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# PRODUCTION OF GLYCOHYDROLASES FROM RESIDUES FROM THE FLOUR INDUSTRY BY *Aspergillus niger*

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## Abstract

The liquid from cassava processing and the peel are agro-waste produced by the flour industry, a sector of great economic importance in Brazil. These residues can cause environmental impacts when disposed of improperly, due to their high concentration of carbohydrates and chemical demand for oxygen - COD. Faced with this demand, the objective of the present study was to carry out the bioremediation of cassava and cassava peel aiming at the production of glycohydrolases by two strains of *Aspergillus niger*, DR02 and AN 400. The tests took place in agitated medium, for 144 hours. The analyzes carried out were: glucose, pH, COD and the enzymes glucoamylase, xylanase and α -amylase. The results obtained showed a maximum COD reduction of 76 and 78% - DR02 and AN 400, respectively. The highest production of enzymes detected during this bioprocess, for the strains of A. niger DR02 and AN 400, were α-amylases (11.78 and 3.64 U/mL), glucoamylases (8.21 and 3.80 U/ml) and xylanases (2.66 and 1.43 U/ml), respectively. In view of the results reported here, it can be inferred that mycoremediation can be an alternative to manage waste from the flour industry, simultaneously generating value-added products that can be used in the food industry.

**Keywords:** biodegradation, remediation, fungi.

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## Introduction

The cassava flour industries have a waste generation problem, responsible for environmental contamination, due to the high amount of garbage accumulation (Li *et al*., 2019). They generate both solid and liquid by-products, which, when disposed in the open field, cause environmental problems. Their rapid decomposition contaminates water bodies, in addition to emanating strong and offensive odors, becoming a nuisance for the producer (Cavalcanti *et al*., 2020). However, the use of these agro-industrial residues is a promising alternative for of low-cost substrate in bioprocesses. These residues represent one of the best carbon reservoirs in nature, considering their high amounts of carbohydrates. Research have been carried out on the effective use of these residues for large-scale biohydrogen production, biosurfactant, bioplastics, citric acid (Cavalcanti *et al*., 2020). Figure 1 presents the most common process for the manufacture of cassava flour.

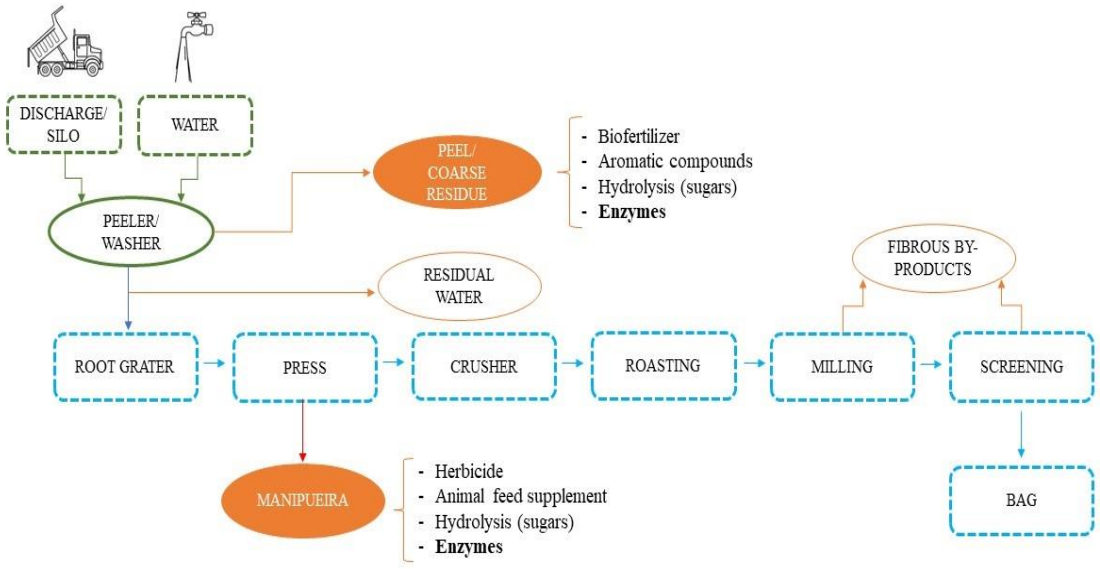


**Figure 1.** Flowchart of cassava flour production and waste generated. Use of cassava peel and manipueira *in natura* residues.

