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Effect of pH and temperature on the production and activity of *Schwanniomyces polymorphus* extracellular proteases in fermentation medium

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Proteases are among the most studied enzymes due to the great interest with regard to their cleavage of peptide bonds in industrial applications. With the goal of maximizing the advantages of these biocatalysts, new producers of proteolytic enzymes are being prospected from various microhabitats. This study aimed to select a yeast strain with high yield of extracellular proteolytic enzyme and characterize the yield and activity of theses enzymes. Among the 521 yeast isolates tested for proteolytic activity on solid medium, 20 isolates were selected for the determination of proteolytic activity in liquid medium. Enzymatic assay was performed using azocasein as substrate, and one unit of protease activity was defined as the amount of enzyme able to produce an increase in absorbance of 0.001/min and expressed in U/mL. A yeast extracted from Jabuticaba fruit, identified as *Schwanniomyces polymorphus* through the analysis of its rDNA sequence, showed the highest proteolytic activity. The optimal conditions for protease production, with a maximum value of 289.9 U.mL^{-1}, were pH 7.8, 28ºC and 72 h of fermentation. The activity of extracellular proteases contained in the culture supernatant was subjected to enzymatic characterization: the optimal pH was 8.0 and the optimal temperature was 35ºC. Therefore, among the yeasts isolated from tropical fruits, *S.* polymorphus presented the highest proteolytic enzyme activity under conditions of minimal fermentation, at temperature of 28ºC and pH of 7.8.

Key words: Proteolysis, fermentation, biochemical characterization, tropical fruits.

INTRODUCTION

The increasing body of knowledge about enzymes has motivated advances in enzymology (Lee and Huang, 2008). With this development of enzyme technology, the interest in industrial enzymes has increased greatly with the goal of maximizing the advantages of catalysis and minimizing the disadvantages to enable the use of these

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catalysts on a large scale (Buchholz et al., 2012). Enzymes like proteases, lipases and pectinases are one of the most important biomolecules with a wide range of industrial applications, having a great impact in the production of textiles, detergents, food, processing of fruit drinks and alcoholic beverages (Gurung et al., 2013).

Proteases, which catalyze the cleavage of peptide bonds in proteins and peptides, are among the most studied, encompassing a class of enzymes with multiple applications in biology and various other fields. Microbial proteases represent one of the three largest groups of industrial enzymes, corresponding to approximately 60% of the total sale of industrial enzymes worldwide (Rai and Mukherjee, 2010).

Due to its wide application in industry, proteases form the most studied group of hydrolases, prompting the exploration of new sources of these enzymes. The industrial demand for proteolytic enzymes with appropriate specificities and stabilities with regard to pH, temperature, metal ions, compatibility with such detergent compounds as surfactants and organic solvents continues to stimulate the search for new supply sources of these enzymes for research. Proteases with high activity and stability in certain ranges of pH and temperature are interesting for applications in bioengineering and biotechnology. In general, microbial proteases are extracellular in nature and are secreted directly into the fermentation broth during industrial production, thus simplifying the processing of the enzyme compared to the proteases obtained from plants and animals (Zavala et al., 2004; Lageiro et al., 2007).

Yeasts are another attractive host strains for cell-surface display systems due to their safety, ease of high-cell density cultivation and the capability of eukaryotic proteins folding and glycosylation (Gai, 2007).These properties make enzyme displayed yeast be of great value in biocatalytic process, especially in the industrial production using displaying yeast as whole-cell biocatalysts (Huang et al., 2012; Tanino, 2009). Yeasts are abundant in habitats in which carbohydrates are present, such as fruits, flowers and tree bark (Kurtzman and Fell, 1998). Yeast species have a great potential for the production of microbial enzymes for industry, and these microrganisms offer an alternative source of these enzymes.

The enzymes from these microrganisms are well distributed in nature, making these microrganisms preferable sources for bioprocess fermentation because they have a rapid growth rate and can be genetically constructed to produce enzymes with desired capabilities or simply for enzyme overproduction (Lucena et al., 2007).

