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Sugarcane Bagasse Hydrolysis Using Yeast Cellulolytic Enzymes

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Ethanol fuel production from lignocellulosic biomass is emerging as one of the most important technologies for sustainable development. To use this biomass, it is necessary to circumvent the physical and chemical barriers presented by the cohesive combination of the main biomass components, which hinders the hydrolysis of cellulose and hemicellulose into fermentable sugars. This study evaluated the hydrolytic capacity of enzymes produced by yeasts, isolated from the soils of the Brazilian Cerrado biome (savannah) and the Amazon region, on sugarcane bagasse pre-treated with H_2SO_4. Among the 103 and 214 yeast isolates from the Minas Gerais Cerrado and the Amazon regions, 18 (17.47%) and 11 (5.14%) isolates, respectively, were cellulase-producing. *Cryptococcus laurentii* was prevalent and produced significant β-glucosidase levels, which were higher than the endo- and exoglucanase activities. *In natura* sugarcane bagasse was pre-treated with 2% H_2SO_4 for 30 min at 150°C. Subsequently, the obtained fibrous residue was subjected to hydrolysis using the *Cryptococcus laurentii* yeast enzyme extract for 72 h. This enzyme extract promoted the conversion of approximately 32% of the cellulose, of which 2.4% was glucose, after the enzymatic hydrolysis reaction, suggesting that *C. laurentii* is a good β-glucosidase producer. The results presented in this study highlight the importance of isolating microbial strains that produce enzymes of biotechnological interest, given their extensive application in biofuel production.

Keywords: *Cryptococcus laurentii*, yeasts, second-generation ethanol, bioethanol, Cerrado, Amazon region

Introduction

Ethanol fuel production from lignocellulosic biomass is emerging as one of the most important technologies for sustainable development. Bioethanol is an alternative for reducing greenhouse gas emissions associated with global warming and climate change [20, 37]. Agricultural residues, such as sugarcane bagasse for bioethanol production, are an interesting alternative and are essential for sustainable development. In addition to not competing with food crops, lignocellulosic residues are cheaper than conventional materials [1]. Cellulose is the most abundant constituent of plant biomass, representing approximately 30% to 50% of plant tissue [28]. Given its renewable nature, cellulose is a potential raw material for the production of biofuels, chemicals, energy, and other materials of industrial interest [30, 47]. The monosaccharides contained in the cellulosic (glucose) and hemicellulosic fractions (xylose, arabinose, mannose, and galactose) represent substrates that can be used for ethanol production *via* fermentation. However, physical and chemical barriers presented by the cohesive combination of the main components of lignocellulosic biomass (cellulose, hemicellulose, and lignin) hinder the hydrolysis of cellulose and hemicellulose into fermentable sugars [38]. Many lignocellulosic materials have been tested for bioethanol production in tropical countries, particularly in Brazil. Sugarcane bagasse, which is a fibrous residue obtained after sugarcane juice extraction during sugar and alcohol production, is one of the main lignocellulosic materials produced in large quantities [7]. Given the importance of bagasse as an industrial residue, there is much interest in developing methods for its use in

the production of fuels and other products of economic interest [28]. The following steps are necessary for ethanol production from lignocellulosic biomass: pre-treatment, hydrolysis, fermentation, and product separation/purification. The task of hydrolyzing lignocellulosic material into fermentable monosaccharides remains technologically difficult because of the low cellulose digestibility due to physical-chemical and structural factors [13]. Thus, pre-treatment is an essential step for obtaining fermentable sugars in the hydrolysis step [24, 41]. Hydrolysis using appropriate enzymes represents another efficient method for releasing sugars from cellulosic materials, and specific enzymes, called cellulolytic enzymes or cellulases, catalyze this process [39]. The enzymatic hydrolysis of cellulose involves the synergistic action of three enzymes of the cellulolytic complex: exo-1,4-β-D-glucanase (E.C. 3.2.1.91), which hydrolyzes the cellulosic chain from its ends; endo-1,4-β-D-glucanase (E.C. 3.2.1.4), which hydrolyzes the cellulosic chain internally at random; and β-D-glucosidase (E.C. 3.2.1.21), which hydrolyzes cellobiose into glucose [47]. Various fungal and bacterial species produce cellulase and transport it across cell membranes into the external environment [21]. Thongekkaew *et al*. [40] reported efficient cellulose degradation by the fungi of the genera *Trichoderma*, *Aspergillus*, *Penicillium*, and *Fusarium*. Asha *et al*. [2] isolated and identified a novel bacterial strain, MG7, as *Paenibacillus barcinonensis* with high cellulase activity, but few studies have identified cellulase-producing yeasts. The recalcitrant nature of lignocellulosic materials and the high cost of these enzymes hinder the widespread use of these materials for biofuel production. Therefore, improving the carbohydrate conversion into fermentable sugars is of critical importance for the commercial production of lignocellulosic ethanol [46]. The first step in developing the industrial production of an enzyme is to isolate potential strains, and in this case, isolating and selecting cellulase-producing microorganisms is immensely important to optimize their performance in biotechnological applications in biofuel production [16]. Therefore, the present study was conducted with the following objectives: isolate cellulolytic enzyme-producing yeasts in soil samples, select the best isolates for producing the enzyme extract, and evaluate the hydrolytic capacity of the enzyme extract on sugarcane bagasse pre-treated with dilute acid.

