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COCOA SHELL FOR THE PRODUCTION OF ENDOGLUCANASE BY *Penicillium roqueforti* ATCC 10110 IN SOLID STATE FERMENTATION AND BIOCHEMICAL PROPERTIES

CÁSCARA DE CACAO PARA LA PRODUCCIÓN DE ENDOGLUCANASA POR *Penicillium roqueforti* ATCC 10110 EN FERMENTACIÓN DE ESTADO SÓLIDO Y PROPIEDADES BIOQUÍMICAS

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Abstract

This paper aims to evaluate the use of the cocoa shell residue as an alternative substrate for the production of endoglucanase by *Penicillium roqueforti* ATCC 10110 in solid state fermentation. In order to do so, the process was optimized and biochemical properties were investigated. The Box-Behnken design was utilized for verify the parameters: incubation time, water activity and fermentation temperature were optimized by response surface methodology, in order to increase the yield of enzymatic activity coupled with an economic process. Temperature, pH, different ions and solvents stability and determination of kinetic parameters were also verified. The maximum activity was 0.89 U/g under the conditions of fermentation time, water activity and incubation temperature were of 72h, 0.964 A_w and 30 ºC, respectively. A higher endoglucanase activity at pH 5 and temperature 50 °C was achieved during the reaction, were stable at pH in the range 5 to 7 and temperatures ≤ 70 ºC. The addition of the Mg^{2+}, Cu^{2+}, Co^{2+} ions, the trolox and SDS compounds to the crude extract resulted in increased enzymatic activity. The dichloromethane and hexane behaved as endoglucanase activators, increasing the activity of the enzyme in 5.42 and 1.18%, respectively. The kinetic parameters K_m and V_{max} were determined using carboxymethylcellulose as substrate, with values of 1.17 mg/mL and 0.90 mg/mL/min, respectively.

Keywords: Box-Behnken design, stability, kinetic parameters, endoglucanase.

Resumen

Este estúdio evaluó el uso del resíduo e cáscara de cacao como sustrato alternativo para La producción de endoglucanasa por *Penicillium roqueforti* ATCC 10110 en fermentación en estado sólido y investigar sus propiedades físicas y químicas. Los parámetros: tiempo de incubación, actividad del agua y temperatura de fermentación se optimizaron utilizando la metodología de superficie de respuesta com la ayuda del diseño Box-Behnken. La actividad máxima fue 0,89 U/g en las condiciones de tiempo de fermentación, actividad de agua y temperatura de incubación de 72h, 0,964 A_w y 30 ºC, respectivamente. Una mayor actividad de endoglucanasa a pH 5 y una temperatura de 60 ºC se logró durante la reacción, fueron adecuadamente estables a pH en el rango de 5 a 7 y temperaturas ≤ 70 ºC. La adición de Mg^{2+}, Cu^{2+}, iones Co^{2+}, los compuestos Trolox y SDS al extracto crudo dio como resultado un aumento de la actividad enzimática. El diclorometano y hexano se comportaron como activadores de la endoglucanasa, aumentando la actividad de la enzima en 5, 42 y 1,18%. Los parámetros cinéticos K_m y V_{max} se determinaron usando carboximetilcelulosa como sustrato, con valores de 1,17mg/ml y 0,90mg/ml/min, respectivamente.

Palabras clave: diseño Box Behnken, estabilidad, parámetros cinéticos, enzima.

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1 Introduction

Cocoa shell is a residue composed of film/husk that surrounds the almonds and is obtained during the processing of the fruit (*Theobroma cacao* L.) (Lessa *et al*., 2018). According to the International Cocoa Organization (2016), approximately 700 thousand tons of this residue are generated, a significant amount that arouses the interest of researchers, in exploring different ways for their application (Okyiama *et al*., 2017). Studies were carried out using the cocoa shell in natura as a substrate, carbon and nitrogen source, by culture of *Penicillium roqueforti* in solid state fermentation (SSF) to obtain lipase (Silva *et al*., 2017) and to obtein ligninases (dos Santos *et al*., 2011) and also cellulases (dos Santos *et al*., 2013) by *Aspergillus níger*. Lessa *et al*. 2018, in turn, investigated the effect of SSF on the composition of secondary metabolites: phenol, carotenoid content, anthocyanins, flavonols and fatty acids and antioxidant activity in the cocoa shell with *Penicillium roqueforti*, verifying the continuity of the presence of most metabolites and reducing the content of saturated fatty acids after fermentation, suggesting the reuse of this residue by the food industry as a substitute for synthetic antioxidants.

