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MICROBIOLOGY

Catalytic properties of amylases produced by *Cunninghamella echinulata* and *Rhizopus microsporus*

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Abstract: The present work aimed to characterize and compare the catalytic properties of amylases from *Cunninghamella echinulata* and *Rhizopus microsporus*. The highest production of amylase by *C. echinulata*, 234.94 U g^{-1} of dry substrate (or 23.49 U mL^{-1}), was obtained using wheat bran as a substrate, with 50–55% initial moisture and kept at 28 °C for 48 h. The highest production of amylases by *R. microsporus*, 224.85 U g^{-1} of dry substrate (or 22.48 U mL^{-1}), was obtained cultivating wheat bran with 65% initial moisture at 45 °C for 24 h. The optimal activity of the amylases was observed at pH 5.0 at 60 °C for *C. echinulata* enzymes and at pH 4.5 at 65 °C for *R. microsporus*. The amylases produced by *C. echinulata* were stable at pH 4.0–8.0, while the *R. microsporus* enzymes were stable at pH 4.0–10.0. The amylases produced by *C. echinulata* remained stable for 1 h at 50 °C and the *R. microsporus* amylases maintained catalytic activity for 1 h at 55 °C. The enzymatic extracts of both fungi hydrolyzed starches from different plant sources and showed potential for liquefaction of starch, however the amylolytic complex of *C. echinulata* exhibited greater saccharifying potential.

Key words: agro-industrial residues, amylase production, amylolytic enzymes, solid-state cultivation.

INTRODUCTION

Amylases comprise an important class of enzymes with numerous industrial applications and represent 25% of the world enzyme market (Paul et al. 2021), thereby highlighting the use of these enzymes in the production of food (syrups and bread), detergents, papers, fabrics and biofuels (Defaei et al. 2018, Al-Dhabi et al. 2020, Farooq et al. 2021). According to the action mechanism, amylases can be classified into four distinct groups: endoamylases, exoamylases, debranching and transferases (Chilakamarry et al. 2021). The first two groups are widely employed in industrial processes, especially α-amylases and glucoamylases.

α-Amylases (E.C. 3.2.1.1) hydrolyze α-1,4 glycosidic bonds inside the starch molecule (endoamylases) and produce linear and branched oligosaccharides as end-product (Farooq et al. 2021). Glucoamylases (EC 3.2.1.3) hydrolyze α-1,4 and α-1,6 linkages, from the non-reducing ends of the starch molecule (exoamylases), and produce β-glucose units as end-product (Karim & Tasnim 2018). These enzymes are used in the liquefaction and saccharification of starch, to the glucose syrup and biofuels production (Lincoln et al. 2018).

The production of industrial enzymes from microbiological cultures is justified owing to the reduction of the final cost of the biocatalyst of

interest and large-scale production capacity, in addition to the wide microbial metabolic diversity, which makes it possible to yield enzymes with different characteristics. Over the past 20 years, solid-state cultivation (SSC) has been used by many researchers and become credible on the part of industrial corporations because it features certain advantages compared to the submerged cultivation process, many of which are related to the morphological and physiological aspects of filamentous fungi, considering that this group of microorganisms are the most adapted to SSC processes (Garcia et al. 2015, Soccol et al. 2017).

Enzymes produced by different microorganisms have varying biochemical characteristics. In general, they are differentiated according to their catalytic function and structural stability at pH and temperature, determining factors for the industrial use of these biocatalysts in multiple processes (Singh et al. 2016).

Given the few studies using the filamentous fungus *Cunninghamella echinulata* for the production of amylolytic enzymes, the present work describes a new microbial source to amylases production and details its catalytic properties. The study also compares the properties of this new biocatalyst to amylases produced by *Rhizopus microsporus*, fungal species with recognized potential to amylolytic enzymes production (Escaramboni et al. 2018, Ranke et al. 2020).

MATERIALS AND METHODS

Microorganisms

The filamentous mesophilic fungus, *Cunninghamella echinulata* was isolated from soil samples collected in Serra da Bodoquena, deciduous seasonal forest vegetation - Atlantic Forest, in the municipality of Bodoquena-MS, and the thermophilic filamentous fungus, *Rhizopus microsporus* was isolated from decomposing Cerrado fruits from the region of Dourados-MS. The lineages were identified by Micoteca URM at the Universidade Federal de Pernambuco (UFPE). The microorganisms were kept on Sabouraud dextrose agar at 4 °C.

Inoculum

The microorganisms were grown in 250 mL Erlenmeyer flasks containing 40 mL of the Sabouraud dextrose agar slants, maintained for 48 h at 28 °C and 45 °C for *C. echinulata* and *R. microsporus*, respectively. The suspension of the microorganisms was obtained by gently scraping the surface of the culture medium using 25 mL of nutrient solution (0.1% ammonium sulfate, 0.1% magnesium sulfate heptahydrate, and 0.1% ammonium nitrate, m/v). The inoculation of fungi on the substrates (agro-industrial residues) was performed by transferring 5 mL of this suspension (10^5 spores/g of dry substrate) (Garcia et al. 2018).