The use of specific yeast strains with high enzyme activity is an important factor in industrial production, and the identification of yeasts with these characteristics is based on various molecular techniques, including the sequencing of D1/D2 domains of the 26S subunit of the rRNA gene (Gildemacher et al., 2006). These techniques are rapid, simple and accurate, making them suitable for the rapid screening and identification of yeast isolates (Kurtzman and Fell, 1998; Lachance et al., 2000; González et al., 2004; Fell et al., 2000).

Northeast Brazil presents a great diversity of tropical fruits that can harbor several species of yeast (Souza Filho et al., 2002), representing an important micro-habitat for a wide variety of yeast species due to the high sugar concentration and various pH values of the fruit (Lachance et al., 1982). The main goal of this study was to select and identify yeast isolates from the surface of tropical fruits that are able to secrete high amounts of proteases and to optimize the culture conditions for the production and activity of protease enzymes.

MATERIALS AND METHODS

Source of organisms

For this work, we used yeast strains belonging to the bank of the Applied Microbiology Laboratory at Universidade Federal de Sergipe, Sergipe, Brazil. The strains had been isolated from the fruits mangaba (*Hancornia speciosa* Gom.), pitanga (*Eugenia uniflora* L.), acerola (*Malpighia punicifolia* L.), umbu (*Spondias tuberosa* Avr. Cam) and jabuticaba (*Myciaria cauliflora* Berg) collected from the Sergipe State, Brazil.

Protease activity selection on plates

For the selection of yeast isolates showing the production of proteolytic enzymes, the different yeasts were grown on plates of milk-gelatin agar medium (5.0 g/L meat peptone, 3.0 g/L yeast extract, 12.0 g/L agar, 10.0 g/L skim milk and 10.0 g/L gelatin powder, pH adjusted to 7.2 with 1 M HCl) sterilized under conditions of 121°C and 15 lb pressure for 15 min. The plates were incubated at 30°C ± 3°C for 48 h. The presence of the proteolytic activity was indicated by formation of clear halos around the colony after the addition of 10% glacial acetic acid to the plates. The enzymatic activity was measured according to the modified method of Price (1982) by the value of the precipitation zone (PZ). Therefore, the enzyme activities were measured by dividing the diameter of the colony by the diameter of the colony plus the precipitation zone. The results were presented in code: the value 1 when PZ = 1.0 (no enzyme activity), value 2 when 0.63 <PZ <1.0 (moderate enzyme activity) and 3 when PZ value <0.63 (strong enzyme activity).

Production medium

For protease production, 1 mL of each isolate selected for extracellular protease activity on the solid medium was inoculated into 250 mL Erlenmeyer flasks containing 100 mL of liquid minimal medium for fermentation (MMF), (20.0 g/L glucose, 7.5 g/L meat peptone and 4.5 g/L yeast extract, 0,1% gelatine powder, pH adjusted to 7.2 with 1 M HCl) sterilized under conditions of 121°C, 15 lb pressure for 15 min. The incubation was performed in an incubator with shaking at 150 rpm and a temperature 28°C for 72 h.

Estimation of total protein

The total protein determination was performed using the Bradford

Method (1976), with bovine serum albumin (BSA) as the standard. The standard curve was prepared from concentrations of 0-100 $\mu g.mL^{-1}$ of BSA stock solution (1 mg/ml).

Enzymatic assay

The proteolytic activity in the culture supernatant was determined using the method of Weckenmann and Martin (1984), with modifications. The reaction mixture contained 100 µL supernatant and 1.0% azocasein (w/v) in 0.2 M Tris-HCl buffer (pH 7.2) containing 1.0 mM $CaCl_2$. The reaction mixture was incubated in a water bath at 37°C for 1 h and reaction was stopped by the addition of 1.0 mL of 20% trichloroacetic acid (TCA). The solution was centrifuged 23000 x *g* for 15 min at 4°C, and 200 µL of 3 M NaOH was added to 1.0 mL of the supernatant (Dosoretz et al., 1990; Leighton et al., 1973; Porto et al., 1996). The absorbance was spectrophotometrically (FEMTO 480) measured at 440 nm. One unit of proteolytic activity was defined as the amount of enzyme capable of producing an increase in absorbance of 0.001/ min and was expressed as U/mL. The specific activity was calculated by the ratio between the total protease activity and total protein concentration (U/mL). The experiments were performed in triplicate. Enzymatic activity was presented as mean and standard deviation of three replicates.