Solid state fermentation (SSF) is a biotechnological process in which microorganisms, mainly fungi, grow in non-soluble material or solid substrates in low free water content (Sadh *et al*., 2018), recognized as a technology with environmental and economic advantages to obtain enzymes (Ferraz *et al*., 2017). The genus Penicillium is known for the production of secondary metabolites and extracellular enzymes (Banu *et al*., 2010) several species have already been characterized by the secretion of cellulases (Guzakov e Sinitsyn, 2012). Although the ability of *Penicillium roqueforti* to adapt to different temperatures and pH and to be considered a GRAS fungus (Generally regarded as safe), there is still a reduced scientific literature on its application in solid state fermentation.

One of the main cellulases, Endoglucanases (EG, endo-1,4-β-endoglucanases, E.C. 3.2.1.4) act in the hydrolysis of the amorphous fraction of the cellulose chain reducing its degree of polymerization (Brito *et al*., 2017; Payne *et al*., 2015). They are enzymes widely used in several industrial applications, such as food, textiles and laundry, paper and cellulose, and agricultural, production of bioethanol from lignocellulosic residues, extraction of fruit and vegetable juices (Acharya e Chaudhary,2012), and for this reason, a growing interest in obtaining these stable and more specific enzymes has been investigated.

Multivariate statistical tools have their increasing use in optimization methods, due to the favorable characteristics in the execution of experiments that reduce the number of experiments and the time of analysis, which results directly in saving time, reagents and later waste disposal. Additionally, such tools may demonstrate interaction effects between variables, often not observed in univariate models (Ferreira *et al*., 2004; Breitkreitzet *et al*., 2009; Ballus *et al*., 2011; Govarthanan *et al*., 2013; Govarthanan *et al*., 2015; Coutinho *et al*., 2015; Dias *et al*., 2016). In this way, the objective of this work was to optimize the production and to determine the fundamental biochemical characteristics of endoglucanase produced by the fungus, *Penicillium roqueforti* (ATCC 10110), using cocoa shell, the residue of a product with high regional value.

2 Materials and methods

2.1 Cocoa shell

Cocoa shells (CSs) were provided by the chocolate industries located in the South of Bahia (Brazil), which were dried in an oven at 50 ºC for 24 h. They were crunched in a mill of knives of the Wiley (ACB LABOR®) type down to a particle size of 2 mm and then stored in plastic container until they were ready to use.

2.2 Penicillium roqueforti

The filamentous fungus *Penicillium roqueforti* was provided by Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, RJ, Brazil) and duly deposited at the Instituto Nacional de Controle de Qualidade em Saúde (INCQS, Rio de Janeiro, RJ, Brazil) under the registration number 40075 and lot number 079840075. This fungus was preserved in silica and glycerol and maintained in an ultra freezer at a temperature of -80 ºC. The spore suspension was prepared with fungus cultivated in Potato-Dextrose-Agar (VETEC®) and Agar Agar (VETEC®), which were placed in 250 mL Erlenmeyer flasks, over a period of 7 days in a bacteriological greenhouse (SL 222, Solab) maintained at 26.5 ºC; the collection of

spores was made by adding Tween 80 (VETEC®) (0.01% v/v) sterile glass beads to the Erlenmeyer flasks. For counting the number of spores in suspension, adoubly mirrored Neubauer Chamber and a binocular microscope (BIOVAL® L1000) were used. (dos Santos *et al*., 2016).

2.3 State solid fermentation (SSF)

5.0 g of CS were autoclaved (121 ºC/1 atm/15 min) in 125mL Erlenmeyer flasks; after cooling, the sterile substrate was inoculated with 10^7 spores/gand moistened with sterile distilled water (Novasinalabswift, TECNAL®) until the value of the desired water activity (aw) was determined. The fermentations were incubated in bacteriological greenhouses under diferent temperatures (T, °C).

2.4 Design experimental

The optimization of the production of endoglucanase by SSF was evaluated using the experimental methodology design with a Box-Behnken Design composed of 15 diferent experiments, 3axial points and 3 central points. The corresponding matrix is represented in Table 1. The independent variables (factors) analyzed and their ranges were time (t =48, 72 and 96), temperatura (T = 20, 30 and 40 ºC) and water activity (Aw= 0.937, 0.964 and 0.984). The dependent variable (response) evaluated was the activity of endoglucanase (U/g), which was measured in the enzymatic extract that was obtained. The data was analyzed with the aid of STATISTICA® v. 10.0 (Statsoft, the USA) software.

