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# Production of β-glucosidase on solid-state fermentation by *Lichtheimia ramosa* in agroindustrial residues: Characterization and catalytic properties of the enzymatic extract

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ABSTRACT

*Background:* β-Glucosidases catalyze the hydrolysis of cellobiose and cellodextrins, releasing glucose as the main product. This enzyme is used in the food, pharmaceutical, and biofuel industries. The aim of this work is to improve the β-glucosidase production by the fungus *Lichtheimia ramosa* by solid-state fermentation (SSF) using various agroindustrial residues and to evaluate the catalytic properties of this enzyme.
*Results:* A high production of β-glucosidase, about 274 U/g of dry substrate (or 27.4 U/mL), was obtained by cultivating the fungus on wheat bran with 65% of initial substrate moisture, at 96 h of incubation at 35°C. The enzymatic extract also exhibited carboxymethylcellulase (CMCase), xylanase, and β-xylosidase activities. The optimal activity of β-glucosidase was observed at pH 5.5 and 65°C and was stable over a pH range of 3.5–10.5. The enzyme maintained its activity (about 98% residual activity) after 1 h at 55°C. The enzyme was subject to reversible competitive inhibition with glucose and showed high catalytic activity in solutions containing up to 10% of ethanol.
*Conclusions:* β-Glucosidase characteristics associated with its ability to hydrolyze cellobiose, underscore the utility of this enzyme in diverse industrial processes.



## 1. Introduction

The pronounced scarcity of fossil fuels related to environmental problems resulting from their processing and consumption has prompted the search for alternative sources of biofuels and renewable energy. This in turn, has generated significant interest in the use of cellulases and other enzymes to convert vegetal biomass into fermentable sugars [1].

Enzymatic hydrolysis of cellulose to glucose requires at least three different enzymes including endo-glucanases (EC 3.2.1.4), that internally hydrolyze cellulose chains, reducing its degree of polymerization; exo-glucanases (EC 3.2.1.91) that attack the non-reducing and reducing extremities of cellulose, releasing cellobiose; and β-glucosidases (EC 3.2.1.21) that hydrolyze cellobiose and oligosaccharides, thereby releasing glucose [2].

The ability of β-glucosidase to utilize different glycosidic substrates renders it suitable for several industrial processes, including the enzymatic hydrolysis of cellulose in order to obtain fermentable sugars and the production of functional foods derived from soy. The enzyme is also used in the juice and beverage industry, where it can improve the aromatic quality of wine and other grape derivatives [3].

The obtainment of industrial enzymes in a sustainable and economically viable manner requires the pursuit of renewable raw materials and processes at low cost. The use of solid-state fermentation (SSF) can reduce the environmental impact and add value to the by-products of agroindustry [4]. The iterative improvement and advantages of SSF have been described in several reports, which studied the influence of different cultivation parameters on the production of microbial enzymes [5,6]. The advantages of SSF include the simplicity of growth conditions, because they are very similar to the environmental systems where many microorganisms develop (especially filamentous fungi); the reduced energy consumption, and that complex equipment or sophisticated control systems are not required. The method also results in higher levels of productivity and low catabolite repression, and favors increased stability of the secreted enzymes [7].

In general, the industrial applicability of an enzyme is closely related to the cost of its production and physicochemical characteristics. The

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production costs can be reduced by screening hyper producer strains, associated with the cultivation process optimized in low-cost mediums [3]. Previous work conducted by our Research Group revealed high β-glucosidase production by the fungus *Lichtheimia ramosa* by SSF using several lignocellulosic materials [6,8]. This study aimed to optimize the β-glucosidase production by this fungus by SSF. The β-glucosidase produced was biochemically characterized and the catalytic properties of the enzymatic extract were evaluated.

## 2. Material and methods

### 2.1. Microorganism

The filamentous fungus *L. ramosa* was isolated from sugarcane bagasse provided by São Fernando Açúcar e Álcool Ltda., Dourados, MS, Brazil [8]. The microorganism was maintained on Sabouraud Dextrose Agar medium; after growth at 28°C for 48 h, the strain was stored at 4°C.

