Endoglucanase production by trichoderma reesei cultivated in solid state fermentation using lignocellulosic waste

Utilização de resíduos lignocelulósicos para a produção de endoglucanases por trichoderma reesei cultivado sob fermentação em estado sólido

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ABSTRACT
Trichoderma reesei is a fungus that has been widely explored for its potential as cellulolytic enzyme producer and has diverse industrial applications. However, obtaining the enzymes is still considered a costly and, sometimes, inefficient process. This study aimed to produce endoglucanases by cultivating *T. reesei* (CCT-2768) in solid state fermentation, using cashew apple bagasse (CAB), sugarcane bagasse (SCB) and green coconut fiber (GCF) residues as substrates. The influence of moisture and pH on enzyme production was evaluated using a factorial design. *T. reesei* showed viability for producing endoglucanases in all of the three lignocellulosic residues tested, with maximum activity (2.29 ± 0.01 U/g) observed when cultivated in the SCB substrate and using moisture of 60% and pH 5.5. Thus, use of lignocellulosic residues proves to be a viable alternative for producing endoglucanases by cultivation of *Trichoderma reesei*, which contributes to the recycling of waste and the reduction of environmental impacts.

Keywords: Cellulolytic enzymes, Carboxymethylcellulase (CMCase), Filamentous fungi, Bioprocess, Factorial design

RESUMO
Trichoderma reesei é um fungo bastante explorado devido ao seu potencial produtor de enzimas celulolíticas, as quais possuem diversas aplicações industriais. No entanto, a obtenção dessas enzimas ainda é um processo de alto custo e, algumas vezes, ineficiente. Este trabalho objetivou produzir endoglucanases por meio do cultivo de *T. reesei* (CCT-2768) sob fermentação em estado sólido, utilizando como substratos resíduos de pedúnculo de caju (PC), bagaço de cana-de-açúcar (BCA) e fibra de coco verde (FCV). A influência do teor de umidade e pH na produção enzimática foi avaliado a partir de um planejamento fatorial. *T. reesei* apresentou viabilidade para a produção de endoglucanases nos três resíduos lignocelulósicos testados, com máxima atividade (2,29 ± 0,01 U/g) quando cultivado no substrato BCA, a 60% de umidade e pH 5.5. A utilização dos resíduos lignocelulósicos demonstra-se uma alternativa viável para a produção de endoglucanases por *Trichoderma reesei*, contribuindo com a reciclagem de resíduos e com a redução de impactos ambientais negativos.

Palavras-chaves: Enzimas celulolíticas, Carboximetilcelulase (CMCase), Fungos filamentosos, Bioprocessos, Design fatorial.

1 INTRODUCTION

Vegetable biomasses are the most abundant source of carbon in the biosphere and consist mainly of carbohydrate polymers such as cellulose (40-50%) and hemicellulose (20-30%), aromatic polymers such as lignin (10-25%), and small amounts of pectin, proteins and lipids. Recently, these materials have emerged as an alternative in the production of renewable fuels, such as bioethanol, as well as providing other value-added products, especially from the conversion of the polysaccharides present in these materials into fermentable sugars (Benevides et al. 2021).

Cellulose is an unbranched polymer of glucose residues, which is linked by β-1,4-glycosidic bonds, and has a highly ordered and crystalline conformation that is resistant to hydrolysis. One of the possible ways to carry out its hydrolysis is through bioconversion mediated by the synergism between several enzymes, especially cellulases, which catalyze the breakdown of cellulose into simple and soluble sugars (El-Naggar et al. 2014). In addition, it is possible to hydrolyze cellulose into simple sugars using conventional chemical methods; however, the enzymatic process is considered more economical and more environmentally friendly (Ibrahim et al. 2021; Nagl et al. 2021).

