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Biodegradation of Cassava Flour Production Wastes in the Brazilian Industry for Industrial Glycohydrolase Enzymes Production by *Aspergillus niger*

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HIGHLIGHTS

- *Aspergillus niger* DR02 produced enzymes from cassava wastewater.
- Higher reaction rates were for amylases > glucoamylase > xylanase.
- The addition of cassava peel flour favored the production of α-amylase by the fungus.

Abstract: The production of the enzymes α-amylase, glucoamylase, and xylanase by *Aspergillus niger* DR02 was studied using waste from the Brazilian cassava flour industry. Cassava wastewater (manipueira) at 30% (v/v) was used, either supplemented (M30F10) or not (M30) with 10 g/L of cassava peel flour as a carbon source. Variables such as chemical oxygen demand (COD), proteins, reducing sugars, glucose, nitrate, ammonia, and enzymatic activities were monitored. The results for M30F10 and M30 at 30°C and pH 5.00 showed similar COD removals of 82.88%±12.00 and 82.41%±7.00, respectively. Despite the significant reduction in organic load, the final effluent still requires post-treatment before being discharged into the receiving water body. The addition of cassava peel flour favored the maximum production of 18.40 U/mL±0.15 α-amylase in 72 hours of cultivation, 14.80 U/mL±0.91 xylanase in 96 hours, and 4.50 U/mL±0.29 pectinase in 24 hours. Without medium supplementation, the highest glucoamylase activity (19.90 U/mL±1.01) was

obtained in 96 hours. The kinetic adjustment to a first-order model (R^2: 0.9998) confirmed the highest efficiency for α-amylase production when the medium was supplemented with peel flour (M30F10), with a production rate of 7.07 x 10^{-2} h^{-1}, while the highest glucoamylase production rate was obtained in M30 (3.251 x 10^{-2} h^{-1}; R^2: 0.9975). These results confirm the effects of mitigating environmental impact and the commercial value enhancement of cassava flour industry waste through biodegradation with the studied strain.

Keywords: *Aspergillus niger*; manipueira; enzymatic activity; cassava flour.

INTRODUCTION

There is a growing interest in the development of processes for the utilization of agro-industrial waste [1, 2, 3], so that, at the same time, proper disposal of agro-industrial effluents can be promoted, and high-value commercial compounds can be obtained from them [4, 5].

Cassava (*Manihot esculenta Crantz*, family: *Euphorbiaceae*) is a tuber widely cultivated in tropical regions of Asia, Africa, and the Americas, where it holds significant economic and industrial relevance. It serves as an important food source in the fight against hunger and malnutrition, as well as unemployment [6].

The Brazilian cassava root processing industry is mainly focused on flour production (60%), with the remainder used for starch extraction and animal feed [7]. There are two types of flour in Brazil: dried flour and water flour, the latter being commonly produced in the state of Maranhão and in the northern region of the country [6]. Water flour differs from dried flour by involving a root fermentation step of up to 5 days to remove cyanide, like the gari flour production in African countries such as Nigeria and Ghana. Among other differences, cassava is grated or ground before fermentation for the preparation of gari flour, while in the production of Brazilian water flour also called "pubada," the cassava is peeled and subjected to fermentation before being grated [8] [9].

Figure 1 presents a flowchart of the dried flour production process in the Brazilian industry, outlining the basic steps and the waste generated in each. The production of dried cassava flour occurs in all regions of Brazil, using a variety of cassava that, along with the degree of mechanization, mode of operation, and type of oven used for roasting, determines variations in the sensory properties of the flour [8].

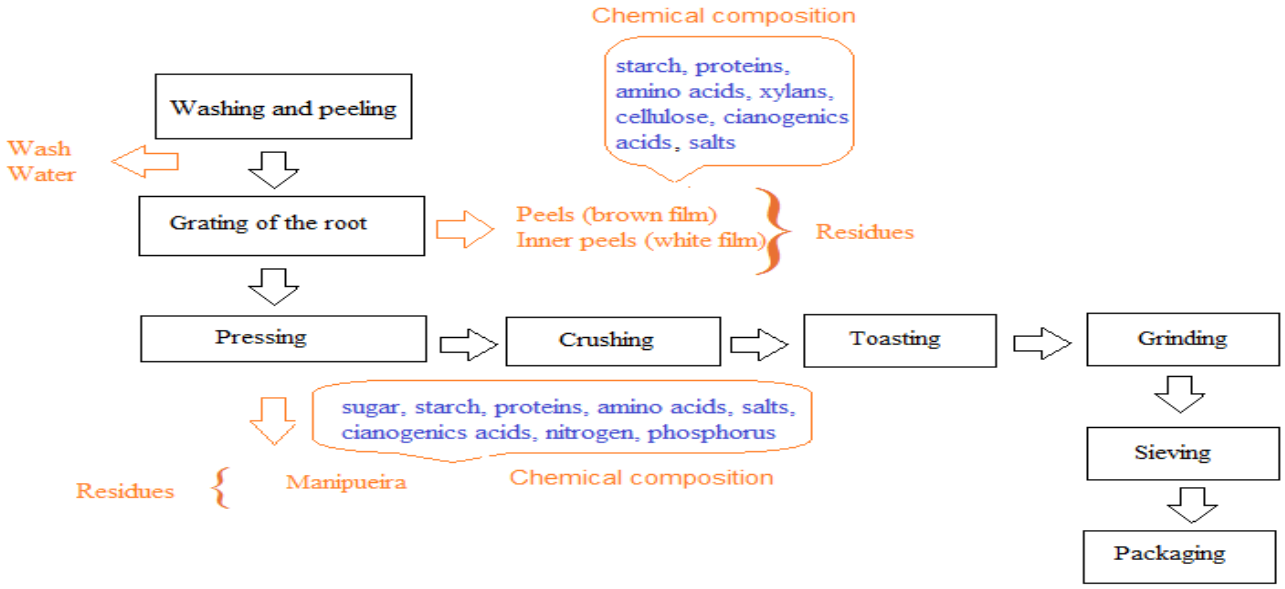

Figure 1. Flowchart of the dried cassava flour production process and the generation of residues.