### 2.2. Inoculum

The organism was cultivated in inclined 250 mL Erlenmeyer flasks containing 40 mL of Sabouraud Dextrose Agar and maintained for 48°C h at 28°C. A fungal suspension was obtained by adding 25 mL of nutrient solution and gently scraping the surface of the culture. The nutrient solution was composed of 0.1% ammonium sulfate, 0.1% magnesium sulfate heptahydrate, and 0.1% ammonium nitrate (w/v) [9]. As inoculum, 5 mL of this suspension was transferred to each 250 mL Erlenmeyer flask containing lignocellulosic material as substrates.

### 2.3. Solid-state fermentation (SSF)

The enzyme was produced by cultivating the fungus in 250 mL Erlenmeyer flasks containing 5 g of substrate (wheat bran, soy bran, corn cob, corn straw, rice peel, or sugarcane bagasse), previously washed and dried at 60°C for 24 h. The optimal substrate for enzyme production was used in subsequent steps to evaluate the effects of varying the pH of cultivation medium, moisture content, temperature, and time of cultivation. The parameter selected in each step was used for further cultivation, in an iterative strategy designed to optimize the fermentation process for β-glucosidase production. All material was previously autoclaved for 20 min at 121°C, and the experiments were performed in duplicate.

### 2.4. Enzyme extraction

The extraction of the enzyme from the fermented substrate was carried out by adding 50 mL of distilled water, and constantly shaking at 100 rpm for 1 h. The sample was filtered and centrifuged at 3000 × g for 5 min. The supernatant was considered the enzymatic extract and was used in the following steps.

### 2.5. Determination of β-glucosidase activity

The β-glucosidase activity was determined with 50 μL of enzymatic extract, 250 μL of sodium acetate buffer (0.1 M, pH 4.5), and 250 μL of p-nitrophenyl-β-D-glucopyranoside (4 mM, pNPβG, Sigma) during a 10 min reaction at 50°C. The enzymatic reaction was stopped with 2 mL of sodium carbonate (2 M), and the liberated product was spectrophotometrically quantified at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of nitrophenol per minute of reaction [10].

### 2.6. Characterization of β-glucosidase produced by the fungus L. ramosa

#### 2.6.1. Effect of pH

The optimum pH for β-glucosidase activity was determined by measuring the activity at 50°C at different pH values (3.0–8.0), with increments of 0.5, using 0.1 M citrate–phosphate buffer solution. The pH stability was determined incubating the enzyme for 24 h at 25°C at different pH values, appropriately diluted with buffer solutions: 0.1 M citrate–phosphate (pH 3.0–8.0), 0.1 M Tris–HCl (pH 8.0–8.5), and 0.1 M glycine NaOH (pH 8.5–10.5), with increments of 0.5, adopting as 100% the highest value of residual activity obtained after the samples treatment. The residual activity was determined under optimal conditions of pH and temperature.

#### 2.6.2. Effect of temperature

The optimum temperature for β-glucosidase activity was obtained by determining the enzymatic activity over a temperature range of 30°C–75°C, with increments of 5°C, at the respective optimum pH. Thermostability was determined by incubating the enzyme for 1 h at different temperatures (30°C–70°C), with increments of 5°C, adopting as 100% the highest value of residual activity obtained after the samples treatment. The residual activities were measured under optimal conditions of pH and temperature.

#### 2.6.3. Effect of glucose and ethanol on β-glucosidase activity

The enzymatic activity was quantified with the addition of glucose or ethanol at different concentrations in the reaction mixture (0–200 mM glucose or 0–30% of ethanol). The activities were measured under optimal conditions of pH and temperature.

### 2.7. Catalytic potential of the enzymatic extract

The CMCase and xylanase activities were quantified using 3% carboxymethylcellulose (Sigma C5678) and 1% xylan (Sigma Birch-Wood), respectively. The reducing sugar released was quantified by the DNS method [11]. The β-xylosidase activity was measured with the synthetic substrate p-nitrophenyl-β-D-xylopyranoside (4 mM, Sigma), following the methodology described in Section 2.5. The potential to hydrolyze cellobiose was evaluated with a glucose-oxidase kit (Glucose-PP Analisa). Specifically, 100 μL of the enzymatic extract was added to 0.9 mL of 50 mM sodium acetate buffer containing 0.5% cellobiose (Fluka). One unit of enzymatic activity was defined as the amount of enzyme capable of producing 1 μmol of product per min of reaction.