The first residue from the cassava flour manufacturing process is cassava husk. Residue with high carbohydrate content, is formed by brown husk, between bark and coarse residue, as cassava butts (Costa *et al*., 2022). Utilization of the peel is limited by its low digestibility and toxicity from high levels of cyanogenic glycosides (Ubalua, 2007). The second residue called *manipueira* in

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Brazil is generated from the pressing of cassava roots and consists of tuber water mixed with root washing water (Li *et al*., 2019). This liquid is rich in sugars, starch, proteins, salts, nitrogen, phosphorus, potassium and others substances (Costa *et al.*, 2022). Cassava root generates at least 300 L of wastewater per ton of processed root and has a high chemical oxygen demand (COD), which can reach 62 g/L (De Carvalho *et al*., 2018; Santos Ribeiro *et al*., 2019).

The residue generated by the manioc processing can then be used as a substrate for enzyme production, as reported by Gusmão *et al.* (2018), where the authors observed the capacity of fungal strains isolated from their own manipulation to produce enzymes of economic interest. The maximum production of pectinase, xylanase, amylase and CMCase was observed for the species: *Aspergillus sp*. B5C; at 120 hours (163.6 ± 0.13 nKat $mL^{-1}$), due to *Aspergillus sp*. B4I; at 144 hours (99.8 ± 0.24 nKat $mL^{-1}$), by Penicillium sp. B3A; e at 48 hours (55.5 ± 0.21 nKat $mL^{-1}$), due to *Aspergillus sp. B4O.* These enzymes can contribute to advances in the bioeconomy, increasing their various uses in biotechnology, as has been reported by Filiatrault-Chastel *et al*. (2021).

The potential use of fungi for the treatment of organic polluted ecosystems has stimulated interest in the exploitation of agricultural waste as substrates for enzyme production (Robl *et al*., 2015). *A. niger* is one of the most important fungi in bioprocess industry and has been applied in agri-food areas (Troiano, Orsat and Dumont, 2020; Li *et al*., 2020). Many of their products are classified as GRAS (Generally Considered Safe) under the Food and Agriculture Organization of The United Nations (FAO) safety examinations of the food industry. Therefore, food processing safety, excellent protein secretion capacity and low-cost culture make *A. niger* one of the most important species to produce food quality products (Li *et al*., 2020).

Faced with the possible problems caused by the inadequate disposal of residues from the flour industry in the environment, some studies have been carried out with proposals for the use of this effluent (Coutinho Rodrigues *et al*., 2021; Cavalcanti *et al*., 2020; Santos Ribeiro *et al*., 2019). In view of this problem, the present study aimed to investigate the decrease in chemical oxygen demand - COD and the production of glycohydrolytic enzymes using manipueira and cassava peel as a carbon source for the fungus *Aspergillus niger* by two trains, AN 400 and DR02.

## Materials and methods

*Waste from the flour industry*

The *manipueira* and solid residue of cassava peeling was transferred by the cassava processing industry Rocha Alimentos, located in the city of Sangão/SC-BR. The manipueira collected was acquired directly at the exit of the pressing process, during the manufacture of the flour, and transported in 5 L bottles. The solid residue was collected from the root peeling tank outlet and transported in 3 kg plastic bags at room temperature to the Laboratory of Microorganisms and Biotechnological Processes (LAMPB) of the Federal University of Santa Catarina (UFSC) and stored in a freezer at -22°C.

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To obtain the flour from the peel, the solid residue was dehydrated at 60ºC in a drying oven with mechanical circulation (Quimis) in trays and crushed in a Willye mill, model TE-650/1-TECNAL, to obtain some undefined granulometry flour.

*Spore production*

Production of spores of the fungus *A. niger*, strains AN 400 and DR02, from LATAM (IFCE) and LAMPB (UFSC), respectively, PDA (Potato Dextrose Agar) sterilized in Erlenmeyer (121 °C for 15 minutes) was used. After being sterilized, the media were transferred to glass petri dishes and then seeded using the spread plate technique. The media were transferred to Petri dishes and incubated in a microbiological oven at 28°C for seven days. At the end of the incubation period, the surfactant Tween 80 was added to each plate, so that spores could be released.

