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Bioprospecting of yeasts for amylase production in solid state fermentation and evaluation of the catalytic properties of enzymatic extracts

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Profiling microorganisms with potential for amylase production in low cost culture media has been widely recognized due to its broad applicability. The aim of this study was to select yeast strains with potential to produce amylolytic enzymes by solid state fermentation. Fifty-four (54) strains were assessed and three exhibited ability to produce amylases: *Candida parapsilosis* with 14.68 U/mL (146.8 U/g substrate); *Rhodotorula mucilaginosa* with 25.0 U/mL (250 U/g substrate), and *Candida glabrata* with 25.39 U/mL (253.9 U/g substrate), in solid state fermentation, for 120 h at 28°C, using wheat bran with 70% moisture. The enzymes exhibited maximum activity at a pH of 7.0 and at 60°C. Amylases demonstrated satisfactory structural stability, maintaining their catalytic activity after 1 h at 50°C. All enzymes were ethanol tolerant and retained more than 70% of their original activities in 15% ethanol solution. Corn starch was efficiently hydrolyzed by enzymes and the extracts produced by *C. parapsilosis* and *C. glabrata* exhibited dextrinizing activity, while those produced by *R. mucilaginosa* exhibited saccharifying activity.

Key words: *Candida parapsilosis*, *Candida glabrata, Rhodotorula mucilaginosa,* dextrinizing and saccharifying activity.

INTRODUCTION

The improvement of ethanol production processes and the development of new biotechnological techniques to use plant-based polysaccharides are important to ensure mankind's supply of energy requirements (Scott et al., 2013).

In Brazil, ethanol is produced by fermenting monosaccharides from the hydrolysis of sucrose by *Saccharomyces cerevisiae* yeast, which metabolizes under appropriate

conditions, releasing ethyl alcohol as the main product of fermentation (Basso et al., 2008). To meet the demand for this biofuel, several studies have focused on devising efficient mechanisms to hydrolyze starch and cellulose, thereby obtaining free glucose that can be fermented into ethanol by *S. cerevisiae*. In this regard, enzymes that catalyze the hydrolysis of these polymers are considered very important in the global energy scenario (Gupta et al., 2003; Sahnoun et al., 2012).

The main polysaccharide of plants is starch, which consists of glucose residues linked by glycosidic bonds (α-1,4 and α-1,6) that are found mainly in rice, corn, wheat, cassava and potato (Zeeman et al., 2010), all of them widely consumed and produced in different Brazilian regions. Starch can be hydrolyzed by chemical or enzymatic techniques and the action of different enzymes is necessary for its efficient conversion into products of lower molecular weight, such as dextrins, maltose and glucose. Enzymatic hydrolysis has some advantages compared to chemical methods, the biocatalysts act under mild conditions of pH and temperature, reducing energy consumption, equipments corrosion and eliminates neutralization steps. However, specificity of enzymatic catalysis can be considered as the main advantage of the enzymes use, preventing the formation of undesirable byproducts commonly observed in reactions by chemical catalysis (Gupta et al., 2003; Sivaramakrishnan et al., 2006).

Amylases account for 25-33% of the international enzyme market and are used in numerous industrial processes that require the partial or total hydrolysis of starch (Özdemir et al., 2011). Endoamylolytic and debranching enzymes (α-amylases and isoamylases, respectively) reduce the degree of polymerization of the starch molecule, producing linear glucose-based dextrins. These enzymes are employed in the starch liquefaction process. Exoamylases are used in subsequent steps of the enzymatic hydrolysis of starch. These enzymes hydrolyze the dextrins from the liquefaction, producing maltose (β-amylases) or glucose (amyloglucosidase, α-glucosidases and glucoamylases) syrups that are used in the food, beverage and biofuel industries (Silva et al., 2005; Sivaramakrishnan et al., 2006; Özdemir et al., 2011).

