Open Access

SCHOLARENA

Journal of Waste Resources and Reprocessing

RESEARCH ARTICLE

Production of cellulases by *Aspergillus niger* IOC 3998 by means of solid-state fermentation (SSF) using as substrate the chestnut seed (*Terminalia catappa Linn.*)

Viviane Hiromi Uchida and Everaldo Silvino dos Santos*

Chemical Engineering Department, Centre of Technology, Federal University of Rio Grande do Norte (UFRN), Natal-Rio Grande do Norte, Brazil

***Corresponding author:** Everaldo Silvino dos Santos, Chemical Engineering Department, Centre of Technology, Federal University of Rio Grande do Norte (UFRN), Natal-Rio Grande do Norte, Brazil, E-mail: everaldo@eq.ufrn.br

Citation: OViviane Hiromi Uchida, Everaldo Silvino dos Santos (2020) Production of cellulases by *Aspergillus niger* IOC 3998 by means of solid-state fermentation (SSF) using as substrate the chestnut seed (*Terminalia catappa Linn.*). J Waste Resources Reprocessing 1: 101

Abstract

Organic residues are widely used for the production of enzymes in industry in general as substrate for producing cellulases by *Aspergillus niger* IOC 3998 on Semi-Solid Fermentation (SSF). The protocols analysis of the National Renewable Energy Laboratory (NREL) were used to caracterize the untreated the chestnut seed. A two-variable central composite rotatable design (CCRD) coupled to response surface methodology (RSM) with four experiments at center point were used to investigate the influence of initial moisture and pH on CMCase and FPase activities produced by the *Aspergillus niger* on Semi-Solid Fermentation (SSF) using chestnut seed (Terminalia catappa linn) as substrate (lignocellulosic biomasses). The source of nitrogen (ammonium sulfate and peptone) on solution of medium was avaiable influence on production cellulases and the variations of pH and temperature on stability enzyme. The results show that the treated waste (content approximately 22.0%) has a better yield in the cellulases production obtaining 13.2 U/g for CMCase and 0.232 U/g for FPase. In addition, the moisture proved to be the factor that most influenced the production of cellulases when analyzed in the CCRD for CMCase and FPase. Stability assays against pH and temperature showed that the activities for CMCase and FPase had best performance at neutral pH. Regarding the temperature, it was observed that its increase reduced the activity of CMCase and FPase at temperatures above 50 °C.

Keywords: Cellulase; Chestnut Seed; Semi-Solid Fermentation

Introduction

The use of raw materials of plant origin to produce molecules with higher benefit, such as enzymes, or even commodities as bioethanol, is increasing mainly because these lignocellulosic materials are a renewable raw material [1-4]. One of the lignocellulosic materials is cellulose, a linear biopolymer formed by glucose linked by beta-1,4-glycosidic bonds, considered to be the most abundant biopolymer and mainly found in vegetables [5-7]. In addition, cellulose can be hydrolyzed by enzymes called cellulases and are classified into 3 classes: endo-cellulases - which hydrolyze the biopolymer chain at random; exo-cellulases, which hydrolyze the reductive and non-reducing ends; and beta-glucosidases which hydrolyze cellobiose and two glucose molecules [8].

In nature, there are several filamentous fungi belonging to genus Trichoderma and Aspergillus that are prolific producers of cellulase, constituting a very accessible source for research and innovation. Aspergilus fumigatos is quite common when used in the production of cellulases. The strains Asppergilus are main applied for β-glucosidase production. However, when using lignocellulosic materials, such as cellulose, in the industry it is necessary to carry out a pretreatment step in order to remove lignin and hemicellulose that inhibits the action of the cellulase and, therefore, its yield [9-12]. This approach makes the process of cellulase production very costly and, as intermediary product in production, can reach until 40% of the total production cost in ethanol fuel, for example (Wayman 1992, Zhang 2006, (Ahamed and Vermette, 2008; Deswal *et al.*, 2011)). Therefore, the development of alternative and optimized industrial scale is critical to maintain the economic viability of cellulase potential users such as: food, beverages, textile, laundry, paper, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment industries.

One possible source for cellulose is the unused fruits of fruiting trees located in urban regions which, if not treated, can cause a quantity of biomass that is irregularly disposed into the environment, generating environmental impact on the soil or even in groundwater, in addition to causing outbreaks of microorganisms or unwanted animals due to rotting fruits [13]. Thus, the use of waste in its entirety can reduce or even eliminate this source of pollution. A possible and interesting alternative would be the use of these in the production of enzymes by solid-state fermentation (SSF) that is a process in which a microorganism grows and develops on the surface of solid materials containing low water activity [14]. In addition of its accessibility of cellulose material along with a simple and economical type of fermentation, it becomes very interesting for industries due the low cost production potential [15-18].

In Brazil, one of these urban fruit trees is the fruit from Terminalia catappa linn, a tree widely used as an ornamental plant and its fruits are not explored, despite being edible, it has no commercial value, even though it has large amounts of lipids and proteins [19]. The fruit has a very fibrous and fine pulp, inside the stone there is an oily almond. In this context, the present study aimed to use the residue of the chestnut seed (*Terminalia catappa Linn.*) as a substrate for the production of cellulases by SSF using the fungus *Aspergillus niger* IOC 3998, evaluating the influence nitrogen sources, pH and moisture in this process. Moreover, it important to highlight that to the best our knowledge there were no reports in the literature for the use of this material for the production of cellulase.

Material and Methods

Lignocellulosic Material

The chestnut fruits were harvested from a small forest located in the Federal University of Rio Grande do Norte (UFRN), municipality of Natal (Rio Grande do Norte / Brazil), from November to January 2016.

Material Pretreatment

After collection, the core was extracted from the pulp and dried at 70 °C for 24 hours. Then, after being powdered using a knife mill (Tecnal / TE-680) about 30 grams of the seed were pretreatment with two concentrations of sodium hydroxide (2% and 4%), with a proportion of the residue and the basic solution of 1:10. After that, the mixture was autoclaved for 30 minutes at 120°C. Then, the mixture was filtered and dried at 70°C during 24 hours in an hot air oven (Tecnal/TE-394/1). The treated residues were used in the production of cellulases using the fungus *Aspergillus niger* IOC 3998 in SSF.