After applying the surfactant, the spores were removed from the plate using a previously sterilized pipette, forming a suspension which was stored in amber glass under refrigeration at 4°C. The spores in the suspension were counted in a Neubauer chamber, according to the methodology by Sampaio (2005).

*Culture medium*

For the production of α-amylase, glycoamylase, xylanase enzymes and determination of chemical oxygen demand, culture medium was prepared with distilled water and unfiltered manipueira (30% v/v); peel flour - 10.0 g/L; peptone - 1.0 g/L; Vishiniac Solution: $ZnSO_4.7H_2O$, 4.4 g/L; $MnCl_2.4H_2O$, 1.0 g/L; $CoCl_2.6H_2O$, 0.32 g/L; $CaCl_2.2H_2O$, 1.47 g/L; $FeSO_4.7H_2O$, 1.0 g/L; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.22 g/L. The medium was sterilized in an autoclave at 121°C for 15 minutes.

*Shake flasks cultures*

The composition of the culture medium was adapted from Marinho *et al*. (2011). For each strain (DR02 and AN 400), assays were done in triplicate. A 100 mL volume of culture medium was transferred to 250 mL Erlenmeyer flask and inoculated with 2.0 x $10^6$ spores/mL. The six flasks were incubated in orbital shaking, TE-421, TECNAL, at 120 rpm, at 28 °C for 144 hours.

Two six vials, three or a half of culture with the handle and the species *A. niger* AN 400 and the other three vials or a half of culture with the handle and the species A niger DR02. Were taken daily and centrifuged at 13000 rpm for 15 minutes (mini-spin - Eppendorf) to remove the biomass. The supernatant was used to determine the enzymatic activity in triplicate by quantifying reducing sugars (glucose) using the Miller (1959) - 3-5-dinitrosalicylic acid (DNS) method.

*Glucose determination*

Glucose concentration was determined with Liquiform Glucose Labtest®.

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*Determination of Chemical Oxygen Demand (COD)*

The COD measurement was carried out only in the liquid fraction of the medium, at the beginning and at the ed of the reactor operation. The COD was analyzed according to Standard methods for the examination of water and wastewater, 21th ed., APHA, AWWA, Washington, DC, USA; 2012.

*Determination of enzyme activity*

The measurement of enzymatic activity was performed in microplate and incubated in thermocycler (Mastercycler Personal 5332 - Eppendorf). For glycoamylase, a mixture containing 20 µL of the enzyme extract, 30 µL of citrate buffer (0.05 M - pH 5), 50 µL of starch (0.5% w/v) and incubated at 50°C for 10 minutes. The reaction was stopped by adding 100 µL of the Miller (1959) reagent, incubated at 95°C for 5 minutes and measured at 540 nm (Bio-TEK EL800). The same procedure was performed to determine xylanase, using Beechwood xylan (0.5% w/v). One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute from the polysaccharide used.

The determination of α-amylase was adapted from Xiao *et al.* (2006). A mixture containing 20 µL of enzyme extract, 20 µL of phosphate buffer (0.1 M - pH 6), 20 µL of starch (2 g/L) and 20 µL of distilled water was incubated at 50°C for 30 minutes. The reaction was interrupted by the addition of 20 µL of HCl (1M), followed by the addition of 100 µL of the iodine reagent (5 mM $I_2$ and 5 mM KI). The mixture was measured at 595 nm (Bio-TEK EL800). One unit (U) for the microplate-based starch–iodine assay is defined as the disappearance of an average of 1 mg of iodine binding starch material per min in the assay reaction.

*Statistical Analysis*

Statistical analyses were performed using the Statistics software (version 13.5). All data collected were subjected to one-way analysis of variance (ANOVA) and the significance of the differences between means was tested using Tukey's ($p<0.05$).

## Results and discussion

*Evaluation of pH and glucose in the medium*

The Figure 2 (a) presents the pH for fermented extracts in the different strains of *A. niger*. The initial pH of the cultures was 4.92 ± 0.02, indicates the acidic nature of the *manipueira*. Magalhães *et al*. (2019) stated that fungi from *Aspergillus* genera growth abundantly in aerobic processes with acidic medium, thus, in this work, the growth of the microorganism may have been favored by the pH of the medium.