The use of yeast to produce enzymes offers certain advantages, such as a moderate temperature for microbial growth, high metabolic diversity and rapid cell growth, which results in shorter fermentation cycles and easy adaptation to different cultivation conditions (Kato et al., 2007). The use of agroindustrial wastes as substrate in solid state fermentation reduces enzyme production costs and contributes to minimize environmental problems caused by the agroindustry (Singhania et al., 2009). These advantages explain the search for microbial strains that exhibit significant enzyme production in low-cost fermentation processes (Alves-Prado et al., 2010). The use of agroindustrial wastes for enzymes production can become economically viable for the application of these biocatalysts in large scale, considering that one of the major problems in the enzymes utilization in industrial processes is the high cost of the microbial culture media, about 30-40% of the cost enzyme production (Romero et al., 2007; García-Martínez et al., 2010).

Therefore, the aims of this study were to select yeast strains having the potential to produce amylolytic enzymes by solid state fermentation, characterize them biochemically, and evaluate their catalytic properties.

MATERIALS AND METHODS

Microorganisms

The present study analyzed 54 yeast strains supplied by the organization RECOL – Rede Centro-Oeste de Leveduras (Central-Western Yeast Network, Brazil), and one strain of *Saccharomyces cerevisiae* supplied by the São Fernando sugar and alcohol mill in the municipality of Dourados, MS, Brazil. The strains from RECOL were isolated in natural and industrial environments such as fruit from the Cerrado, poultry litter and sugarcane must.

Inoculum preparation

The strains were cultivated in test tubes containing 5 mL of YEPD medium (yeast extract 1%, peptone 2%, glucose 2% and agar 1.5%) for 48 h at 28°C. The yeast suspension was obtained by scraping off the surface of the medium using 3 mL of nutrient solution (0.5% ammonium sulfate, 0.5% magnesium sulfate heptahydrate and 0.5% ammonium nitrate). Yeast was inoculated in the substrate by transferring 3 mL of the microbial suspension (at 10^6 cells/mL) to Erlenmeyer flasks containing wheat bran.

Selection of strains for amylase production by solid state fermentation

The yeasts were cultivated in solid state in Erlenmeyer flasks (250 mL) containing 5 g of wheat bran (ground to 2-3 mm size) with 70% moisture content (mass of dry substrate per volume), using the above described nutrient solution to moisten the substrate. Prior to the inoculation of microorganisms, all the material was autoclaved at 121°C for 20 min. After inoculation, the Erlenmeyer flasks were stored at 28°C for 120 h. Due to the high number of strains evaluated in this study, the cultivation parameters (substrate, moisture, temperature and fermentation time) were fixed near values described as optimal, for amylases production by different fungal strains, in previous works as a comparison parameter

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(Ellaiah et al., 2002; Bhatti et al., 2007). All the assays were performed in duplicate.

Enzyme extraction

Enzymes were extracted by adding 50 mL of distilled water to the flasks containing the ferment medium. The flasks were kept under agitation at 150 rpm for 1 h, after which they were filtered through Whatman paper (n.1) and centrifuged at 3000x *g* for 10 min at 25°C. The supernatant was used for the enzyme assays.

Determination of amylase activity

The enzyme activity was determined by adding 0.1 mL of enzymatic extract to 0.9 mL of sodium acetate buffer (0.1 M, pH 5.0, 1% corn starch). After 10 min of reaction at 50°C, the reducing sugar released was quantified by the 3,5-dinitrosalicilic acid method (DNS) (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of the product per minute of reaction.

Identification of selected strains

High level amylase-producing strains were subjected to molecular identification (DNA sequencing). Genomic DNA was extracted from three days cultures grown in yeast-malt extract agar at 25°C (Sampaio et al., 2001). The D1/D2 domains of the 26S rRNA gene were amplified using the universal primer pair NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). This region is generally used as the molecular marker for yeast identification (Kurtzman and Robnett, 1998; Schoch et al., 2012). PCR conditions were as follows: 96°C for 3 min, 35 cycles at 96°C for 30 s, 61°C for 45 s, and an extension step at 72°C for 1 min (Rodrigues et al., 2009). The expected size of amplicons (500-600 base pairs) were checked after electrophoresis in 1% agarose gel supplemented with GelRed® (Biotium). Amplicons were purified and then quantified in NanoDrop® 2000 (Thermo Scientific). Sanger cycle sequencing reactions were performed with BigDye Terminator v. 3.1 (Life Technologies), using 20 ng of amplicon template and the same primers used in the PCR. Both forward and reverse sequences were generated in ABI3330xl (Life Technologies) and assembled in BioEdit v.7.0.5.3 (Hall, 1999). Consensus sequences were queried in the NCBI – GenBank and CBS (Centraalbureau voor Schimmelcultures) databases. We adopted the criterion used by Kurtzman and Robnett (1998), and query sequences that showed 99% identity with those deposited in the database were considered conspecific.