Materials and Methods

Isolating and Selecting Cellulolytic Yeasts

Sampling site and sample collection. Soil samples were collected in Brazil from the Alto Solimões region, western Amazonas State, municipality of Benjamin Constant, and from the Minas Gerais Cerrado in the municipalities of Arcos, Passos, and Luminárias, during periods of high rainfall. In the Cerrado (CE), five composite samples were collected (CE1, CE2, CE3, CE4, and CE5) from each municipality, totalling 15 samples. Each composite sample consisted of 12 simple subsamples, collected at a 20 cm depth. There were a total of 19 Amazon soil samples, including seven samples from primary rainforests (RF1, RF2, RF3, RF4, RF5, RF6, and RF7), two samples from agroforestry (AF1 and AF2), five samples from old secondary forests (OF1, OF2, OF3, OF4, and OF5), two samples from young secondary forest (YF1 and YF2), and three samples from pastures (PA1, PA2, and PA3). For each sampling point in both regions (Amazon and Cerrado), the subsamples were collected in two concentric circles at 3 and 6 m radii from the center. The soil samples were extracted using a flame-sterilized auger, packed in sterile plastic bags, transported to the laboratory, and stored in a freezer for later use [23].

Yeast isolation. Yeasts were isolated from the soil samples using an enrichment technique. A total of 10 g of each sample was suspended in 90 ml of yeast extract peptone dextrose (YEPD) medium containing 2% glucose, 2% peptone, and 1% yeast extract and incubated at 28°C on a rotary shaker (130 rpm) for 2 to 7 days. Serial dilutions of the suspension were surface-plated on YEPD agar, pH 3.5, to prevent bacterial growth. The plates were incubated under aerobic conditions at 28°C for 2 to 3 days. Morphologically different colonies appearing on the plates were purified through repeated streaking on YEPD agar, pH 3.5. The purified isolates were stored in 20% glycerol at –80°C and used during the course of the study.

Screening and identification of cellulase-producing yeasts. Aliquots of 10 µl of 10^7 cells/ml of each isolate were used to inoculate plates containing CMC agar (per liter), 0.2% carboxymethylcellulose (CMC), 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.02% peptone, and 1.7% agar. The plates were incubated at 28°C for 48 h. After incubation, the size of the colony was measured, and the surface of the medium was flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 ml of distilled water) for 3 to 5 min [22], leaving a clear halo where the CMC had degraded. Cellulase-producing strains were selected based on the relationship between the diameter of the halo and the diameter of the colony, expressed as an enzyme index (EI). Cellulase-producing yeasts were selected for identification and used in further studies. Internal transcribed spacer (ITS) region sequencing was used to identify the yeasts following the methodology of Ramos *et al*. [31]. The ITS region was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplification products were separated by electrophoresis in 0.5% agarose gel at 60–65 V in 0.5× TAE for 1 h. The DNA was purified before sequencing, and the amplification products were detected through electrophoresis to confirm the presence of DNA. The sequencing of portions of the ITS region was performed at Macrogen, Seoul, Korea. Sequence similarity searches were performed using the

BLAST database from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

Enzyme production. We selected five yeast strains that exhibited the highest enzyme indices (EI > 3.0) (UFLA CES 523, UFLA CES 526, UFLA AMS 95.1B, UFLA AMS 98.1, and UFLA AMS 99.2). The inoculum of each isolate was prepared in 250 ml Erlenmeyer flasks containing 50 ml of YEPD medium. The yeast was grown at 28°C under 150 rpm agitation for 24 h.

Cellulase was produced under submerged fermentation (SmF) using three different carbon sources. The medium for cellulase production consisted of the following: 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.02 g of peptone, and 0.2% of different inducers. Carboxymethylcellulose (CMC), Avicel, and cellobiose were used as inducers to produce endoglucanase, exoglucanase, and β-glucosidase enzymes, respectively. SmF was conducted in 250 ml Erlenmeyer flasks containing 50 ml of production medium with the respective inducers. The flasks were inoculated using enough inoculum volume to obtain a concentration of 10^7 cells/ml in the fermentation medium. The Erlenmeyer flasks were incubated in a rotary shaker at 28°C and 150 rpm, for 48 h. After fermentation, the content of each flask was centrifuged at 3,600 ×*g* at 4°C for 10 min. The enzymatic activities were determined in the cell-free supernatant, which was labeled as crude enzyme.