Characterization of the Lignocellulosic Material

The chestnut seed (*Terminalia catappa Linn.*) was characterized in terms of cellulose, hemicellulose, lignin, ash, moisture and extractability content according to the protocol adopted by NREL (National Renewable Energy Laboratory - USA) [20].

Microorganism and Maintenance

This study used a strain of *Aspergillus niger* IOC 3998 and the fungus was stored on sterilie sand and kept at -80 °C.

Inoculum

For the inoculum the fungus were transferred to petri plates containing 20g of medium (PDA) and incubated during 5 days at 30ºC using a biochemical demand of oxygen (BDO) equipment (Tecnal/TE-391), with two transfer. Them, the cells were collected by using 5 mL of a 0.5% Tween 80 solution and a scraping of the spores was carried out with the help of a steel handle. The spore counting was performed using a Neubauer chamber to an initial inoculum concentration reaching 5.0x107 cells/mL. That suspension was used as inoculum.

Cellulases Production by SSF

In the present study, the chestnut fruit was used as the carbon source for the production of cellulases by SSF using the fungus *Aspergillus niger* IOC 3998. Thus, 3.5 g of the substrate were transferred into 125 mL Erlenmeyers along with a humidifying solution that was prepared according to Urbánszki, *et al.* [21]. This solution consisted of (g/L): 5g (KH_2PO_4), 5g ($(NH_4)_2SO_4$), 1g NaCl, 5mg ($FeSO_4.7H_2O$), 1,6mg ($MnSO_4$), 3,45 ($ZnSO_4$), 2mg ($CoCl_2.6H_2$). The tests were carried out in BOD (Tecnal/TE-391) at 35 °C for 6 days (conditions defined after carrying out a previous kinetic study, data not shown). After the fermentation time, the Erlenmeyers were removed from the BOD and the extraction of the enzymes was performed by adding 25 mL of the phosphate buffer (pH 6.0) in each Erlenmeyer, followed by shaking in a shaker (Tecnal/TE-421), 150rpm for 30 minutes. Then, the samples were filtered with filter paper (Whatman n°1) and stored for later use.

Effect of Nutrients in the Culture Medium on Cellulolytic Activity

The analyses occurred by adding nitrogen sources to the nutrient solution in order to evaluate the growth of the fungus and the production of scellulases by means of the cellulolytic activity, both the endo and total type (CMCase and FPase). Peptone and ammonium sulfate were used as a nitrogen source at a concentration of 0.03g/mL in 4.2 mL of the nutrient solution (NS). In addition, experiments were also performed containing only the NS for comparison [21].

Influence of pH and Moisture on the Production of Cellulases by SSF

A central rotational composite design (DCCR) with replicate at central point was used to assess the influence of pH and humidity on cellulase production (Table 1). For the DDCR, cellulase activity (CMCase and FPase) was used as a response when using the nut from the chestnut seed as a substrate for FES. The Response Surface Methodology (RSM) and the analysis of variance (ANOVA) were used to analyze the production of cellulases during cultivation by FES.

Factors	Levels				
	-α	-1	0	1	+α
pH	3	3.5	4.5	5.5	6
Moisture (%)	52.4	54	58	61.5	63.6

Table 1: Factors and levels codified for the DCCR

Influence of pH and Temperature on Enzyme Stability

The stability of cellulases against changes in pH was achieved by adding 0.5mL of the enzymatic extract, obtained from FES, in 1mL of buffer. The mixture was placed in in a water bath for 1h at 50 °C. After that time, the activities were analyzed for CMCase and FPase. In the analysis, two buffers were used with different proportions of the concentration of the components in the mixture to obtain different pHs. The buffers used were the citrate buffer (pH 3, 3.8 and 5) and the phosphate buffer (pH 7 and 8). In order to assess the effect of temperature on the enzymatic extract, the temperature was varied from 30 °C to 70 °C with an increase of 5 °C, and for each temperature the sample remained at rest for 1 hour. The stability was analyzed using the relative residual activity, where the value of the enzyme activity of the extract before the process was used as reference value.

Quantification of Enzyme Activity

The activity of endoglucanases (CMCase) and activity of exoglucanase (FPase), were estimated according to Ghose (1987). For the analysis of CMCase a solution of carboxymethylcellulose (CMC) was used and for FPase, filter paper (Whatman n°1) was used as substrate in citrate buffer (50 mM, pH 4.8). The reducing sugars were quantified according to Miller (1959), based on the reagent 3,5-dinitrosalysilic acid (DNS). For CMCase and FPase activities, one unit of enzyme activity was defined as the amount of enzyme required to release the equivalent to 1.0 µmol of glucose per minute. In both cellulases, CMCase and FPase, the results were expressed in U/g of enzymatic activity per dry substrate used in the SSF.

Statistical Analysis

The results of the DCCR were analyzed using the software Statistica 7.0 (Statsoft, USA) at a confidence level of 95% and significance ($p < 0.05$) was assumed to establish a statistically significant relationship between the variables. All tests were performed in duplicate.

Results and Discussion

Influence of Residue Pretreatment on the Production of Cellulases

In the present study, the influence of residue pretreatment on the production of cellulases produced by *Aspergillus niger* IOC 3998 using the SSF was evaluated. Table 2 shows the composition of cellulose, lignin, ash, moisture and extractables for the treated and untreated waste. As shown in Table 2, residue after pretreatment resulted in a mass loss of 32.44%, leaving 67.56% of the final mass for fermentation. The chestnut seed (*Terminalia catappa Linn.*) treated with 2% (v/v) NaOH showed a greater amount of cellulose when compared to the chestnut without treatment, with an increase of almost 22%. The high reduction of hemicellulose (79.1%) and lignin (85.1%) in the treated waste is notable when compared with the waste without treatment. In conclusion, most of the material that was lost in the pretreatment was lignin and hemicellulose.