Biochemical characterization of the amylases produced

Effect of pH and temperature

The optimum pH was determined by measuring the enzyme activity at 50°C with different pH conditions using McIlvaine buffer (0.1 M), due to its wide buffering range (from 3.0 to 8.0). The optimum temperature was defined by measuring the enzyme activity in different temperature conditions (from 30 to 75°C) at the respective optimal pH of each enzyme. The pH stability of enzymes was assessed by incubating them at 25°C for 24 h at different levels of pH. The following buffers were used: McIlvaine 0.1 M (3.0 - 8.0); Tris-HCl 0.1 M (8.0 to 8.5) and glycine-NaOH 0.1 M (8.5 - 10.5). Thermostability was assessed by incubating the enzymes for 1 h at different temperatures (30 to 75°C). The residual activity was determined under optimum conditions of pH and temperature for enzymes (Leite et al., 2008).

Effect of ethanol on enzyme activities

Enzyme activity was quantified by adding different concentrations of ethanol (0 to 30%) to the reaction mixture. The assays were performed at 50°C in McIlvaine buffer 0.1 M (pH 7.0, 1% corn starch) (Leite et al., 2008).

Catalytic potential for different sources of starch

Enzyme assays were performed using potato, corn, wheat and cassava starch (1%) as enzyme substrate, vegetal cheap sources and available in Brazil. The enzymatic reactions were performed in McIlvaine buffer 0.1 M (pH 7.0). The sugar released was quantified using the DNS method (Miller, 1959).

Dextrinization potential of enzymatic extracts

Dextrinizing activity was assessed using corn starch (1%) as enzyme substrate in McIlvaine buffers 0.1 M (pH 7.0) and the iodometric methods described by Fuwa (1954) and Pongsawadi and Yagisawa (1987). The reaction mix contained 0.1 mL of extract enzymes in 0.3 mL of buffer solution containing starch. After 10 min at 60°C, the reaction was stopped by adding 4 mL of HCl solution 0.2 M. Finally, 0.5 mL of reactive iodine and 10 mL of distilled water were added. The absorbance was quantified at 700 nm. One unit of activity was defined as the amount of enzyme required to reduce the intensity of the blue of the starch iodine complex by 10% per minute of reaction.

Saccharification potential of enzymatic extracts

Saccharifying activity was assessed using corn starch (1%) as enzyme substrate in McIlvaine buffer 0.1 M (pH 7.0) and employing the glucose-oxidase/peroxidase method (Bergmeyer and Bernt, 1974). The reaction mix contained 0.1 mL of the extract enzymatic in 0.4 mL of buffer solution containing starch. After 10 min at 60°C, the reaction was stopped in an ice bath. The glucose released was quantified using an enzymatic colorimetric kit (*Glicose-PP Analisa*). The absorbance was quantified at 505 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute of reaction.

RESULTS AND DISCUSSION

Selection and identification of strains with potential for amylase production

To determine their potential for amylase production by solid state fermentation, the 54 yeast strains were grown for 120 h at 28°C, using wheat bran with 70% moisture. Wheat bran was chosen as the substrate for the selection of promising strains due to its complexity nutritional, favoring microbial growth and the production of different fungal amylases; fact described by several authors in previous works (Ellaiah et al., 2002; Bhatti et al., 2007).

Table 1. Production of amylase by solid state fermentation for 120 hours at 28°C, using wheat bran (70% moisture content) as substrate.