Enzyme activity assays. All enzymes in this study were analyzed according to Lever [17], with slight modifications. For endoglucanase (CMCase) and exoglucanase activities, the assays were conducted in reaction tubes (1,000 µl) containing 450 µl of 1% (w/v) carboxymethyl cellulose (CMC) or 1% (w/v) Avicel, respectively, in 0.05 mM sodium acetate buffer, pH 5.0, with 50 µl of crude enzyme solution. The tubes were incubated at 50°C for 30 min. To measure the glucose released through enzyme action, we used 1% ρ-hydroxybenzoic acid hydrazide (PAHBAH). One unit (U/ml) of enzyme activity was defined as the amount of enzyme that produces 1 µmole of glucose per minute under the assay conditions. The β-glucosidase activities were assayed using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate. The reaction tubes (1,000 µl) contained 300 µl of pNPG in 0.05 mM sodium acetate buffer, pH 5.0, and 200 µl of crude enzyme extract. The reaction was performed at 50°C for 30 min and subsequently stoped through the addition of 1 ml of 1 M Na_2CO_3, and the amount of *p*-nitrophenol was measured spectrophotometrically at 405 nm. One unit (U/ml) of enzyme activity was defined as the amount of enzyme that produces 1 µmole of *p*-nitrophenol per minute under the assay conditions**.** For specific activity determination, the protein content of each sample was determined, using bovine serum albumin as a standard [5].

Statistical Analysis

The statistical analysis was performed with SISVAR 5.1 software (Statistical Analysis System) [10] using the Skott Knott test at 5% significance to compare the best isolates that produce endoglucanase, exoglucanase, and β-glucosidase enzymes.

Enzymatic Hydrolysis of Pre-Treated Sugarcane Bagasse

Sugarcane bagasse sample pre-treated with diluted H_2SO_4. Sugarcane bagasse was obtained from a local farm and ground in a knife mill after drying to room temperature and subsequently sieved (20 mesh). The sugarcane bagasse was subjected to pre-treatment with dilute H_2SO_4 in a 2 L stainless steel reactor (Parr Marks, model 4848). The following previously defined pre-treatment conditions were used: solids content of 15% (w/w), loading of 2.0% (w/w) H_2SO_4, temperature of 150°C, and hydrolysis time of 30 min. The compositions of raw materials *in natura* and in pre-treated bagasse were determined according to Van Soest [42]. The obtained slurry was filtered into a liquid hydrolysate and a fiber residue. The levels of monomeric and oligomeric sugars, cellobiose, and by-products (acetic acid and furfural) were analyzed in the liquid fraction. The fibrous residue, after several washes with water, was dried in an oven at 65°C until reaching a constant weight for subsequent use in the enzymatic hydrolysis step.

Enzymatic hydrolysis of the pre-treated bagasse. Enzymatic hydrolysis assays of the pre-treated bagasse were performed in 250 ml Erlenmeyer flasks, in triplicate. The reaction mixture (150 ml) for the enzymatic hydrolysis of sugarcane bagasse comprised 75 ml of sodium acetate buffer (0.05 M, pH 5.0) with 2% of pre-treated biomass, which had been autoclaved for 15 min at 121°C. Then 75 ml of an enzyme cocktail was added (consisting of 35 ml of exoglucanase, 25 ml of endoglucanase, and 15 ml of β-glucosidase, equivalent to an enzyme load of 42.22, 71.64, and 369.24 U/mg, respectively), produced by the isolate UFLA AMS 95.1B from the Amazon region.

Similarly, a substrate without the enzyme cocktail was used as a control, using sodium acetate buffer (0.05 M, pH 5.0) to complete the final reaction volume. The hydrolysis was performed at 45°C under mechanical agitation at 150 rpm for 72 h. At 0, 24, 48, and 72 h of enzymatic hydrolysis, 2 ml aliquots were removed and frozen for subsequent chromatographic analyses. The remaining fibrous residue was characterized according to levels of cellulose, hemicellulose, and lignin, consistent with the methodology of Van Soest [42].

Chromatographic Analysis

The analyses for glucose and acetic acid content were performed using a high-performance liquid chromatography (HPLC) system (Model LC-10Ai; Shimadzu Corp., Tokyo, Japan) equipped with a dual detection system comprising a UV detector and a refractive index detector (RID-10A SPD-10Ai). A Shimadzu ion-exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) operated at a temperature of 50°C was used to achieve chromatographic separation. The acids were detected *via* UV absorbance (210 nm), whereas the sugars were detected *via* RID. To determine the concentrations of cellobiose, xylose, and arabinose, a 5 µm Spherisorh NH_2 column (4.6 × 150 mm) was used with a 37°C temperature, an acetonitrile/water (80:20) solution, a 1 ml/min flow rate, and a 20 µl injection volume.