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Until 72 h of the assay, the probable accumulation of acids in the culture medium seemed to be responsible for the pH variation from 4.92 to 4.76 ± 0.09 and 4. 86 ± 0.04, DR02 and AN 400, respectively. Acidification seems to be an important strategy for filamentous fungi to facilitate or even improve growth conditions (Melzer *et al*., 2007).

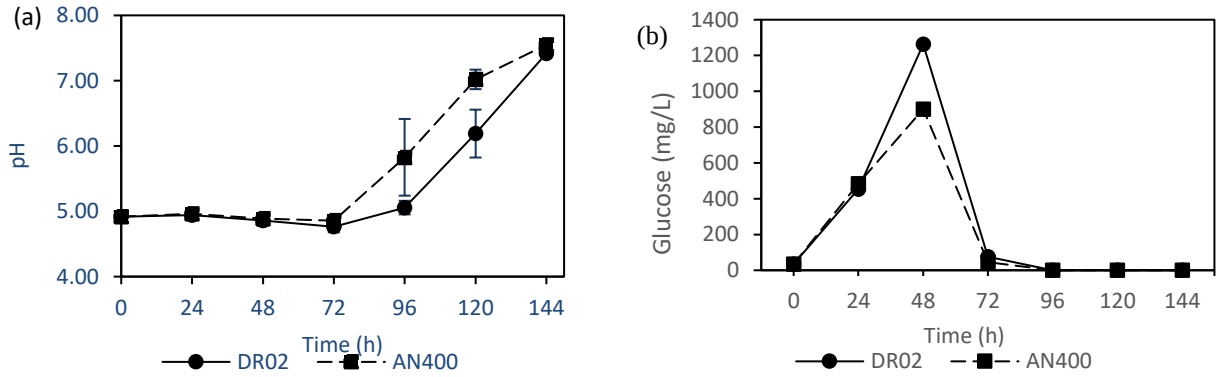


**Figure 2.** a) pH values for fermented extracts in the different strains of *A. niger*; b) Glucose consumption for the different strains of *A. niger.*

After 96 h of cultivation, the pH (5.06 ± 0.10 and 5.83 ± 0.59) increased until 144h (7.42 ± 0.02 and 7.55 ± 0.07) in the DR02 and AN 400 strains, respectively, at the same time points of glucose exhaustion occurs, Figure 2(b). An adaptation phase of fungal growth can be seen in the first 24 h with glucose production in 32% and 41% by the DR02 and AN 400 strains, respectively. This may be caused by cassava polysaccharides hydrolysis.

The highest glucose production occurred in 48 h (1262.9 mg/L ± 5.1 - DR02 and 900.6 ± 3.2 mg/L – AN 400) when a greater amount of biomass formed was already visually observed. *A. niger* strains grew vigorously at 28°C at this time, but cell mass could not be determined due to the insoluble carbon source. However, it is observed a decrease in glucose concentration from 72 h of 94% for the two strains (76.6 ± 1.1 mg/L - DR02 and 45.7 ± 0.0 mg/L – AN 400).

*Biotreatment performance by A. niger DR02 and AN 400 strains*

To improve the process of bioremediation of manipueira by *A. niger*, the mixture of manipueira (30%) with cassava peel flour (10 g/L) with different strains of *A. niger* was evaluated.

COD was measured at the beginning and end of the experiments (0h and 144h). At 0 h the value determined was 10.09 ± 0.36 g/L. Coutinho Rodrigues *et al*. (2021) worked with a sample of pure manipueira from three agro-industries located in cities in the state of Santa Catarina and obtained

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an initial COD of 90 g/L (Imaruí), 79 g/L (Jaguaruna) and 92 g/L (Paulo Lopes). The value observed in the literature for this variable was higher than the initial value in this study. The compilation of results from other studies demonstrates that the composition of manipueira will change according to it's the form of cultivation of the cassava species.

At 144 h, a total removal COD of 76% (2.38 ± 0.48 g/L) and 78% (2.19 ± 0.33 g/L) was observed for the strains DR02 and AN 400, between pH 7.42 and 7.55, respectively. These results showed that the strains presented the same reduction profile and a high COD removal.

In line with the present study, Arikan *et al.* (2020) evaluated the decrease in chemical oxygen demand (COD) using filamentous fungi to biodegrade effluent from the wheat industry, with a pH of 4.5. Among the tested species, A. parasiticus showed the highest COD removal efficiency (77.5%) at the end of the incubation period (9 d), which may indicate the fungi affinity for the acidic medium.