2.5 Multienzymatic extract

After fermentation, 50 mL of sodium phosphate buffer (0.1 M/pH 5.0) was added to the fermented substrate and the mixture was stirred (shake incubator, TECNAL) at 35 ºC and 170 rpm for 10 min. The liquid phase was separated by mechanical pressing was permeation achieved gauze followed by centrifugation (704.34 g) for 10 minutes to remove impurities.

2.6 Determination of enzymatic activity

The method chosen to determine the endoglucanase activity is based on the measurement of reducing sugars produced by the degradation of 2% (w/v) carboxymethyl cellulose (CMC) (Cromoline, Diadema, Brazil) diluted in 50 mM sodium citrate buffer (pH 4.8) (10). The dinitrosalicylic acid (DNS) (Sigma-Aldrich, St. Louis, MO, USA) was used for the quantification of reducing sugars (11). Reaction assays were conducted by adding 0.5 mL of 50 mM sodium citrate buffer (pH 4.8), 0.5 mL of enzyme extract, and 0.5mL of 2% (w/v) CMC (cromoline). The reaction control was carried out in another tube, to which 0.5 mL of the same buffer solution and 0.5 mL of enzyme extract have been added. The blank assay contained 0.5 mL of DNS (Sigma-Aldrich) and 0.5mL of buffer solution. The samples were incubated in a bacteriological incubator (SL 222; Solab) at 50 ºC and centrifuged at 10×g for 10 min (CT-6000R; Cientec). The reaction was interrupted by the addition of 0.5 mL of DNS (Sigma-Aldrich). After that, the tubes were heated in boiling water for 5 min and short lyafter, 6.5 mL of distilled water were added into the tubes for measuring absorbance at 540 nm carried out using aspectrophotometer (SF200DM-UV Vis; Bel Photonics,Osasco, Brazil).

2.7 Enzymatic characterization

2.7.1 Effect of temperature on activity and stability

The reaction temperature for maximum activity was determined by conducting the assay to determine endoglucanase activity at different temperatures (40-80ºC) at pH 5.0. The responses were expressed as relative activity (%), the highest result being considered as 100%. Thermal stability was assessed by incubating the crude endoglucanase extract in sodium citrate buffer (0.1 M, pH 5.0) under different temperatures (40-80 ºC) for 5h. Aliquots were taken at 1h intervals and subjected to determination of endoglucanase activity.

2.7.2 Effect of pH on activity and stability

The reaction pH for maximum activity was determined by conducting the xylanase activity assay at different pH (4 to 8) at 50 ºC. To reach these pH values it was necessary to dilute the crude endoglucanase extract 10 times in sodium citrate buffer (0.1 M, pH 4 to 5) and sodium phosphate buffer (0.1 M, pH 6 to 8). For pH stability assays, the enzyme extract (diluted 10-fold in suitable buffer) was incubated at different pH (3-8) at 50 ºC for 5h and aliquots were withdrawn at 1h intervals and subjected to the activity assay of endoglucanase. The results were expressed as relative activity.

2.7.3 *Effect of metal ions*

The effects of the metal saltsMgCl$_2$, Pb(C$_2$H$_3$O$_2$)$_2$, Co(C$_2$H$_3$O$_2$)$_2$, Zn(C$_2$H$_3$O$_2$)$_2$, CuSO$_4$, Al(NO$_3$)$_3$ and Na$_2$CO$_3$ were evaluated on the enzymatic activity, all at the concentration of 2 mmolL-1 of reaction medium. The assays consisted of incubating the enzyme extract in sodium citrate buffer (0.1 M, pH 5.0) with the additives for 15 minutes followed by determination of endoglucanase activity. The results were expressed as relative activity (%).

2.7.4 *Effect of organic compounds*

The organic compoundsácido etileno-diamino tetra-acético (EDTA), dodecil sulfato de sódio (SDS), T-octil-fenoxi-polietoxi-etanol (Triton X-100) e 6-hidroxi-2,5,7,8-tetrametil-cromano-2-ácido carboxílico (Trolox) were evaluated on the enzymatic activity, all at the concentration of 2 mmolL^{-1} of reaction medium. The assays consisted of incubating the enzyme extract in sodium citrate buffer (0.1 M, pH 5.0) with the additives for 15 minutes followed by determination of endoglucanase activity. The results were expressed as relative activity (%).

2.7.5 *Effect of solvents*

The organic solvents acetone, dichloromethane, ethyl ether, hexane and methanol were evaluated on the enzymatic activity. The assays consisted of incubating 1.0 μL the solvent in enzyme extract for 15 minutes followed by determination of endoglucanase activity. The results were expressed as relative activity (%).