## 3. Results and discussion

### 3.1. Production of β-glucosidase by solid-state fermentation

#### 3.1.1. Selection of substrates for β-glucosidase production

Among the tested substrates, the cultivation of the fungus *L. ramosa* in wheat bran provided higher β-glucosidase production (162.2 U/g or 16.22 U/mL) (Table 1). The wheat bran has suitable nutritional composition as a substrate for microbial growth; it contains

**Table 1**
β-Glucosidase production in different agroindustrial substrates (75% of initial moisture) by *L. ramosa* by solid state fermentation, at 96 h of incubation, under 28°C.

| Substrate | U/mL | U/g |
|---|---|---|
| Wheat bran | 16.22 ± 0.42 | 162.2 ± 4.2 |
| Soy bran | 1.15 ± 0.07 | 11.5 ± 0.7 |
| Corn cob | 0.35 ± 0.04 | 3.5 ± 0.45 |
| Corn straw | 0.27 ± 0.02 | 2.7 ± 0.2 |
| Rice peel | 0.068 ± 0.00 | 0.68 ± 0.00 |
| Sugarcane bagasse | 1.11 ± 0.025 | 11.1 ± 0.25 |

appropriate quantities of carbohydrates, proteins, fats, fiber and ashes (Ca, Mg, P, K, S), favoring enzymes production [12].

High level β-glucosidase production can be achieved during the culture of microorganisms by solid-state fermentation, using wheat bran as either the main substrate, or as a substantial component of the mixture [3,8,12,13]. Thus, wheat bran was selected for subsequent assays in order to optimize the cultivation process for β-glucosidase production.

*3.1.2. Influence of fermentative parameters on β-glucosidase production using wheat bran as substrate*

The greatest β-glucosidase production by the fungus was obtained from the cultivation where the initial pH of the nutrient solution was adjusted to 5.0 (Fig. 1a). However, the microorganism showed considerable enzyme production for all pH values evaluated. Previous reports indicate that filamentous fungi produce cellulases at pH values below neutrality [14]. Most filamentous fungi show optimal growth in slightly acidic pH. In general, values of pH higher than 7.0 reduce fungal growth and, thereby reducing the enzyme production [15].

The pH was not controlled during the cultivation process due to the heterogeneity of the process of solid-state fermentation. According to Pandey et al. [16], the difficulty of monitoring and controlling fermentation parameters in solid-state fermentation is perhaps, the main drawback of this process. Variations of pH during the fermentation process are due to the metabolic activity of the microorganisms, and may be increased or decreased according to the by-products released or the nutrients consumed during the process.

Among the moisture values evaluated, the highest enzyme production was obtained in wheat bran with 65% of initial moisture (Fig. 1b). Values between 60 and 70% of moisture are often used for cultivation of filamentous fungi when the aim is to produce β-glucosidase. Leite et al. [3] reported the β-glucosidase production (70 U/g of substrate) by the cultivation of *Thermoascus aurantiacus* in wheat bran with 60% of moisture. Brijwani et al. [13] obtained higher production of β-glucosidase (10.71 U/g) using soybean peel and wheat bran with 70% of moisture, during co-cultivation of *Trichoderma reesei* and *Aspergillus oryzae* by solid-state fermentation.

The moisture in solid-state fermentation can influence the synthesis and secretion of extracellular enzymes. The presence of free water between the particles of the substrate reduces the porosity of the medium, interfering with the gas transfer and temperature. On the other hand, the low moisture content can decrease the solubility of nutrients, disfavoring microbial metabolic activity [17].

The ideal temperature for β-glucosidase production by *L. ramosa* was 35°C, about 249.0 U/g (24.9 U/mL) (Fig. 1c). Fig. 1c reveals that a higher amount of enzyme was produced in cultures carried out at 30°C–40°C. This optimal temperature for enzyme production is higher than the range most commonly considered optimal for the cultivation of mesophilic microorganisms, which is usually between 28°C and 30°C [13,18]. This characteristic favors the use of this strain in industrial processes, where variations in process temperature are acceptable, considering that the control of fermentation parameters on a large scale is not as precise as in laboratory conditions.