The potential of cellulases is not limited only to biomass conversion. Cellulases can also be made use of by various industrial segments, including food, textiles, detergents, pulp, and paper industries (Wang et al. 2005). However, the structure of these enzymes is diversified, and includes the group of endoglucanases (EC 3.2.1.4), which are responsible for the hydrolysis of internal bonds, exoglucanases (EC 3.2.1.91), which produce cellobiose from chain-end hydrolysis, and β-glucosidases (EC 3.2.1.21) that act on cellobiose hydrolysis for its final conversation into glucose (KUHAD et al., 2016; Srivastava et al. 2021). In this sense, endoglucanases are particularly important in the breakage of cellulose fibers, in addition to reducing the size of the molecule and forming the reducing end, thus allowing other enzymes access to the cellulose structure (Nagl et al. 2021).

In general, microorganisms are the main source of cellulases, and are naturally specialized in degrading and recycling different types of plant biomass. As such, fungi are of the great importance for the industrial production of cellulases, due to their ability to produce a large amount and variety of these enzymes, which are secreted in the fungal growth substrate and improve the extraction and purification of these molecules (Huang et al. 2015).

Trichoderma reesei is a fungus that has been widely studied in relation to its efficiency in degrading cellulose and for secreting a large amount of endoglucanases (20-36%) and exoglucanases (60-80%) (Li et al. 2013; Zhang et al. 2018). However, *T. reesei* needs an inducer for enzymatic synthesis, thus making the process more expensive given the high commercial cost of microcrystalline cellulose, for which the carbon source represents about 40 to 50% of the total cost of enzyme production (Singh et al. 2021). Thus, an alternative that would aid in solving this problem is the use of low-cost lignocellulosic residues, which can act as a source of nutrients and physical support for fungal growth, in addition to inducing the production of enzymes (Martins et al. 2020; Oliveira et al. 2018; Mejias et al. 2018; Subramanian et al. 2017).

One way to obtain fungal enzymes from waste is through solid-state fermentation (SSF), which can be described as a biotransformation that occurs in a solid matrix in the minimal presence of water, and whose use is widely studied for producing cellulases, lipases, chitinases, proteases and other enzymes (Singhania et al. 2015). The advantages of using this method are the minimal or non-existent pre-treatment of the waste used (recycling), less generation of effluents, and the ability to simulate microenvironments that are favorable to the growth of microorganisms, thus mimicking their natural growth process and, as a result, obtaining the product in high concentrations (Abu Yazid et al. 2017; Hemansi et al. 2018).

Considering the need for new sources for producing cellulases, which have a wide range of applications and great commercial value, the objective of this work was to evaluate the production of endoglucanases by a strain of *Trichoderma reesei* that was cultivated in different lignocellulosic residues, varying the moisture and pH.

2 MATERIAL AND METHODS

2.1 LIGNOCELLULOSIC WASTE

The residues used as the substrate for the solid-state fermentation were obtained from agro-industries in the northeast of Brazil. Sugarcane bagasse was obtained from the Estivas factory (Arês, Rio Grande do Norte), fresh green coconuts were collected near Ponta Negra beach Natal, Rio Grande do Norte, and the cashew apple bagasse came from the Cione Co. factory (Fortaleza, Ceará). After collection, the residues were washed with water to remove dirt and then dried at 70 °C for 48 h. Subsequently, they were ground in

a knife mill (Wiley TE-680, Tecnal, Brazil), sieved with a 20 mesh sieve and stored in plastic bags at room temperature (± 28 °C) (Oliveira et al. 2018).

2.2 MICROORGANISM

Trichoderma reesei, strain CCT-2768, was commercially obtained from the Tropical Cultures Collection of the André Tosello Foundation, Campinas, São Paulo, Brazil. The strain was maintained in Petri dishes at ± 28 °C and reactivated every 3 months to maintain the microorganism.