Residue	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ashes (%)	Moisture (%)
Untreated	20.5± 0.0	10.70± 0.0	23.6± 0.0	1.62± 0.0	18.70± 0.10
Pretreated with com 2.0% (v/v) NaOH	25.0± 0.0	2.24± 0.0	3.52± 0.0	1.46± 0.0	8.78± 0.78

Table 2: Composition of cellulose, lignin, ash, moisture and extractables for the chestnut seed (*Terminalia catappa Linn.*) untreated and pretreated with 0.2% (v/v) NaOH

Thus, it remains evident that the treated chestnut has a greater potential for the production of cellulases, since it has a lower amount of lignin and hemicellulose after treatment, and also has a good amount of cellulose that can induce the synthesis of cellulases by the fungus. The pre-treatment has been common for some substrates before SSF, facilitating access to the interece nutrient and helping the growth of the microorganism [22]. In fact, when using the SSF for 6 days, the untreated residue was able to produce 2.54 U/g and 0.053 U/g of CMCase and FPase, respectively. However, for residues pretreated with 2.0% (v/v) NaOH the values obtained were much higher, reaching 11.04 U/g of CMCase and FPase 0.34 U/g while achieved 11.08 U/g of CMCase and 0.36 U/g for FPase

when the residue was pretreated using 4.0% (v/v) NaOH. As previously mentioned, the pre-treatment with NaOH when removing hemicellulose and lignin, components that block the access of cellulose by the fungus, favors the expression of cellulases, thusthe use of 2.0% (v/v) NaOH is sufficient. Thus, this concentration was used to pre-treat the residue in subsequent tests.

Figure 1 shows the production of cellulases produced by *Aspergillus niger* IOC 3998 using the SSF and the chestnut seed (*Terminalia catappa Linn.*) pretreated with 2% (v/v) NaOH. For CMCase in 48 hours there was a rapid cellulase production with 12.42 U/g, followed by a reduction reaching the lowest value of 9.77 U/g in 96 hours, however increasing afterwards and reaching 12.73 U/g in 144h, or 6 days. This rapid decrease in enzyme activity may be due to an increase in the concentration of residues secreted in the medium during fermentation. The decrease in enzyme activity that occurs shortly after the rapid increase in enzyme activity at the beginning of fermentation is due to the formation of sugars produced by cellulose degradation [5,23].

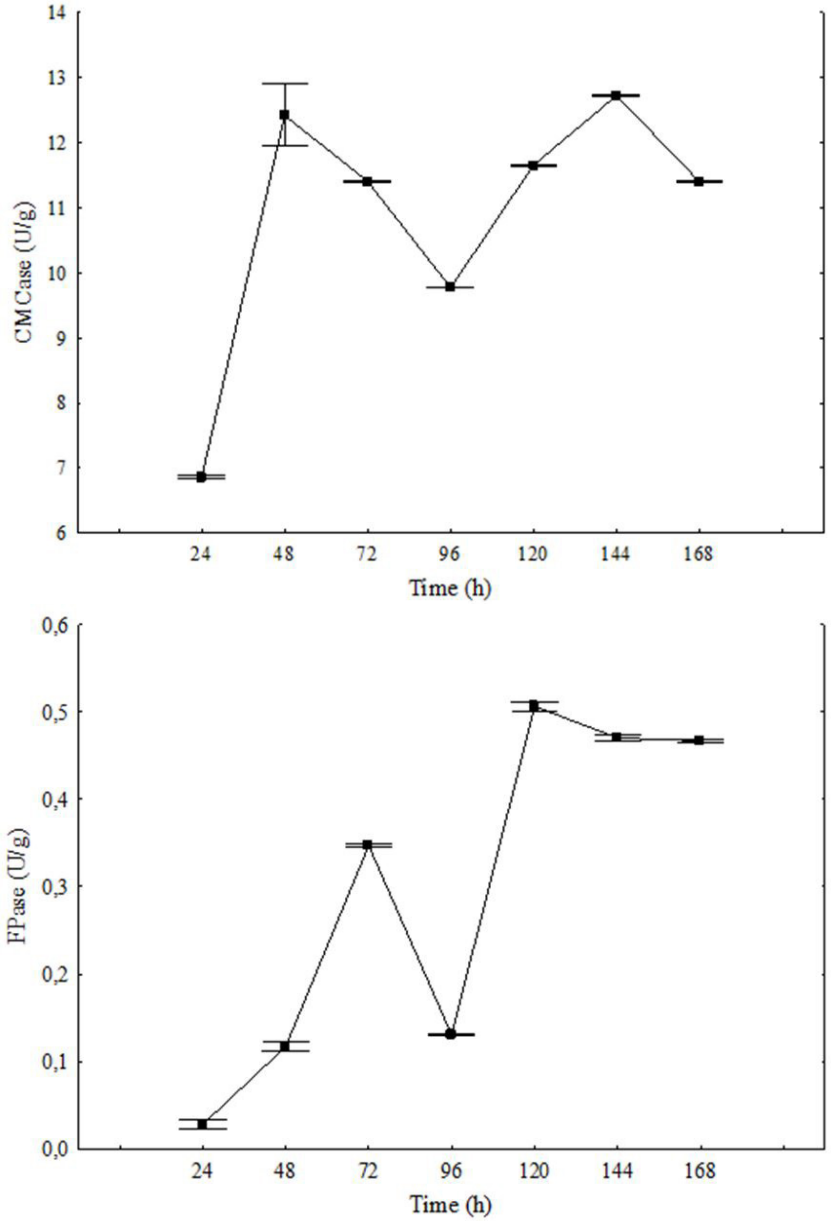


Figure 1: Production of CMCase and FPase during SSF cultivation of *Aspergillus niger* IOC 3998 using chestnut seed (Terminalia catappa linn) as substrate and pretreated using 2% (v/v) NaOH

Regarding FPase, the maximum production occurred in 120h, reaching a value of 0.51 U/g, however the following values of enzymatic activity are little changed, keeping cellulases production almost constant for up to 168h with 0.46 U/g. The slow decrease in enzyme activity may be related to the mixture of secondary compounds formed over the fermentation time, hindering the interaction of the substrate and microorganism [42]. CMCase has two hours for maximum value production, 48 hours and 144 hours (Figure 1), resulting 12.8 U/g while for FPase has a peak production of 0.51 U/g with 120 hours. In a similar way, Rodríguez-Zúñiga, *et al.* [25] using wheat bran also reported that there was a point of maximum enzymatic activity of endoglucanases and in the activity of total cellulose after 72 hours. The work of Cavalcante, *et al.* [26] also reported 72 hours time when using *Aspergillus niger* in their fermentations in FES whilst Santos, *et al.* [27] reported fermentation time for production of CMCase and FPase ranging from 70 to 80

hours when using *Aspergillus niger*. The use of the treated chestnut as a substrate may have been the cause of rapid production in 48 hours, since its consumption by the microorganism becomes easier.