A considerable reduction in enzyme production was evident in cultures performed at 25°C and 45°C (Fig. 1c). Temperatures that are significantly lower than the optimal for microbial growth disfavor nutrient transport and the exchange of products between the intracellular and extracellular environment. This is because low temperatures reduce both the permeability of the plasma membrane and the speed of the metabolic reactions. On the other hand, very high temperatures cause the collapse of membranous structures and denature structural proteins and enzymes. Accordingly, both cases result in reduced enzyme production [12].

Finally, the influence of cultivation time was investigated. The highest enzyme production was obtained at 96 h of incubation at 35°C using wheat bran as the substrate with 65% of moisture and pH 5.0 (Fig. 1d). The overall optimization of the process increased the β-glucosidase production from 162.2 U/g (16.22 U/mL) to 274.0 U/g (27.4 U/mL) (Table 1 and Fig. 1d, respectively). In addition to increased enzyme production, the optimization permitted a reduction in cultivation time to less than the duration used in preliminary assays carried out by our research group [4,8]. Gonçalves et al. [8] reported the production of 17.26 U/mL of β-glucosidase in cultivation of the fungus *L. ramosa* for 120 h by solid-state fermentation. Our current results reinforce the importance of optimizing the culture parameters, as evidenced by a 68.9% increase in the β-glucosidase production when compared to the initial values.

The reduced cultivation time, achieved in the present work, is also a key improvement for fermentation techniques that use *L. ramosa*, since the cost of enzyme production is proportional to incubation time. A

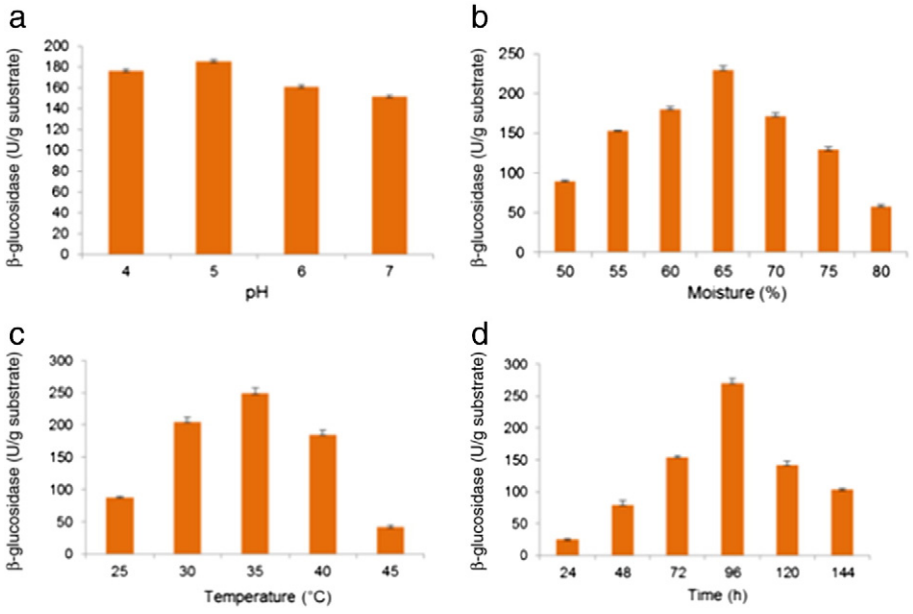


**Fig. 1.** Influence of fermentation parameters on β-glucosidase production by the fungus *L. ramosa*, by SSF in wheat bran. (a) Influence of initial cultivation pH; (b) Influence of initial substrate moisture; (c) Influence of cultivation temperature; (d) Influence of cultivation time. Conditions: pH 5.0; moisture 65%; temperature 35°C.

reduced amount of enzyme was found in extracts obtained after 96 h of cultivation (Fig. 1d). This is likely explained by the consumption of the culture medium nutrients and the excretion of by-products by the microorganism used for fermentation. Such by-products, which may interfere with protein synthesis as well as enzymatic activity, include proteases and substances that reduce macro and micronutrients, alter the pH, and decrease water availability [19].

