoglucanase (CMCase) activity

CMCase activity was based on the amount of reducing sugars produced by the degradation of carboxymethyl cellulose (Cromoline, Paraná, Brazil). 2% (p/v) carboxymethyl cellulose was diluted in 50 mM sodium citrate solution, pH 4.8. The dinitrosalicylic acid (DNS) (Sigma-Aldrich, Missouri, EUA) method was used for quantification of enzyme activity (MILLER, 1959; GHOSE, 1987). Reaction assays were conducted by incorporating 0.5 mL of 50 mM sodium citrate buffer (pH 4.8), 0.5 mL enzyme extract and 0.5 mL carboxymethyl cellulose (2% per volume) to an assay tube. A negative control reaction did not contain CMC. The blank assay contained 0.5 mL of DNS and 0.5 mL sodium citrate buffer. The reactions were incubated in a bacteriological incubator SL 222/CFR (Solab, Piracicaba, Brazil) at 50ºC for 10 min. The reactions were stopped by the addition of 0.5 mL DNS. After that, the tubes were submerged in boiling water for 5 min. Samples were diluted with the addition of 6.5 mL distilled for absorbance measurements at 540 nm in a BEL PHOTONICS SF200DM spectrophotometer (BEL, Osasco, Brazil).

Determination of total cellulase (FPase) activity

The activity of total cellulase (FPase) (exoglucanase and endoglucanase complex), expressed as filter paper activity, was determined by measuring sugars released during the degradation of a Whatman nº. 1 filter paper strip (1.0×6.0 cm). 1 mL 50 mM sodium citrate buffer (pH 4.8), 0.5 mL enzyme extract and a filter paper strip were added to a tube. Another tube contained 1 mL of the same buffer solution and 0.5 mL of enzyme extract. A 3rd tube (negative control) contained 1.5 mL buffer and a filter paper strip. The blank contained 0.5 mL buffer and 0.5 mL of DNS. The samples were incubated at 50ºC for 1 h (SL 222; Solab). The reaction interrupted by adding 3 mL of DNS (Sigma-Aldrich). The tubes were heated in boiling water for 5 min. Samples were diluted with the addition of 20 mL distilled for absorbance measurements at 540 nm in a BEL PHOTONICS SF200DM spectrophotometer.

Calibration curve

The DNS method was used to establish a standard curve for CMCase and FPase activity using 0.1-2.0 g/L glucose standards. One unit of enzyme activity (U) was defined as the amount of enzyme capable of releasing 1 μmol reducing sugar/min at 50ºC. Enzyme activity was expressed as U/mL. CMCase and FPase activity were measured by spectrophotometry at an absorbance of 540 nm.

Determination of thermostability and pH stability

Thermal stability was determined by measuring enzymatic activity after incubation of the enzymes in a water bath at 20, 40, 60, 80 and 100°C for 0-240 min. Aliquots were removed after 10, 30, 60, 90, 180 and 240 min and immediately placed on ice for enzymatic activity assays. The results are expressed as residual activity (%), the enzymatic activity that remained active after the incubation period.

pH stability was determined by measuring the enzymatic activity after incubation of the enzymes in sodium citrate buffer at pH 3, 4, 5, 6, 7 and 8. Aliquots were removed after 10, 30, 60, 90, 180 and 240 min and kept on ice until analysis. The results are expressed as residual activity (%). The activity of each enzyme was measured in triplicate.

Enzymatic deactivation kinetics

Aliquots of the enzymatic extracts were stored at 4ºC and −25ºC and removed at 24, 48, 72, 96, 120 and 144 h to determine enzymatic activity. The activity of each enzyme was evaluated in triplicate.

Statistical analysis

All statistical analyses were conducted using the Statistical Analysis System, version 9.3, with RSREG procedure (SAS Institute Inc., Cary, NC, USA). To validate the adjusted model, the optimized values of the independent variables X_1 (fermentation time), X_2 (water activity) and X_3 (fermentation temperature) were used in the same initial experimental procedure in order to verify the prediction power of the developed models by comparing the theoretical predicted data with the experimental data.

RESULTS AND DISCUSSION

Analysis of variance (ANOVA) was used to evaluate which factors had a significant effect on the enzymatic activity of CMCase and FPase from *Aspergillus niger* and *Rhizopus sp.* (Table 3) and parameter estimation analyses were conducted for Central Composite Rotatable Design.