2.3 INOCULUM ACTIVATION

T. reesei was inoculated in plates containing PDA (Potato Dextrose Agar) medium and incubated at 28 ± 2 °C for 5 days. In the Petri dish containing the microorganism, 5 mL of Tween 80 solution (0.3% v/v) was added, and the spores contained in the dish were removed by scraping. The inoculum for the SSF was obtained from the propagation of the fungus spores, standardized at 1 x 10^6 spores/g of biomass by counting in a Neubauer chamber. The inoculum was produced in 125 mL Erlenmeyer flasks that contained 4.6 g of previously ground corncob (dried at 55 °C and autoclaved) and 6 mL of nutrient solution (peptone 56 g/L, monobasic potassium phosphate 0.2 g/L, zinc sulfate 0.396 g/L and iron II sulfate heptahydrate 0.46 g/L) and incubated in a BOD-incubator at 30 °C (± 2 ºC) for 7 days (Oliveira et al. 2018).

2.4 SOLID STATE FERMENTATION (SSF)

SSF was carried out for 72 h in a BOD incubator at 30 °C (± 2 ºC), using 5 g of each substrate (cashew apple bagasse, sugarcane bagasse and green coconut fiber) in 250 mL Erlenmeyer flasks, under the conditions established in accordance with experimental design of the factorial type (2^2), and with three repetitions of the central point to evaluate the influence of the moisture content and pH on the enzymatic activity. The inoculum concentration used was 1.0 x 10^6 spores/g of residue, on dry basis, and a sterile nutrient saline solution was added in order to vary the moisture and pH of the waste (Table 1). The solution was composed of KH_2PO_4 (5 g/L); $(NH_4)_2SO_4$ (5 g/L); $MgSO_4 \cdot 7H_2O$ (1 g/L); NaCl (1 g/L); $FeSO_4 \cdot 7H_2O$ (5 mg/L); $MnSO_4$ (1.6 mg/L); $ZnSO_4 \cdot 7H_2O$ (3.45 mg/L); $CoCl_2 \cdot 6H_2O$ (2 mg/L) (Urbánszki et al. 2000). Moisture values were standardized from the estimate of water activity (a_w) for each residue (Oliveira Júnior 2018).

2.5 ENZYME EXTRACTION

The enzymatic extraction was performed after 72 h of fermentation, with 6 mL of sodium acetate buffer (200 mM, pH 5.0) being added for each gram of post-fermentation material. The flasks were shaken in an orbital shaker at 160 rpm, 4 °C, for 30 min, then filtered and centrifuged at 2,000 rpm, 4 °C, for 10 min. The supernatants (enzyme extracts) were stored at -18 °C, until the quantification of enzymatic activity (Oliveira et al. 2018).

2.6 ENDOGLUCANASE ACTIVITY

The endoglucanase activity was determined from the hydrolysis of carboxymethylcellulose (CMC), according to methodology adapted from Ghose (1987), and was prepared at 4% (w/v) in sodium citrate buffer (50 mM, pH 4.8). The quantification of the reducing sugars released from the hydrolysis of the CMC was performed by adding 3,5-dinitrosalicylic acid (DNS) to the reaction mixture and subsequent spectrophotometric reading at 540 nm. Reducing sugars were estimated from a standard curve of glucose, and enzyme activity was expressed in activity units (U) per gram of substrate, where one unit was defined as the amount of enzyme needed to release 1 µmol of glucose-equivalent reducing sugars.

2.7 STATISTICAL ANALYSIS

The experiments were carried out in triplicate. The STATISTICA program (v. 7.0 TIBCO software Inc., Palo Alto, California, USA) was used for the design and analysis of the results obtained from of the experimental matrix. The treatments were submitted to analysis of variance (ANOVA) and their means to the Tukey test ($p < 0.05$). The results were also expressed in the form of Pareto diagrams and three-dimensional surface response graphs in order to illustrate the main and interactive effects of the independent variables (moisture and pH) of the substrate on the response variable (enzymatic activity).