Yeast	Collection site	Amylase activity (U/mL)
01	Cereja do Rio Grande**	5.07 ± 0.08
29*	Uvaia**	14.68 ± 0.5
30*	Uvaia	25.00 ± 0.16
37*	Pêssego do Mato**	14.10 ± 0.44
39	Acerola**	5.39 ± 0.19
41	Acerola	11.22 ± 0.27
43*	Pequi**	25.39 ± 0.23
44	Pequi	10.17 ± 0.27
46	Uvaia	5.54 ± 0.18
53	Sugarcane must	10.30 ± 1.25
54	Sugarcane must	11.77 ± 0.20
S. cerevisiae	Commercial strain	0.39 ± 0.05

*Strains 29 and 37 (*Candida parapsilosis*); Strain 30 (*Rhodotorula mucilaginosa*); Strain 43 (*Candida glabrata*).**Cereja do Rio Grande *(Eugenia involucrata DC);* Uvaia *(Eugenia pyriformis);* Pêssego do Mato *(Hexachlamys edulis);* Acerola *(Malpighia glabra L.);* Pequi *(Caryocar brasiliense)*.

Table 2. Molecular identification of amylase-producing yeast strains using the D1/D2 rRNA gene marker.

Yeast	Identification	Size[1]	GenBank closest relative[2]		CBS closest relative[2]	
			%	Accession #	%	Accession #
29	*Candida parapsilosis*	571	99	*Candida parapsilosis* (AB370926)	100	*Candida parapsilosis* CBS 10947
30	*Rhodotorula mucilaginosa*	571	100	*Rhodotorula mucilaginosa* ATCC 4056 (KC881063)	100	*Rhodotorula mucilaginosa* PYCC 5995
37	*Candida parapsilosis*	570	100	*Candida parapsilosis* (AB370926)	100	*Candida parapsilosis* PYCC 2545
43	*Candida glabrata*	581	100	*Candida glabrata* CBS 138 (AY198398)	100	*Candida glabrata* CBS 858

[1]In base pairs; [2]Results obtained in the databases.

Solid state fermentation was adopted aiming to lower the cost of enzymes production, considering that one of the major problems for the enzymes application in industrial scale, is the high cost of these biocatalysts, given the high price of formulated culture media (Romero et al., 2007; García-Martínez et al, 2010). In this way, the use of agroindustrial wastes for the microorganisms cultivation and enzyme production is one alternative to reduce the final cost of these biological catalysts (Singhania et al., 2009; Alves-Prado et al., 2010), especially in countries such as Brazil that have agriculture as one of the main economic activities.

Under the culture conditions used in this study, the strains that displayed the highest production of extracellular amylase were strains 29 (14.68 U/mL or 146.8 U/g substrate), 30 (25 U/mL or 250 U/g substrate), 37 (14.10 U/mL or 141 U/g substrate) and 43 (25.39 U/mL or 253.9 U/g substrate) (Table 1). These results are very promising when compared to previous results reported in the literature. Bhatti et al. (2007) cultivated the fungus *Fusarium solani* in solid state, using wheat bran as substrate, and obtained glucoamylase (about 61.35 U/g). Anto et al. (2006) reported the production of α-amylase by *Bacillus cereus* MTCC1305 (about 122 U/g), also grown in solid state. Similar results were reported by Ellaiah et al. (2002), who cultivated *Aspergillus* species in solid state. The authors obtained the maximum glucoamylase production (247 U/g), after 120 h of cultivation.

High level amylase-producing strains were selected for molecular identification. Using the criterion established by Kurtzman and Robnett (1998), our results show that the D1/D2 sequences of strains 29 and 37 were 99% identical to a sequence of *Candida parapsilosis* found in the database (Table 2). On the other hand, sequences

Table 3. Effect of pH and temperature on amylase produced by the selected yeast strains.