The effects of pH and incubation temperature on protease production

The yeast that was selected based on its high production of protease was propagated in the minimum liquid medium for fermentation (MMF) at pH values ranging from 6.5 to 7.8 and temperatures between 26 to 35°C in an incubator with shaking at 150 rpm. The production of protease by the yeast was determined as described above.

The effects of pH and incubation temperature on protease activity

To determine the optimal incubation temperature, we evaluated the activity of the enzyme at temperatures between 25 to 45°C in a water bath for 1 h. To determine the optimal pH, the protease was incubated at different pH values. Buffers used were: phosphate (pH 6.2 to 6.8) and Tris-HCl (pH 7.2 to 8.0) and reactions were incubated in a water bath at 37°C. The protease activity was determined as described above.

Electrophoretic procedures

Electrophoresis was performed by SDS-PAGE (polyacrylamide gel in sodium dodecyl sulfate) to identify the protease isoenzymes produced by the selected strain. The analysis followed the method of Laemmli (1970). The gel system consisted of a resolving gel (12%) plus 1% (w/v) gelatin and a stacking gel (5%). The proteolytic activity was detected according to Li et al. (1997). Briefly, after electrophoresis, the gel was washed twice in 50 mM Tris-HCl (pH 9.0) containing 5% (v/v) Triton X-100 for 15 min at 4°C. The gel was then incubated in 50 mM Tris-HCl (pH 9.0) for 12 hat 56°C to allow the degradation of the gelatin. The gel was stained with 0.1% Coomassie Brilliant Blue G250 (w/v) in 45% (v/v) methanol and 10% (v/v) acetic acid and destained by 30% (v/v) methanol and 10% (v/v) acetic acid. The band with proteolytic activity was observed as a clear colorless area.

Taxonomy of selected of yeast

The strain of yeast selected due to protease activity was identified based on the sequence of the conserved D1 and D2 domains of the large subunit 26S rRNA gene (Lachance and Starmer, 1998; Kurtzman and Suzuki, 2010). The code yeast sequence was analyzed using the ABI 3730 DNA Analyzer (Applied Biosystems), with the BigDye® Terminator Cycle Sequencing v3.1 kit, as a service provided by Centro de Estudos do Genoma Humano - USP, São Paulo, Brazil. BLAST (Basic Alignment Search Tool Locus) and nucleotide-nucleotide (BLASTn), which are available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/), and was used for a comparison with the sequences deposited in GenBank. The matrix of the sequence was determined by a similarity greater than or equal to 99%.

RESULTS

Screening for the enzymatic production of extracellular proteases on solid medium

The screening was performed using the collection of strains isolated from tropical fruits and was composed of 521 isolates. Of these, several yeast isolates were identified by their morphology and physiology as belonging to the following genera: *Candida*, *Cryptococcus*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces*, *Saccharomyces*, *Trichosporon*, *Geotrichum*, *Pseudozyma*, *Leucosporidium*, *Kluyveromyces*, *Issatchenkia*, *Kloeckera*, *Myxozyma*, *Metschnikowia*, *Torulaspora*, *Zygosaccharomyces* and *Black Yeast*.

Through the production of extracellular proteases in plate containing milk-gelatin agar were halos of degradation that were detected and measured by testing PZ. Out of 521 isolates, only 20 presented Pz classified as moderate proteolytic activity (Table 1).

Identification of the selected isolates

The D1/D2 domains of the 26S subunit of the isolated 8Cb6 code yeast were sequenced. The nucleotide sequence obtained was compared with sequences deposited in the GenBank database using the BLASTn program 2.2.26 + (Altschul et al., 1997). According to the results, the selected microrganism in this study showed 100% similarity with *S. polymorphus* (accession number 319439558|FR774544.1).