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ANOVA for the models was performed and model significance was examined using Fisher's statistical test (F-test) applied to significant differences between sources of variation in experimental results, i.e., the significance of the regression (SOR), the lack of fit (LOF), and the coefficient of multiple determination (R^2). Since the full second order models (models containing both parameter interactions) not accepted by the mentioned tests, they improved by the elimination of the model terms until the determined conditions were met. All factors that were not significant at 10% were then pooled into the error term and a new reduced model was obtained for response variables by regression analysis using only the previously listed significant factor.

Table 3. ANOVA for response surface quadratic models for cellulolytic enzymes productions using the *Aspergillus niger* and *Rhizopus sp.* in solid state fermentation of prickly palm cactus.

	DF	SS	MS	F-value	p-value
Aspergillus niger					
Endoglucanase					
Model	4	16.83383	4.20846	11.52	0.0003
Error	13	4.74997	0.36538		
Lack of fit	14	21.45092	1.53221	34.59	
Pure error	3	0.13288	0.04423		
Total	17	21.58380			
R^2	0.78				
Total celulase					
Model	6	1569.50352	261.58392	20.20	<0.0001
Error	11	142.47331	12.95210		
Lack of fit	14	1711.90944	122.27925	5443.34	
Pure error	3	0.06739	0.02246		
Total	17	1711.97683			
R^2	0.92				
Rhizopus sp.					
Endoglucanase					
Model	4	55.45346	13.86336	10.19	0.0006
Error	13	17.68501	1.36039		
Lack of fit	14	73.05206	5.21801	181.18	
Pure error	3	0.08640	0.02880		
Total	17	73.13846			
R^2	0.76				
Total celulase					
Model	4	207.97351	51.99338	23.62	<0.0001
Error	13	21.02796	1.61754		
Lack of fit	14	228.97498	16.35536	1851.83	
Pure error	3	0.02650	0.00883		
Total	17	229.00147			
R^2	0.91				

ANOVA outcomes are shown in a Pareto chart (Figure. 1). The absolute value of the magnitude of the standardized estimated effect (the estimate effect divided by the standard error) of each factor is plotted in decreasing order and compared with the minimum magnitude of a statistically significant factor with 99% confidence (p=0.01), represented by the vertical dashed line.

As shown in figure 1, temperature and fermentation time had a significant effect on the enzymatic activity of endoglucanase and total cellulase in *Rhizopus sp.* and *Aspergillus niger*. On the other hand, in *Aspergillus niger*, the Pareto chart showed that water activity had a significant effect on total cellulase activity. The interactions between temperature, time, and water activity were not significant in the presented models. A second-order polynomial model can describe the behaviors of the studied systems, where the interaction term between the independent variables was not significant (p>0.01).

Temperature affects the microbial metabolism, regulates growth, spore formation, germination and product formation. At lower temperatures, molecules move slower, enzymes cannot mediate chemical reactions. As the temperature increases, molecules move faster, enzymes speed up metabolism and cells rapidly increase in size. However, after a certain value, all of these activities occur at overly high rates, and enzymes start to denature.