Influence of the Nitrogen Source on the Production of Cellulases

An ideal substrate would offer necessary nutrients for the growth and good functioning of the microorganism, however some nutrients, in the substrate, may be in very low concentrations or not be present in the substrate and still be removed when the pretreatments are needed and, in many cases, supplementation of nutrients is needed. Therefore, different additional sources of nitrogen were used in the present study to evaluate the influence of this important nutrient on the enzymatic activity of CMCase and FPase. Table 3 shows the values of CMCase, FPase and total proteins obtained during cultivation. It is noteworthy that in this study, a carbon source (glucose) was also added to the nutrient solution (NS) with a concentration of 0.03g/mL.

Source	CMCase (U/g)	Fpase (U/g)	Protein (mg/mL)
Glucose + SN	**5.88± 0.67**	**0.04± 0.01**	**0.26± 0.03**
Ammonium sulfate + SN	12.92± 0.04	0.12± 0.01	0.48± 0.01
Peptone	9.13± 0.09	0.10± 0.01	0.44± 0.01
Nutrient solution	15.68± 0.99	0.13± 0.03	0.17± 0.02

Table 3: Influence of nitrogen during FES using *Aspergillus niger* IOC 3998 and chestnut seed (*Terminalia catappa Linn.*) as substrate

According to Table 3, none of the additional nitrogen sources increased the cellulase production when compared to the nutrient solution (NS). On the other hand, the addition of some of these sources resulted in a significant decrease in the production of cellulases. The addition of ammonium sulfate in the nutrient solution did not improve the cellulase production yield at the end of the fermentation. The addition of peptone did not improve the production of cellulase, a probable cause can be that it is a source of nitrogen and carbon, resulting in a decrease of cellulase production, leading the microorganism to preferentially consume the peptone instead of substrate, as observed when glucose was added according to Table 3. This efffect can be seen when glucose is added to NS resuting in values only 5.88 U/g and 0.04 U/g for CMCase and FPase, respectively. The addition of glucose made the fungus to consume this source, easily metabolizable, for its growth without the need to express cellulases [25,28,29]. The relationship between carbon and nitrogen in the substrate is important in SSF. Based on the results of the present study, it is evident that the nutrient solution used in the present study has enough nitrogen and other micronutrients to meet the needs of the fungus Aspergilus niger IOC 3998.

Optimizing the Production of Cellulases by SSF using the Central Rotational Composite Design (DCCR)

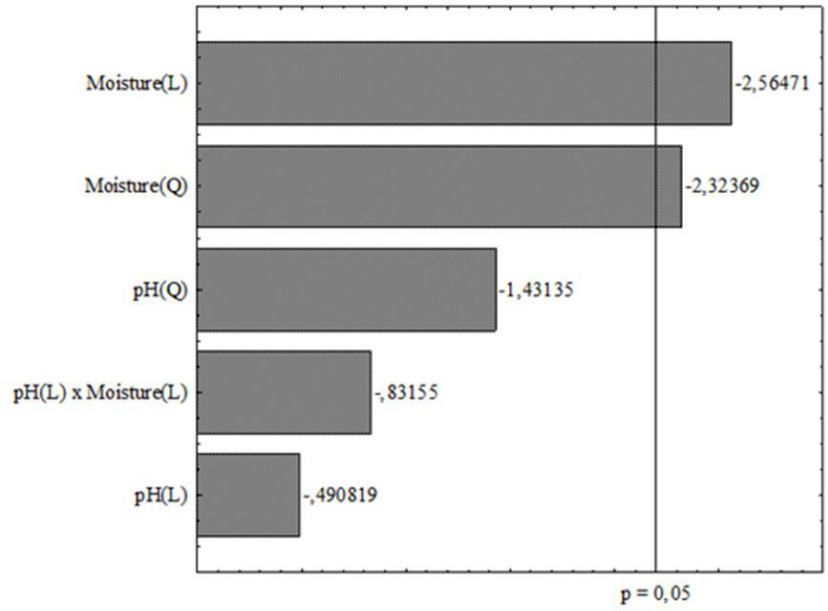
Run	pH	Moisture (%)	CMCase(U/g)	FPase(U/g)
1 (10)	**3.5**	**54**	**12,91 ± 0,44**	**0,08 ± 0,00**
			10,65 ± 0,04	0,07 ± 0,00
2 (7)	3.5	61,5	10,10 ± 0,03	0.00 ± 0.00
			10,34 ± 0.04	0.06 ± 0.00
3 (2)	5.5	54	10,13 ± 0,02	0,06 ± 0,00
			13,20 ± 0,02	0,12 ± 0,00
4 (9)	5.5	61,5	8,61 ± 0,02	0,19 ± 0,00
			9,16 ± 0,01	0,23 ± 0,00
5 (1)	3	58	10,06 ± 0,05	0.14 ± 0,00
			10,08 ± 0,04	0,14 ± 0,00
6 (5)	6	58	10,95 ± 0,02	0,07 ± 0,00
			9,70 ± 0,02	0,07 ± 0,00
7 (8)	4.5	52	10,02 ± 0,06	0,06 ± 0,00
			9,82 ± 0,04	0,08 ± 0,00
8(4)	4.5	63.5	9,34 ± 0,02	0,15 ± 0,00
			9,36 ± 0,02	0,22 ± 0,01
9(3)	4.5	58	11,44 ± 0,02	0,13 ± 0,01
			12,03 ± 0,01	0,09 ± 0,00
10 (6)	4.5	58	11,82 ± 0,03	0,09 ± 0,00
			10,06 ± 0,04	0,08 ± 0,00

Table 4: DCCR to evaluate the influence of pH and moisture on the substrate in the production of cellulases (CMCase and FPase) during FES using *Aspergillus niger* IOC 3998 and the chestnut seed (*Terminalia catappa Linn.*) as substrate

In this study, the use of an experimental design (DCCR) allowed the investigation of the influence of pH and moisture on the production of cellulolytic enzymes (CMCase and FPase) by SSF with *Aspergillus niger* IOC 3998 using as substrate the chestnut seed (*Terminalia catappa Linn.*). Table 4 illustrates the results of the SSF operating at 35 °C after 6 days. The experimental planning was carried out in duplicate in order to increase the system's degrees of freedom in estimating deviations. The pH range was based on the fact that for *Aspergillus niger*, acidic pH is more favorable for fermentation [30,31].