Production of amylases by SSC

Microbial cultivation occurred in 250 mL Erlenmeyer flasks with 5 g of agro-industrial residues (corn straw, corn cob, rice peel, soybean meal, sugarcane bagasse, and wheat bran) featuring 70% moisture (moistened with nutrient solution described for the previous item), maintained for 48 h at 28 °C and 45 °C, respectively, for *C. echinulata* and *R. microsporus*. The substrates were properly washed with distilled water and then dried in oven at 50 °C for 48 h. The material was next sterilized at 121 °C for 20 min. The substrate used in the crops that resulted in the greatest production of amylase by the fungi was selected for the evaluation of other cultivation parameters, such as: initial moisture (50–80%) and cultivation time (24–96 h), being the adopted optimum condition of

each experiment in subsequent assays (Costa et al. 2019).

Extraction of the enzyme

For the extraction of the enzymes, 50 mL of distilled water was added to the cultivation. The flasks were kept under agitation for 1 h at 100 rpm. Subsequently, all content was filtered through synthetic fabric (nylon) and centrifuged at 1500 × *g* for 5 min at 10 °C. The supernatant was used as the enzyme extract and used in subsequent assays (Garcia et al. 2018).

Determination of amylase activity

Amylase activity was determined by adding 0.1 mL of enzyme extract in 0.9 mL of 0.1 M sodium acetate buffer, pH 4.5 containing 1% corn starch. After 10 min at 50 °C, the reaction was stopped with 1 mL of 3,5-dinitrosalicylic acid (DNS). The control tubes contained the same composition as the enzymatic reaction tubes, but the enzymatic extract was later added to the DNS, to prevent the enzyme activity. The tube used to reset the equipment (white tube) consisted of 1 mL of distilled water and 1 mL of DNS. The samples were boiled for 10 min and then cooled in an ice bath. After adding 8 mL distilled water, the amount of product released was determined using a spectrophotometer at 540 nm (Miller 1959). A unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of product per minute of reaction.

Effect of pH and temperature on enzyme activity

The optimum pH of the enzymes was determined by measuring the enzymatic activity in McIlvaine buffer solution, ranging from pH 3.0–8.0, at 50 °C. The optimum temperature was determined by dosage of the enzymatic activity under different temperature conditions (30–85 °C) in the respective optimum pH of each enzyme. The stability of enzymes at pH was evaluated by incubating them for 24 h at 25 °C at different pH values; the buffers used were McIlvaine (pH 3.0–8.0), 0.1 M Tris-HCl (pH 8.0–8.5) and 0.1 M Glycine-NaOH (pH 8.5–11). Enzymatic thermostability was assessed by incubating the enzymes for 1 h in different temperature ranges (30–75 °C). Residual activities (%) were determined under the optimum pH and temperature conditions for each enzyme, the highest catalytic activity obtained in these assays were adopted as 100%, and the other values were calculated proportionally (Oliveira et al. 2015). To determine the half-life ($t_{1/2}$) of the enzymes, the enzymatic extracts were incubated at 55 °C in 0.1 M sodium acetate buffer at the respective optimum pH for each enzyme. Samples were removed at different incubation times and the residual catalytic activity was quantified under the optimum conditions for each enzyme (Morais et al. 2018). The half-life ($t_{1/2}$) of the enzymes was defined as the time during which the residual activity of the enzyme was 50% of the original activity following thermal treatment (Tomazic & Klibanov 1988).

Effect of ethanol on enzyme activity

Enzyme activity was quantified with the addition of ethanol towards different final concentrations (0–30%) in the reaction solution. The assays were carried out in 0.1 M sodium acetate buffer under the optimum pH and temperature conditions for each enzyme. The values were expressed in residual activity (%), the assays performed without ethanol were considered controls (100% activity) and the other values were calculated proportionally (Oliveira et al. 2016).

Evaluation of the catalytic potential for starches from different sources

The enzymatic extracts were evaluated for the potential to hydrolyze starches from different

plant sources. The enzymatic assays were performed using starch (1%) from several sources, including: potato, corn, cassava, rice, wheat, and oat. The reactions were carried out at the optimum pH and temperature values for each enzyme (Cavalheiro et al. 2017). The amount of reducing sugar released was quantified by the DNS method (Miller 1959).

Dextrinizing potential of enzymatic extracts

Dextrinizing activity was conducted using the iodometric method described by Fuwa (1954) and Pongsawadi & Yagisawa (1987). The reaction mixture was composed of 0.1 mL of enzyme added to 0.3 mL of sodium acetate buffer solution containing 1% starch. After 10 min, the reaction was stopped by adding 4 mL of 0.2 M HCl. Subsequently, 0.5 mL of iodine reagent and 10 mL of distilled water were added. The absorbance was quantified at 700 nm. The assays were carried out under the optimum pH and temperature conditions for each enzyme. One unit of activity was defined as the amount of enzyme needed to reduce the intensity of the blue color of the iodine-starch complex by 10% per minute of reaction.

Saccharifying potential of enzyme extracts

Saccharifying activity was carried out using the glucose-oxidase/peroxidase method described by Bergmeyer & Bernt (1974). The reaction mixture was composed of 0.1 mL of enzyme added to 0.9 mL of 0.1 M sodium acetate buffer solution containing 0.5% starch. After 10 min, the reaction was stopped in an ice bath. The assays were conducted under the optimum pH and temperature conditions of each enzyme. The glucose released was quantified with the colorimetric enzyme kit (Glucose-PP Analisa). The absorbance was quantified at 505 nm. A unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute of reaction.

Statistical analysis

All experiments were performed in triplicate and the results are presented as the average of three independent assays. The statistical analysis of the data included a unidirectional analysis of variance (ANOVA) followed by Tukey's test with a 5% significance level.