High moisture leads to decreased substrate porosity, which prevents oxygen penetration. On the other hand, low moisture may lead to poor accessibility of nutrients, resulting in poor microbial growth (PANDEY, 2003). Although the water activity of the medium could be attributed to the mass transfer of water and solutes across the microbial cells, its effect on enzyme production was lower compared with temperature and fermentation duration (Figure 1).
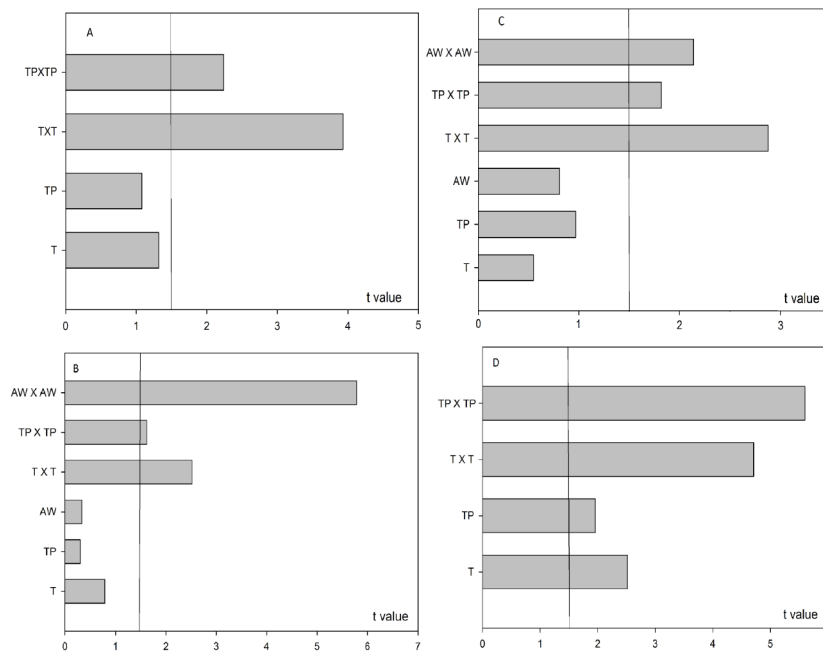
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Figure 1. Pareto chart showing the significance of fermentation time (T), fermentation temperature (TP), and water activity (Aw) during solid-state fermentation of cactus. The endoglucanase (A) and total cellulases (B) of *Aspergillus niger*. The endoglucanase (C) and total cellulases (D) of *Rhizopus sp.*

Nutrient levels and culture conditions required for microbial growth and enzyme production are affected by fermentation. The production of enzymes is slow at the start of fermentation and then accelerates until it reaches a maximum. From there the high concentration of products inhibits enzyme activity through negative feedback (GOMES et al., 2000; CAMASSOLA et al., 2004; OMEMU et al., 2005).

The reduced models can be described by equations 2-5, in terms of uncoded values.

$$E_1 = -4,436 + 0,101X_1 - 0,0007X_1{}^2 + 0,290X_3 + 0,291X_3{}^2 \quad (2)$$

$$E_2 = -3082,32 + 0,326X_1 + 7038,565 X_2 + 8,481X_3 - 0,00240 X_1{}^2 - 4163,338 X_2{}^2 - 0,142X_3{}^2 \quad (3)$$

$$E_3 = -59,3372 + 0,140X_1 + 0,042X_3 - 0,001 X_1{}^2 - 0,065X_3{}^2 \quad (4)$$

$$E_4 = -71,17559 + 0,256X_1 + 5,051X_3 - 0,002X_1{}^2 - 0,085X_3{}^2 \quad (5)$$

in which E_1 and E_2 represent the activity of endoglucanase and total cellulases for *Aspergillus niger*, respectively; E_3 and E_4 represent the activity of endoglucanase and total cellulases for *Rhizopus sp*, respectively; and X_1, X_2 and X_3 correspond to fermentation time, activity and fermentation temperature, respectively.

Using the response surface method (RSM), with the water-activity value fixed in the optimal condition, the relationship between factors and response can be better understood. The derivatives of equations 2-5 show that temperature and fermentation duration affect enzymatic activity (Figure 2).

In *Aspergillus niger,* the greatest endoglucanase and total cellulase activity were 4.165 U/mL and 30.923 U/mL after 70.35 and 74.27 h, respectively. In *Rhizopus sp.* these values were 68.12 h and 7.859 U/mL for endoglucanase and 72.48 h and 13.571 U/mL for total cellulases.

To validate the models, three repeated experiments were performed under the optimal conditions to test the suitability of the model. The enzyme activities identified by the tests were 3.921 U/mL for endoglucanase and 29.377 U/mL for total cellulases produced by *Aspergillus niger* and 7.545 U/mL for endoglucanase and 12.721 U/mL for total cellulases produced by *Rhizopus sp.* These models are reliable for predicting endoglucanase and total cellulose production from these fungi.