It is noteworthy that enzymatic activity for CMCase higher than 8.8 U/g was observed while for FPase a maximum of 0.75 U/g was reached (run 8). Moisture was the only significant factor in the production of CMCase according to the Pareto diagram, shown in Figure 2A, being the best activity for CMCase ranging from 11.78 to 12.04 U/g) obtained with moisture at 54%-58%. It is observed that there is a negative correlation between moisture and CMCase activity since its increase induces lower values for enzyme activity. The work of Oliveira, *et al.* [14] also reported when using coconut fiber as a substrate in SSF that moisture influenced the production of cellulases. Pandey [22] commented that the increase in moisture can cause its compaction of the solid matrix causing the microorganism and nutrients to fail to penetrate the substrate, thus causing a decrease in the production of cellulases.

While regarding pH, the best results were obtained at pH 5.5, that is, the best pH range for cellulase production is in low acid conditions, close to neutral pH. However, as illustrated by Figure 2 and by the response surface (Figure 3), there was a wide range for pH that guarantees good CMCase activity.



(A)

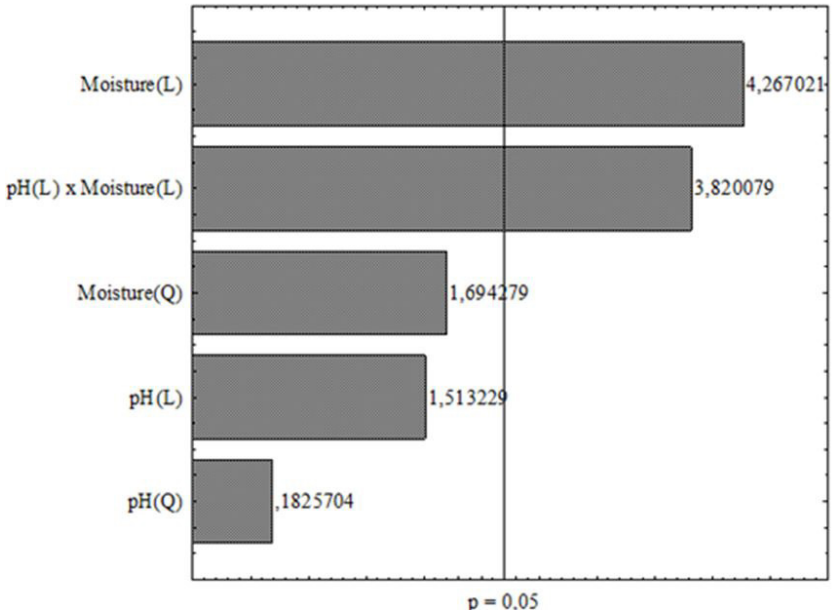


Figure 2: Pareto chart for DCCR investigating the influence of pH and moisture on CMCase (A and B) during SSF cultivation of *Aspergillus niger* IOC 3998 using chestnut seed (*Terminalia catappa Linn.*) as substrate

Regarding the activity of FPase (Figure 2B), it is noteworthy that the moisture had a positive influence on the enzymatic activity, that is, the higher the moisture, the greater the enzymatic activity of FPase. In this case, the interaction of pH with moisture was also significant, which is in accordance with the response surface shown in Figure 3. The increase in moisture increases the production of FPase within the studied humidity chamber. The production of FPase will also depend on the pH, when increasing the pH it is necessary to increase the humidity so that the production reaches its maximum value within the studied moisture and pH range. When lowering the pH, a smaller amount of moisture is necessary for the production of the enzyme.