Enzyme assay for extracellular proteolytic enzyme production into fermentation media

To analyze the production capacity of extracellular proteolytic enzymes in fermentation, 20 isolates were selected and their production capacities of total protein and proteolytic activities were measured. However, according to the results, the production of total proteins was not correlated with proteolytic activity, because not all isolates that produced high protein concentration also showed high activity proteolytic (Table 2). The largest production of proteolytic enzymes was observed in the

Table 1. Screening of yeast with extracellular proteolytic activity by Pz Method in plate containing solid medium Agar-gelatine-milk under a temperature of 30ºC ± 3° C for 48 h.

Species	Order code	Source substrate isolates	Precipitation zone (halo) (mm)	Enzyme activity	Pz
Candida valida	R03	Umbu	10	0.75	2
Kloeckera apis	R37	Acerola	05	0.75	2
Candida sergipensis	R78	Acerola	02	0.83	2
Black yeast	R474	Acerola	05	0.83	2
Cryptococcus humicolus	R301	Pitanga	05	0.86	2
Cryptococcus yarrowii	R354	Pitanga	05	0.87	2
Candida vartiovaarae	R374	Pitanga	05	0.75	2
N.I	3C1-4	Jabuticaba	05	0.83	2
N.I	4Ab2	Jabuticaba	04	0.90	2
N.I	4Ab7	Jabuticaba	02	0.91	2
N.I	6Ca3	Jabuticaba	02	0.83	2
N.I	8Aa4	Jabuticaba	02	0.91	2
N.I	8Ab4	Jabuticaba	02	0.84	2
N.I	8Ab12	Jabuticaba	02	0.79	2
N.I	8Ab13	Jabuticaba	04	0.83	2
N.I	8Ca1	Jabuticaba	02	0.80	2
N.I	8Ca3-4	Jabuticaba	02	0.83	2
N.I	8Cb4	Jabuticaba	03	0.79	2
N.I	8Cb6	Jabuticaba	05	0.75	2
N.I	10Aa12	Jabuticaba	05	0.75	2

NI = Not identified. The results were presented in the code value 1 determining PZ=1.0 (No enzyme activity), value 2 when 0.63 <PZ<1,0 (moderate activity) e value 3 when PZ≤0,63 (strong enzymatic activity).

Table 2. Proteolytic activity of yeast isolates in minimal medium for fermentation (MMF) under a temperature of 28ºC and pH 7.2 for 72 h.

Order code isolates	Source substrate isolates	Total protein ($\mu g.mL^{-1}$)	Proteolytic activity ($U.mL^{-1}$)	Specific activity ($U.\mu g^{-1}$)
R03	Umbu	11.7	4.77 ±4.80	0.41
R37	Acerola	10.4	8.0 ±2.50	0.77
R78	Acerola	49.4	ND	ND
R301	Pitanga	71.7	5.3 ±2.39	0.10
R354	Pitanga	54.3	23.3 ±7.72	0.43
R374	Pitanga	124.5	4.8 ±4.22	0.04
R474	Acerola	65.7	16.6 ±1.98	0.25
3C1-4	Jabuticaba	17.2	8.30 ±1.21	0.48
4Ab2	Jabuticaba	37.2	ND	ND
4Ab7	Jabuticaba	-	ND	ND
6Ca3	Jabuticaba	-	8.58 ±1.62	ND
8Aa4	Jabuticaba	181.9	76.1 ±3.18	0.42
8Ab4	Jabuticaba	158.0	77.2 ±5.46	0.49
8Ab12	Jabuticaba	3.7	6.86 ±3.23	1.85
8Ab13	Jabuticaba	21.6	15.6 ±3.72	0.73
8Ca1	Jabuticaba	-	9.0 ±0.33	ND
8Ca3-4	Jabuticaba	10.5	2.1 ±3.63	0.20
8Cb4	Jabuticaba	-	15.7 ±0.40	ND
8Cb6	Jabuticaba	139.0	257.4 ±9.4	1.85
10Aa12	Jabuticaba	58.6	ND	ND

Total proteins concentration below 3.0 $\mu g.mL^{-1}$; ND = no proteolytic activity detected.