Yeast	Optimum pH	Optimum temperature (°C)	Stability pH	Stability temperature (°C)
Candida parapsilosis	7.0	60	5 -10	30 - 50
Rhodotorula mucilaginosa	7.0	60	3.5 - 9.5	30 - 50
Candida glabrata	7.0	60	3 - 8.5	30 -50

derived from strains 30 and 43 were 100 and 99% identical to a sequence of *Rhodotorula mucilaginosa* and *Candida glabrata,* respectively (Table 2). Reports about the use of these strains to produce enzymes of industrial interest are scarce, which underlines the importance of the present study. According to Saran et al. (2007), the prospection of microorganism producers of industrially-relevant enzymes has become increasingly important since it enables an understanding and improved application of these enzymes in industrial processes, as well as the discovery of new catalytic properties that may be used to develop new biotechnological processes.

Biochemical characterization of the amylases produced

Effect of pH and temperature

Amylases were characterized using enzymatic extracts obtained by growing the yeasts under the previously described conditions. The enzymes exhibited maximum activity at pH 7.0 and 60°C (Table 3). In general, fungal amylases have optimum pH values ranging from acidic to neutral (Gupta et al., 2003). Giannesi et al. (2006) obtained amylases from several microbial sources, exhibiting an optimum pH from 4.5 to 7.0. Rahardjo et al. (2005) reported pH 7.0 as optimal for amylases produced by *Aspergillus oryzae.* Figueira and Hirooka (2000) found optimum pH values of around 6.7 for amylases produced by the fungus *Fusarium moniliforme*. With respect to temperature, Aquino et al. (2003) reported that 60°C was the optimal temperature for α-amylase produced by *Scytalidium thermophilum*. Similar results were found by Li et al. (2007) for amylase produced by the yeast *Aureobasidium pullulans* N13d.

The enzymes were stable in a broad range of pH: *C. parapsilosis* (5 to 10); *R. mucilaginosa* (3.5 to 9.5) and *C. glabrata* (3 to 8.5). Amylases produced by selected strains were stable for 1 h at 50°C (Table 3). The enzymes exhibited higher pH and temperature-related stability than amylases produced by different microbial species. The amylase produced by *Vibrio* sp. was stable at pH 6.0 to 7.5, maintaining only 50% of its activity at pH 4.5 and 8.5. The same enzyme was stable for 30 min at 50°C (Najafi and Kembhavi, 2005). Sahnoum et al. (2012) found that the amylases produced by *Aspergillus oryzae* remained stable after 48 h at pH 5.6. However, when incubated for the same period at other levels of pH, they exhibited a considerable decrease in their catalytic potential. In the same study, the authors reported that the enzymes exhibited about 60% of residual activity after 1 h at 50°C. Gomes et al. (2005) reported a decrease of approximately 30% in the catalytic activity of amylase produced by the fungus *Thermomyces lanuginosus* when incubated for 24 h at pH 4.0 and 10.0. However, the enzyme remained stable after 1 h at 60°C.

The presence of enzymatic activity at extremes of pH and temperature confirms the high structural stability of the amylases produced by the yeasts assessed in the present study, highlighting their biotechnological potential. Previous studies reported high stability for extracellular enzymes produced by yeasts (Fossi et al., 2005; Leite et al., 2008). Enzymatic action in a wide range of pH and temperatures is advantageous for their industrial application, considering that only minor adjustments are needed in the processes (Leite et al., 2008).

Effect of ethanol on enzymatic activity

Enzymes may be exposed to alcoholic solutions in various industrial applications; therefore, the inhibition of ethanol is a trend in the study of some enzymes (Sun and Cheng, 2002). When incubated in solutions containing up to 15% ethanol, the enzyme activity was higher than 70% of the original (Figure 1). The increased catalytic potential of the amylases produced by the yeasts *C. parapsilosis* and *R. mucilaginosa* may be associated with the use of ethanol as the acceptor of intermediate glycosylation during the hydrolysis of the substrate, increasing the speed of the reaction (Villena et al., 2006).