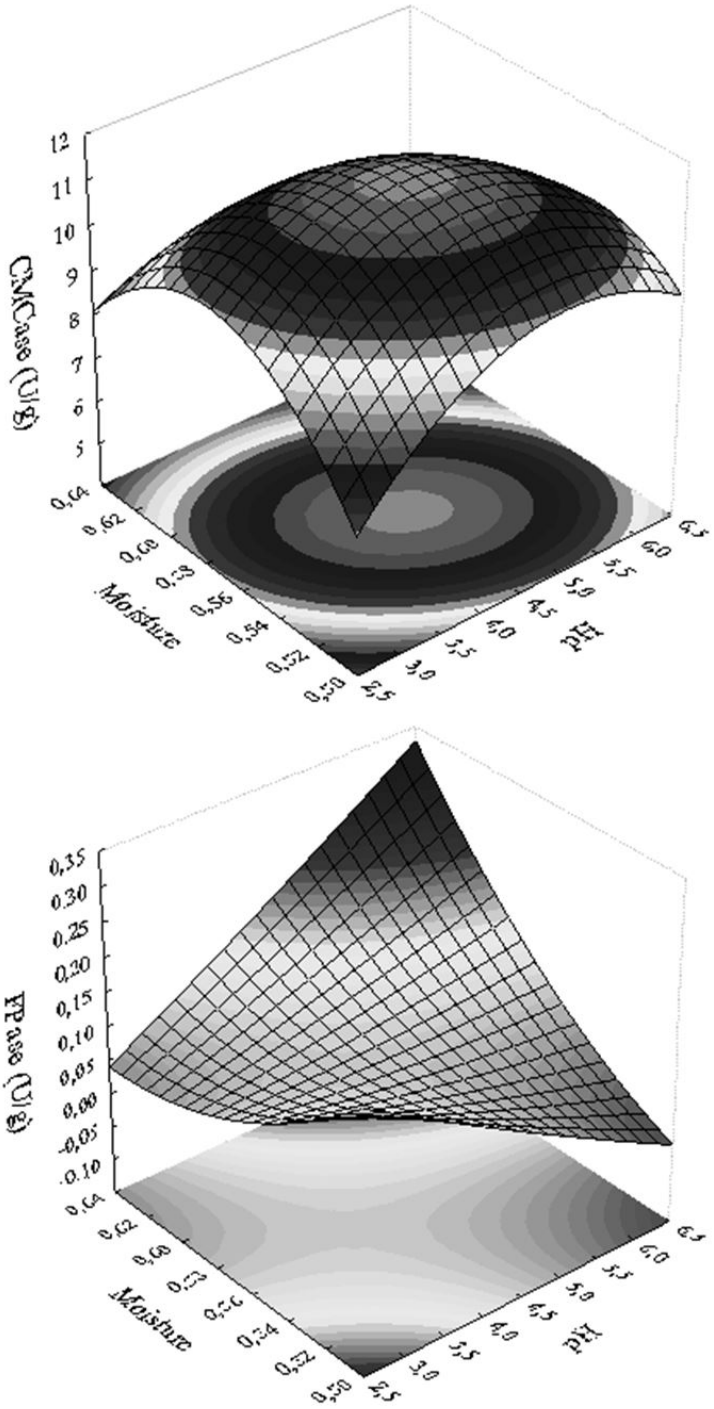


Figure 3: Response surface to the DCCR investigating the influence of pH and moisture on CMCase (left) and FPase (right) during SSF using *Aspergillus niger* IOC 3998 and castanet seed (*Terminalia catappa Linn.*) as substrate.

Evaluation of the Stability of CMCase and FPase Activities

Figure 4 shows the values of the relative activity of CMCase and FPase at different pHs after 1h of incubation at 50 °C. CMCase was more stable at pH 5.0, maintaining 74% of the relative activity. At the same pH, FPase showed 27% of the relative activity. Regarding FPase, it remained more stable at pH 7.0, maintaining 43% of its activity, while CMCase with 46% of relative activity. Kang *et al.*

(2004) also reported that the best value of FPase enzyme activity was achieved at pH 7 when A. niger cellulase was produced. The work of Oliveira, *et al.* [14] observed that the activity of CMCase and FPase were more stable at very low pH (pH 2.0) and FPase was more sensitive to pH change, when using sugarcane bagasse as a substrate for the fungus A. fumigatus using SSF. Inforsato and Porto [32] observed that the highest quantity of cellulases by Aspergillus sp. occurred at pH 6 [33-35] (Figure 4).

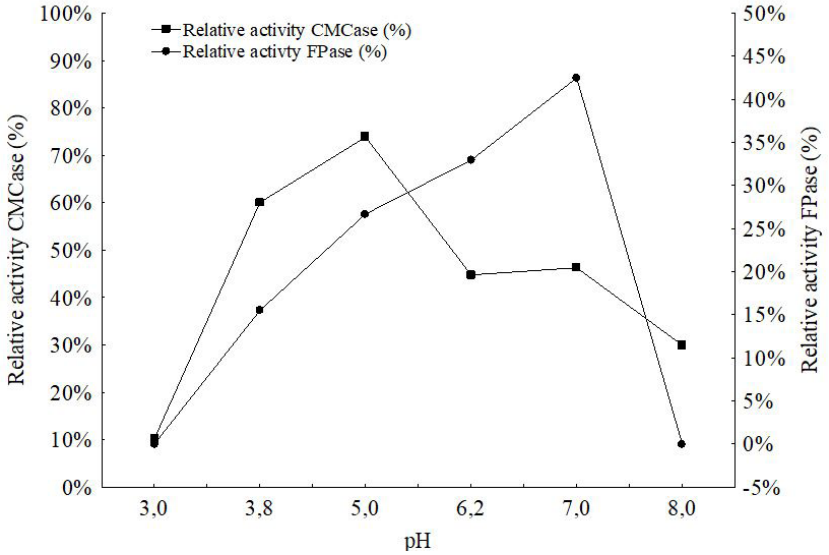


Figure 4: Influence of pH on enzyme stability for cellulases (CMCase and FPase) produced by SSF cultivation of *Aspergillus niger* IOC 3998 using chestnut seed (Terminalia catappa linn) as substrate

Figure 5 shows the values of the relative activity for CMCase and FPase at different temperatures. The activities of CMCase and FPase proved to be stable from 30 °C to 50 °C, maintaining values of relative activity between 80% and 100% [36,37]. After 50 °C, the denaturation of the enzymes was observed. However, even at 70 °C a residual activity of about 38% is shown for CMCase while about 20% was found to FPase. Oliveira, *et al.* [14] reported in their study that the best condition found for the CMCase and FPase was at 72% at 60 °C and 33% at 50 °C respectively. The work of Dos Santos, *et al.* [27] observed that the residual activity obtained by Aspergilus niger remained stable at a temperature of 60 °C for 90 minutes. Stati [38] used *Aspergillus niger* in submerged fermentation to produce cellulase from alkaline residues reporting the best production was at 60 °C whereas for acid residues it was at 45 °C. Therefore as shown in the present study the use of a residue can be better used for cellulases production that current play a key role on the bioethanol production.