The processing of cassava in the production of dried flour involves the following stages: washing and peeling, grating, pressing, crushing, toasting, grinding, sieving, and packaging, generating both solid and liquid waste. Solid waste is produced in the initial stage of cassava processing, coming from the peeling of the roots, consisting of peels (brown film) and inner peels (white film) [8] [9]. The peel is the outermost layer that covers the cassava root and the inner peel is the layer between the peel and the pulp of the root. Both are primarily composed of fibers, residual starch, proteins, lipids, phenolic compounds, minerals, and cyanogenic glycosides. Additionally, cassava peels also contain lignocellulosic compounds that contribute to

their fibrous structure [10] [11]. During the peeling process, the removal of the peels and intermediate peels nearly eliminates the phenolic compounds and most of the potentially cyanogenic compounds from the root, reducing the toxicity of the final product [9].

When mechanized peeling is used, only the outermost brown peel is removed, and a second peeling may be necessary to eliminate small pieces still attached before proceeding to grating. Grating is essential in flour production to obtain a wet mass, the excess moisture of which is removed before toasting, during the pressing phase, facilitating its drying. The toasting is of the most critical stages in flour production. The toasting stage directly influences its color, flavor, texture, and preservation. The flour can be ground after toasting, sieved to separate fibers and other larger aggregates, and classified based on the dimensions of its granules using standardized mesh screens and packaged [8].

Liquid waste consists of wash water and cassava wastewater (manipueira), a residual liquid obtained during cassava pressing, which has a high organic load. Both types of waste are generated in large quantities in artisanal and industrial processing of this tuber, leading to various environmental challenges [12].

In the mechanical and manual peeling of one ton of cassava, 20 to 300 kg of waste is generated, resulting in at least approximately 4 to 6 billion kg of waste annually in Brazil alone, which is the fourth-largest cassava producer in the world. This activity plays a crucial role in the country's agricultural economy, producing more than 20 million tons in 2020 [13]. Regarding liquid waste, at least 600 liters of wastewater, including 279 liters of manipueira, are generated for every ton of roots processed. Manipueira is rich in carbohydrates, with a high total solids content and a chemical oxygen demand (COD) of up to 56 g/L. The pollutant load also includes cyanogenic glycosides, resulting in a significant environmental impact due to its toxicity [14] [15].

New technologies have been explored to treat toxic compounds in industrial effluents efficiently and economically. Among these techniques, biodegradation plays a crucial role in reducing the harmful effects of pollutants on the environment, transforming polluting compounds into less dangerous or non-hazardous substances through the action of microorganisms [16]. Among the mentioned residues, manipueira, a rich source of sugars, starch, proteins, salts, nitrogen, and phosphorus, could be valorized by not only promoting the reduction of its organic load during biological treatment but also by simultaneously obtaining economically valuable enzymes with the potential to meet part of the demand for these enzymes in different industrial sectors [9].

Both the solid residues (peels, inner peels) and the liquid residues (manipueira and wash waters) generated during cassava processing need mitigation due to their polluting nature, which can be addressed using fungi in a biological treatment process [17]. At the same time, it is particularly interesting to investigate the potential of residues such as manipueira, cassava peels and inner peels for enzyme generation and utilization during the biological treatment process [5]. During the reduction of the organic load present in these residues, inducing substances in their composition, such as starch, proteins and amino acids, xylans, and cellulose, respectively favor the production of amylase, protease, and xylanase enzymes by microorganisms. These enzymes help break down more complex molecules into simpler compounds during the degradation process [9].

In the context of sustainability, it is important to optimize this process to make it feasible for these enzymes to be recovered and utilized, meeting the market demand for enzymes conventionally produced from specific substrates and processes. According to Khvedelidze and coauthors [18], cellulase, xylanase, amylase, protease, inulinase, and lipase enzymes comprise more than 80% of all commercially relevant enzymes due to their potential applications in various industrial sectors, medicine, and agriculture. Among these, amylases are particularly notable, representing 20 to 30% of the entire global market, with α-amylases and glucoamylases standing out due to their wide applicability in converting starch into fermentable sugars and other derivative products. α-amylase is very versatile and used in a variety of industrial processes, while glucoamylase is used in the production of pure glucose and biofuels [19] [20].

However, the process is expensive, especially concerning the fermentation medium employed [21]. In a global market where the demand for these enzymes is high and growing exponentially, driving billions of dollars in various industrial activities, the substrate used for their production represents a high cost [22]. Therefore, utilizing the large volume of manipueira generated during the pressing stage of cassava flour production can reduce production costs. Valuing the waste could make the activity a sustainable practice, and the search for cheaper production methods is highly significant for enzyme production, given the large number of agricultural by-products that can be utilized, representing savings in raw materials, and generating returns [23].

The production of enzymes involves the use of microorganisms, among which various species of *Aspergillus* are notable. *Aspergillus niger* is a filamentous fungus belonging to the class of Eurotiomycetes and holds significant importance in the biotechnological industry, playing a crucial role in agro-food

applications and the biodegradation of industrial effluents [24] [25] [26]. It is known for secreting a variety of hydrolytic enzymes, such as amylases, hemicellulases, and pectinases [27] [28].

The costs of agricultural waste treatment can be reduced through the reuse of organic matter produced in the treatment process itself. Furthermore, reuse is a key concept in a circular economy (CE) that aims to minimize waste production by converting it into valuable resources, reintroducing them into the market, and mitigating environmental impacts [14] [29].