The furfural was analyzed using gas chromatography (GC) on a Shimadzu model 17A equipped with a flame ionization detector (FID) and silica HP FFAP capillary column (30 m × 0.25 mm i.d. × 0.25 µm) (J&W Scientific, Folsom, USA). For GC analysis, 100 µl of each sample was diluted 20-fold in milli-Q water and filtered using a nitrate-cellulose membrane (0.20 µm pores) before injection into the GC. Operating conditions were as follows: oven temperature was kept at 60°C for 3 min, programmed to 75°C at 2°C/min, kept at 100°C for 3 min, programmed to 184°C by increasing 3°C/min, then kept at 184°C for 30 min, and then programmed to 220°C in 15 min. Injector and detector temperatures were kept at 240°C and the carrier gas (N_2) was kept at a flow rate of 1.2 ml/min [9].

Scanning Electron Microscopy of Sugarcane Bagasse

For the structural characterization, scanning electron microscopy was performed on samples of *in natura* sugarcane bagasse pre-treated with H_2SO_4 after enzymatic hydrolysis (72 h), using a LEO scanning electron microscope (model EVO 40). The samples were mounted on aluminium "stub" supports and subsequently subjected to gold metal plating (Bal-Tec SCD 050 metallizer) [25].

Results and Discussion

Isolation of Yeasts

A total of 103 yeast isolates were purified from Cerrado soil samples collected from the following cities: Passos, Arcos, and Luminárias, Minas Gerais State, Brazil. The yeast populations (log CFU/g of soil) ranged from 3.34 log CFU/g (point CE2p at Passos) to 8.90 log CFU/g (points CE1a and CE4a at Arcos) (Fig. 1). A total of 214 yeast isolates were obtained from native forest soils of the Amazon region. The largest population was identified at point RF6 in a rainforest site, with 7.34 log CFU/g of soil, and the smallest population was obtained at point PA1 in a pasture site, with 3.00 log CFU/g of soil (Fig. 2). The Amazon region contains large areas without human intervention, which can be exploited to study their biodiversity [19] and potential for biotechnological applications. As these are environmental samples, no result is unexpected in terms of microbial population size and diversity. The yeast populations in soil vary and are influenced by several factors, such as species longevity, ability to compete with other soil organisms, soil composition, season, climate, temperature, exposure to sunlight, and moisture, among others. Evidence for the variability being influenced by these factors includes quantities ranging from a few up to thousands of cells per gram of soil in different samples from the same environment [8].

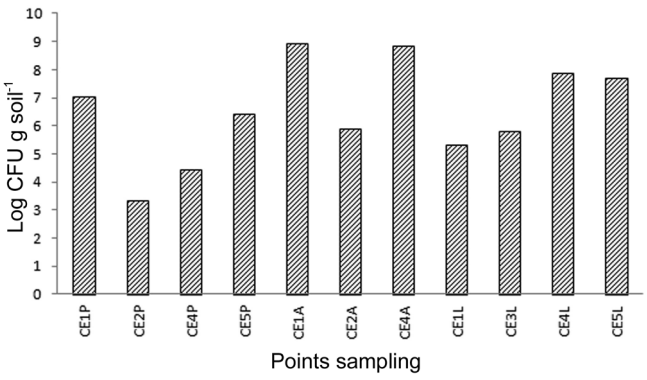

Fig. 1. Microbial density (log CFU g/soil) of the yeasts from Minas Gerais Cerrado.

CE1, CE2, CE3, CE4, and CE5 refer to the five composite samples from each municipality Arcos (A), Passos (P), and Luminárias (L).

Screening and Identification of Cellulase-Producing Yeasts

Of the 103 isolates obtained from Minas Gerais Cerrado, 18 (17.5%) exhibited a hydrolysis halo surrounding the colony and were considered positive for cellulase production. Out of a total of 214 isolates from the Amazon region, 11 (5.14%) were cellulase-producing. Isolates that exhibited positive results for cellulase production in solid medium were identified as *Cryptococcus laurentii* based on ITS region sequencing and subsequent comparison with GenBank reference sequences, using the BLAST. The sequences exhibited greater than 98% similarity (Accession No. FN428926.1).

The *Cryptococcus laurentii* yeast identified in the present study was previously described in Amazonian soils [44].