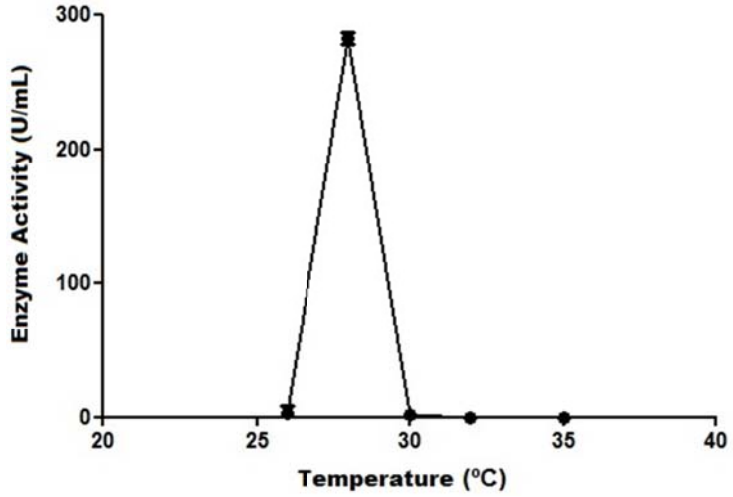

Figure 1. Influence of temperature on the production of extracellular proteolytic enzymes by *Schwanniomyces polymorphus* with minimal medium for fermentation (MMF) at different temperatures for 72 h.

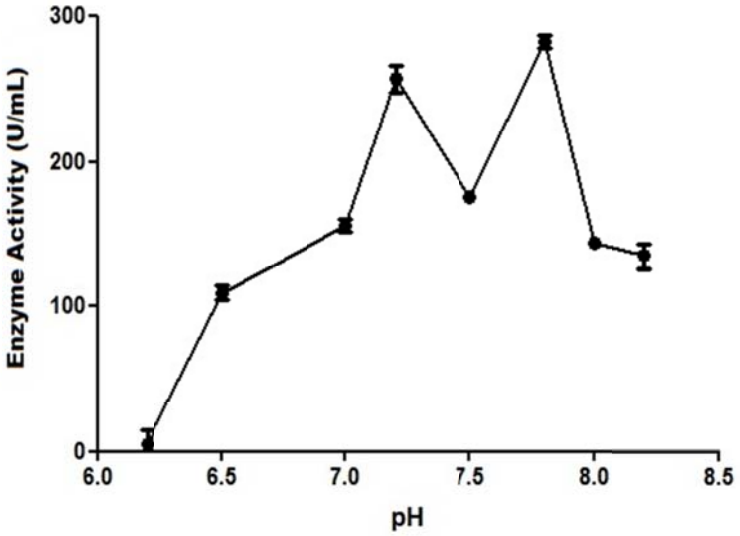

Figure 2. Influence of pH on the production of extracellular proteolytic enzymes by species *S. polymorphus* in different pH of incubation with minimal medium for fermentation (MMF).

isolate extracted from jabuticaba with order code 8Cb6 and was, therefore, selected for enzyme characterization and species identification.

Effects of temperature and pH on protease production in the selected isolate *S. polymorphus*

Effect of temperature

The optimum temperature for the maximum production of proteolytic enzymes was 28°C after 72 h of incubation with agitation (Figure 1). Other temperatures presented low or no production, with a temperature of 28ºC being ideal for growth and the secretion of proteolytic enzymes in the fermentation media.