To enhance the economic feasibility of utilizing enzymes produced during the biological treatment of cassava waste, it has been proposed to use them within the industry itself. Thus, the amylase produced during the treatment of these wastes could be used in the flour production process to reduce the viscosity of the cassava dough, which would improve the quality of the final product, or even in the production of other cassava derivatives, such as bakery products, thereby making the economic activity even more profitable [30]. Therefore, it is necessary to explore alternatives for reusing these wastes, as they are generated in large quantities in the cassava flour industry [31].

Since manipueira, cassava peels, and cassava inner peels are wastes that cannot be simply discarded into the environment, this study aimed to treat them by reducing their organic load through the action of the *Aspergillus niger* strain DR02. This fungus produces hydrolytic enzymes (α-amylase, glucoamylase, pectinase, and xylanase) that are induced by the compounds present in these wastes and are highly valued as commercial products. Thus, the objective of the study was to reduce the pollutant load of manipueira, peel, and inner peel, mitigating potential environmental damage caused by their improper disposal, while simultaneously obtaining additional benefits during the treatment process by utilizing the enzymes generated by the strain in biodegradation. This approach addresses an emerging and significant issue in the field of Environmental Engineering.

MATERIAL AND METHODS

Samples from the flour industry

The peels and barks obtained from cassava peeling, along with manipueira, were collected at the Rocha Alimentos cassava processing plant in Sangão, Santa Catarina, Brazil. The peels and barks were gathered at the exit of the root peeling tank and transported in 3 kg plastic bags, while the manipueira was obtained directly from the press during flour production, then stored in polyethylene containers (4 L) and frozen at -20°C. To produce peel flour, solid residues were placed on trays and dehydrated in a forced air circulation dryer (QUIMIS® brand) at 60°C for 48 hours. After drying, the peels were crushed in a Willye mill (model TE-650/1-TECNAL) to obtain flour with undefined granularity.

Cultivation of the microorganism

A. niger DR02 provided by Laboratory of Microorganisms and Biotechnological Processes (LMBP) at the Federal University of Santa Catarina, Florianópolis, Santa Catarina and it was inoculated on Petri dishes containing BDA medium (Potato Dextrose Agar - Sigma Aldrich) for fungal colony growth, which were then incubated in a microbiological incubator at 28 ± 2°C for seven days. After this period, the spores were harvested in Tween 80 solution (0.5%, v/v), and their concentration was estimated using a Neubauer chamber for later use as the inoculum for batch reactors.

Fermentation medium

The medium composition was adapted from Marinho [26] and utilized Vishiniac solution (1 mL/L) containing $ZnSO_4.7H_2O$ (4.4 g/L), $MnCl_2.4H_2O$ (1.0 g/L), $CoCl_2.6H_2O$ (0.32 g/L), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.22 g/L), $CaCl_2.2H_2O$ (1.47 g/L), $FeSO_4.7H_2O$ (1.0 g/L) and peptone (1.0 g/L). Cassava wastewater (manipueira) was added to the medium at different concentrations. The medium was sterilized in an autoclave at 121 ± 2°C for 15 minutes.

Batch assay

The effect of manipueira concentration on fungal growth was assessed in a batch assay using 250 mL Erlenmeyer flasks containing 100 mL of basal medium with manipuera. The flasks were grouped into M30, M50, and M70, designated according to the concentration of cassava wastewater added to the medium, namely, 30%, 50%, and 70% (v/v), respectively. The flasks were inoculated with *A. niger* DR02 (2 x 10^6 spores/mL) and subsequently transferred to an orbital shaker, where they were maintained at 28 °C and 120 rpm for 144 h, as described by Marinho [26]. The entire assay was conducted in triplicate.

Cultivation in stirred tank reactor

A stirred tank reactor (TEC BIO FLEX II - TECNAL) was inoculated with 2 x 10^6 spores/mL of *A. niger* DR02 and fed with 4.5 L of the fermentation medium containing 30% (v/v) manipueira, with (M30) and without (M30F10) the addition of 10 g/L of cassava peel flour, made from the brown peel and inner peel, as an additional carbon source. The bioreactor featured automatic temperature control (30±2°C). The pH of the medium was adjusted to 5 by the automatic addition of H_2SO_4 (0.4 M) or NaOH (2 M). The dissolved O_2 level was maintained above 30% air saturation through automatic adjustment of aeration (0.3-1 L/min) and agitation (200-400 rpm). Foam formation was manually controlled by the addition of polyglycolic antifoaming agents (15%, v/v) (Fluent Cane 114, DOW Chemical, Brazil) [28]. A heating blanket that surrounded the bioreactor vessel was placed so that the cultivation temperature was maintained at 30°C.The studied reaction times were 24, 48, 72, 96, 120, and 144 h, with a sample aliquot taken at each intermediate time for monitoring physicochemical parameters.

Analytical methods

The samples were centrifuged for 15 minutes at 5,000 rpm to remove biomass, the supernatant was used for the determination of the physicochemical parameters and methods described below. All analyses were conducted in triplicate.

Free cyanide

Cyanide determination was based on the spectrophotometric method of Essers [32]. Readings were performed at 578 nm, and results were obtained from a calibration curve using sodium cyanide solution (KCN) as a standard.

Starch, total carbohydrates, and fixed mineral residue

Starch content, total carbohydrate and was estimated using the standard method described in AOAC [33]. Total carbohydrate content was determined according to Analytical Standards of Adolfo Lutz Institute [34]. Fixed mineral residue content was determined using the Brazilian Compendium of Animal Feed [35].