RESULTS AND DISCUSSION

SSC amylase production

The highest production of amylase by the microorganisms assessed in this study was obtained on wheat bran, 157.66 and 144.80 U g^{-1} of dry substrate for *C. echinulata* and *R. microsporus*, respectively (Table I). Several studies have pointed out wheat bran as an excellent substrate for the cultivation of filamentous fungi for the production of industrial enzymes (Kumar et al. 2018, Costa et al. 2019, Garbin et al. 2021, Sanguine et al. 2022).

Table I. Production of amylases by fungi *C. echinulata* and *R. microsporus* in agro-industrial residues, maintained for 96 h at 28 °C and 45 °C, respectively, containing 70% of initial moisture.

	Amylolytic activity (U g^{-1} of dry substrate)	
Agro-industrial residues	***C. echinulata***	***R. microsporus***
Wheat bran	157.66±0.37[a]	144.80±0.77[a]
Soybean meal	15.88±0.0[b]	3.46±0.06[b]
Corn straw	7.78±0.05[c]	8.55±0.02[b]
Rice peel	5.84±0.04[cd]	6.10±0.00[b]
Corn cob	5.62±0.56[cd]	5.36±0.13[b]
Sugarcane bagasse	2.14±0.21[d]	3.31±0.00[b]

[a,b,c,d] **Different letters indicate a significant difference according to the Tukey test ($p < 0.05$). The values (±) represent the standard deviations of the triplicate in the experiment.**

The composition of wheat bran comprises approximately 13–18% protein, 3.5% fat and 56% carbohydrates (Apprich et al. 2014). Zimbardi et al. (2013) pointed out that the use of wheat bran as a substrate can supplement the use of nitrogen sources and some minerals as it has a rich nutritional composition, containing B vitamins, proteins, carbohydrates, lipids and minerals.

Meijer et al. (2011) demonstrated the production of a wide variety of hydrolases by the microorganism, *Aspergillus*, using wheat bran as a substrate. Fernández Núñez et al. (2017) obtained higher amylase production, when they used wheat bran as a substrate for the SSC of the fungus *Rhizopus miscrosporus* var. *oligosporus*.

In view of the reports in the literature and the observation of the results obtained in the present study, wheat bran was adopted as a substrate for subsequent crops, considering that other cultivation parameters were also evaluated for optimization of amylase production by fungi *C. echinulata* and *R. microsporus*, such as moisture and cultivation time (Figure 1).

The highest production of amylase by the fungus, *C. echinulata* was obtained in cultures with initial moisture between 50–55% (179.61 U g^{-1} of substrate). Low moisture values decrease the risk of bacterial contamination during the cultivation processes, which is an important characteristic of SSC (Soccol et al. 2017, Garcia et al. 2018). Different results were found for *R. microsporus*, with higher production of the enzyme observed in crops with 65% of initial

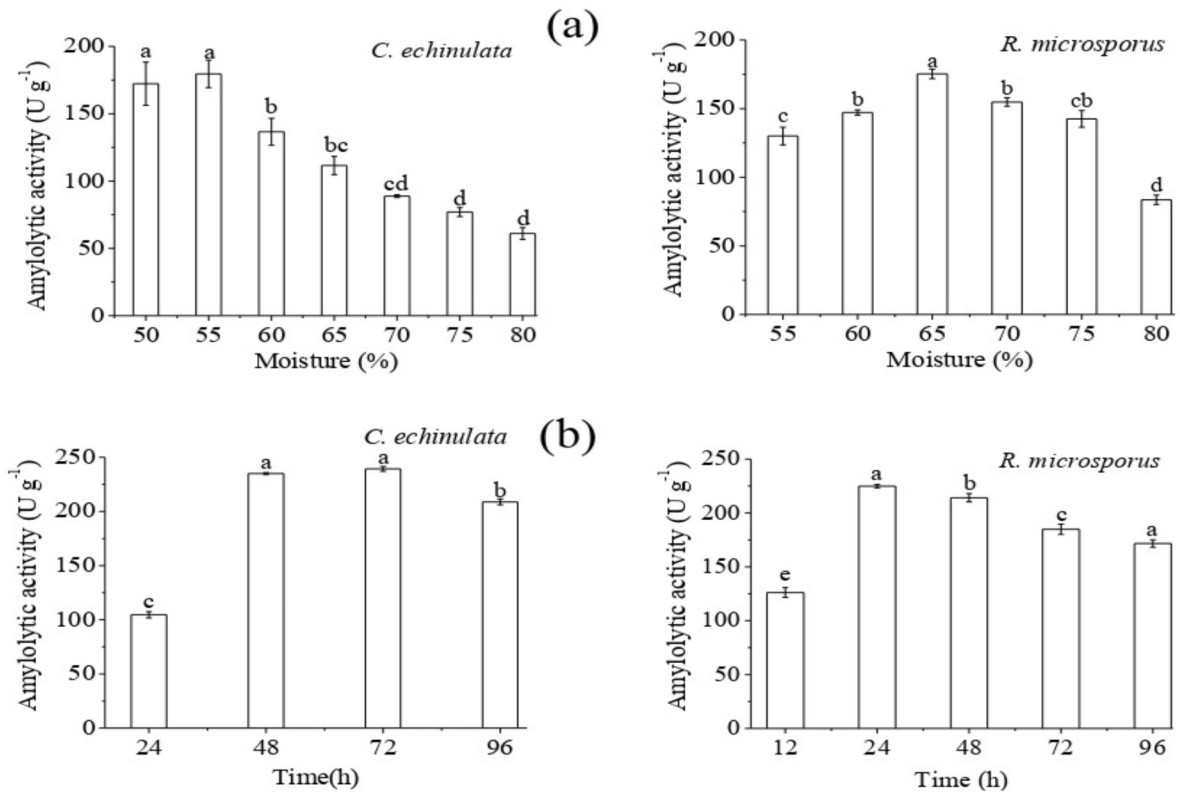

Figure 1. Production of amylases by SSC of fungi *C. echinulata* and *R. microsporus* in wheat bran at 28 °C and 45 °C, respectively. a) initial moisture of the substrate; b) cultivation time. [a,b,c,d] Different letters indicate a significant difference according to the Tukey test (p <0.05).

moisture at 175.28 U g^{-1} of substrate (Figure 1a and b).