The optimum temperature was between 27 and 32ºC, which may have been related to the metabolic characteristics of the microorganisms. In *Aspergillus niger*, the optimum temperature for endoglucanase and cellulase production were 29.56 and 31.22 ºC, respectively. In *Rhizopus sp*, the optimum temperatures were 30.41ºC and 27.86ºC for endoglucanase and total cellulose production, respectively. In filamentous fungi, the temperature directly influences spore germination, growth and formation of products. In practically all the solid-state fermentation processes, temperature was a critical factor in enzyme production due to the metabolic heat generated during the reaction, because in addition to the difficulty to mix the solid medium, most of the substrates utilized have low thermal conductivity (SANTOS et al., 2012).
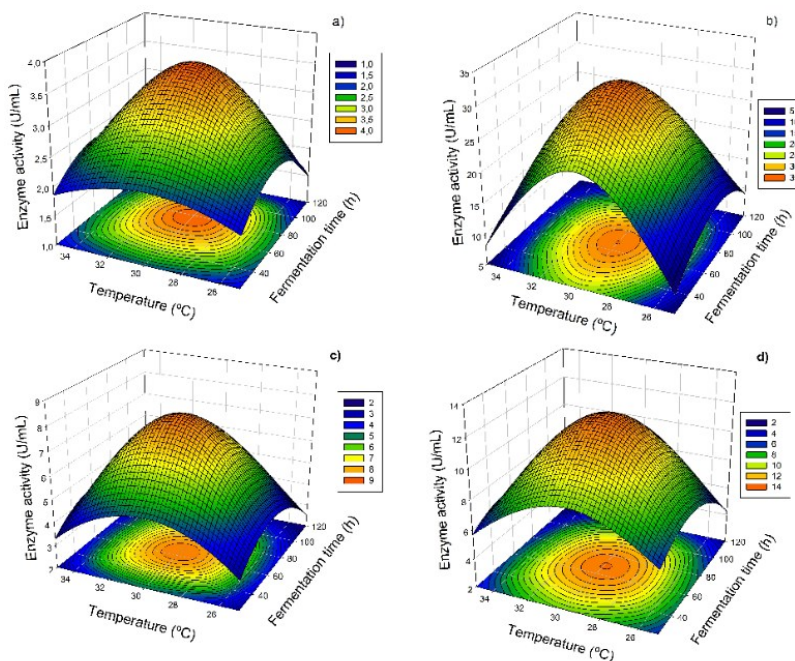
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Figure 2. Response surface plots of (A) endoglucanase, (B) total cellulases of *Aspergillus niger*, (C) Endoglucanase and (D) total cellulases of *Rhizopus sp* production during solid-state fermentation of cactus.

In solid-state fermentation, water activity is a critical factor for fungi growth and controlling its activity is essential for bioprocess optimization. The appropriate water content for the substrate enables the formation of a water film on the surface, which facilitates the dissolution and transfer of nutrients and oxygen. However, spaces between the particles should remain free to allow the diffusion of oxygen and the dissipation of heat (GERVAIS; MOLIN, 2003; SANCHEZ, 2009;). In this study, the optimal water activity for total cellulase production from *Aspergillus niger* was 0.875.

The water activity values in this study were ideal for the growth of fungi; therefore, the evaluated values were suitable for enzyme production. Water activity influences microbial growth and biological and enzymatic processes (GERVAIS; MOLIN, 2003). Each microorganism has a minimum water activity limit for metabolic activities to occur.

The presence of pre-existing nutrients in the prickly pear contributed to the growth of the microorganism, and when nutrient levels decreased due to consumption by the fungi, enzyme production decreased. Enzyme production starts out slow and then accelerates until it reaches a peak. Other microorganisms can also produce enzymes for industrial use. For example, endoglucanases are also produced by actinomycetes such as *Streptomyces* T3-1, which can produce 40.3 U/mL in 1.5% CMC, ammonium sulfate, urea and peptone. Furthermore. 0.59 U/mL total cellulases were produced by *Penicilium brasilianum* IBT 20888 cultivated in a bioreactor in medium containing yeast extract and wood from a type of pine tree subjected to steam explosion (JANG; CHEN, 2003; JORGENSEN;

OLSSON, 2006; SANTOS et al., 2011;).

In all the aforementioned studies, incubation times from 7 to 10 days, which is much longer than those utilized in the present study. It is worth noting that no inductors or supplements other than fresh prickly pear were used. The substrate was also not pre-hydrolyzed, demonstrating that the enzymes were constitutive.