Sugars determination

Reducing sugar determination was based on the analytical method of Somogyi-Nelson [36]. The supernatant (1 mL) was mixed with SN-1 reagent (Somogyi-Nelson 1) (2 mL) and boiled in a water bath for 6 minutes. The mixture was cooled in an ice bath for 5 minutes. Subsequently, SN-2 reagent (Somogyi-Nelson 2) (2 mL) was added to the mixture and allowed to rest for 5 minutes at room temperature. Distilled water (25 mL) was added, and absorbance was measured at 540 nm using the Bio-Tek EL800 spectrophotometer (USA). For glucose determination, the Labtest® Liquiform Glucose kit was used.

Total Protein

For protein determination, the Bradford method [37] was employed using bovine serum albumin as a standard. Absorbance was measured at 595 nm using the Tecan Infinite® 200 spectrophotometer (USA).

COD (Chemical Oxygen Demand), ammonia and nitrate

The standard method used to determine COD, ammonia and nitrate followed APHA [38]. For the determination of COD, the following reagents were used: potassium biphthalate for calibration curve; 3.5 mL of catalyst solution; 1.5 mL of digester solution, and 2.5 mL of the supernatant. The digestion process was carried out in an autoclave at 121°C for 60 minutes. Absorbance readings were taken with the samples at room temperature, using a Bio-Tek EL800 (USA) instrument, at a wavelength of 600 nm, and compared to a predetermined standard curve. For ammonia determination, 10 mL of the supernatant were diluted in a 100 mL flask and transferred to an Erlenmeyer flask. To the solution, 1 mL of zinc sulfate (100 g/L) and 1 mL of sodium hydroxide (6 N) were added, left to rest for 15 minutes at room temperature. An aliquot of 10 mL from the mixture was removed, transferred to a test tube, and 2 drops of Rochelle salt and 200 µL of Nessler reagent were added, left to rest for 10 minutes. Absorbance was measured at 450 nm using a Bio-Tek EL800 (USA) instrument. The analysis to determine nitrate was done by adding to the porcelain capsule 10 mL of

the supernatant and 1 mL of sodium salicylate were added and heated in a water bath at 80 °C until dry. Then, 2 mL of pure sulfuric acid was added to the dry material and left to rest for 10 minutes. Subsequently, 15 mL of a double sodium and potassium tartrate solution was added and left to rest again for 15 min. Absorbance was measured at 420 nm using a Bio-Tek EL800 (USA) instrument.

α-amylase

The α-amylase determination was adapted from Xiao [39]. A mixture containing 20 µL of enzymatic 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 stopped by adding 20 µL of hydrochloric acid (1 M), followed by 100 µL of the iodine reagent (5 mM I_2 and 5 mM KI). The absorbance was measured at 595 nm using the Tecan Infinite® 200 spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute from soluble starch under the assay conditions.

Glucoamylase, pectinase and xylanase

For the enzymatic activity of glucoamylase, pectinase, and xylanase, the dinitrosalicylic acid method (DNS) was used, with starch, pectin, and Beechwood's xylan as standard substrates, respectively. Absorbance was measured at 540 nm using the Tecan Infinite® 200 spectrophotometer. The assays included 20 µL of supernatant, 30 µL of citrate buffer (0.05 M and pH 5), and 50 µL of the standard substrate solution (0.5% w/v). The mixture was incubated for 10 minutes at 50°C, except for pectinase, which was assayed at 37°C. The reactions were stopped by adding 100 µL of DNS and incubated again at 95°C for 5 minutes. One unit of enzyme activity (U/mL) was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute from soluble substrates under the assay conditions.

Kinetic parameters

The kinetic parameters were estimated using the first-order model (Eq 1) through nonlinear regression of experimental data for COD and enzymatic activities. Analyses to verify enzyme production were performed starting from 24 h of reaction, which was used as time zero for obtaining the kinetic profiles in this study.

$$C = C_o . e^{\pm kt} \quad (1)$$

Where: C_0 and C represent the initial and final concentrations for COD (mg O_2/L) or enzymatic activity (U/mL); k_1 is the first-order rate constant (h^{-1}), is the first-order rate constant (h^{-1}), indicating (-) for reagent consumption and (+) for product formation and t is the reaction time in hours.

RESULTS AND DISCUSSION

Physicochemical characterization of fresh cassava

The results of the characterization of manipueira are presented in Table 1. Its potential for use as a carbon and micronutrient source in the cultivation with *A. niger* DR02 in a stirred tank bioreactor is evidenced by the high content of chemical oxygen demand, total carbohydrates, ashes, proteins, and others.

Table 1. Physicochemical characterization of manipueira – number of samples (n):1.

Parameters	Values
COD (g/L)	37,5
pH	5,7
Cyanide (mg/L)	11,9
Total carbohydrates (g/kg)	34,5
Starch (g/L)	9,8
Total reducing sugars (g/kg)	12,9
Proteins (mg/L)	78,2
Fixed mineral residue (%)	0,6

The values observed in fresh cassava wastewater can vary depending on factors such as soil composition, cultivation practices, root maturation, climatic conditions, among others [40]. The cyanide concentration in the sample exceeds the allowed maximum total CN^- release limit by about 60 times according to CONAMA Resolution nº 430 [41], indicating that the effluent should not be discharged directly into water bodies without proper treatment. The wastewater from the flour industry has a low protein content compared to other industrial effluents such as pig manure, rubber, and palm oil [39].

Batch assay

There was visual biomass growth in M30, M50 and M70 reactors (Figure 2), regardless of the presence of cyanide in the medium, with the formation of pellets of various sizes and quantities at the studied concentrations. Growth in the form of pellets can compromise oxygen transfer and decrease the lifespan of the biofilm if biomass growth occurs excessively, as there are many empty spaces between the cluster of hyphae, and in this biofilm form, there is a compression effect on air bubbles, leading to increased coalescence, affecting the efficiency of the biological process [42].