The moisture of the culture medium is one of the main factors that considerably influences the SSC process. The substrate must contain moisture that favors the solubilization of nutrients and the exchange of oxygen and carbon dioxide between the microorganism and the medium. Thus, high levels of moisture hinder gas exchange during the cultivation process and increase the risk of bacterial contamination, while on the other hand, the low moisture content reduces microbial metabolic activity, which results in a drop in growth speed and a decrease in the production of the enzyme of interest (Singhania et al. 2009, Cavalheiro et al. 2017).

The highest production of amylase by the fungus *C. echinulata* (234.94 U g^{-1} of substrate) was observed between 48–72 h of culture, with no significant difference by statistical analysis. Thus, 48 h was adopted as the optimum cultivation time for the production of the enzyme. The maximum production of amylase by the fungus, *R. microsporus* (224.85 U g^{-1} of substrate), was obtained in 24 h of culture (Figure 1b).

The values of amylase production between the two fungi were very similar. However, the reduced time of enzyme production by *R. microsporus* should be highlighted, reached in 24 h of cultivation. This becomes even more evident if the results were expressed in productivity, 4.89 U g^{-1} h^{-1} from *C. echinulate* and 9.36 U g^{-1} h^{-1} from *R. microsporus*.

The most suitable cultivation time for the production of amylases by the fungi, *C. echinulata* (48 h) and *R. microsporus* (24 h), is less than the time found by Mazumdar & Maumdar (2018) for the thermotolerant fungus, *Aspergillus oryzae*, which was 96 h of cultivation in banana peel as the substrate. The cultivation time obtained in the present study was shorter than that reported by Chaturvedi et al. (2018), with the yeast *Saccharomyces pastorianus* grown in Yeast Peptone Dextrose (YPD) medium, which showed greater production of amylase with 216 h of cultivation.

According to Santana et al. (2012), the fungus, *Aspergillus niger*, featured the cultivation time of 24 h as the most suitable for the production of amylase by SSC in cocoa bran, a value similar to that obtained for *R. microsporus*. Previous work confirms the reduced cultivation time for the production of amylolytic enzymes by fungi of the genus *Rhizopus*. Ferreira et al. (2015) reported the production of amylases at 63.5 U g^{-1} of substrate by the fungus, *Rhizopus oryzae*, with 24 h of SSC using wheat bran as a substrate. For the production of industrial enzymes, the shorter the microbial cultivation time, the greater the economic viability of the process owing to the lower energy demand, which considerably reduces the production cost of the biocatalyst of interest (Bernardes et al. 2014).

Data observed in the scientific literature show values lower or close to those described in the present study, for the production of amylases by several fungal strains. However, productions greater than the ones obtained in this work were also described (Table II).

After the adjustments made to the cultivation parameters, the production of amylase by *C. echinulata* in wheat bran increased from 157.66 to 234.39 U g^{-1} of substrate, which represents a gain of 48% with the production of this enzyme. The production of amylase by *R. microsporus* in wheat bran increased from 144.80 to 224.85 U g^{-1} of substrate, equivalent to a 55% increase in amylolytic activity (Table I and Figure 1b).

It is worth mentioning that the evaluation of the cultivation parameters allowed obtaining of enzymatic extracts with high amylolytic activity from crops in mediums of low commercial value (agro-industrial residues) with diminished

Table II. Production of amylases by different fungal strains in SSC.

Strain	Substrate	Amylolytic activity (U g^{-1} of dry substrate)	Author (s)
C. echinulata	Wheat bran	234.39	This work
R. microsporus	Wheat bran	224.85	This work
Trametes polyzona	Wheat bran	1.16	Acheampong et al. (2021)
Rhizomucor miehei	Broken corn	13.0	Bernardes et al. (2014)
Fusarium oxysporum	Wheat bran	17.8	Prasoulas et al. (2020)
Gongronella butleri	Wheat bran	63.25	Cavalheiro et al. (2017)
Rhizopus oryzae	Wheat bran	63.5	Ferreira et al. (2015)
Rhizopus oryzae	Bread waste	100.0	Benabda et al. (2019)
Thermoascus aurantiacus	Wheat bran	144.5	Oliveira et al. (2016)
Candida parapsilosis	Wheat bran	146.8	Oliveira et al. (2015)
Aspergillus sp.	Wheat bran	164.0	Chimata et al. (2010)
Lichtemia ramosa	Wheat bran	417.2	Oliveira et al. (2016)
Rhizopus microsporus var. oligosporus	Wheat bran	392.5	Núñez et al. (2017)

growth time. The characteristics described contribute to the use of these biocatalysts in different industrial processes owing to the decrease in the production costs of the enzymes of interest.