*Production of enzymes by A. niger DR02 and AN 400 strains*

In this study, submerged cultures were used to bioremediate and simultaneously produce α-amylase, glucoamylase and xylanase by *A. niger* strains in medium prepared with agro-industrial cassava residues. Despite strains DR02 and AN 400 belonging to the same genus, and having similar morphologies (rough dark brown conidia, spherical vesicles and biseriate conidiophores), they had different enzymatic profiles. Figure 3 shows the activities of α-amylase, glucoamylase and xylanase over 144 h of fermentation.

It was found that both strains presented different times for higher enzyme production. The DR02 strain showed its peak production in 96 h and the AN 400 strain showed its production peak at 48 h, except for α-amylase production. The fungi amylolytic and xylanolytic titers differed among strains under the same conditions tested. The DR02 strains showed the highest activity for α-amylase and glycoamylase. However, this was not observed when the AN 400 was used. Low levels of xylanase activity were detected for the two strains, although the strain DR02 highlighted. The xylanase production is associated with hemicellulose presence in media.

The production of glycohydrolases is closely related to the nature of the carbon source, since microbial metabolism is greatly influenced by pH and composition of the medium. Each strain has a distinct metabolic profile, while the enzymatic profile is also distinct and depends on the medium and cultivation time. Physiological variations are the result of the adaptation and evolution of microorganisms, considering original habitats and other factors (Robl *et al.*, 2015). A potential strategy for the glycohydrolase production high added value products, is the use of different carbon sources that induce the production of different specific enzymes.

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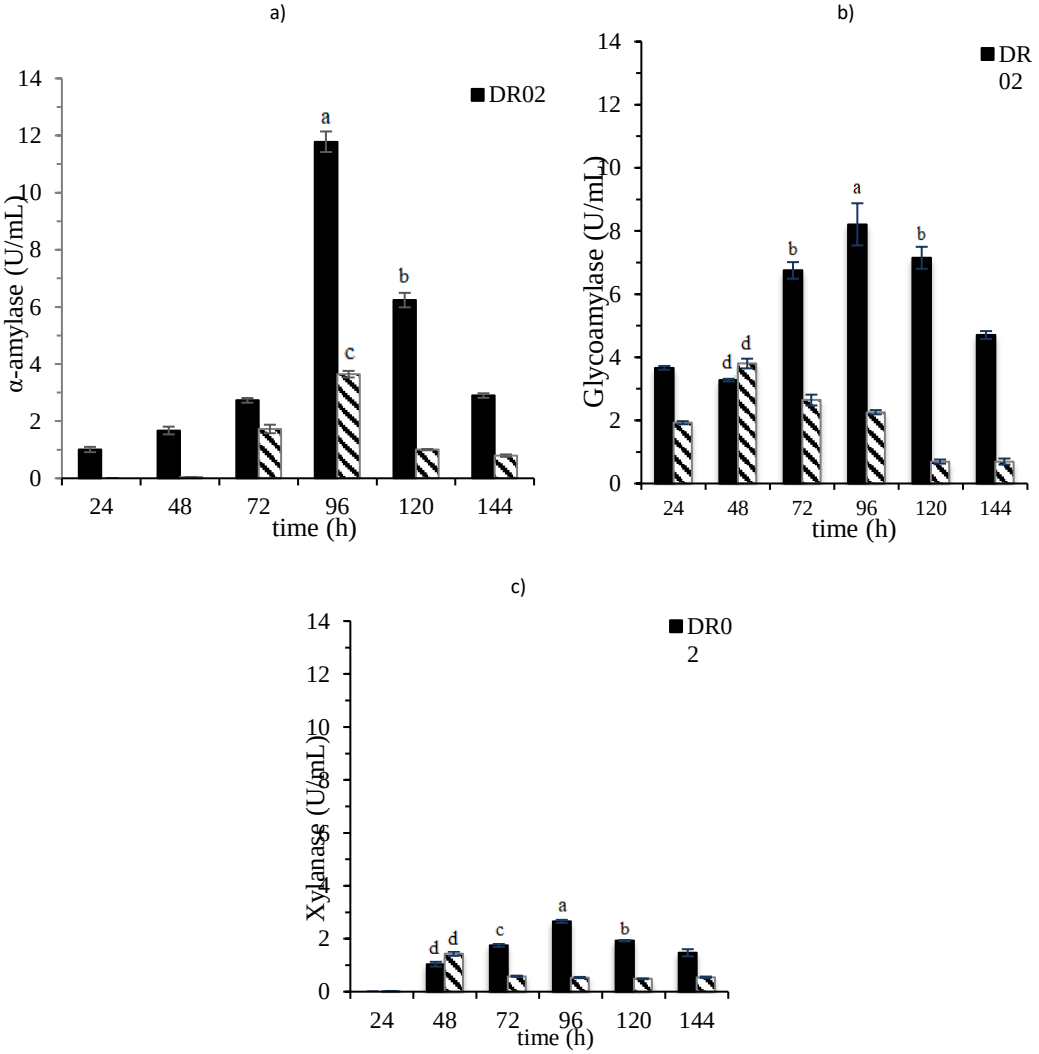