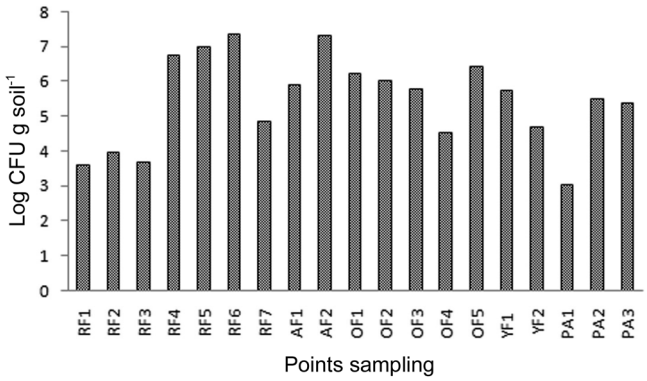

Fig. 2. Microbial density (log CFU g/soil) of the yeasts from the Amazon region municipality of Benjamin Constant, including samples from primary rainforests (RF1, RF2, RF3, RF4, RF5, RF6, and RF7), agroforestry (AF1 and AF2), old secondary forests (OF1, OF2, OF3, OF4, and OF5), young secondary forest (YF1 and YF2), and pastures (PA1, PA2, and PA3).

Sláviková and Vadkertiová [34, 35] also identified this species in agricultural and temperate forest soils and characterized *Cryptococcus laurentii* as being typical in soil. Reports on cellulase activity in yeast are scarce. The main genera and species related to the production of cellulase are *Cryptococcus flavus* [29], *Aureobasidium pullans* [18], and *Candida, Rhodothorula, Pichia,* and *Debaryomyces* [15]. Besides the cellulases, the genus *Cryptococcus* is reported as a producer of other economically important enzymes such as β-xylosidase, xylanase [3, 11, 4, 29], α-amylase [12, 45], and phytase [43].

Enzyme Assay

After submerged fermentation, we assessed the production of endoglucanase, exoglucanase, and β-glucosidase in five yeast isolates that exhibited the highest enzymatic indices (EI > 3.0); three isolates were obtained from the Amazon region, and two isolates were obtained from the Minas Gerais Cerrado region. As shown in Fig. 3, endoglucanase, exoglucanase, and β-glucosidase activities were detected. All selected isolates were capable of producing cellulolytic enzymes, but the activity levels varied in different induction media. There was no significant difference in the production of endo- and exoglucanase among the isolates (UFLA CES 523, UFLA CES 526, UFLA AMS 95.1B, UFLA AMS 98.1, and UFLA AMS 99.2). However, the UFLA 95.1B isolate exhibited the highest endo- and exoglucanase activities, at 2.8 and 1.3 U/mg, respectively. The data presented in Fig. 3 clearly indicate that β-glucosidase had the highest enzyme activity in all isolates and had a higher activity than the endo- and exoglucanases. However, a few isolates exhibited significant differences in β-glucosidase activity. The UFLA AMS 95.1 B isolate was the best producer, with 24.61 U/mg activity, followed by UFLA CES 523 (18.89 U/mg), UFLA AMS 98.1 (18.38 U/mg), and UFLA AMS 99.2 (16.37 U/mg), and UFLA CES 526 had the lowest activity at 10.11 U/mg. This difference in β-glucosidase activity might reflect the fact that most yeast species occurring in soils possess a broad spectrum of metabolic capacity, facilitating the use of various products resulting from the degradation of plants and other microorganisms. Moreover, almost all soil yeasts grow in the presence of cellobiose as a carbon source, which is produced during cellulose breakdown [36]. Thus, even if β-glucosidases do not directly act on cellulose, these enzymes convert cellobiose and cello-oligosaccharides, which are produced by endo- and exoglucanase, into glucose [13]. The specific activities of isolates from the Amazon region were generally higher than isolates from the Cerrado region (Table 1). Notably, the yeast strains used in this study were isolated from two regions that used different cover crops during different seasons. Thus, these factors could directly influence the results obtained with the different isolates of the same species, which exhibited different enzymatic activities. One isolate from the Amazon region (UFLA AMS

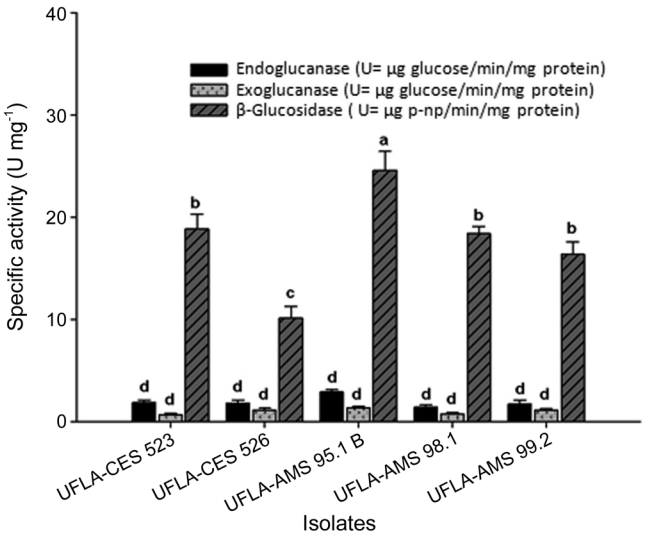

Fig. 3. Specific enzymatic activity of endoglucanase, exoglucanase, and β-glucosidase (U/mg) of yeasts.