2.7.6 *Determination of kinetic parameters*

The kinetic parameters for the Michaelis-Menten model (K_m and V_{max}) were estimated. The medium employed and the reaction conditions were the same as those applied to the determination of endoglucanase activity, except for contractions of the carboxymethyl-cellulose substrate (Sigma®) ranging from 1.0 to 20 mg mL^{-1}. The Michaelis-Menten constant (K_m, mg mL^{-1}) and the maximum reaction rate (V_{max}, μmol min^{-1}mL^{-1}) were estimated by the Lineweaver and Burk regression method (1934).

3 Results and discussion

3.1 *Optimization of endoglucanase production*

The experimental results obtained from the Box-Behnken design conditions applied to optimize the production of endoglucanases using cocoa shell, can be seen in Table 1.

Table 1. Box-Behnken design matrix for the analysis of fermentation factors: Time (t, h), Water activity (A_w), Temperature (T, °C) on Endoglucanase activity (End, U/g) produced by *P. roqueforti* ATCC 10110 grown in the cocoa shell.

Run	**Factors**			**Response**
	t (h)	**AW**	**T (°C)**	**End (U/g)**
1	- 1 (48)	-1 (0.937)	0 (30)	0.585 ± 0.003
2	1 (96)	-1 (0.937)	0 (30)	0.439 ± 0.004
3	-1 /(48)	1 (0.984)	0 (30)	0.536 ± 0.001
4	1 (96)	1 (0.984)	0 (30)	0.565 ± 0.007
5	-1 (48)	0 (0.964)	-1 (20)	0.526 ± 0.001
6	1 (96)	0 (0.964)	-1 (20)	0.546 ± 0.001
7	-1 (48)	0 (0.964)	1 (40)	0.634 ± 0.004
8	1 (96)	0 (0.964)	1 (40)	0.423 ± 0.017
9	0 (72)	-1 (0.937)	-1 (20)	0.526 ± 0.013
10	0 (72)	1 (0.984)	-1 (20)	0.731 ± 0.004
11	0 (72)	-1 (0.937)	1 (40)	0.682 ± 0.001
12	0 (72)	1 (0.984)	1 (40)	0.263 ± 0.006
13	0 (72)	0 (0.964)	0 (30)	0.829 ± 0.016
14	0 (72)	0 (0.964)	0 (30)	0.829 ± 0.005
15	0 (72)	0 (0.964)	0 (30)	0.897 ± 0.009