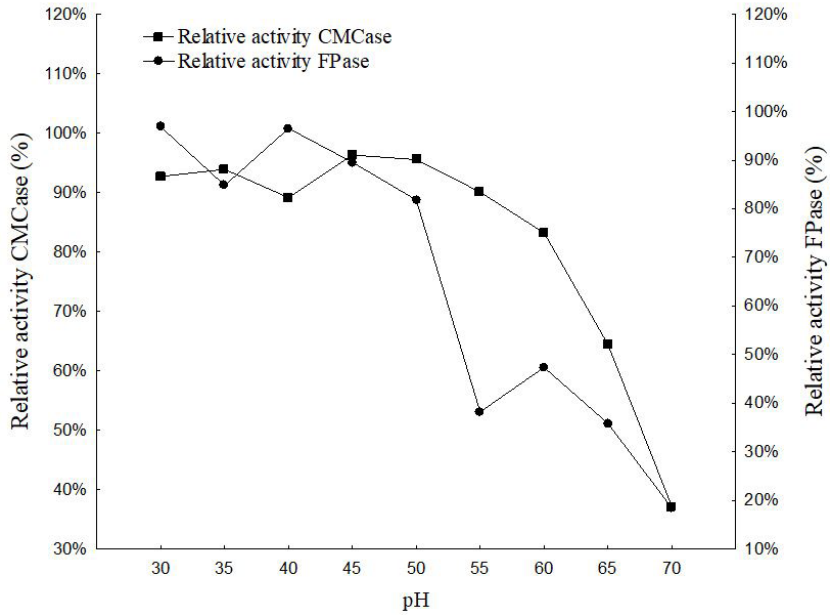


Figure 5: Influence of pH on enzyme stability for cellulases (CMCase and FPase) produced by SSF cultivation of *Aspergillus niger* IOC 3998 using chestnut seed (Terminalia catappa linn) as substrate

Conclusion

Secondary occupation did not have a significant relationship with crop farmers safe farming practices ($\chi 2$ =6.06, p=0.108). This implies that the secondary occupation crop farmers got themselves involved, did not influence their farming safety practices other than providing alternative sources of income for better social and economic well-being. The farm size of the respondents did not have a significant relationship with respondents' safe farming practices ($\chi 2$ =4.05, p=0.105). This indicates that respondents' farm size did not influence the safe farming measures adopted by them to prevent possible farm hazards. Respondents average monthly income had no significant relationship on their farming safety practices ($\chi 2$=5.53, p=0.137). This implies that respondents' average monthly income did not influence their safe farming practices. This could be that the respondents paid little or no priority to cropping safety practices as a result of overwhelming financial and social respondents as pressing needs, limiting their financial commitments to cropping safety practices.

Based in the results obtain, the treatment of chestnut seeds (*Terminalia catappa Linn.*) favored the induction of cellulases by SSF when using *Aspergillus niger* IOC 3998. The pretreatment with NaOH 2% proved to be very efficient since most of the material that was lost in the pretreatment was lignin and hemicellulose, leaving most of the cellulose present in the material after treatment. Furthermore, the humidifying solution was effective in the production of cellulases without the need for adding other nutrient sources to the substrate. The activity of CMCase and FPase are mainly influenced by moisture, and the best result for CMCase was 13.2 U/g when using pH 5.5 and 54% moisture. Moreover, it must be highlight that for CMCase the pH did not show any significant influence. In assessing stability at different temperatures, both CMCase and FPase were more stable from 30°C to 50°C. In addition, the CMCase was more resistant to temperature increase while FPase maintained 81.83% of relative activity at 50°C.

Acknowledgment

The authors thank CAPES and Brazilian National Council for Research (CNPq) for the financial support (Grants: 305211/2017-1). CAPES supported the scholarship of Viviane Hiromi Uchida (Finance Code 001).

References

1. Sudhanshu SM, Rao N, Menganhi E (2012) Phytochemical and Antimicrobial Screening against effect of Environmental Pollution. Int J Pharm Sci Res 3: 1000-4.

2. Araujo CKC, Campos AO, Padilha CEA, Sousa Junior FC, Nascimento RJA, et al. (2017) Enhancing enzymatic hydrolysis of coconut husk through Pseudomonas aeruginosa AP 029/GLVIIA rhamnolipid preparation. Bioresour Technol 237: 20-6.

3. Silva FL, Campos AO, Santos DA, Oliveira Junior SD, Padilha CEA, et al. (2018) Pretreatments of Carnauba (Copernicia prunifera) straw residue for production of cellulolytic enzymes by Trichoderma reesei CCT-2768 by solid state fermentation. Renew Energ 116: 299-308.

4. Padilha CEA, Nogueira CC, Souza DFS, Oliveira JA, Santos ES (2019) Valorization of green coconut fibre: Use of the black liquor of organosolv pretreatment for ethanol production and the washing water for production of rhamnolipids by Pseudomonas aeruginosa ATCC 27583. Industrial Crops and Products 140: 111604.

5. Goncalves FA, Ruiz HA, Nogueira CC, Santos ES, Teixeira JA, et al. (2014) Comparison of delignified coconuts waste and cactus for fuel-ethanol production by the simultaneous and semi-simultaneous saccharification and fermentation strategies. Fuel 131: 66-76.

6. Souza Filho PF, Ribeiro VT, Santos ES, Macedo GR (2016) Simultaneous saccharification and fermentation of cactus pear biomass—evaluation of using different pretreatments. Ind Crops Prod 89: 425-83.

7. Guilherme AA, Dantas PVF, Soares JCJ, Santos ES, Fernandes FAN, et al. (2017) Pretreatments and enzymatic hydrolysis of sugarcane bagasse aiming at the enhancement of the yield of glucose and xylose. Braz J Chem Eng 34: 937-47.

8. Linton SM (2020) Review: The structure and function of cellulase (endo-β-1,4-glucanase) and hemicellulase (β-1,3-glucanase and endo-β-1,4-mannase) enzymes in invertebrates that consume materials ranging from microbes, algae to leaf litter. Comp Biochem Physiol B 240: 110354.

9. Abubakar FA, Oloyede OB (2013) Production and activity of cellulase from *Aspergillus niger* using rice bran and orange peel as substrates. Int J Sci Res Managet 1: 285-91.

10. Pothiraj C, Balaji P, Eyini M (2006) Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste. Afr J Biotechnol 5: 1882-85.

11. Nogueira CC, Padilha CEA, Leitão ALS, Rocha PM, Macedo GR, et al. (2018) Enhancing enzymatic hydrolysis of green coconut fiber-Pretreatment assisted by tween 80 and water effect on the post-washing. Ind Crops Products 112: 734-40.

12. Nogueira CC, Padilha CEA, Jesus AA, Souza DFS, Assis CF, et al. (2019) Pressurized pretreatment and simultaneous saccharification and fermentation with in situ detoxification to increase bioethanol production from green coconut fibers. Ind Crop Prod 130: 259-66.