**Figure 3.** Evolution with time of enzymatic activities for *A. niger* DR02 and AN 400 strains. The shake flask cultivation using manipueira supplemented with cassava peel at 28°C and 120 rpm. (a) α-amylase, (b) glycoamylase and (c) xylanase. Means calculated from three replications. Data not transformed. Means followed by the same small letter do not differ among them by Tukey test at 5%.

*α-amylases*

The *α-amylase* enzyme was the one that the fungi produced most efficiently using cassava as a substrate. At 96 h, amylase production reached the peak of production for both strains, however, strain DR02 showed the greatest significant effect in relation to AN 400.

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Also, for the DR02 strain, at 120 h also showed significant effect. To produce amylases, the time of 96 hours could be given at the end of cultivation, since it was the shortest time that provided higher activity values. α-Amylase by *A. niger* AN 400 and DR02 using cassava residues as a carbon source was affected by culture medium pH. It is known that pH affects the enzymatic activity of α-amylases. This is perhaps not surprising, since most α-amylases of fungi are known to have optimal pH in the acid to neutral range (Salgado *et al*., 2016). Adeniran, Abiose and Ogunsua (2010) investigated the production of α-amylase by *A. niger* in submerged cultivation, using five agricultural residues (cassava peels, yams, banana and brewery grains). *A. niger* produced the highest amount of α-amylase (8.4 EU/mL) in the medium supplemented with cassava peels. The actual study presented better performance with *A. niger* DR02, thus being a good producer of α-amylase using the submerged cultivation method.

Silva *et al*. (2009) used cassava peel as a carbon source for the production of amylolytic enzymes by *A. niveus* in submerged cultivation. The optimized culture medium conditions were initially at pH 5.0, 35°C for 48 h. The amylolytic activity was further improved (50%) with a mixture of cassava peel and soluble starch in a 1:1 (w/w) ratio. However, the crude extract showed optimum temperature and pH of approximately 70°C and 4.5, respectively. Under static conditions the production of α-amylase was superior to agitated condition (50 U/mL and 28 U/mL). The results mentioned were higher than those of this study, probably due to pH adjustment and addition of soluble starch, factors that favor enzymatic activity. It is important to highlight that the purpose of this research was to treat and investigate the potential of the organic and nutritional load of *manipueira* and cassava flour peel, without supplementation of nutrients or reagents to produce these enzymes, since the addition of nutrients would increase production costs.

França and Silva (2021) carried out studies for amylase production with the potato powder and cassava peels, using *Aspergillus niger* UCP 1095, in submerged cultivation. The independent variables evaluated were pH and the tuber peels. The experiments were carried out during 144 h, 150 rpm, temperature of 37°C. The results showed that amylase production occurred using both residues tested, but the best enzymatic result obtained was 0.91 U/mL with the residue of potato peel (11 g).

*Glycoamylases*

The *A. niger* strains showed promising performance to produce glycoamylases using submerged cultivation, the type of culture used in this study. Glycoamylases are among the most important fungal products used in the food industry, being most active in the range between pH 5 and 6, which improves the starch conversion process (Li *et al*., 2020).