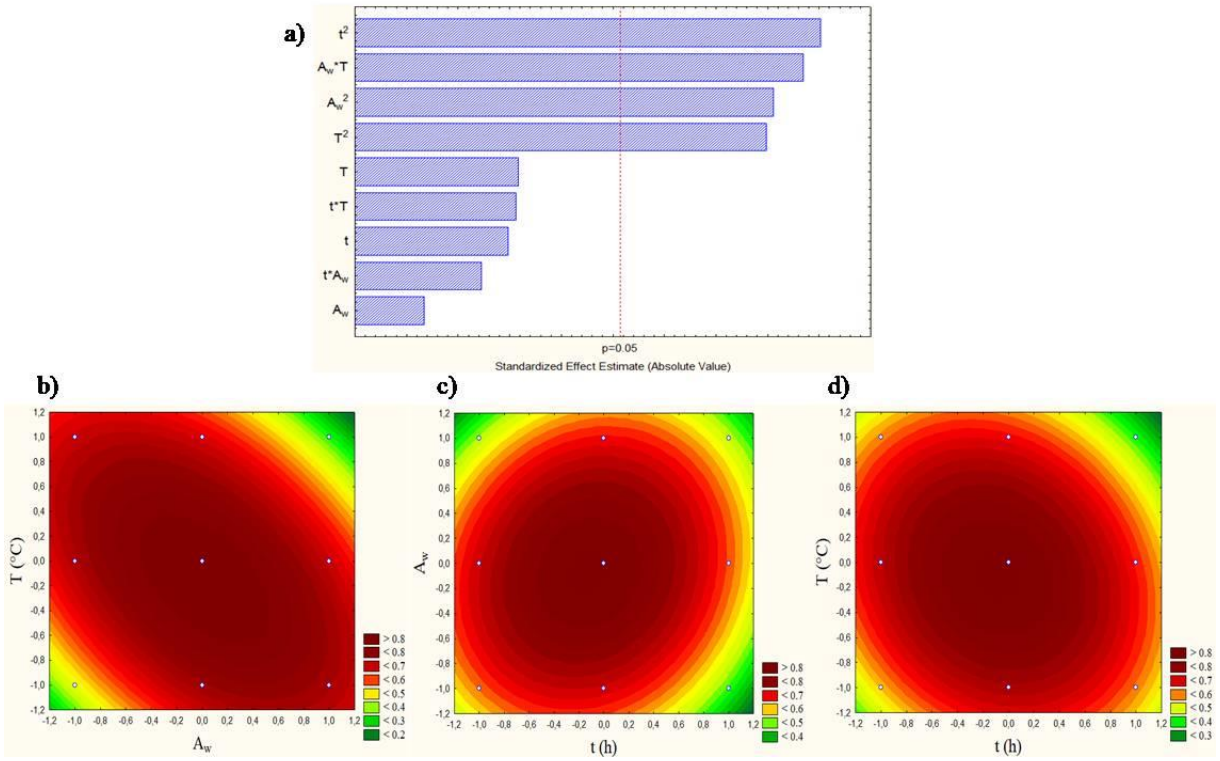

Fig. 1. **a)** Pareto chart showing the significance of the terms of the variables: fermentation time (t), water activity (A_w) and incubation temperature (T); **b)** Contour surface graph: $T \times A_w$; **c)** Contour surface graph: $T \times t$; **d)** Contour surface graph: $A_w \times t$, having as response the endoglucanase activity.

The response values are the average result of triplicate carried out in the determination assays of the enzymatic activity. In Figure 1a the significant statistical effects of quadratic, linear and interaction terms on enzyme activity were analyzed using the Pareto graph. The vertical line representing the minimum significant factor of 90% of reliability allows observing the variables of greater and smaller effect, where all significant quadratic terms suggested that the three variables investigated: time, water activity and temperature, were critical for the production of endoglucanase, being the time variable the one of greater influence, corroborating with other results of the literature, as reported by dos Santos *et al*. (2013), to optimize the process of obtaining endoglucanase in cocoa shell by Aspergillus niger, varying the factors time and water activity. They emphasized the relationship between the effect of the fermentation time and the presence of macro and micronutrients in the fermentation medium.

The analysis of variance (ANOVA) (Table 2), in turn, was used to prove the efficacy of the mathematical model through the Fisher's test (F test), considering the significant difference between the sources of variation in the results and only the coefficients statistically significant.

With the results presented in Table 2, is possible to observe that the mathematical model was statistically validated (p<0,05). The values of R^2 and R^{2-adj} (0.9364 and 0.8219, respectively) indicate a good fit for the experimental results, mainly, being a biological system. The non-significant terms were discarded resulting in Equation 1, generated only, from the significant factors.

$$Y_{(U/g)} = 0.814 - 0.168(t^2) - 0.152(A_w^2) - 0.149(T^2) - 0.156(t * T) \quad (1)$$

For cocoa shell substrate, the highest activity of the enzyme was achieved when the conditions were maintained at the central level, according to the contour characteristics in Figure 1, in this case, time (72h), water activity (0.964) and temperature (30 ºC).

Table 2. ANOVA for the adjusted reduced model. R^2 = 0.9364; R^{2-adj} = 0.8219; DF, degree of freedom; SS, sum of squares; MS, middle square; F, Fisher's test; P, probability.

	SS	*DF*	*MS*	*F*	*P*
Regression	0.38	1	0.379982	147.2981	p < 0.05
Residue	0.0258	10	0.00258		
Total	0.406	11	0.036889	0.5	
Lack of Fit	4.36E+11	4	1.10E+11		
Pure Error	4.36E+11	2	2.18E+11		

R^2 = 0.9364; R^{2-adj} = 0.8219; DF, degree of freedom; SS, sum of squares; MS, middle square; F, Fisher's test; P, probability.

The results were validated, with the triplicate repetition of the critical values indicated by the model through Equation 1, and an endoglucanase activity of 0.8514 ± 0.007 U/g was found, a value close to that predicted by the model. Marques *et al*. (2017) produced the enzyme endoglucanase from *Penicillium roqueforti* in rice husk, at an optimum temperature at 32 °C and in a time of 72h, similar to that found in this work. Kim *et al*. (2015) used *Penicillium* sp. GDX01, at an optimum temperature at 30 ºC,but in a higher time, 240 h. Dutta *et al*. (2008), used *P. citrinum* to produce endoglucanase in wheat bran in a time of 168 hours and a temperature of 28 °C.

Very low temperatures tend to negatively affect the microbial development, being able to occur decrease in it is metabolism and consequently reduction in the enzymatic production.