Considering that the final concentration of ethanol in traditional fermentation processes stabilizes at around 10%, the results obtained here are very promising. Ethanol concentrations higher than 12% are harmful to the fermenting microorganism, hindering the recycling of yeast for a new fermentation cycle (Gu et al., 2001). Therefore, it can be inferred that the enzymes produced by the selected strains may be applied in alcohol production processes from amylaceous sources. Enzymatic stability in ethanol is an advantageous feature in simultaneous saccharification and fermentation (SSF) processes in which fermentable sugars released by the action of enzymes on vegetable polysaccharides are converted simultaneously into ethanol by fermenting micro-

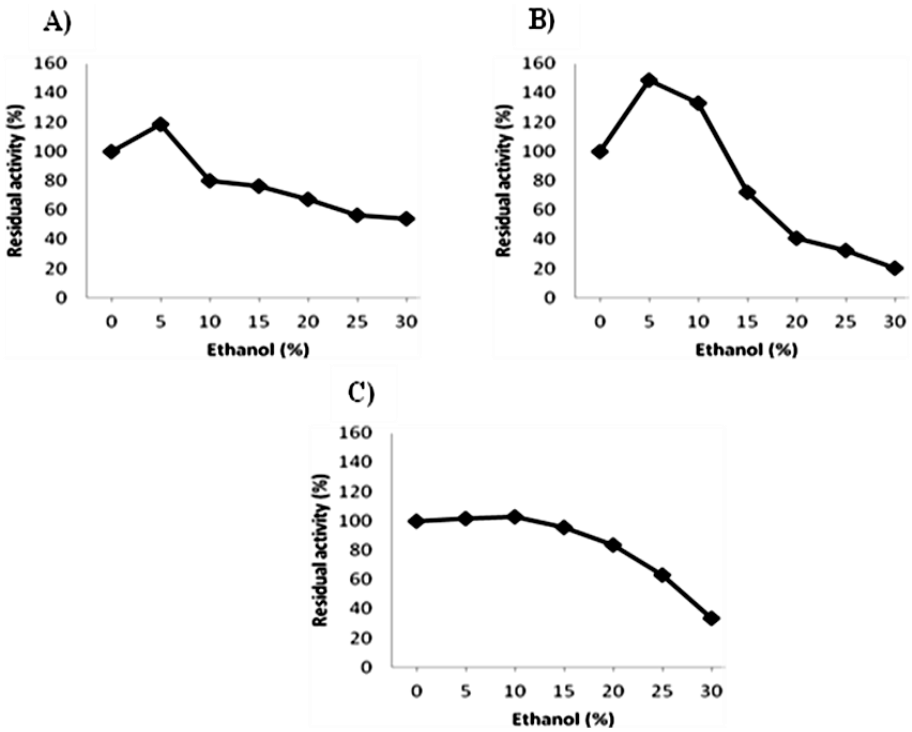

Figure 1. Effect of ethanol on enzymatic activity at pH 7.0 and 50°C. **A)** *Candida parapsilosis*. **B)** *Rhodotorula mucilaginosa*. **C)** *Candida glabrata*.

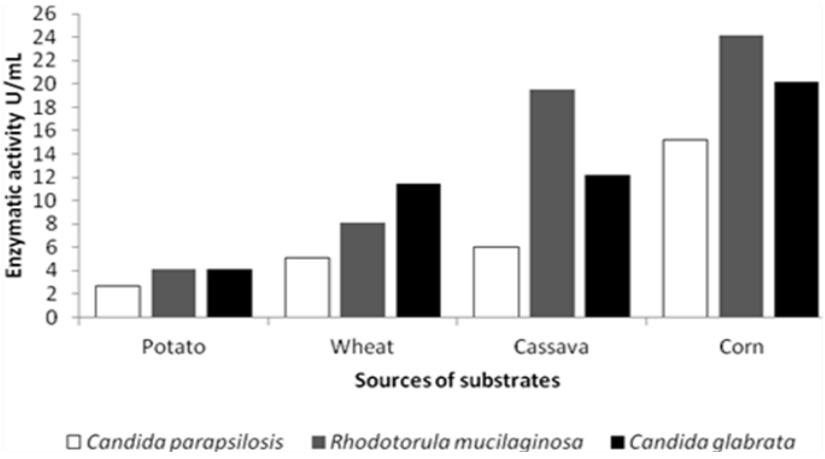

Figure 2. Catalytic potential of the enzymes on starches from different vegetable sources, quantified by the DNS method (reducing sugar).

organisms (Leite et al., 2008; Scott et al., 2013).