Biochemical characterization of amylases produced

Effect of pH and temperature

Amylase from *C. echinulata* showed greater activity in the pH range 4.5–6.0 with an optimum pH of 5.0 (27.75 ± 0.23 U mL^{-1}). The enzyme from *R. microsporus* showed optimal activity at pH 4.5 (26.15 ± 0.41 U mL^{-1}; Figure 2a). The results indicate that the studied fungi produce amylases that can be applied in processes that tend to have more acidic pH values, especially between 4.5 and 6.0. Obafemi et al. (2018) established pH 6.0 as the optimum for partially purified amylase from *Aspergillus niger*. According to Singh & Kayastha (2014), the *Aspergillus fumigatus* amylase exhibited greater catalytic activity at pH 6.0.

The enzymatic activity is directly influenced by the pH and the optimization of this parameter is indispensable for the efficient use of a biocatalyst (Neina 2019). Variations in pH can cause changes in protein conformation (Tang et al. 2019), therefore, it is essential to determine the optimal pH of an enzyme in order to direct its industrial application (Fincan et al. 2021).

The amylase of *C. echinulata* showed an optimum temperature at 60 °C (40.30 ± 0.35 U mL^{-1}) and that of *R. microsporus* at 65 °C (42.66 ± 0.15 U mL^{-1}; Figure 2b). The optimum temperatures observed for the enzymes evaluated in the present study were considerably high, especially when considering the mesophilia of the fungus, *C. echinulata*. However, previous work confirmed high temperatures as optimal for catalytic activity of amylases produced by mesophilic fungal strains. *Aspergillus awamori* amylase showed greater activity at 60 °C (Karam et al. 2017). Cavalheiro et al. (2017) reported 55 °C as the optimum temperature for *Gongronella butleri* amylase.

Regarding pH stability, *C. echinulata* amylase maintained an activity higher than 85% of the initial one for 24 h in the pH range 4.0–8.0 (Figure 3a). The amylase produced by *R. microsporus* maintained an activity greater than

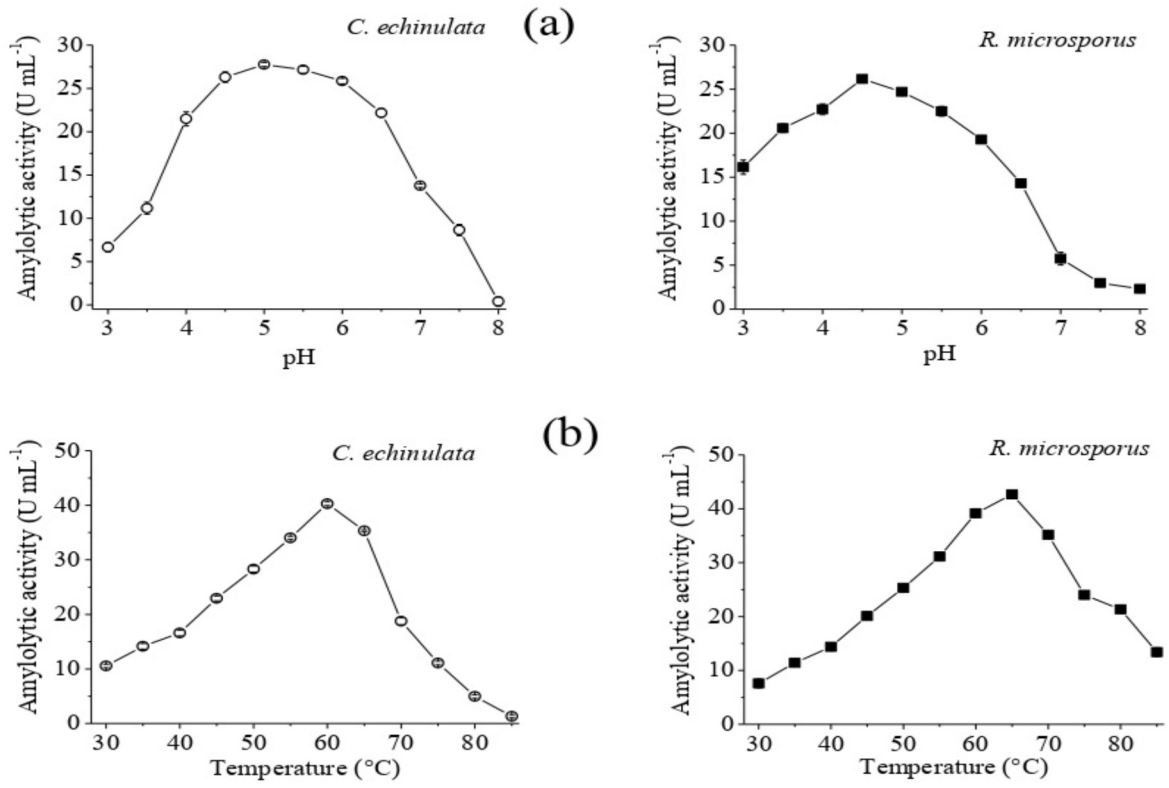

Figure 2. Effect of pH and temperature on enzyme activity. a) optimum pH; b) optimal temperature. The assays were performed in triplicate and error bars were expressed in the figure. *C. echinulata* (○); *R. microsporus* (■).

82% of the original for 24 h in the pH 3.0–10.0 range (Figure 3a).