The utilization of solid-state fermentation using lignocellulosic biomass and inducers has also contributed to increase the cellulolytic enzymes production in order to reduce cost, emphasizing the importance of optimizing the fermentation process and the ability of microorganisms to express enzymes (FOROOTANFAR et al., 2011; OSMA et al., 2011; AHMAD et al., 2013; MANAVALAN et al., 2013).

The design of the bioreactor is also important for maximizing cellulolytic enzyme production via solid-state fermentation. The bioreactor should also provide the optimum environment for microorganism growth and consider parameters such as aeration, humidity, agitation and temperature (COUTO; SANROMÁN, 2005; RABELO et al., 2011).

In general, the use of enzyme extracts in industrial processes reduces the risk of contamination by microorganisms, promotes solubility of substrates and products, increases reaction rates and increases the diffusion coefficient of the substrates; however, under elevated temperature and pH conditions, enzymatic activity is significantly reduced (PALMA-FERNANDEZ et al., 2002; GOMES et al., 2007).

Enzymatic characterization is important for

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optimizing variables such as temperature and pH, which are the most relevant for industrial applications.

According to the thermal stability results, endoglucanase (Figure. 3A) produced by *Aspergillus niger* maintained its activity for 60 minutes at 60ºC, and then the activity decreased to 20% of its relative activity after 240 minutes. However, in endoglucanase produced by *Rhizopus sp.* (Figure. 3B), its activity increased until 90 minutes,

considering the temperature interval of 20 to 60ºC; at 60ºC, the relative enzymatic activity did not reduce until the end of the analysis at 240 minutes. At 80ºC, the relative enzymatic activity reduced by 20% after 60 minutes for endoglucanase produced by the fungus *Aspergillus niger* (Figure. 3A), whereas at the same temperature this 20% reduction was only observed at 240 minutes in the enzyme produced by *Rhizopus sp.*
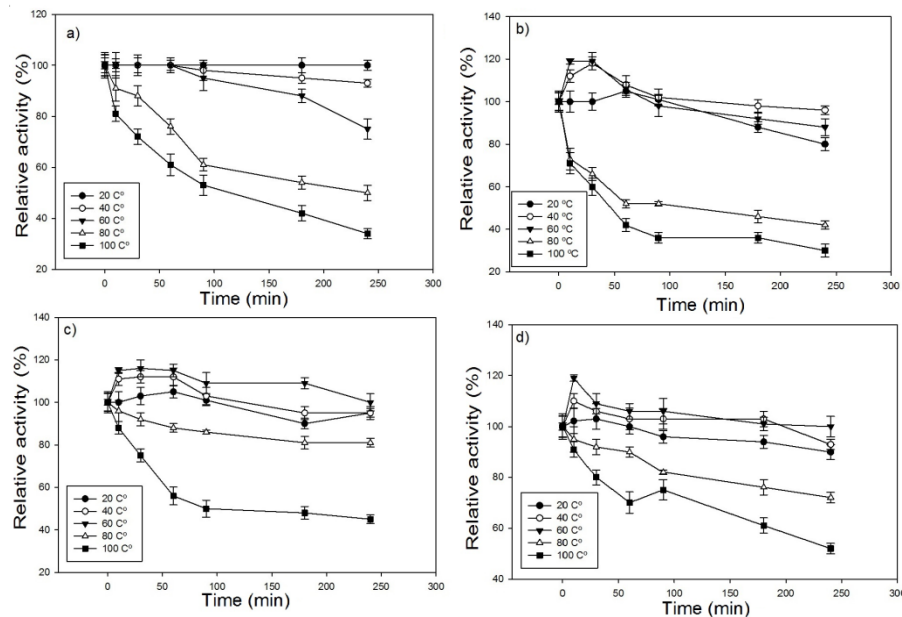

Figure 3. Thermostability of the enzymes (A) Endoglucanase, (B) total cellulases extracted of *Aspergillus niger* and (C) Endoglucanase, (D) total cellulases extracted *Rhizopus sp.*

At 60ºC, the residual activity of total cellulase from *Aspergillus niger* was 100% at 90 minutes of incubation, with an increase of approximately 20% during the 40 initial minutes of incubation. However, at 60ºC, the residual activity of total cellulose from *Rhizopus sp.* remained at 100% until 250 minutes. After 20 minutes at 100ºC, the residual activity of total cellulase from *Rhizopu*s sp. was approximately 70%, whereas the residual activity was 30% when produced by *Aspergillus*.