At high temperatures the microorganisms channel the energy obtained from the substrate to synthesize only a small amount of proteins necessary for their growth and physiological processes, and the denaturation of the enzyme caused by the high temperatures can lead to changes in its three-dimensional structure (Pal and Khanum, 2010). Temperature affects microbial metabolism, regulates growth, spore formation, germination and product formation. However, the spaces between the particles must remain free to allow diffusion of oxygen and heat dissipation (dos Santos *et al*., 2011).

3.2 *Enzymatic characterization*

3.2.1 *Effect of temperature on activity and stability*

The temperature effect was evaluated for different temperatures according to Figure 2a. where it was possible to observe that the relative activity of endoglucanase was maximal at 50ºC and that there was a decline from that value. Marques *et al*. (2017) analyzing the enzyme obtained by *Penicillium roqueforti* ATCC 10110 in rice husk residue, also observed preservation of activity above 100% at a temperature of 50 ºC. In relation to thermostability (Figure 2b), the activities remained stable, between 40 and 60 ºC, whose activities at the end of the first incubation time for each were above 100%, mainly at 50 °C.

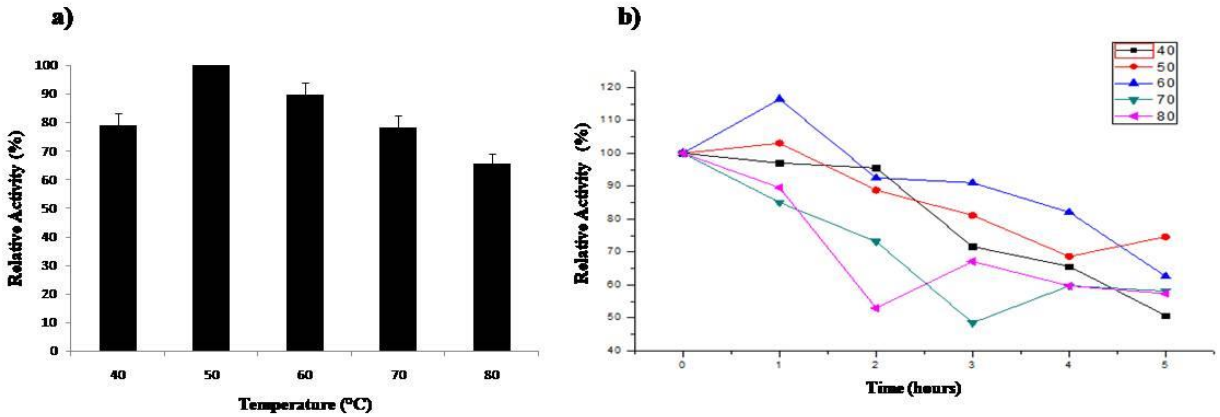

Fig. 2. **a)** Activity of endoglucanase by *P. roqueforti* ATCC 10110 in SSF grown on residue of cocoa shell, obtained in different temperatures; **b)** Temperature stability profile.

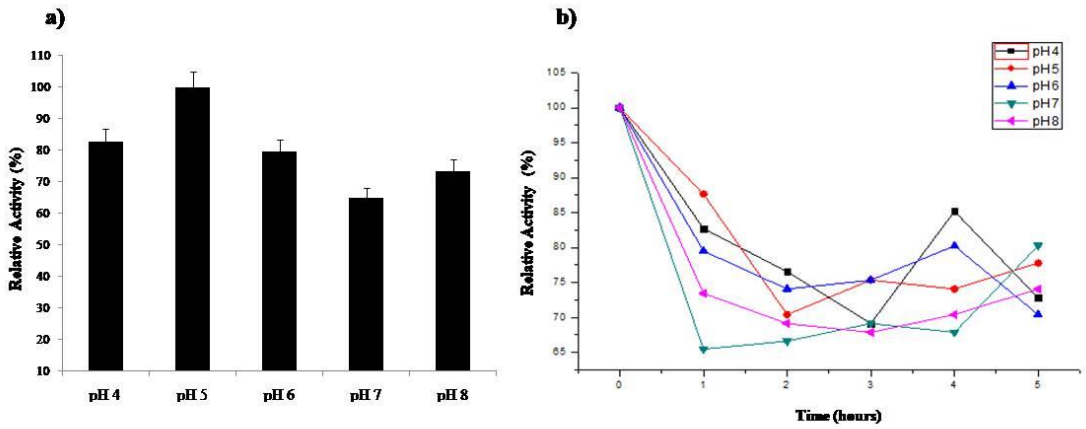

Fig. 3. **a)** Activity of endoglucanase by *P. roqueforti* ATCC 10110 in SSF grown on residue of cocoa shell, obtained in different pH ranges; **b)** pH stability profile.