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Glycoamylase production by DR02 strain showed a greater significant difference according to the Tukey test when compared to the AN 400 strain. The highest production of glucoamylase occurred at 96 h by DR02, although 72 h and 120 h were also significant for this same strain. When analyzing the pH of this culture medium, it was found that the medium had a pH between 4.76 ± 0.09 and 6.19 ± 0.37, considered optimal to produce this enzyme.

The *A. niger* 400 strain reached peak production at 48 h with a value of 3.8 U/mL, this value being obtained when the pH of the medium was 4.89 ± 0.06. It was observed that in less important media the enzyme values were lower, as at 120 and 144 h, where pHs 7 and 7.6 were recorded, respectively, and reaction times with lower production of glycoylase.

Moshi *et al*. (2016) studied the effect of temperature and pH on the production of glucoamylase by *Aspergillus* MZA-3 that could be used to produce bioethanol. This process used wild cassava flour (210 g/L) as nutrient medium and incubated at 30°C, 110 rpm. During the process, the best pH and temperature for higher enzyme yield (3.3 U/mL) were 5.5 and 30°C, which showed that the fungal species needs a more acidic environment for the production of the enzymes in question.

*Xylanases*

The experiments conducted in this work using only the *manipueira* did not present satisfactory data to induce xylanase production (data not shown). Thus, the use of cassava peel, another residue with lignocellulose composition was tested in the media formulation. According to Acheampong *et al.* (2021) cassava peels present an abundance of sugar polymers in the form of holocellulose (cellulose and hemicellulose) and for the hydrolysis of hemicellulose, a complex system of enzymes, called hemicellulases, which includes xylanase is required.

The results indicated that the 96 h cultivation period was optimal for xylanase production by the DR02 strain, while the AN 400 strain showed maximum production in 48 h of fermentation. For the other times, significantly different results were obtained to produce the enzyme. Significant differences in xylanase production were found between DR02 and AN 400 species, which reached mean values of 2.66 ± 0.06 and 1.43 ± 0.08 U/mL, respectively.

The DR02 strain was also used to produce xylanase in the studies by Robl *et al*. (2013). The authors verified a maximum production of xylanase in 120 h of cultivation (4.5 U/mL) by *A. niger* DR02 in sugarcane bagasse. The higher concentration produced by the authors can be attributed to the fact that sugarcane has more xylan than manipueira with cassava husk flour and the authors use thermochemical treatment, which may have facilitated the access of enzymes in the degradation of the lignocellosic material.

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Acheampong *et al*. (2021), in one of their experiments, studied the production of xylanase by the *Trametes polyzone* in submerged cultivation using, among others, cassava peel as a substrate. The reactors were incubated for 14 days, at 180 rpm, at 30°C, pH 6, 60% humidity and were supplemented with urea and magnesium. According to the results obtained, the xylanase activity was significantly higher when cassava peel was used, which was approximately 0.87 U/mL. It is important to emphasize that in the present study the averages obtained by the two fungal species were higher than those of Acheampong *et al*. (2021), which presents itself as an attraction, also because there was no increase in the supplement.

Xylanases are produced for the degradation of hemicellulose, present in the constitution of the substrate used in the present study. The lower production of these enzymes compared to amylases can be attributed to the constitution of the fungal growth medium, since one of the main components of the plant cell wall is hemicellulose, which has a content of 0.45% in fresh cassava root. and 1.07% in the bark, while the starch content is 79.30% and 80.43% in the fresh root and bark, respectively (Poonsrisawat *et al*., 2014). This fact justifies the lower amount of xylanase activity in relation to amylases.

## Conclusions

The bioremediation of residues generated by the flour industry such as manipueira and cassava peels by fungal culture of *A. niger* strains AN 400 and DR02 demonstrated the potential of these microorganisms to treat high concentrations of organic matter present in these residues. The COD removal efficiency for these strains showed the same profile. Furthermore, the bioremediation processes of these effluents allowed the simultaneous production of α-amylase and glucoamylase. A positive effect of the mixture of cassava flour and peel was also observed on the production of xylanases. The results obtained in this study demonstrated that submerged culture for 96 h is sufficient time to produce amylolytic enzymes by *A. niger*. Therefore, manipueira mixed with cassava peel flour, residues from the flour industry and rich sources of carbohydrates, can induce the production of enzymes through low-cost production, in addition to minimizing the disposal of residues in the environment without previous treatment.

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