The level of β-glucosidase production obtained during the current study is significantly higher than that described by other groups. Leite et al. [3] obtained 7.0 U/mL of β-glucosidase with 72 h of *T. aurantiacus* cultivation in wheat bran. Delabona et al. [20] obtained 105.82 U/g of substrate by cultivation of the fungus *Aspergillus fumigatus* in wheat bran for 96 h. Xin and Geng [21] obtained 61.6 U/g of substrate with *T. reesei* cultivated at 26°C for 192 h on woodchips. Ng et al. [2], reported the production of 159.1 U/g of substrate by the fungus *Penicillium citrinum* YS40-5 after cultivation in rice bran for 96 h. Zimbardi et al. [22] optimized the β-glucosidase production by the fungus *Colletotrichum graminicola* in wheat bran, with a maximum production substrate of 159.3 U/g, after 168 h. Silva et al. [6] reported the production of 0.061 U/mL of β-glucosidase by the fungus *L. ramosa* in pequi residue (typical fruit of the Cerrado vegetation), after 48 h in solid-state fermentation.

The enzymatic extract obtained, under optimal culture conditions, was used in subsequent steps for the biochemical characterization of β-glucosidase.

### 3.2. Characterization of β-glucosidase produced by the fungus L. ramosa

#### 3.2.1. Effect of pH and temperature

The β-glucosidase produced by the fungus *L. ramosa* showed higher catalytic activity at pH 5.5 and temperature of 65°C (Fig. 2a and Fig. 2b).

Surprisingly, the enzyme showed higher catalytic activity at temperatures above 50°C, not observed routinely in enzymes produced by mesophilic microorganisms. Belancic et al. [23] obtained the best activity at pH 5.0 and optimum temperature of 40°C for β-glucosidase produced by *Debaryomyces vanrijiae*. Most fungal β-glucosidases show optimum activity between 40°C and 50°C and at pH between 4.0 and 6.0 [24,25]. However, some studies have reported the production of extremely stable β-glucosidase by mesophilic strains [3].

The β-glucosidase produced by the fungus *L. ramosa* showed remarkable structural stability. The enzyme retained its original activity after 24 h of incubation over a pH range of 3.5–10.5 (Fig. 2c). Regarding the thermal stability of the enzyme, about 90% of the catalytic activity was recovered after 1 h at 55°C (Fig. 2d).

The results are more significant when compared with previously published data. β-glucosidase produced by different species of *Penicillium* showed stability from pH 4.0 to 6.0 [26,27]. The enzyme produced by the fungus *Trichoderma harzianum* was stable at temperatures below 55°C for 15 min, maintaining only 36% of initial activity after 15 min at 60°C [28]. Delabona et al. [5] describe the stability of β-glucosidase produced by the fungus *A. fumigatus* P40M2 at temperatures from 40°C to 60°C and at pH 3.0 to 5.5. The β-glucosidase produced by the yeast *Sporidiobolus pararoseus* maintained its catalytic activity for 1 h at 40°C; at higher temperatures, only 30% of the initial activity was recovered [24].

#### 3.2.2. Effect of glucose and ethanol on β-glucosidase activity

Evaluation of the effect of ethanol on enzymatic activity is essential in studies with β-glucosidases, since these enzymes are frequently exposed to substantial concentrations of alcohol in many industrial applications [29].

Fig. 3a shows the effect of different concentrations of ethanol (0–30%) on the enzymatic activity. Ethanol concentrations up to 5% potentiated the enzymatic activity, and elicited an increase of up to 20% compared to the initial activity. Increasing ethanol concentration to 15% dramatically reduced enzymatic activity to only 22% of the original level. However, at a concentration of 10% of ethanol, β-glucosidase retained a level of catalytic activity similar to the control. Considering that the final ethanol concentration in fermented broths obtained by traditional processes is around 10% [30], we infer that the enzyme is sufficiently stable to be applied in industrial fermentation processes containing ethanol.