3 RESULTS AND DISCUSSION

3.1 ACTIVITY OF ENDOGLUCASES PRODUCED BY *T. REESEI*

The extracts obtained from solid state fermentation (SSF) of the fungus *Trichoderma reesei* CCT-2768, using residues of green coconut fiber (GCF), sugarcane bagasse (SCB) and cashew apple bagasse (CAB) showed positive activity for

endoglucases, which indicates that the residues studied were suitable for the induction of cellulolytic enzyme synthesis, and that there were significant differences between the growth substrate tested (Table 1). In general, GCF showed better performance as an enzymatic synthesis inducer, with an average value of 1.70 U/g between the different conditions, against an average of 1.63 and 1.24 U/g for SCB and CAB, respectively. However, it is observed that, within the ranges studied, the peak of enzymatic activity was observed in SCB, with 2.29 ± 0.01 U/g, at the level coded as "+1", with values of 60% moisture and pH 5.5 (Table 1).

Oliveira et al. (2018), when working with *Penicillium chrysogenum* and the same residues as used herein, observed percentage values for cellulose of 39.25 ± 5.49 in SCB, 36.23 ± 0.09 in GCF e 21.02 ± 0.31 in CAB. These data justify the results obtained in the present study, in which there was a greater production of endoglucanases in SSF with SCB, since the cellulose contained in the substrate acts as a natural inducer for producing cellulolytic enzymes (Oliveira et al. 2018; De Paula 2017).

Table 1. Coded levels (in parentheses), real values and responses from solid-state fermentation for endoglucanase production by *Trichoderma reesei* CCT-2768 on different substrates.

Experimental run	**Green coconut fiber (GCF)**			**Sugarcane bagasse (SCB)**			**Cashew apple bagasse (CAB)**		
	Moisture (%)	**pH**	**Activity (U/g)**	**Moisture (%)**	**pH**	**Activity (U/g)**	**Moisture (%)**	**pH**	**Activity (U/g)**
1	(-1) 70	(-1) 3.5	1.25^{f}	(-1) 40	(-1) 3.5	1.14^{e}	(-1) 60	(-1) 3.5	1.22^{c}
2	(+1) 80	(-1) 3.5	1.55^{d}	(+1) 60	(-1) 3.5	1.11^{f}	(+1) 70	(-1) 3.5	1.46^{a}
3	(-1) 70	(+1) 5.5	1.36^{e}	(-1) 40	(+1) 5.5	2.19^{b}	(-1) 60	(+1) 5.5	1.35^{b}
4	(+1) 80	(+1) 5.5	1.18^{g}	(+1) 60	(+1) 5.5	2.29^{a}	(+1) 70	(+1) 5.5	1.17^{d}
5*	(0) 75	(0) 4.5	2.25^{a}	(0) 50	(0) 4.5	1.59^{c}	(0) 65	(0) 4.5	1.17^{d}
6*	(0) 75	(0) 4.5	2.20^{b}	(0) 50	(0) 4.5	1.55^{d}	(0) 65	(0) 4.5	1.17^{d}
7*	(0) 75	(0) 4.5	2.15^{c}	(0) 50	(0) 4.5	1.59^{c}	(0) 65	(0) 4.5	1.15^{e}

*Central points. Results are mean values of the three replicates. Letters indicate differences by the Tukey test at 0.05 significance.

Ambrozim et al. (2018) performed a similar fermentation process using the microorganism *Trichoderma harzianum* IOC-3844. In their study, greater enzymatic activity (4.95 U/g) was obtained in 72 h of cultivation in a substrate composed of 40% cocoa husk and 60% sugarcane bagasse with 70% moisture. However, Carvalho et al. (2018) used coffee bean husks for cellulase production by *T. reesei* BTF-0948 and observed an endoglucanase activity of 12.92 U/g, with 70% moisture and pH 5.0 in 96 h of cultivation. Silva et al. (2018) found that the fungus *T. reesei* CCT-2768 showed

maximum activity for endoglucases (13 U/g) when using carnaúba (*Copernicia prunifera*) residue, which had been pretreated with alkaline hydrogen peroxide, 60% moisture and a pH of 5.0 in 96 h of fermentation.