Despite a decay at temperatures 70 and 80 ºC up to 3 h and 2 h, respectively, the activities of the enzyme, at the end of the 5 h time, remained for both approximately 65%, revealing an enzyme that acts over a wide temperature range. Because it is a crude enzyme extract, other enzymes are likely to exert protective behavior over endoglucanase against the effect of increasing temperature and time. These values are similar to the results found for other enzymes from *Penicillium citrinum*. Peña-Maravilla *et al*. (2017) found endoglucanase by *Penicillium citrinum* optimal activity values at 60 °C, but stability at 40 °C. While, Santa-Rosa *et al*. (2018) analyzed endoglucanase from *Penicillium* sp. LMI01, which presented optimum temperature at 60 °C and stability similar to 50 and 60 °C.

3.2.2 Effect of pH on activity and stability

The effect of pH on the activity of endoglucanase obtained from *P. roqueforti* ATCC 10110 was verified in the range of 4.0 to 8.0 and the maximum enzymatic activity was observed at pH 5.0 (Figure 3a). Peña-Maravilla *et al*. (2017) observed optimal pH at 5.5 for *Penicillium citrinum* endoglucanase using coffee pulp in solid state fermentation, partially purified endoglucanase from *P. citrinum* showed optimum activity at pH 5.5 with a second peak activity at pH 8.0 (Dutta *et al*., 2008). The (Figure 3b) shows that the best stability was achieved using pH values in the range (4-8), with the enzyme activity being maintained at about 80% after 5 h of incubation at 50 °C. At pH values 6, 7 and 8, about 66, 68 and 71% of the enzymatic activity were maintained, respectively, after the maximum incubation time investigated. The results demonstrated stable endoglucanase at a broad pH range when compared to endoglucanases obtained from other Penicillium species. Pol *et al*. (2012) and Ire *et al*. (2018) found *Penicillium pinophilume* and *Penicillium* sp. endoglucanase, respectively, maximum activity and stability at pH 5.

3.2.3 Effect of metal ions

The results of the effect of different metal ions on the activity of the endoglucanase extract are represented in Table 3. Among the seven metallic ions three had positive effects. The Co^{2+} ion was the species that exerted the greatest effect, increasing about 67% of the activity. The Cu^{2+} and Mg^{2+} species increased activity by about 27.97 and 5.14%, respectively. When compared with literature data, Co^{2+} and Mg^{2+} ions also activated the enzyme endoglucanase from Aspergillus niger NRRL 567, as reported Ghori *et al*. (2012). Huang *et al*. (2015) found the activating effect of the Mg^{2+} ion on endoglucanases of *Penicillium simplicissimum* H-11 and *Arthrobacter* sp. HPG166, respectively. The presence of the Pb2+ ion markedly modified the endoglucanase activity of the present study, since the species behaved as the major inhibitor. The presence of Mn2+, Na2+ and Zn2+ also reduced their activity, corroborating with that found for the same enzyme by Sadhu *et al*. (2013) e Azzeddine *et al*. (2013). Some ions and heavy metals can cause oxidation of groups present in the side chain of amino acids resulting in a drastic decrease in activity, that is, their presence can modulate the catalytic site of the enzyme, causing structural changes in the same (Manavalan *et al*., 2015).

Table 3. Endoglucanase activity from *P. roqueforti* ATCC 10110 against different metal ions; organic compounds and solvents.

	Relative Activity (%)
CONTROL	100
$MgCl_2$	105.14 ± 0.02
$CuSo_4$	127.97 ± 0.10
$Co(C_2H_3O_2)_2$	167.58 ± 0.10
$Pb(C_2H_3O_2)_2$	70.23 ± 0.00
$Zn(C_2H_3O_2)_2$	86.34 ± 0.00
Na_2CO_3	82.32 ± 0.03
$MnSO_4$	90.37 ± 0.00
EDTA	38.00 ± 0.02
TRITON X	55.46 ± 0.00
TROLOX	118.87 ± 0.00
SDS	106.48 ± 0.00
Methanol	87.73 ± 0.008
Acetone	98.70 ± 0.005
Ethyl eter	99.76 ± 0.003
Dichloromethane	105.42 ± 0.003
Hexane	101.18 ± 0.005

3.2.4 *Effect of organic compounds*

Among the organic compounds investigated and presented in Table 3, Trolox behaved like an activator increasing the catalytic activity in about 118%. This compound has antioxidant activity, which suggests a protective effect for the endoglucanases studied, possibly avoiding the oxidation of groups of the enzymes and allowing the maintenance of its active form (Pereira *et al*., 2017). The presence of SDS resulted in a small increase of 6% in the catalytic activity. On the other hand, Triton X and EDTA presented an inhibitory effect, resulting in a reduction of 55% and 38%, respectively, in the enzymatic activity, as reported for the same enzyme in the literature by other authors (Li and Yu, 2013; Marques *et al*., 2017).