Effect of pH

Using the optimum temperature of 28°C for extracellular enzyme production, the optimum pH for the maximum production of proteolytic enzymes was found to be pH 7.8 after 72 h of incubation with shaking (Figure 2). The proteolytic activity increased from pH 6.2, reaching a

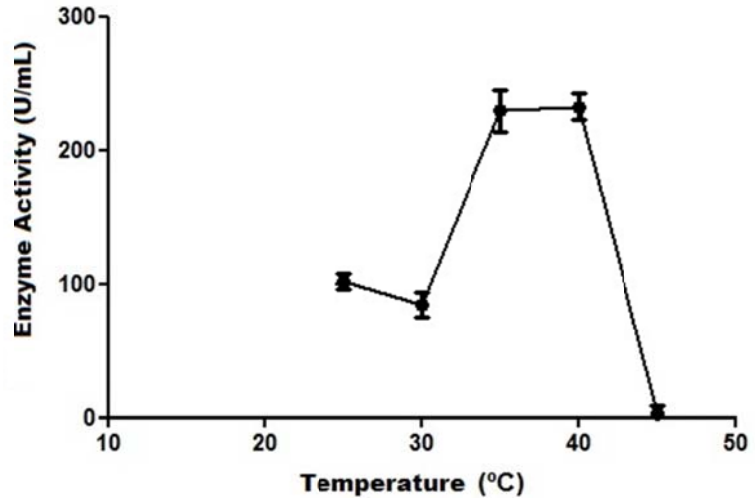

Figure 3. Effect of temperature on the activity of extracellular proteolytic enzymes produced by species *Schwanniomyces polymorphus* in incubation. The optimum temperature was determined by assay of protease activity in different incubation temperatures in a water bath.

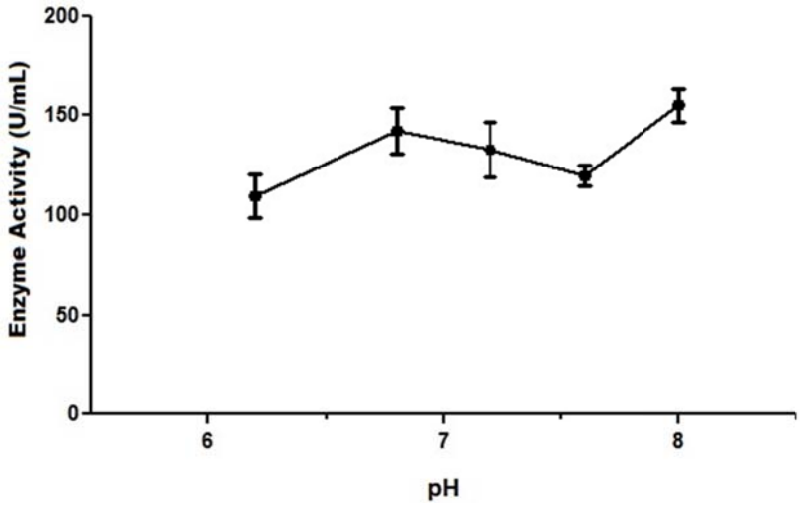

Figure 4. Effect of pH on the activity of extracellular proteolytic enzymes produced by species *Schwanniomyces polymorphus* in incubation. The optimum pH was determined by assay of protease activity in different pHs on incubation at 37° C in a water bath.

peak at pH 7.2 which was followed by a decrease at pH 7.5. Increase in pH values above 7.8 resulted in decreased proteolytic activity.

Enzymatic characterization

The activity of the proteolytic enzymes in the culture supernatant was evaluated regarding the optimum temperature. Reaction mixture was incubated in temperatures between 25 and 45ºC and the optimum activity was observed at temperatures between 35 and 40ºC with 229.9 and 232.8 U/mL, respectively (Figure 3). Above this temperature, the enzymes may denature, losing their catalytic activity. The enzymatic activity of the extracellular proteases was also evaluated at pH values from 6.2 to 8.0. Very similar activities were observed between pH 6.8 and 8.0, with enzymatic activity varying from 142.4 to 155.0 U/mL, respectively (Figure 4). These data allow us to conclude that the extracellular proteolytic enzymes remain active in environments in which the temperature is maintained at approximately 37.5 ± 2.5°C and near-neutral pH.