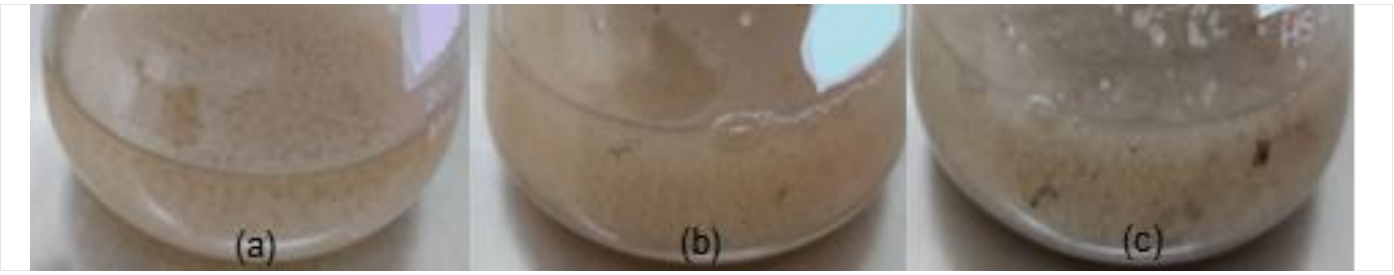

Figure 2. Cultures in batch reactors (a) M30, (b) M50 and (c) M70 with *A. niger* DR02 after 144 h at 28°C.

The concentrations of 50% and 70% cassava wastewater (17.3 g/kg and 24.2 g/kg of total carbohydrates) apparently showed higher pellet formation. When using reactors for the treatment of this residue or the production of enzymes, this could lead to a critical concentration of oxygen, causing damage to the process. For M30, there were approximately 10.4 g/kg of total carbohydrates. Therefore, when using a stirred tank bioreactor for the treatment of the residue and enzyme production, it was decided to work with the lowest concentration of cassava wastewater studied (30%, v/v).

Cultivation in stirred tank reactor

The variation of organic matter, in terms of COD, glucose and reducing sugar over the 144 hours of fermentation of wastewater with cassava wastewater, with (M30) and without (M30F10) the addition of cassava peel flour by *A. niger* DR02, is presented in Figure 3.

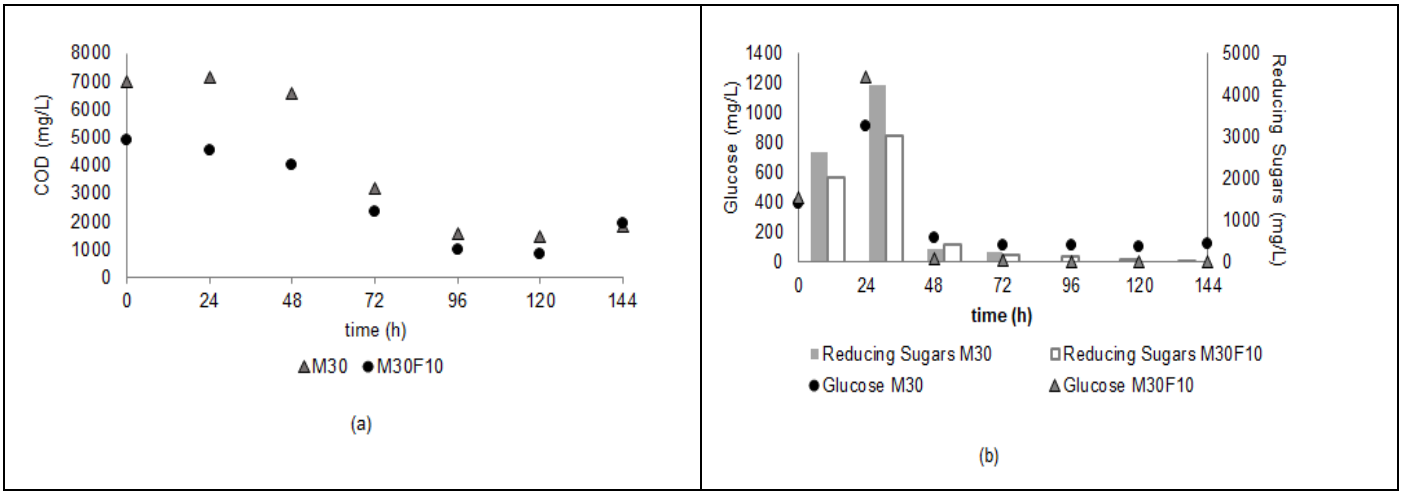

Figure 3. Variation in the concentration of (a) COD and (b) glucose and reducing sugar over the 144 h of fermentation of wastewater with cassava wastewater, with (M30) and without (M30F10) the addition of cassava peel flour by *A. niger* DR02.

The highest COD removals were recorded in the last reaction time (144 h), reaching 82.88%±12.00 and 82.41%±7.00, respectively, for M30 and M30F10. It was observed that in the first 24 hours, there was no removal of organic matter, but rather glucose production in the medium, as reported by Potivichayanon and coauthors [43]. After 24 hours of fermentation, the biodegradation of cassava effluent occurred, with COD removals of 27.95%±7.00 and 28.86%±5.00 in M30 and M30F10, respectively. Concurrently, during this period, *A. niger* DR02 produced more glucose when cassava peel flour was available (74.00%±12.00), while in the absence of flour, the production was 27.00%±2.50.

Cassava wastewater is a rich source of carbon, containing a mixture of sugars such as sucrose, glucose, and fructose, as well as mannose, xylose, arabinose, fructose, and rhamnose. In a medium with different sugars, their consumption can occur simultaneously or sequentially and *A. niger* has a higher preference for glucose and the consumption of other sugars occurring only after its depletion from the medium [44]. The initial glucose content in the M30F10 medium (432.40 mg/L±19.00) was higher than in M30 (397.10 mg/L±49.00), attributed to the addition of cassava peel flour to the culture medium. According to Makelã and coauthors [45], little is known about sugar absorption systems in fungi, except for extensive studies in the yeast model *Saccharomyces cerevisiae*. However, sugar absorption is vital for their growth, reducing the adaptation phase and favoring cell growth, and not all sugars can induce spore germination [46].