In *Aspergillus niger* in purple mombin waste, the maximum activity of endoglucanase and total cellulase occurred at 50 and 60ºC, respectively (SANTOS et al., 2013). The increased enzyme activity was due to temperature-induced activation, in which atoms acquired more energy and thus a greater tendency to move. After this period, there is denaturation or thermal deactivation caused by the rupture of weak interactions holding the globular protein structure together. Under the conditions studied, the optimal working temperature of the cellulase complex was between 50 and 60ºC.

From Figure 3, A–D, we can assume that the thermostability of *Rhizopus sp.* enzymes were superior to those produced by *Aspergillus niger*. Endoglucanase and total cellulase produced by the fungi *As-*

pergillus niger and *Rhizopus sp.* can be utilized in catalytic steps at temperatures of up to 60ºC for 240 minutes. *Rhizopus* endoglucanase can be utilized at temperatures of up to 80ºC. Although incubation at 100ºC caused a marked reduction in activity for all the evaluated enzymes, the residual activity was higher than 30% after 180 minutes (Figure. 3 A–D).

At a pH of 5 and 6, the residual activity of *Aspegillus niger* endoglucanase (Figure. 4) was approximately 100% for 240 minutes, with an elevation of approximately 10% throughout the assay, and residual activity above 80% was observed at pH of 4, 7 and 9. The residual activity of *Rhizopus sp.* endoglucanase, however, was 100% at pH 5 after 240 minutes over the course of the assay and at pH of 4, 6 and 7, the residual enzymatic activity was greater than 70%.

The residual activity of *Aspergillus niger* total cellulases (Figure. 4B) was greater than 100% until 250 minutes of incubation. At pH of 5, its activity increased by 30% at 90 minutes of incubation. At pH 6, a decrease of approximately 10% was observed. The relative activity exceeded 70% at pH of 4, 7 and 8. However, the residual activity of total cellulases produced by *Rhizopus sp.* (Figure. 4D) was 100% for only 90 minutes at pH 5 and 6. After this time, a

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decrease of approximately 10% was observed until 250 minutes of incubation. At pH 4, 7 and 8, the residual enzymatic activity reduced by approximately 30%.
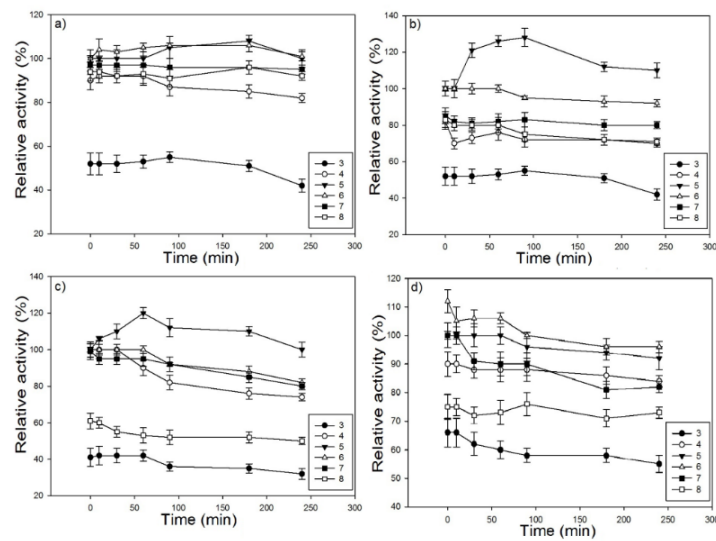

Figure 4. pH stability of the enzymes (A) Endoglucanase, (B) total cellulases from *Aspergillus niger* and (C) Endoglucanase, (D) total cellulases from *Rhizopus sp.*

The optimal pH for endoglucanase and total cellulases activity was 5.0 or 6.0, similar for other *Aspergillus* species. The presence of endoglucanase with an optimal pH of 5.0 and 6.0 in total cellulases was reported for *A. nidulans* (BAGGA et al., 1990). *Melanocarpus sp.* also produced enzymes with higher activity at pH 5.0 and 6.0 (GOMES et al., 2000). Total cellulase from *P. echinulatum*, however, had higher activity at pH 4.5 (CAMASSOLA et al., 2004).