The same letters means that the data did not differ by Skott–Knott test at 5% probability. The bars indicate standard deviation.

Table 1. Specific β-glucosidase activity of the different *Cryptococcus laurentii* strain isolated from Minas Gerais Cerrado and Amazon region.

Region	Strain	β-Glucosidase activity (U/mg)*
Minas Gerais Cerrado	*Cryptococcus laurentii* UFLA CES 523	18.89 ± 2.05[b]
	Cryptococcus laurentii UFLA CES 526	10.11 ± 1.63[c]
Amazon region	*Cryptococcus laurentii* UFLA AMS 95.1B	24.61 ± 2.05[a]
	Cryptococcus laurentii UFLA AMS 98.1	18.38 ± 1.01[b]
	Cryptococcus laurentii UFLA AMS 99.2	16.37 ± 1.72[b]

*Different letters indicate significant differences ($P < 0.05$).

Table 2. Composition of sugarcane bagasse untreated and after pre-treatment in g/100 g dry matter[a].

Content	Sugarcane bagasse untreated	Sugarcane bagasse pre-treated
Cellulose	42.10 ± 0.40	27.78 ± 0.70
Hemicellulose	24.90 ± 0.70	12.45 ± 0.20
Lignin	21.30 ± 0.30	18.41 ± 0.10
Ash	2.65 ± 0.26	0.88 ± 0.01

[a]Data are the mean values of triplicate measurements ± standard deviation.

95.1B) was selected for enzyme extract production and subsequent sugarcane bagasse hydrolysis.

Chemical Composition and Effect of Pre-Treatment on Bagasse

Table 2 shows the results of the chemical characterization of the sugarcane bagasse used in this study, both before (*in natura*) and after pre-treating with 2% H_2SO_4 at 150°C for 30 min. The *in natura* sugarcane bagasse composition consisted of 42.10 ± 0.40% cellulose and 24.90 ± 0.70% hemicellulose before pre-treatment, and pre-treatment resulted in levels of 27.78 ± 0.7% cellulose and 12.45 ± 0.2% hemicellulose (g/100 g dry matter). Therefore the solubilization reduced the levels of cellulose and hemicellulose in the biomass by approximately 34% and 50%, respectively. The glucose and xylose yields following different pre-treatments indicated the efficient conversion of cellulose and hemicellulose into the corresponding monosaccharides [22]. After pre-treating with H_2SO_4, 9.07 g of glucose/100 g DM and 30.14 g of xylose/100 g DM were released into the liquid fraction. In decomposing hemicelluloses, 12.34 g of acetic acid/100 g DM and 0.59 g of furfural/100 g DM were also produced. During the degradation of the lignocellulosic structure, many inhibitory compounds are released, in addition to fermentable sugars [26]. The presence of acetic acid and furfural suggests pentose degradation, generating inhibitory compounds [6]. Martín *et al*. [22] pre-treated the same residue with the same catalyst used in the present study (sugarcane bagasse and sulfuric acid), but under conditions of 1% sulfuric acid at 205°C for 10 min. After pre-treatment, higher glucose (22.6 g/100 g dry bagasse) and lower xylose (3.6 g/100 g) and arabinose (0.4 g/100 g) yields were obtained in the liquid fraction. However, we detected high concentrations of fermentation-inhibiting compounds, such as furfural (4.4 g/100 g dry bagasse), hydroxymethylfurfural (1.8 g/100 g dry bagasse), and acetic acid (6.5 g/100 g dry bagasse). The results of Martín *et al*. [22] were similar to those obtained in the present study, which showed furfural and acetic acid as compounds that negatively influence the subsequent steps of the process [27]. Saha *et al*. [32] did not detect furfurals in the hydrolysates of wheat straw pre-treated at 140°C and 160°C, but 11 and 32 mg of furfural/g DM were produced at 180°C with sulfuric acid concentrations of 0.25% and 0.50% (v/v), respectively. Furthermore, these authors did not detect hydroxymethylfurfural in the hydrolysates but rather detected acetic acid in all hydrolysates, and the acetic acid concentration increased significantly with increasing temperature.