The higher glucose production in M30F10 would be related to the utilization of lignin present in cassava peel [47] and by the hydrolysis of the present polysaccharides, mainly starch. However, it is important to note that, simultaneously with glucose production, it can also be consumed to provide energy for the fungus [45]. Similarly, there was an increase in the concentration of reducing sugars in the first 24 hours, from 2,038.60 mg/L±89.00 to 3,003.70 mg/L±88.00 for M30, and from 2,640.50 mg/L±14.20 to 4,023.80 mg/L± 304 for M30F10. In 48 hours, in both cultures, there was a removal of 87.80%±4.00 for M30 and 79.00%±8.50 for M30F10 concerning reducing sugars. The production of enzymes by *Aspergillus niger* is accompanied by a significant removal of reducing sugars [46]. After 48 hours, a decline in the concentration of reducing sugars was observed, reaching 15.70 mg/L±2.00 (MR30) and 14.10 mg/L±6.00 (M30F10) at 144 hours.

However, regardless of the flour addition, the final effluent did not comply with the standard discharge limit currently in effect in the country [41], with a COD concentration of 1,820.00 mg/L±5.51 (M30) and 1,949.00 mg/L± 30.35 (M30F10), necessitating additional treatment before its disposal into the environment.

The nitrate removal by the culture showed a similar behavior to that of organic matter, with the highest nitrate removals occurring after 144 hours of treatment, reaching 87.20%±0.97 and 81.30%±0.24 for M30 and M30F10, respectively. For ammonia, the maximum removal of 66.50%±1.30 (24 h) occurred when flour was not added to the medium, while in M30F10, the maximum removal efficiency was 63.2%±0.00 (48 h). After the attainment of the maximum ammonia removal, an increase in its concentration was observed, reaching 39.70±1.70 mg/L (M30) and 39.20±4.60 mg/L (M30F10) at the end of the process.

The maximum value for ammonia nitrogen in effluent discharge into water bodies is 20 mg/L [41]. Therefore, the effluent is slightly above this value, although it is predominantly in the form of NH_4^+ ions [47], given that the pH of the medium was controlled in the acidic range (pH 5±0.2). Ammonia as well as organic matter can be adjusted through the application of post-treatment.

The enzymatic activity of *A. niger* DR02 displayed in the presence (M30F10) and absence (M30) of cassava peel flour during the treatment of cassava wastewater is shown in Figure 4.

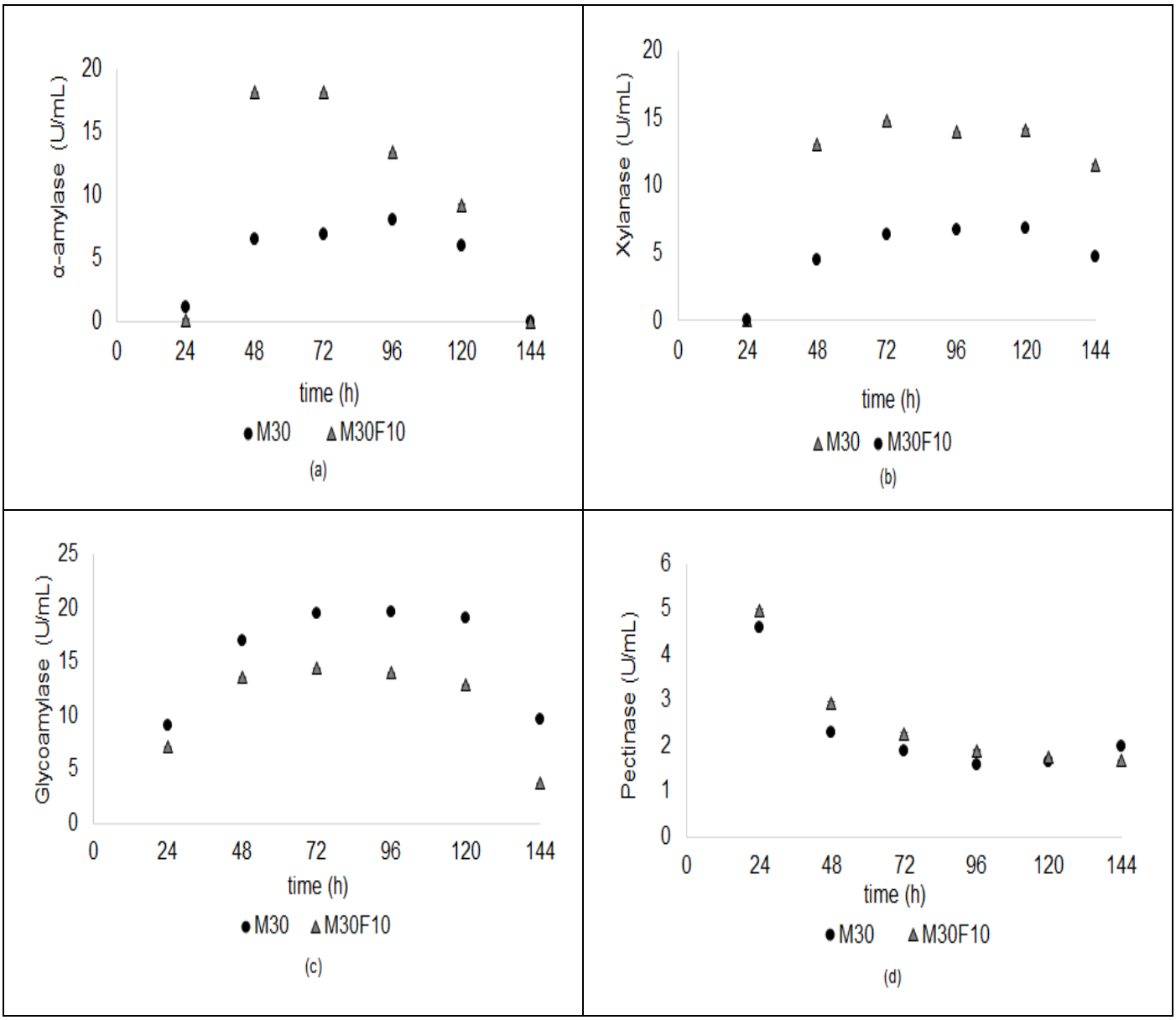

Figure 4. Enzymatic production of α-amylase, xylanase, glucoamylase and pectinase in cassava wastewater with (M30F10) and without cassava bark flour (M30) using *A. niger* DR02 in stirred tank reactor.