The enzymes produced *C. echinulata* and *R. microsporus* were stable over a wide pH range compared to amylases produced by other microbial species. Pasin et al. (2017) described the pH stability of glucoamylase of the fungus, *Aspergillus jabonicus*, the enzyme showing 65% of its initial activity when incubated in the pH range 3.0–6.0. The amylase produced by the bacterium, *Bacillus* sp., was stable in the pH range 6.5–10.5 (Kiran & Chandra 2008).

Regarding thermostability, *C. echinulata* amylase exhibited roughly 80% of the original activity after being incubated for 60 min at a temperature of 30–45 °C, and when the temperature was raised to 50 °C for the same incubation period, 50% of the initial enzyme activity was recovered (Figure 3b).

The enzyme of *R. microsporus* showed activity above 90% after 60 min at a temperature of 30–50 °C, and when the temperature was raised to 55 °C, catalytic activity was reduced to 50% of the initial value (Figure 3b). Structural stability is an extremely important characteristic for industrial application of an enzyme considering that industrial conditions often differ from a controlled laboratory environment, which allows the exposure of this biocatalyst to extremes of pH and temperature (Souza & Magalhães 2010).

The enzymes evaluated in the present study showed similar or even greater thermostability compared to amylases produced by other fungal species. Pasin et al. (2017) described the glucoamylase thermostability of the fungus,

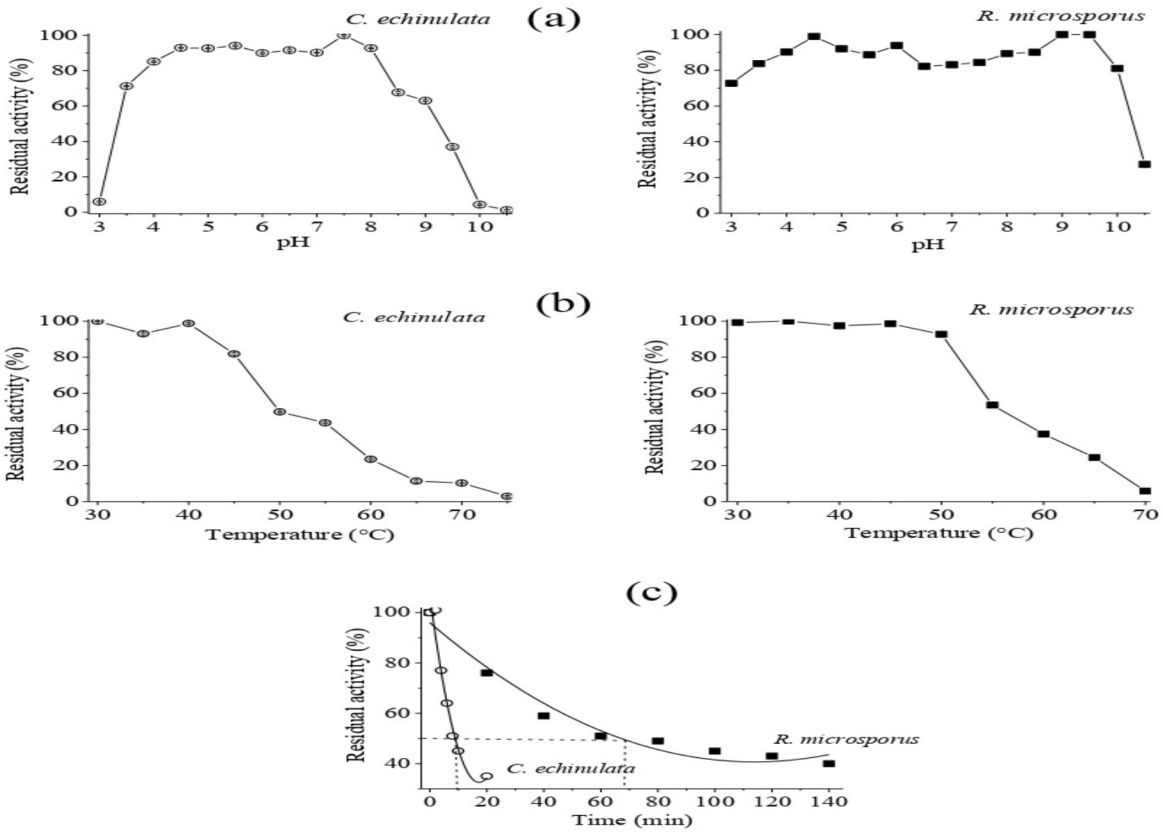

Figure 3. Effect of pH and temperature on enzyme activity. a) pH stability; b) temperature stability; c) half-life $t_{(1/2)}$ at 55 °C. The assays were performed in triplicate and error bars were expressed in the figure. *C. echinulata* (○); *R. microsporus* (■).

Aspergillus jabonicus, approximately 70% of its initial activity was recovered after 60 min at 50 °C. Cavalheiro et al. (2017) recovered only 70% of the original activity of amylase produced by the fungus, *Gongronella butleri*, when incubated for 60 min at 40 °C.

The amylases described in the current work were progressively inactivated when incubated at 55 °C (Figure 3c). However, the amylase produced by the thermophilic fungus, *R. microsporus* showed greater thermostability compared to the enzyme of the mesophilic fungus, *C. echinulata*. The half life $t_{(1/2)}$ at 55 °C of the enzyme produced by *R. microsporus* was obtained with 70 min of heat treatment, while the amylase from *C. echinulata* showed 50% of its original activity with 10 min of incubation (Figure 3c).