The increase in the catalytic potential of β-glucosidases observed by the ethanol addition is related to the glucosyltransferase activity [31]. Ethanol can increase the rate of reaction by acting as preferred acceptor of glycosyl residues during enzymatic catalysis [32]. The

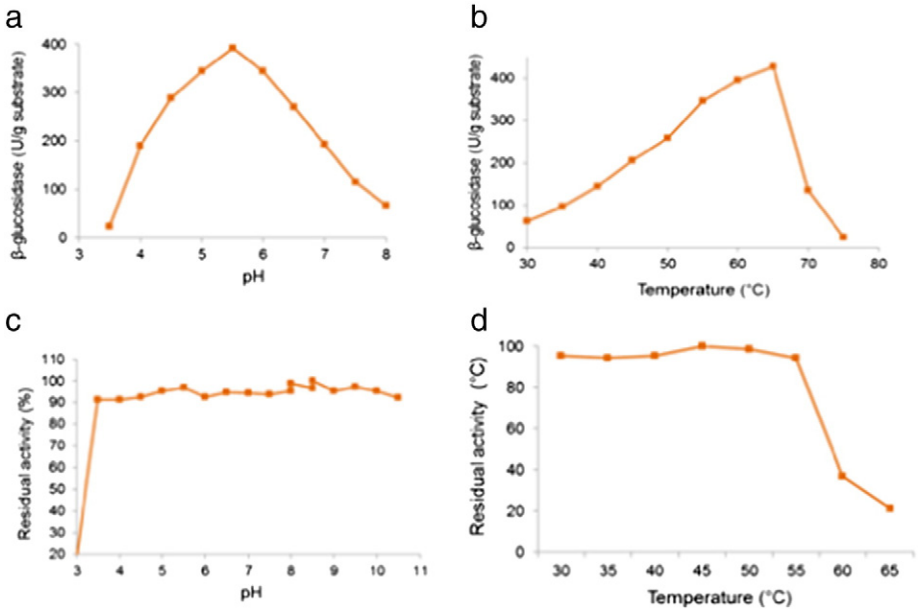


**Fig. 2.** Effect of pH and temperature on the activity (A and B) and stability (C and D) of β-glucosidase produced by the fungus *L. ramosa* by SSF in wheat bran, with 65% of moisture and initial pH 5.0, incubated for 96 h at 35°C.

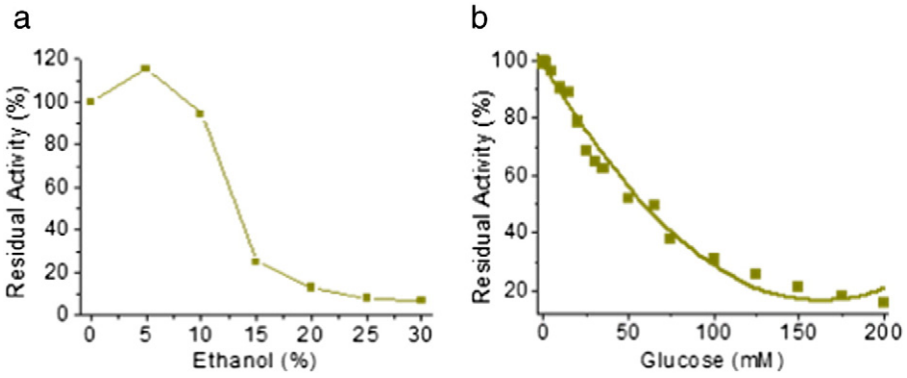


**Fig. 3.** (a) effect of ethanol, and (b) glucose, on the activity of β-glucosidase produced by *L. ramosa* by SSF in wheat bran, with 65% of moisture and initial pH 5.0, incubated for 96 h at 35°C.

hydrolysis and transglycosylation occur through the same biochemical pathway, differing only in the nature of the final acceptor [25].

Different concentrations of glucose were added to the reaction mixture to evaluate the behavior of β-glucosidase in the presence of this inhibitor. The enzyme was strongly inhibited by glucose, maintaining approximately 30% of its original activity in the reaction carried out with 100 mM glucose (Fig. 3b).

The majority of microbial β-glucosidases are inhibited by glucose, which is a major limitation of the use of these enzymes in industrial processes [3]. High glucose concentrations can interfere directly or indirectly with substrate binding to the enzyme active site, reducing the reaction rate [33]. The inhibition of β-glucosidase produced by *L. ramosa* was completely reversed when the substrate concentration was increased to the same glucose concentration, indicating that the interaction of the enzyme with the inhibitor is competitive.