The addition of cassava peel flour favored the production of α-amylase and xylanase, with maximum enzymatic activity recorded for these enzymes at 18.40±0.15 U/mL (72 h) and 14.90±0.91 U/mL (96 h), respectively. Without the addition of flour, the maximum activity of these enzymes was 8.29±.0.29 U/mL and 6.90±.0.36 U/mL, respectively, both at 72 hours of fermentation.

After reaching the maximum activity, the values of α-amylase and xylanase decreased until the last reaction time (144 h), when they were recorded as 0.50±0.06 U/mL (M30) and 0.33±0.01 U/mL (M30F10). The decrease in enzymatic activity over the cultivation time may be attributed to the production of byproducts resulting from microbial metabolism, as well as nutrient depletion, inhibiting the formation of enzymes [48] [49].

The activity of glucoamylase reached its maximum value at 96 h in both cultures, with 19.90±1.01 U/mL and 14.70±0.19 U/mL, respectively, in M30 and M30F30. Starch is a compound that induces production of amylases, and the type of starch present in the environment determines its higher or lower activity [50]. This is because starch consists of various sequences with just two polysaccharides, amylose, and amylopectin, densely packed with a high degree of laminar ordering in a semi-crystalline state [51]. This structural characteristic can hinder its hydrolysis due to its morphology (size, surface characteristics, peripheral pores, and channels) and its crystalline arrangement, thus impeding its availability in the environment for enzyme

activation [50]. Nevertheless, in this research, among all the enzymes studied, amylase exhibited the highest recorded activity.

In addition to promoting amylase production, cassava flour exerted a positive influence on xylanase activity, possibly due to the presence of hemicelluloses in the structural composition of cassava peel, especially xylans, which may have stimulated the production of xylanase [52]. Acheampong and coauthors [49] used cassava peels (10 g) to produce hydrolytic enzymes, including xylanases, and observed a maximum xylanase production of 0.87 U/mL by *Trametes polyzona* at pH 6, with solid-state cultivation at 30°C for 14 days, with urea added as the nitrogen source. It is important to highlight that in the present study, the average obtained by *A. niger* DR02 exceeded that found by this author, making cassava wastewater and cassava peel flour an attractive substrate.

Furthermore, factors such as pH, temperature, and nitrogen source significantly influence enzyme production, and each enzyme has its optimal conditions and variations in these factors, which can compromise production [29].

Perhaps due to the reactive medium not being in ideal conditions for pectinase activity, this enzyme exhibited the lowest activities in the medium. Its production was virtually indifferent to the presence or absence of cassava flour, reaching maximum activity of 4.10±0.18 U/mL and 4.33±0.29 U/mL at 24 hours of treatment and minimum values of 0.90±0.09 U/mL and 1.10±0.07 U/mL at 144 hours, respectively, in M30 and M30F10. Although amylases require optimal conditions for production at pH 5 and 30°C, pectinases show better activity in media with pH 4 and temperatures between 50 and 60°C [53].

Pectins are complex oligosaccharides found in the cell walls of all plants and tend to precede the production of other hydrolases to demethylate pectin, thus facilitating its subsequent depolymerization. However, enzyme production requires special attention to the composition of the culture medium, as it may contain components involved in the induction, repression, or inhibition of enzyme formation and secretion for each strain of interest [53].

In general, the highest enzymatic activities occurred between 48 and 96 h, which would be related to the decrease in glucose in the medium. In media where glucose is present in higher concentrations, as observed in this study (~1,200 mg/L), enzymes related to the hydrolysis of more complex compounds are produced, resulting in the formation of simple sugars. Indeed, in the first 24 h, both in M30 and M30F10, low activity was observed for α-amylase, glucoamylase, and xylanase, with a simultaneous increase in glucose and reducing sugars. Therefore, except for pectinase, the maximum activity of the other studied enzymes occurred upon the removal of most of the initially present organic matter in the medium, with a decrease in COD of around 80%.

In this study, after 120 h, when there was possibly nutrient depletion in the medium and accumulation of inhibitory by-products and reductions in enzymatic activities were observed. Interestingly, Robl [28] reported in their studies that *A. niger* DR02 reached a maximum xylanase activity of 4.50 U/mL after 120 h in submerged cultivation, but with other substrates, namely delignified sugarcane bagasse and soybean meal. This highlights the importance of the medium composition on enzymatic activity and reaction time.

In the industrial process of enzyme production using conventional culture medium and under controlled conditions of pH and temperature, enzymatic activities of up to 200 U/mL can be achieved, typically obtained at higher temperatures, up to 70°C, as mentioned by Khvedelidze and coauthors [18]. According to these authors, temperature and pH strongly influence enzyme stability, but the microorganism involved in the process is crucial for optimal yield. They highlighted that, from a selection of fungi extracted from the soil, approximately 16 and 11 strains of the genus *Aspergillus* were the most viable for obtaining glucoamylase and α-amylase, respectively. Primarily, *Aspergillus niger* achieved the highest glucoamylase activity, reaching 140 U/mL at 35°C, in 72 hours of submerged fermentation in 750 mL Erlenmeyer flasks, using mineral medium containing sodium nitrate as the nitrogen source and supplemented with starch (6%), under agitation at 160 rpm, compared to 5 U/mL of α-amylase under the same conditions, showing that the higher or lower activity results from a combination of factors that will favor the production of a specific enzyme, and it is important to seek the best fit for its production.