Enzymatic hydrolysis of starch from different sources

The action of enzymes on starch from different vegetable sources was assessed using starch extracted from potatoes, wheat, cassava and corn as substrate. These are among the main starch sources commercialized in the world, being all cultivated expressively in Brazil.

The enzymatic extracts exhibited potential to hydrolyze all the starch assessed in this study. However, the best catalytic efficiency was achieved with corn starch (Figure 2).

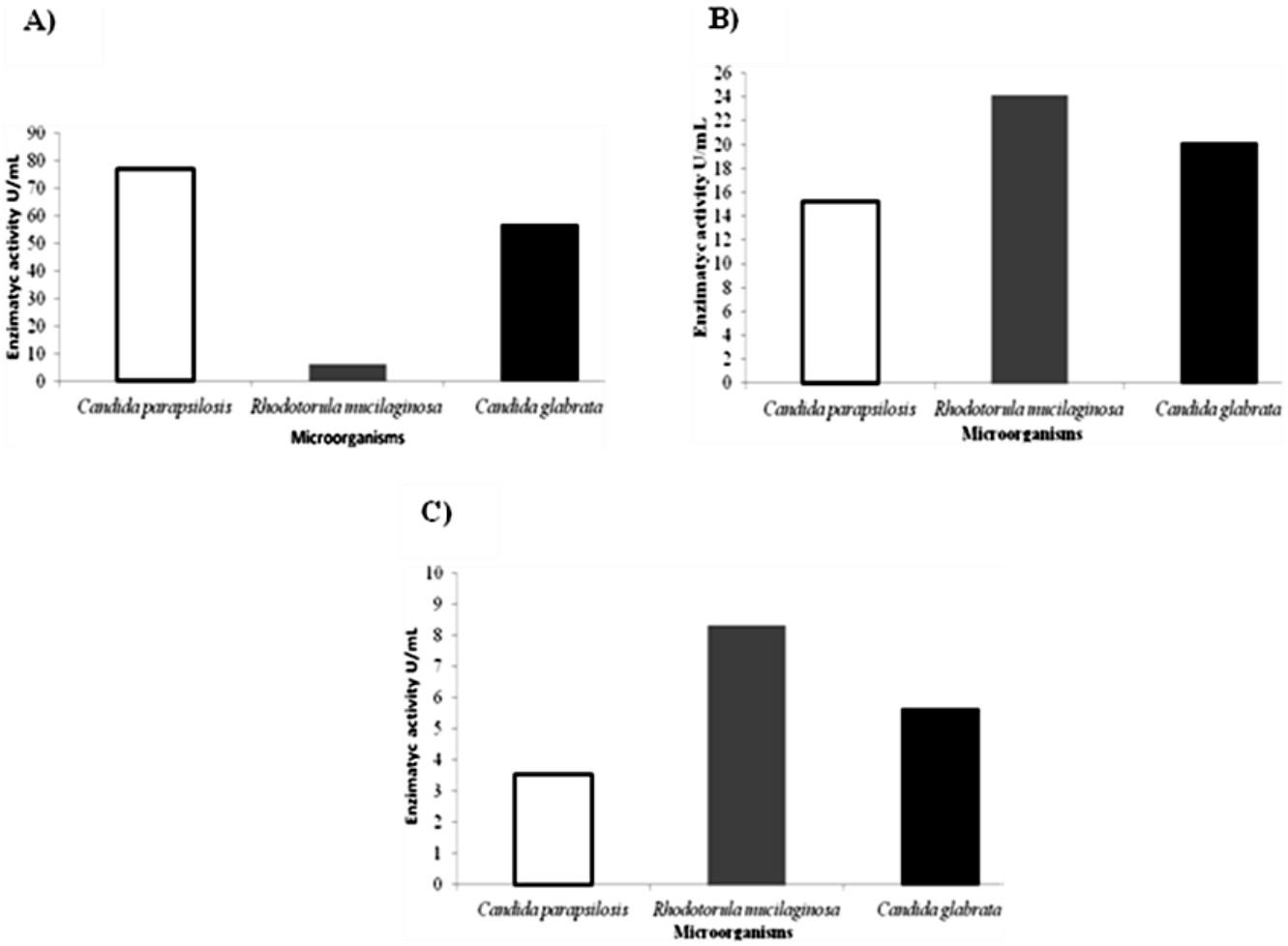

Figure 3. Enzymatic modifications of corn starch. **A)** Quantification of dextrinizing activity using the iodometric method. **B)** Quantification of sugars and reducing ends using the DNS method. **C)** Quantification of free glucose using the glucose-oxidase method.

The catalytic action of different enzymes in starch from different sources may be related to the structure and composition of the substrate molecule, particularly the content and length of the amylose chains. The structural characteristics of starch vary according to its botanical origin. The proportions of amylose and amylopectin affect the texture and architecture of the starch granule and are therefore reflected in the catalytic properties of amylases. Furthermore, the proportions of contaminants, such as lipids, proteins and minerals, also differ according to vegetable origin, and may affect enzymatic reactions (Thomas and Atwell, 1999; Tester et al., 2004).

Corn starch has a higher amount of amylose, and consequently lower levels of amylopectin, favoring enzymatic hydrolysis. The amylopectin molecule is larger than the amylose molecule and exhibits a branched structure of high molecular weight with α-1.4 and α-1.6 glycosidic bonds, which hinder the catalytic performance of amylases (Tester et al., 2004).

Dextrinization and saccharification potential of enzymatic extracts

Based on the above described results, the changes caused by each enzymatic extract in starch molecules were assessed using corn starch as substrate. The enzymes produced by *C. parapsilosis* and *C. glabrata* caused a significant reduction in the degree of polymerization of the starch molecule, which was reflected in the increase of reducing chain ends. However, the presence of free glucose was also observed after enzymatic treatment, indicating that the extracts have the synergistic action of dextrinizing and saccharifying enzymes, despite the predominance of depolymerizing potential (Figure 3). The enzyme extract produced by *R. mucilaginous* was not efficient in reducing