In general, enzymes produced by thermophilic microorganisms have greater thermal stability compared to enzymes produced by mesophilic microorganisms. Thermostable enzymes tend to have a greater number of covalent bonds (disulfide bonds) and non-covalent interactions, such as hydrophobic, electrostatic, ionic and hydrogen bonds. However, there is no structural model that significantly differentiates a stable protein from another non-stable one, and small differences in the number of bonds and interactions, as mentioned previously, considerably alter structural protein stability (Gomes et al. 2007, Morais et al. 2018).

Effect of ethanol on enzyme activity

The amylases of the fungi, *C. echinulata* and *R. microsporus*, maintained roughly 60% of their respective catalytic activities in solutions containing 10% v/v ethanol (Figure 4).

The results permit us to infer that the amylases produced by microorganisms have the potential to be applied in simultaneous saccharification and fermentation processes. With these types of process, fermentable sugars released by saccharification of starch are simultaneously converted into ethanol by fermenting microorganisms. However, for this to be possible, biocatalysts must withstand the presence of ethanol in the reaction mixture (Santos et al. 2016).

Considering that concentrations above 10% of ethanol are harmful, even to the fermenting microorganism (*Saccharomyces cerevisiae*) (Cot et al. 2007), it is possible to infer that the enzymes evaluated in the present work are sufficiently stable to be applied in the types of processes described herein.

Evaluation of the catalytic potential for starches from different sources

Enzymatic extracts have potential to hydrolyze starches from different plant sources and can be used in a wide range of industrial processes (Figure 5). Among the starches evaluated, the most susceptible to the action of the enzymatic extract produced by the fungus, *R. microsporus* were potato, corn, cassava and rice starches. The enzymatic extract of *C. echinulata* showed greater catalytic potential for starches from potatoes, corn and cassava. However, the enzymes of both microorganisms exhibited a robust ability to degrade the other starches used in the present study (Figure 5).

The results indicated that the enzymatic extracts produced by *C. echinulata* and *R. microsporus* are capable of degrading starches with different structural characteristics. Depending the origin and variety of the starch, they have considerable differences in the degree of branching of their internal chains, a fact that influences their crystallinity and enzymatic hydrolysis processes. The greater the crystallinity of the starch molecule, the greater the structural stability of the granule and its resistance to gelatinization, which hinders the access of amylolytic enzymes to specific regions of hydrolysis (Hoover 2001, Singh et al. 2003, Lobo & Silva 2003).

In general, corn starch is hydrolyzed more easily by the action of amylolytic enzymes owing to its structure having a reduced number of

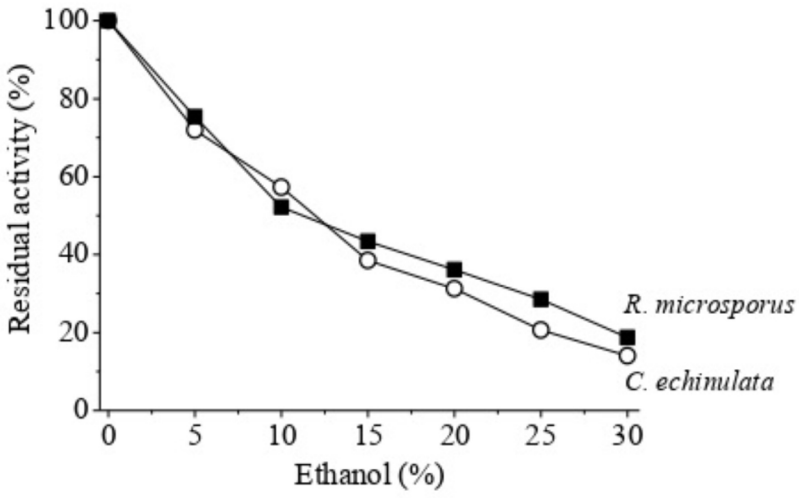

Figure 4. Effect of ethanol on the activity of amylases produced by *C. echinulata* and *R. microsporus*. The assays were performed in triplicate and error bars were expressed in the figure. *C. echinulata* (○); *R. microsporus* (■).

branches (Tester et al. 2004). Oliveira et al. (2015) assessed the catalytic potential of enzymatic extracts produced by the yeasts, Candida parapsilosis, Rhodotorula mucilaginosa and Candida glabrata. The authors reported greater efficiency in the enzymatic hydrolysis of the assays that used corn starch as a substrate.

However, different results were obtained in the present study. The enzymatic extracts produced by *C. echinulata* and *R. microsporus* hydrolyzed starches from different botanical sources with similar efficiency. Cavalheiro et al. (2017) reported a catalytic profile similar to that observed in the present study for the enzymatic extract produced by the fungus, *Gongronella butleri*. The authors suggest that *G. butleri* produces amylases with distinct catalytic actions and these enzymes act synergistically on starch molecules, drastically diminishing the degree of branching and polymerization of this polysaccharide.

Dextrinizing and saccharifying potential of enzymatic extracts produced by C. echinulata and R. microsporus

The enzymatic extracts of both fungi exhibited similar dextrinizing potential as evidenced by the iodometric method, which allows the evaluation of the depolymerization of the starch molecule caused by the action of endoamylases and debranching enzymes (Figure 6). However, the assays carried out with the enzymatic extract produced by *C. echinulata* showed greater conversion of starch to glucose, indicating the catalytic action of exoamylases (glucoamylase and/or α-glucosidase), quantified by the glucose/oxidase method (Figure 6). The synergistic activity between endo- and exoamylases has also been described for the enzymatic extract produced by the fungus, *Lichtemia ramosa* by cultivation in a solid state using wheat bran (Oliveira et al. 2016).