Although the works cited present higher values of enzymatic activity, they used mixes of residues and/or pre-treatment processes. According to Tian et al. (2018), it is important to reduce the production costs of endoglucanases and, therefore, it is recommended to use waste with low cost and high availability, as in the case of sugarcane bagasse. Furthermore, pre-treatments that may also increase the cost of the process and should be avoided.

Regarding moisture, the material used in SSF is generally porous in nature, and thus allows water retention through hygroscopicity or capillary action, which varies according to the substrate used. As such, it is important to standardize the moisture content of the substrate according to the intrinsic water activity of each residue (Singh et al. 2020). Starchy substrates, such as rice bran, wheat, rye, barley, corn and cassava, retain about 60% moisture; on the other hand, lignocellulosic substrates, such as sawdust, straw, bark and bagasse, permit fermentations with higher initial moisture contents, usually of around 80% (Manan and Webb 2017; Selo et al. 2021).

It is important to highlight that very high moisture contents can also harm the process, since the high availability of free water present in the substrates can reduce the porosity of these materials, which generates particle agglomeration, reduces gas exchange and temperature. Notwithstanding, low moisture levels can negatively influence the metabolism of the microorganism, which hinders the solubilization and absorption of nutrients present in the waste (Lodha et al. 2020; Sala et al. 2020).

Despite the importance of moisture during the process, other factors, such as temperature, pH, type of biomass, and characteristics, such as particle diameter, surface area and the interstices between particles, are important for adequately productive fermentation. Therefore, a number of different materials have been tested in order to optimize the production of cellulolytic enzymes (Gonçalves et al. 2015; Guilherme et al. 2015).

3.2 INFLUENCE OF MOISTURE AND PH ON THE PRODUCTION OF ENDOGLUCANASES BY *T. REESEI*

The effect of moisture (A), pH (B) and the interaction between these two variables (A x B) on the production of endoglucanases by *T. reesei* CCT-2768 under SSF is

represented numerically by the results of the analysis of variance (ANOVA) (Table 2) and graphically by Pareto diagrams of each fermentation process (Figure 1).

It is possible to observe that the proposed statistical model presents linearity only for the SCB substrate, with a coefficient of determination $R^2 = 0.98486$, which suggests an agreement between the values that are theoretically expected by the model, as well as in the values observed experimentally. In the other substrates, the variables and interaction effects were insignificant compared to the error limit (Table 2). Additionally, by evaluating the different conditions of the tested fermentation process, it can be observed that the only significant influencing factor was the variation in pH (B), as indicated by the significant *p* value ($p = 0.0008 < 0.05$) and the *F* value that was considerably higher than in the other conditions (Table 2) and which is represented in the diagram by the only bar that exceeds 5% of significance (Figure 1b).

Therefore, the results show that the pH is directly related to the production of endoglucanases by *T. reesei*, under the culture conditions evaluated. In general, the production of enzymes by a given microorganism may be affected by several physical and chemical parameters, among which pH is one of the most important and highly relevant factors in the optimization and prediction of the fermentation process. However, in SSF, pH is one of the most difficult parameters to control, due to the very nature of the solid substrate, lack of free water and heterogeneity, in addition to the difficulty in carrying out continuous monitoring (Manan and Webb 2017). In this study, a mineral solution based on phosphate (KH_2PO_4) was used to control pH and provided adequate conditions for microbial metabolism and enzyme synthesis.