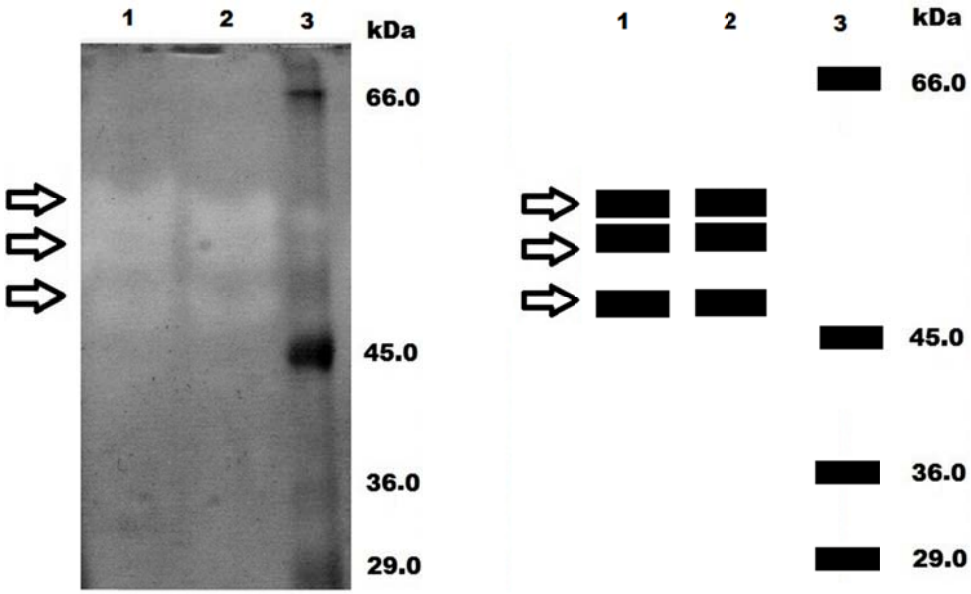

Figure 5. Polyacrylamide gel electrophoresis (12%) containing 1% gelatine for determination of the proteolytic activity of *Schwanniomyces polymorphus*. The bands with proteolytic activity present apperars clear and colorless in the gel. Line 1 and 2, supernatant from fermented; Line 3, Molecular marker.

Determination of the profile of proteolytic enzymes from *S. polymorphus*

The use of SDS-PAGE containing gelatin to determine the profile of *S. polymorphus* extracellular proteolytic enzyme activity presented a protease profile of three well-defined bands between 45 and 60 kDa (Figure 5). The bands suggest the presence of at least three proteases in the crude extract because the transparent gel bands indicate the enzymatic action of proteases on the substrate in the gel.

DISCUSSION

This study describes the production of extracellular proteases from isolates of yeast extracted from tropical fruits. Through the method described by Riffel and Brandelli (2002), we selected 20 of 521 isolated microorganisms based on activity of PZ, through the formation of clear halos in solid culture medium. Selected microorganisms demonstrated moderate production of extracellular proteases.

After this selection, the isolates that showed extracellular proteolytic activity were subjected to fermentation in nutrient broth for growth and the production of proteolytic enzymes (Porto et al., 1996; Alves et al., 2005). The effectiveness of protease production in fermentation medium was determined by an enzyme spectrophotometric assay using azocasein. The data obtained in this step were critical for the screening of the isolate with the highest production of extracellular proteolytic activity. To obtain optimal performance with respect to enzyme production during the development of microrganisms, different types of media are utilized to increase the amount of cells and also to increase the production of enzymes of industrial interest (Brumano et al., 1993; Papagianni and Moo-Young 2002). In this study, a medium with minimum requirements for fermentation was used to evaluate the potential of the isolates under conditions similar to their natural habitat. Under these culture conditions, an isolate with code 8Cb6 provided the largest activity of proteolytic enzymes extracellularly.