the polymerization of the starch molecule. However, an increase in the concentration of total reducing sugars and glucose was confirmed after enzymatic hydrolysis. Therefore, it can be concluded that this enzymatic extract exhibits predominantly saccharification potential (Figure 3).

The enzymatic hydrolysis of starch includes liquefaction and saccharification. Endoamylases and debranching enzymes such as α-amylase and isoamylase are used during liquefaction, drastically reducing the degree of polymerization of starch and releasing dextrins. During saccharification, dextrins are hydrolyzed by exoamylases to produce maltose (β-amylase) or glucose (amyloglucosidase, α-glucosidase and glucoamylases) syrup (Van Der Maarel et al., 2002). Thus, the results obtained in this study indicate that the enzymatic extracts of *C. parapsilosis* and *C. glabrata* exhibited catalytic exoamylase and endoamylase activity, while the extract of *R. mucilaginosa* exhibited mainly exoamylase activity. The scientific literature describes similar results. Silva et al. (2005) reported the dextrinizing and saccharifying activity of enzymatic extract produced by the filamentous fungus *Rhizomucor pusillus*. The production of exoamylases has been described for the yeasts *Aureobasidium pullulans* (Li et al., 2007) and *Schizosaccharomyces pombe* (Okuyama et al., 2005). Furthermore, predominantly dextrinizing activity has been reported for several species of *Aspergillus*, such as *A. flavus*, *A. niger* and *A. oryzae* (Shafique et al., 2009; Sahnoun et al., 2012).

Conclusions

Our results indicate that the evaluated yeast strains have potential for amylase production in low cost culture media. The enzymes exhibited high structural stability, maintaining their catalytic potential at different levels of pH, temperatures and ethanol concentrations. In view of the described characteristics, we highlight the biotechnological potential of these enzymes and intend to apply them in processes to obtain ethanol from starch sources in future studies.

Conflict of interests

The authors did not declare any conflict of interest.

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