Enzymatic Hydrolysis

The steps involved in processing sugarcane bagasse, after pre-treatment, are shown in Fig. 4. The obtained fibrous residue consisting of 27.78 g of cellulose/100 g DM, and 12.45 g of hemicellulose/100 g DM was subjected to enzymatic hydrolysis for 72 h.

The enzyme extract used to hydrolyze the fibrous residue of sugarcane bagasse was prepared from *Cryptococcus laurentii* yeast. The enzyme load used for hydrolysis comprised 42.22 U/mg of exoglucanase, 71.64 U/mg of endoglucanase, and 369.24 U/mg of β-glucosidase.

Fig. 5 shows the results of glucose released during 72 h of enzymatic hydrolysis. The cellulose conversion after this period was approximately 32%, resulting in the formation of 148 mg of glucose/L.

As cellobiose was not detected in the hydrolysate, the amount of glucose detected might be a consequence of low endo- and exoglucanase activities, which resulted in low cellobiose concentrations for β-glucosidase action. In contrast, the β-glucosidase activity was effective in converting cellobiose into glucose. Santos *et al*. [33] assessed different enzyme loads for the enzymes obtained from *Trichoderma reesei* and β-glucosidase from *Aspergillus* sp. to hydrolyze sugarcane bagasse pre-treated with H_2SO_4. These authors observed that lower cellulose conversion values are associated with low enzyme load. These results demonstrate the importance of enzyme load in the saccharification of the cellulose contained in lignocellulosic materials. Not all commercial enzymes exhibit excellent activities for the three

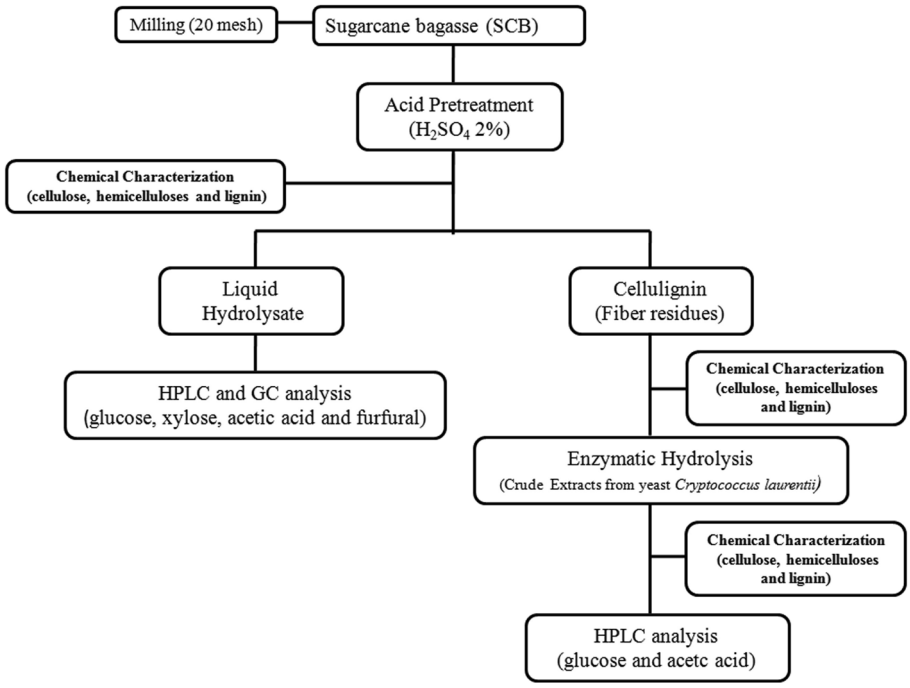

Fig. 4. A general flowchart of the steps involved in processing the sugarcane bagasse.

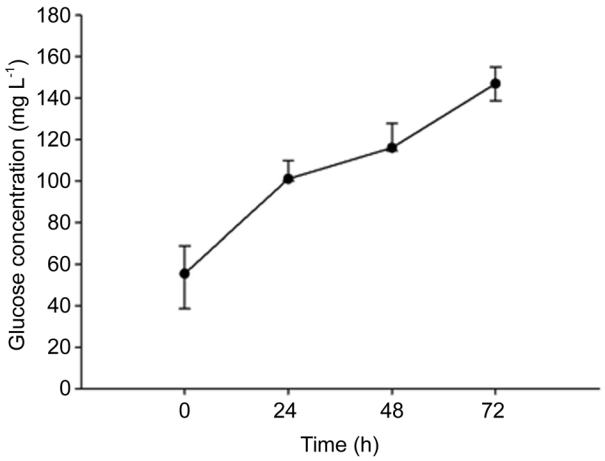

Fig. 5. Concentration of glucose released during 72 h of enzymatic hydrolysis of the acid-treated sugarcane bagasse using enzymes produced by yeast *Cryptococcus laurentii*.
The bars indicate standard deviation of the average.

cellulolytic complex enzymes, which require supplementing the enzyme load using an enzyme cocktail to obtain a more efficiently degraded lignocellulosic biomass.