The use of alternative substrates, such as agricultural residues, results in lower enzymatic yields compared to those obtained using traditional culture media, with varied conditions regarding substrate type, cultivation, and involved microorganism. However, the activities recorded in this study for amylase, glucoamylase, and xylanase are within the expected range, as reported by Da Silva [54], who used soluble starch (1:1) extracted from cassava peel as a substrate for amylase production by *A. niveus* (1×10^7 spores/mL) in 125 mL Erlenmeyer flasks containing 25 mL of Khanna fermentative medium, at 40°C and pH 6.5 for 72 hours, under static and agitated conditions, obtaining 50.00 U/mL and 28.00 U/mL, respectively. In another study, Moshi [55] used *Aspergillus* MZA-3 for glucoamylase production at 30°C, pH 5.50, and 110

rpm for 24 hours, in a basal aqueous medium containing wild cassava flour (210 g/L) (10% v/v), resulting in 3.30 U/mL.

Under the conditions proposed for enzyme production, cassava wastewater also demonstrated better results than another agricultural residue under study, corn stover, which was used by Ire, Chima, and Ezebuiro [56] for xylanase production by *A. niger*, *A. flavus*, and *A. fumigatus*, using an inoculum concentration of 1 x 10^6 spores/mL. These authors employed 10 g of corn cob as a carbon source in submerged fermentation, supplemented with 30 mL of mineral medium, in Erlenmeyer flasks (250 mL), maintaining them under agitation at 120 rpm. They achieved a maximum activity of 9.36 U/mL at pH 5.00 and 25°C over 120 hours for a mutant strain of *A. niger*, with ammonium sulfate as the nitrogen source. This value was lower than those found in the present study (14.90±0.91 U/mL, 96 h), indicating the feasibility of both cassava byproduct and the employed microbial strain.

However, even though the use of agro-industrial residues as alternative substrates for enzyme production results in lower enzymatic activity compared to using conventionally employed substrates, the viability of employing residues to compete in this market cannot be disregarded. These are materials that can alleviate the production process, being available in large quantities and easily accessible, thus becoming a potentially sustainable and economically viable option to meet the growing demand in this market.

The kinetic analysis of data from the bioconversion of cassava wastewater and enzyme production by *A. niger* DR02 indicated a good fit to the first-order model, as evidenced by the coefficient of determination (R^2) values ranging from 0.987 to 0.997. According to Ahmed and coauthors [46], minimum R^2 values of 0.80 are recommended for a well-fitted model, as observed in this study.

The significant potential of using cassava wastewater as a medium, particularly to produce α-amylase and glucoamylase, was endorsed in this research by the higher values of the first-order constant (k) shown in Table 2.

Table 2. First-order kinetic constants at 25°C in cultivation with *A. niger* DR02 using cassava wastewater (M30) and cassava wastewater supplemented with cassava peel flour (M30F10) as substrates.

Variable	M30		M30F10	
	***k* x 10^{-2} (h^{-1})**	**R^2**	***k* x 10^{-2} (h^{-1})**	**R^2**
COD	1,622	0,9929	1,512	0,9974
α-amylase	4,845	0,9953	7,070	0,9998
Glucoamylase	3,261	0,9975	2,665	0,9872
Xylanase	1,514	0,9941	1,585	0,9955

For COD, the reaction rate (k) was slightly higher in M30 compared to the culture containing cassava peel flour (M30F10) (Table 2), indicating that *A. niger* DR02 was able to consume biodegradable organic matter more rapidly. According to Morais [57], the easier the assimilation of organic matter in the medium, the higher the value of k, provided it does not contain inhibitory substances. The slightly lower *k* value (h^{-1}) for the observed decrease in COD in M30F10 can be explained by the presence of lignocellulosic compounds that are difficult to degrade due to the addition of cassava peel flour. However, on the other hand, the addition of cassava flour is interesting when aiming to produce α-amylase and xylanase, based on the data obtained in this research.

Significantly, the reaction rate value for α-amylase production was almost 1.5 times higher in the M30F10 culture than in the M30 system, endorsing the positive effect of cassava peel flour as a supplement to produce this enzyme by *A. niger* DR02. Conversely, the opposite behavior was observed in M30 for glucoamylase production, while regarding xylanase, the reaction kinetics were slightly faster in the presence of cassava peel culture (M30F10).

CONCLUSIONS

A. niger DR02 can produce glycohydrolase enzymes (α-amylase, glucoamylase, and xylanase) from cassava flour industry wastewater at a concentration of 30% (v/v) cassava wastewater. Notably, when the

medium was supplemented with cassava peel flour, there was an increase in the production of xylanase and α-amylase, while glucoamylase exhibited its highest activity in the non-supplemented medium.

Simultaneously, treatment of the effluents under the proposed conditions resulted in a significant reduction in the initial organic load, but the effluent from the process still requires post-treatment for final disposal. It is expected to increase enzymatic production through the development of strategies based on new studies to optimize the utilization of cassava flour production waste, given the demonstrated potential of these as an alternative medium, making cassava flour production an eco-sustainable activity by utilizing its waste in another high-demand sector, which is the enzyme production sector, thereby also reflecting in lower environmental impact.

Furthermore, the high demand for these enzymes in the industrial market, coupled with the ample supply of these residues, which require proper management, can result in a viable and less costly process, as there is both a decrease in environmental impacts and a gain in obtaining commercially valuable enzymes.

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