The enzymatic extract produced by *R. microsporus* exhibited reduced potential to convert starch into glucose, with a small amount of this monosaccharide being recovered at the end of enzymatic treatment. The decreased amount of glucose suggests negligible exoamylase activity in the enzyme extract, with a predominance of endoamylase activity, usually measured by α-amylases. Vijayaraghavan et al. (2011) confirm the production of α-amylase by the fungus, *R. microsporus*, which corroborates the results described in the current work.

Thus, it is possible to infer that the enzymatic extract produced by *C. echinulata* can be applied

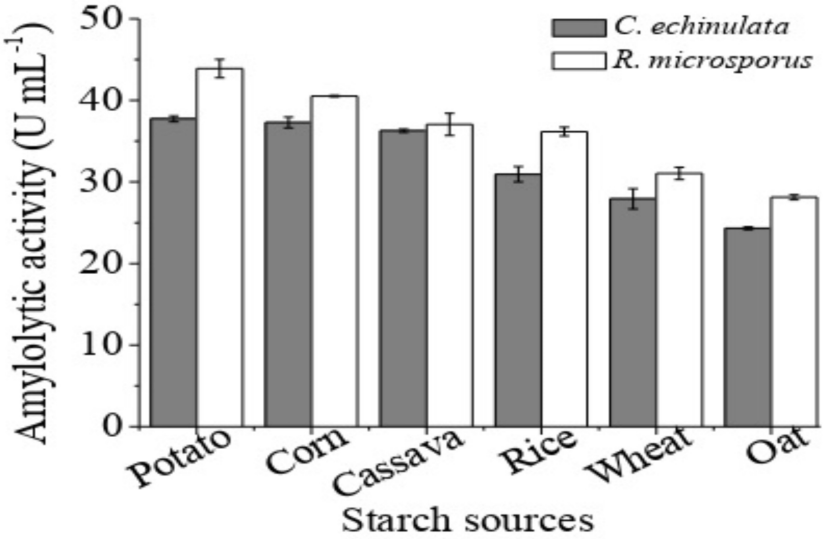

Figure 5. Evaluation of the catalytic potential of enzymatic extracts of *C. echinulata* and *R. microsporus* on starches from different plant sources. Quantified by the DNS reducing sugar method (3,5-dinitrosalisilic acid, Miller (1959)).

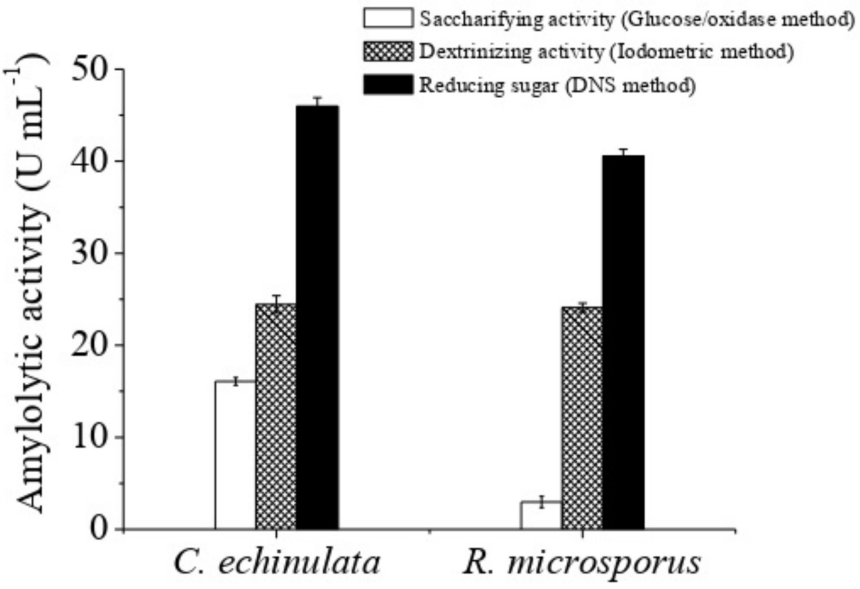

Figure 6. Hydrolysis of corn starch by enzymatic extracts produced by *C. echinulata* and *R. microsporus*, quantified by different colorimetric methods.

in processes that involve the liquefaction and saccharification of starch in order to obtain fermentable sugars, while the enzymatic extract produced by *R. microsporus* is more efficient in processes that involve the liquefaction of starch and can be applied in processes with higher temperatures.

CONCLUSIONS

The results yielded herein permit us to conclude that the fungi, *C. echinulata* and *R. microsporus*, were able to produce high concentrations of amylolytic enzymes during a short cultivation time, alternatively using agro-industrial residues as low-cost substrates. However, the typical heterogeneity of solid-state cultivation hinder the monitoring and controlling the fermentative parameters during microbiological growth. This characteristic limits the use of this type of bioprocess in industrial scale, which encourages studies in this area in order to overcome this difficulty. The enzymes were stable over a wide range of pH and temperature, with emphasis on amylase produced by the thermophilic fungus, *R. microsporus*. The enzymes showed satisfactory catalytic activity in ethanolic solutions, which enables the use of these biocatalysts for production of biofuels. Both enzymatic extracts hydrolyzed starches from different plant sources, favoring application within several industrial processes. However, the enzymatic extracts have different hydrolysis profiles, showing saccharifying and dextrinizing activity (endo and exoamylase) from *C. echinulata* and predominantly dextrinizing activity (endoamylase) from *R. microsporus*.

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