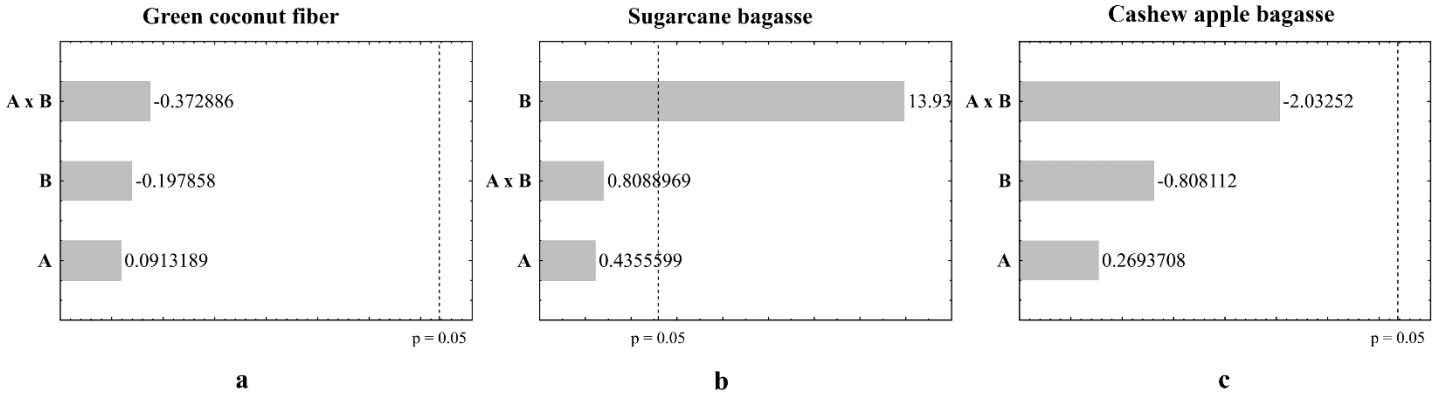
Several studies demonstrate the direct influence of pH on the production of cellulases and xylanases, in which the production of xylanases is favored in higher pH ranges than for cellulases, and the ideal pH for synthesis and activity of endoglucanases, exoglucanases and β-glycosidases is also different (Zhang et al. 2019). According to Li et al. (2013), pH plays an important role in the process of cellulase synthesis by *T. reesei*, with the production rate of these enzymes being maintained in an optimal state by development of control strategies for this parameter, and the yield of saccharification is increased by 26.2% when compared to the process without pH control.

Table 2. Analysis of variance (ANOVA) for endoglucanase activity by *T. reesei* CCT-2768 using different substrates.

Variable	Green coconut fiber (GCF)					Sugarcane bagasse (SCB)					Cashew apple bagasse (CAB)				
	SS	Df	MS	*F*	*P*	SS	Df	MS	*F*	*p*	SS	Df	MS	*F*	*p*
A	0.003600	1	0.003600	0.008339	0.932995	0.001225	1	0.001225	0.1897	0.692591	0.000756	1	0.000756	0.072561	0.805109
B	0.016900	1	0.016900	0.039148	0.855804	1.254400	1	1.254400	194.2655	0.000800	0.006806	1	0.006806	0.653045	0.478163
A × B	0.060025	1	0.060025	0.139044	0.734021	0.004225	1	0.004225	0.6543	0.477774	0.043056	1	0.043056	4.131157	0.135015
Error	1.295096	3	0.431699	-	-	0.019371	3	0.019371	-	-	0.031267	3	0.010422	-	-
Total	1.375621	6	-	-	-	1.279221	6	-	-	-	0.081886	6	-	-	-
R^2	0.58540	-	-	-	-	0.98486	-	-	-	-	0.61816	-	-	-	-