The use of yeast strains with high enzyme activity is an important factor in the industrial production of enzymes, as they are responsible for the transformation of raw materials into products for use in food, medicine or pharmacology. Therefore, the identification of yeast strains suitable for the fermentation and production of extracellular enzymes in certain industrial processes is critical (Fernández-González et al., 2004). After the screening of extracellular proteolytic activity, the identification of this strain was determined by sequencing the conserved D1/D2 region of the large 26S rDNA subunit, as described ((Fell et al., 2000; Lachance et al., 2000; Kurtzman and Suzuki, 2010), with 100% similarity with order Saccharomycetales and 100% similarity with *S. polymorphus*. This species was first reported in 2010 by Kurtzman and Suzuki (2010) in his work on the phylogenetic analysis of ascomycetes. Studies focused on the characterization of extracellular proteolytic enzymes

produced by this isolate which have not been reported to date.

The optimal growth conditions to protease activity and production of this isolate were determined. Temperature is one of the most important factors influencing the growth and survival of organisms, and temperatures of approximately 30 to 40ºC are generally used in the cultivation of fermenting microrganisms. The temperature is a critical parameter that influences relevant enzyme production and requires control (Chaloupka, 1985). The amount of total protein in the strains may rather demon-strate the ability to form zymogens, non-proteolytic enzymes or enzymes that degrade other substrates, in addition to enzymes that hydrolyze protein substances. The largest rates of enzyme production in this study occurred in the range of 28ºC. For the results presented for proteolytic activity in the media of the fermentation temperature of 28ºC (Table 2), it may be noted that some of the isolates showed low rates of total protein production, but with proteolytic activity, it was positive but without specific activity detection. However, the isolated 8Ab12 presented low rate of total protein, and proteolytic activity, but a low specific activity similar to the selected 8Cb6. However, this work prioritize the selection of yeast that has the highest proteolytic enzymes production considering a crucial factor in industrial production on a large scale and by the benefits considered in its specific activity. In similar studies, it was reported that some species of *Candida* sp. showed maximum proteolytic activity in a range of temperature close to 30°C (Chantawannakul et al., 2002; Kanekar et al., 2002; Neves et al., 2006). Another important factor is the pH of the fermentation culture medium. We found that the optimal range of enzyme production for the isolated strain was near pH 7.8, similar to that of the medium, pH 7.2. The pH affects the ionization of amino acids, which dictate the primary and secondary structure of enzymes and, therefore, control enzyme activity (Savitha et al., 2011). Proteases with distinct optimal values of pH have been reported, and the value presented in this paper describes proteases that are active at a near-neutral pH. The data presented are similar to those of the alkaline proteases from *Bacillus subtilis* CN2 reported in the pH range of 7-11 (Uchida et al., 2004). Bolumar et al. (2005) showed that neutral-alkaline proteases ranging in pH 6.0 to 12, showed beat around pH 8.0 with stability at temperatures above 75ºC, while there was decrease of stability at 37°C. It is possible that in this study, alkaline neutral isozymes exhibited optimum pH around 7.2 and 7.8 approaching that that has been reported in other studies.

It was observed that the optimal pH for most fungal proteases was in the pH range from 7.0 to 9.0. These observations showed that the optimum pH for most alkaline proteases was between pH 7.0 and 10.0. Typically, the proteases in this pH range generate a lower bitterness in protein-hydrolyzed food when compared to proteases of animal origin and are, therefore, quite valuable for use in the food industry (Rao et al., 1998). Focusing only on the characteristics of the enzymes produced by incubation at different pH and temperatures, our results indicate proteases belonging to the alkaline or neutral classes. Thus, the number of proteases produced in fermentation was determined by zymography and three proteases were observed with apparent molecular weights between 45 - 60 kDa.

Conclusions

Therefore, it is possible to conclude from this study that *S. polymorphus*, extracted from jabuticaba, showed the highest productivity of extracellular proteases among the yeast isolates used in this study. Optimum conditions to protease production in fermentation medium minimum were 28ºC, pH 7.8 and 72 h of growth. Biochemical characterization of supernatant proteases showed that the enzymes have optimum catalytic activity around 35°C and pH 8.0.

Conflict of interests

The authors did not declare any conflict of interest.

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