3.2.5 *Effect of solvents*

Also, by table 3, it is observed that dichloromethane and hexane behaved as activators of endoglucanase, increasing the activity of the enzyme in 5.42 and 1.18%, respectively. Ethyl ether and acetone did not significantly influence the activity of the enzyme, maintaining it at 99.76% and 98.7%, respectively, while methanol inhibited the enzymatic activity (Table 3). Stability of enzymes in the presence of organic solvents is an extremely important feature, since most enzymes are easily denatured and inactivated in the presence there of, depending on the polarity of the solvent, it may create an interface in the enzyme molecule and allow a open conformation for binding, stimulating its activation (Irfan *et al*., 2017).

3.2.6 *Determination of kinetic parameters*

Figure 4a shows the kinetic profile, relating the substrate concentrations to the values of endoglucanase activity, the linear behavior of the enzyme produced by *P. roqueforti* in cocoa shell confirms that it is a Michaeliana enzyme. To obtain the K_m and V_{max} values, the graph of Figure 4b was generated from the trend line equation, where the values for K_m and V_{max}, were 1.17 mg/mL and 0.90 mg/mL/min, respectively.

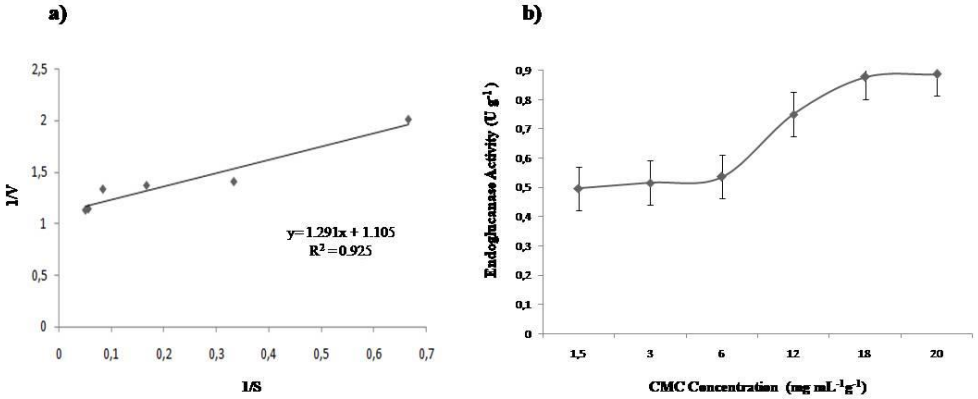

Fig. 4. **a)** Effect of substrate concentration on the enzymatic activity of endoglucanase produced by *P. roqueforti* ATCC 10110 in SSF grown on residue of cocoa shell; **b)** Lineweaver-Burk chart obtained for estimation of K_m and V_{max}.

The low value of K_m found suggests a high affinity of the enzyme to the substrate carboxymethylcellulose, the result exceeds that found in the literature for other fungal endoglucanases, for example; Pol *et al*. (2012) found the value of K_m 4.8 mg/mL for *Penicillium pinophilum* endoglucanase. Bai *et al*. (2013) presented for *Penicillium simplicissimum* H-11 endoglucanase, K_m in the value of 14.88 mg/mL and V_{max} 0.36. Bakare *et al*. (2005) found the value of K_m 3.6 mg/mL for *Pseudomonas fluorescens* endoglucanases. Already, Bai *et al*. (2017) found values for K_m 18.45 mg/mL and V_{max} 18.18 mg/mL/min in *Penicillium ochrochloron* ZH1 endoglucanase.

Conclusions

From the study, it was possible to obtain endoglucanases from *Penicillium roqueforti* ATCC 10110 by solid-state fermentation using the cocoa shell as a residue and the only source of nutrients. The application of the response surface methodology using a Box-Behnken design matrix allowed the conditions of the variables to be successfully obtained: fermentation time, water activity and incubation temperature, necessary to maximize endoglucanase production. The enzyme of the fungus P. Roqueforti ATCC 10110 presented stability in a wide range of pH (5-8) and temperature (50 °C), besides having its active activity against metallic ions, organic compounds and hydrophilic and hydrophobic solvents, being able to be efficiently applied in diverse industries, mainly in the food industry.

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