13. Gouveia N (2012) Urban solid waste: socio-environmental impacts and perspective of sustainable management with social inclusion. Sci Collective Health (Resíduos sólidos urbanos: impactos socioambientais e perspectiva de manejo sustentável com inclusão social. Ciência & saúde coletiva) 17: 1503-10.

14. Rolim PM, Oliveira Júnior SD, Oliveira ACSM, Santos ES, Macedo GR (2018) Nutritional value, cellulase activity and prebiotic effect of melon residues (Cucumis melo L. reticulatus group) as a fermentative substrate. J Food Nutr Res 57: 315-27.

15. Prado FC, Vandenberghe LPS, Woiciechowski AL, Rodrigues-Leon JA, Soccol CR (2005) Citric acid production by solid-state fermentation on a semi-pilot scale using different percentages of treated cassava bagasse. Braz J Chem Eng 22: 547-55.

16. Cerda A, El-Bakry M, Gea T, Sanchez A (2016) Long term enhanced solid-state fermentation: inoculation strategies for amylase production from soy and bread wastes by Thermomyces sp. in a sequential batch operation. J Environ Chem Eng 4: 2394-401.

17. Lizardi-Jimenez MA, Hernández-Martínez R (2017) Solid state fermentation (SSF): diversity of applications to valorize waste and biomass. 3 Biotech 7: 44.

18. Ezeokonkwo CA, Dodson WL (2004) The potential of Terminalia catappa (tropical almond) seed as a source of dietary protein. J Food Qual 27: 207-19.

19. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, et al. (2008) "Determination of Structural Carbohydrates and Lignin in Biomass," Technical Report NREL/TP-510-42618. Laboratory Analytical Procedure (LAP) NREL 2008, National Renewable Energy Laboratory, Golden, Colorado.

20. Urbánszki K, Szakacs G, Tengerdy RP (2000) Standardization of the filter paper activity assay for solid substrate fermentation. Biotechnol Lett 22: 65-9.

21. Pandey A (2003) Solid-state fermentation. Biochem Eng J 13: 81-4.

22. Goncalves FA, Ruiz HA, Santos ES, Teixeira JA, Macedo GR (2016) Bioethanol production by *Saccharomyces cerevisiae, Pichia stipitis* and *Zymomonas mobilis* from delignified coconut fibre mature and lignin extraction according to biorefinery concept. Renew Energy 94: 353-6.

23. Ye Z, Hatfield KM, Berson RE (2014) Relative extents of activity loss between enzyme–substrate interactions and combined environmental mechanisms. Bioresource Technol 164: 143-8.

24. Rodriguez-Zuniga UF, Farinas CF, Bertucci Neto V, Couri S, Crestana S (2011) Production of cellulases by *Aspergillus niger* by solid state fermentation. Brazil Agri Res (Produção de celulases por *Aspergillus niger* por fermentação em estado sólido. Pesquisa Agropecuária Brasileira) 46: 912-9.

25. Cavalcante PAW, Coelho DF, Silva CF, Abud AKS, de Souza RR (2018) Use of lignocellulosic residues in the production of cellulases by *Aspergillus niger* in solid state fermentation. (Utilização de resíduos lignocelulósicos na produção de celulases por *Aspergillus niger* em fermentação em estado sólido). Scientia Plena 14(6).

26. dos Santos TC, Rocha TJO, Oliveira AC, Abreu Filho G, Franco M (2013) Aspergellus niger as a producer of cellulolytic enzymes from cocoa bran (Theobroma cacao). Arch Biol Institute (Aspergellus niger como produtor de enzimas celuloliticas a partir farelo de cacau (Theobroma cacao). Arquivos do Instituto Biológico) 80: 65-71.

27. Chandra MS, Viswanath B, Reddy BR (2007) Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. Indian J Microbiol 47: 323-8.

28. Olsson L, Christensen TMIE, Hansen KP, Palmquist EA (2003) Influence of the carbon source on production of cellulases, hemicellulases and pectinases by Trichoderma reesei Rut C 30. Enzyme and Microbial Technology 33: 612-9.

29. Palma-Fernandez ER, Gomes E, Silva R (2002) Purification and characterization of two β -glucosidases from the thermophilic fungus Thermoascus aurantiacus. Folia Microbiologica 47: 685-90.

30. Gomes E, Guez MAU, Martin N, Silva R (2007) Thermostable enzymes: sources, production and industrial application. New Chemistry (Enzimas termoestáveis: fontes, produção e aplicação industrial. Química Nova) 30: 136-45.

31. Inforsato FJ, Porto ALM (2016) Enzymatic activity of cellulases by the DNS method of fungi Isolated from germinating seeds. Brazilian Journal of Renewable Energies. (Atividade enzimática de celulases pelo método DNS de fungos Isolados de sementes em germinação. Revista Brasileira de Energias Renováveis) 5: 444-65.

32. Calado VMM, Dillon AJP, Salgueiro AA (2007) Cellulases production by Humicola grisea strains under submerged culture. (Produção de celulases por linhagens de Humicola grisea sob cultivo submerse). R Cienc Tecnol 1: 1-6.

33. Ghose TK (1987) Measurement of cellulase activities. Pure and Appl Chem 59: 257-68.

34. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chem 31: 426-8.

35. Oliveira SD, Padilha CEA, Asevedo EA, Pimentel VC, Araujo FR, et al. (2017) Utilization of agroindustrial residues for producing celulases by Aspergillus fumigatus on Semi-Solid Fermentation. J Environ Chem Eng 6: 937-44.

36. Siqueira TMO, Abreu MJ (2016) Closing the cycle of organic waste: composting inserted in urban life. Sci Cult (Fechando o ciclo dos resíduos orgânicos: compostagem inserida na vida urbana. Ciência e Cultura) 68: 38-43.

37. Stati VF (2018) Optimization of cellulase production using cellulosic waste and Environmental Technology) - University of the Sacred Heart, Bauru. (Otimização da produção de celulase empregando resíduos celulósicos e Tecnologia Ambiental) - Universidade do Sagrado Coração, Bauru).