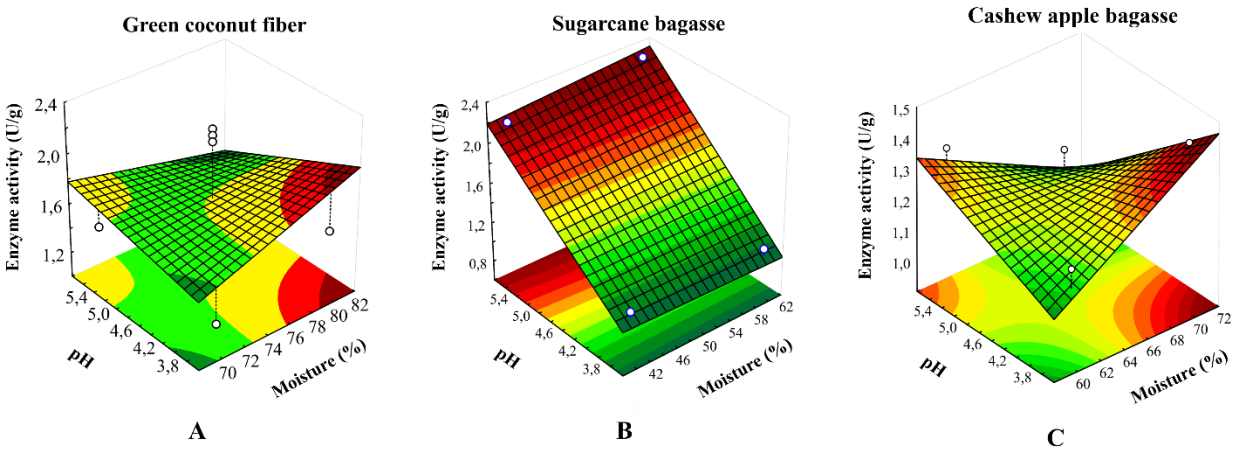
A: Moisture. B: pH. R^2: Coefficient of determination. SS: Sum of squares. Df: Degrees of freedom. MS: Mean square. *F*: *F* value. *p*: *p* value (0.05)**ure 1.** Pareto diagrams of the

Figure 1. Pareto diagrams of the main effects and interactions of moisture and pH on the activity of endoglucanases produced by *T. reesei* CCT-2768, using residues of green coconut fiber, sugarcane bagasse and cashew apple bagasse as substrates.



The influence of the independent variables (moisture and pH) on the dependent variable (enzymatic activity of endoglucanases) is graphically represented by the response surface plot, by which it is possible to verify the high influence of pH on the SCB (Figure 2B). Although pH was the only statistically significant parameter in the production of endoglucanases by *T. reesei*, when cultivated in SCG (Figure 2B), through the response surface (three-dimensional shape) and the associated contour map (temperature and color intensity), a small trend towards the regions that indicate the highest pH and moisture values can be observed (Figure 2A and 2C). Thus, it is possible to infer that pH ranges greater than 5.5 and moisture above 70% allow the achievement of greater endoglucanase activities.

Figure 2. Response surface plot showing the interaction of moisture and pH, using residues of green coconut fiber, sugarcane bagasse and cashew apple bagasse as substrates in solid state fermentation (SSF) for production of endoglucanases by *T. reesei*, in 72 h.



The commercially available endoglucanases produced by. *T. reesei* have optimal activity in pH ranges from 4 to 6 and are generally inactive in higher ranges, which limits their applications in industrial process that require neutral or alkaline pH (Wang et al. 2005). Therefore, a strain with optimal production of this group of enzymes with pH values above 5.5 may be of great industrial interest and open the way for studies aimed at exploring new pH ranges and physicochemical characterization of these enzymes.

4 CONCLUSIONS

Trichoderma reesei CCT-2768 was able to produce endoglucanases in all the lignocellulosic residues tested, with emphasis on sugarcane bagasse (SCB) at 60% moisture and pH 5.5, thus indicating the potential of these materials as the support and carbon source for fungal development. Moreover, through statistical and response surface analysis, the present work proposes that *T. reesei*, when cultivated in SCB, represents a source of endoglucanases with activity in pH ranges higher than 5.5, which is a factor of great interest in industrial processes. Nevertheless, future studies aiming to confirm this hypothesis are recommended, as well as the purification and evaluation of the physicochemical characteristics of the enzyme.

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