Over time, the reduction in hydrolysis may have been due to enzyme deactivation by temperature and/or the absence of substrate factors in the medium. On the other hand, very short reaction times are not sufficient for the degradation of either the amorphous fraction or the crystalline fraction of cellulose. Thus, the hydrolysis time should be sufficient to ensure that the degradation of total cellulases occurs without enzyme deactivation (ZHANG et al., 2006; RABELO et al., 2011).

Overall, regardless of the fungus, at pH 5 and 6 it was observed that the residual activities of endoglucanase and total cellulases were greater than 95% during after 240 minutes of incubation, which suggests these enzymes maintained their structural stability, indicating that they are acid-tolerant and, thus, can be used in industrial processes with acid characteristics.
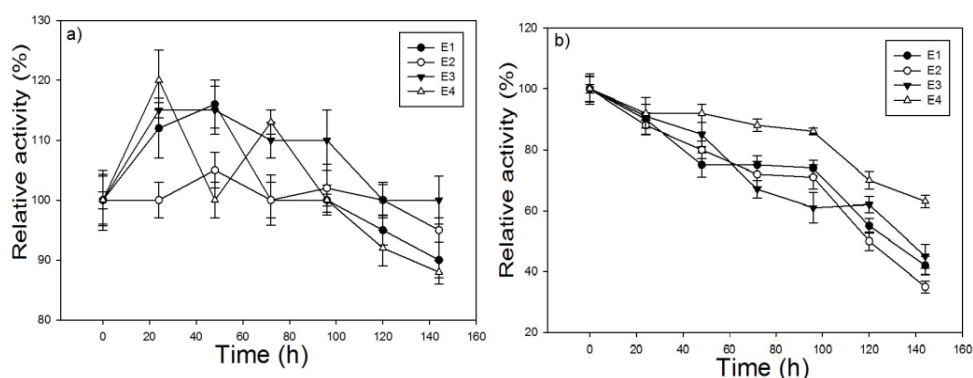

Figure 5. Kinetics of enzyme inactivation after storage at 4°C (A) or -25°C (B). E_1 and E_2 represent endoglucanase and total cellulose, respectively from *Aspergillus niger*. E_2 and E_3 represent endoglucanase and total cellulases, respectively from *Rhizopus sp.*

To evaluate the activity profile of enzymes after storage, enzymatic deactivation experiments were performed at two different temperatures (Figure. 5). Enzymatic deactivation may be influenced by factors such as temperature, pH, hydrodynamic forces, and absence of substrate, among others (AGUIAR; SERGIO, 2011). At a freezing temperature (–25ºC), there was no sudden loss in endoglucanase or total cellulase activity. Even after 144 hours, the relative activity of the crude enzyme extract was over 85%.

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Freezing led to an approximately 15% increase in the relative activity at a given moment; this can be explained by the effect of freezing on the original structure of the proteins. Freezing interferes with water activity, causing the polypeptide chain to restructure; therefore after thawing, the enzyme may have acquired a new configuration that affected the enzymatic activity. Freezing also interrupts chemical reactions and biological activities (AGUIAR; SERGIO, 2011). However, at coolant temperature (4ºC), there was degradation due to microbial growth in the enzyme extract (COLLA; PRENTICE-HERNANDE, 2003).

CONCLUSION

In this study, the Box-Benhnken Design was employed to predict the combined effects of cultivation conditions on cellulolytic enzyme production from of filamentous fungi. Based on the results, the use of prickly pear as substrate and *Aspergillus niger* and *Rhizopus sp.* was satisfactory for the production of endoglucanase and total cellulase. Experiments carried out in optimal conditions validated the statistical models. The enzymes were highly tolerant to a wide range of temperature and pH, which indicates great relevance for industrial applications. The cellulase complex is not significantly deactivated if frozen at − 25ºC for up to 144 hours, but loses approximately 60% of its activity if stored at 4ºC. Since prickly pear is a low-cost organic substrate with high incidence in the Brazilian Northeast semi-arid region, it is a potential nutrient source for the production of enzymes of industrial interest.

ACKNOWLEDGEMENTS

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