Competitive inhibition can be reversed by increasing substrate concentration; the same fact is not observed in non-competitive inhibition. In competitive inhibition, the inhibitor and the substrate compete for the same binding site of the enzyme (the active site, in this case). Thus, increasing the substrate concentration to equal or greater values than those of the inhibitor, favors the binding of the enzyme to the substrate, which is reflected in the reversibility of enzymatic inhibition [3].

The reversibility of inhibition by glucose and stability to ethanol confirm the potential of this β-glucosidase for applications that require simultaneous saccharification and fermentation processes [3], where the monosaccharides released by enzymatic hydrolysis are simultaneously converted to ethanol by fermenting microorganisms [34,35].

### 3.3. Catalytic potential of the enzymatic extract

The production of other cellulases and also hemicellulases by *L. ramosa*, in optimized culture conditions, was evaluated in this work (Table 2). The enzymatic extract exhibited CMCase (152.1 U/g or 15.21 U/mL), xylanase (28.5 U/g or 2.85 U/mL), and β-xylosidase (115.7 U/g or 11.57 U/mL) activity.

The production of the CMCase and hemicellulases by *L. ramosa* is not very impressive when compared with hyper-producing strains. However, relatively little is known regarding the characteristics of these enzymes, and thus further studies in this area are required. Moreover, there is the possibility to improve these enzymes production in new works of culture optimization. Silva et al. [36] reported the production of 60 U/mL of CMCase and 107 U/mL by the fungus *T. aurantiacus* by solid-state fermentation, using corncob as substrate. Delabona et al. [20] report the production of 160.1 U/g of CMCase and 1055.62 U/g of xylanase by the fungus *A. fumigatus* cultivated in agroindustrial residue products.

Another interesting aspect of the current study, shown in Table 2, is the impressive potential of the enzyme to hydrolyze cellobiose, as it yields values similar to those obtained with a synthetic substrate (pNPG). The microbial β-glucosidases can be classified into three major groups: (1) Aryl β-glucosidases, which exhibit high specificity to hydrolyze aryl-glycosides substrates, (2) true cellobiases, which are enzymes with that hydrolyze cellobiose with high specificity and (3) enzymes with low specificity: enzymes that act on different types of glycosides substrates [25,31]. Apparently, β-glucosidase expressed from the fungus *L. ramosa* has low specificity; that is, it has the potential to hydrolyze different glycosides substrates. However, to confirm this hypothesis, further studies should be performed with the purified enzyme. According to Bhatia et al. [25], most of microbial β-glucosidases are classified in the third group.

**Table 2**
Catalytic potential of the enzymatic extract obtained by solid-state fermentation by *L. ramosa* in wheat bran, with 65% of moisture and initial pH 5.0, incubated for 96 h at 35°C.

| Enzyme | Substrate | U/mL | U/g |
|---|---|---|---|
| CMCase | Carboxymethylcellulose | 15.21 | 152.1 |
| β-Glucosidase | p-nitrophenyl-β-D-glucopyranoside (pNPG) | 23.47 | 237.7 |
| β-Glucosidase | Cellobiose | 23.45 | 234.5 |
| Xylanase | Xylan | 2.85 | 28.5 |
| β-Xylosidase | Xylopyranoside p-nitrophenyl-β-D (pNPX) | 11.57 | 115.7 |

## 4. Conclusions

*L. ramosa* has proven to be a remarkable fungus for use in β-glucosidase production when cultivated by solid-state fermentation using wheat bran as the substrate. This fungus is capable of producing several enzymes, including CMCase and β-xylosidase. β-Glucosidase was highly stable across a range of pH and temperatures and retained its original activity in solutions containing 10% of ethanol. Furthermore, the inhibitory effects of glucose were completely reversed at high substrate concentrations. The enzyme can hydrolyze different glycoside substrates; due to these characteristics, the β-glucosidase produced by *L. ramosa* can be used for the production of second-generation ethanol as well as for the improvement of food and beverage quality.

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## Conflict of interest

The authors declare that there is no conflict of interests.

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