Structural Characterization of Sugarcane Bagasse Using Scanning Electron Microscopy

A scanning electron microscope was used to visualize the samples of sugarcane bagasse *in natura* (Fig. 6A), following pre-treatment (Fig. 6B) and after enzymatic hydrolysis (Fig. 6C).

As shown, *in natura* sugarcane bagasse exhibited a fibrous structure that was completely covered with parenchymal cells. Pre-treating with H_2SO_4 did not destroy the fibrous structure of bagasse; however, pre-treatment reduced its compact appearance (*i.e.*, fragmented the morphological structure of the lignocellulosic material), which can also be observed after enzymatic treatment.

Pre-treatment processes are performed to alter the structural features of lignocellulosic biomass (cellulose, hemicellulose, and lignin). The primary objective of acid pre-treatment is to solubilize the hemicellulosic fraction, altering the lignin structure and increasing the surface area of cellulose to provide action sites for enzymes [1].

According to the literature, material with a higher degree of modification can be obtained when using pre-treatments aimed at removing lignin, which might reduce the possible physical barriers and thereby increase the contact surface area to improve enzymatic hydrolysis [14].

In conclusion, Brazilian biomes contain large areas that can be exploited to study their biodiversity. The isolation and selection of microorganisms in natural environments have been important tools for obtaining strains that produce industrially important enzymes. Yeasts isolated from the Cerrado and Amazon rainforest regions are

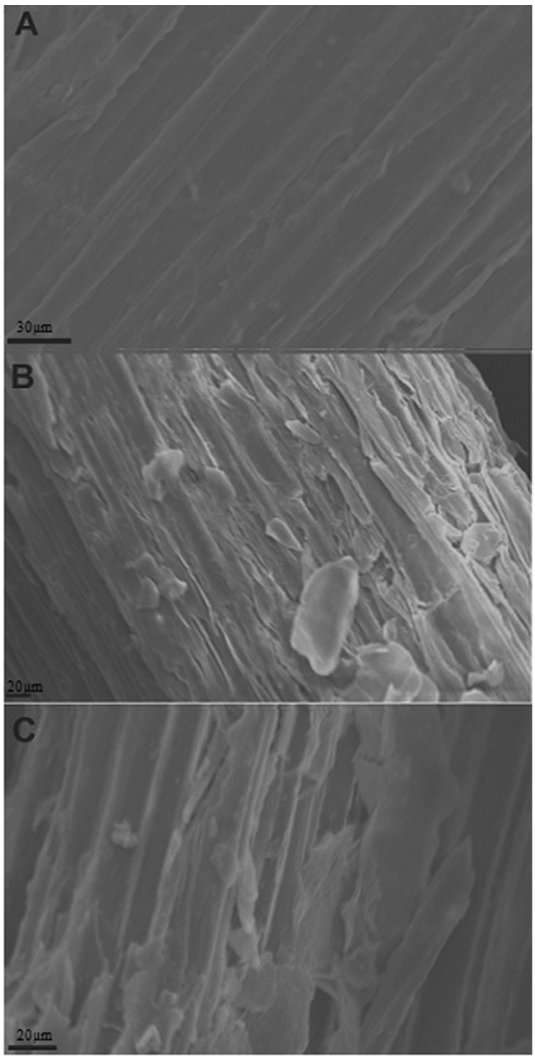

Fig. 6. SEM images of sugarcane bagasse.
(**A**) Untreatred; (**B**) pretreated with 2% H_2SO_4; (**C**) after enzymatic hydrolysis for 72 h; The bars in A, B, and C are equivalent to 30, 20, and 20 μm, respectively.

potential sources of the hydrolytic enzymes necessary for converting biomass and producing lignocellulosic ethanol. The differences in the enzymatic activity of *Cryptococcus laurentii* strains likely reflect sampling from two regions with different cover crops and during different seasons. The β-glucosidase activity level (24.61 U/mg) detected in this study indicated that yeasts isolated from natural sources (Cerrado and Amazon rainforest) might be promising for the production of this enzyme, as yeast cellulases have been poorly studied. The enzyme extract promoted the conversion of approximately 32% of the cellulose, and 2.4% of the extract was in the form of glucose after the enzymatic hydrolysis reaction, suggesting that the *C. laurentii* microorganism is a good β-glucosidase producer. Future studies will be conducted to increase the production of this enzyme by optimizing relevant parameters for improving the enzymatic hydrolysis efficiency.

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