

Evaluation of the milk clotting potential and characterization of proteases from *Aspergillus* sp. and *Pleurotus albidus*

Mariene Marques Noll¹, Alex Graça Contato^{1,2,3*}, Tatiane Brugnari^{1,4}, Ana Júlia dos Reis Buzzo¹, Guilherme Mauro Aranha⁵, Fabíola Dorneles Inácio^{1,6}, Rosane Marina Peralta¹ and Cristina Giatti Marques de Souza¹

¹Departamento de Bioquímica, Universidade Estadual de Maringá, Maringá, Paraná, Brasil. ²Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brasil. ³Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma, United States of America. ⁴Departamento Acadêmico de Química e Biologia, Universidade Tecnológica Federal do Paraná, Curitiba, Paraná, Brasil. ⁵Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brasil. ⁶Instituto Federal do Paraná, Jacarezinho, Paraná, Brasil. *Author for correspondence. E-mail: alexgraca.contato@usp.br

ABSTRACT. The study evaluated the production of proteases in solid-state fermentation using wheat bran as a substrate. The best producing isolates were used to obtain crude extract which was evaluated for optimal pH and temperature, thermostability, effect of salts and activity against inhibitors. The studied fungi were *Aspergillus* sp. 125, *Fusarium* sp. 132, *Fusarium* sp. 206, *Pleurotus albidus* 018 and *Pleurotus pulmonarius* CCB20. The isolates with better results (*Aspergillus* sp. and *P. albidus*), showed protease activity with an optimum pH of 7.0, and an optimum temperature of 50°C with good thermostability between 40 and 50°C. As for salts, the protease activity was inhibited in the presence of ZnSO₄, and the activity of the proteases from the crude *Aspergillus* extract, strongly inhibited by PMSF, indicating the presence of a fraction of serine protease in the extract. The extracts of the two selected isolates showed considerable inhibition by EDTA. The milk clotting activity was 240 U mL⁻¹ for the *Aspergillus* extract and 153 U mL⁻¹ for the crude *P. albidus* extract. Proteases are important enzymes widely used in the food industry, including cheese. The data suggest that these fungi have the potential to produce these enzymes for usage in cheese making.

Keywords: Proteases; milk coagulation; cheese; *P. albidus*; *Aspergillus* sp.

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Introduction

Proteases are hydrolytic enzymes that degrade proteins into peptides and amino acids, playing an important role in the physiological conditions of living beings. They act in many metabolic reactions such as cell growth and death, blood clotting and immune defense. Because it is a large complex group, the properties differ a lot from one protease to another (Vandeputte-Rutten & Gros, 2002; Inácio et al., 2018). Despite this, characteristics like thermal stability, high catalytic activity and wide range of temperature and pH make them suitable to be used in biotechnology, especially the microbial proteases. Even though few microorganisms are known as industrial producers, 90% of the commercially available proteases are obtained from microbial sources (Rao, Tanksale, Ghatge, & Deshpande, 1998; Gimenes, Silveira, & Tambourgi, 2019).

In the industries, protease contributes to the development of processes or products of high added value. In the food industry, for example, they are applied to obtain products with higher digestibility and sensorial quality. The main application is in the cheese production, where it is used to hydrolyze the peptide bond of the milk, generating casein and other peptides (Souza et al., 2015). Due to ethical reasons, microbial proteases are a good alternative to cheese industry in comparison with calf renin (Vishwanatha, Rao, & Singh, 2010).

Traditionally, proteases are obtained through submerged cultures, but solid-state cultures have some advantages. In general, the production of an enzyme can be affected by the type of culture medium, by the pH and temperature of the medium, among other factors. The main factor that affects production of proteases is the C/N variation of the medium (Souza et al., 2015).

Among protease-producing fungi, one of the most studied genus is *Aspergillus* (Alvarez-Vasquez, González-Alcón, & Torres, 2000). The genus *Fusarium* is active in the decomposition of cellulosic substrates and has recently been studied as producer of protease (Leslie, Zeller, & Summerell, 2001; Yang et al., 2001).

Mushrooms of the genus *Pleurotus* are edible and capable of degrading lignin (Contato et al., 2020a) and for this reason many studies explore their ligninolytic enzymes, but little is known about their hydrolytic enzymes. The potential of *Pleurotus* hydrolytic enzymes in milk coagulation was demonstrated in the literature (Martim, Silva, Alecrim, Teixeira, & Teixeira, 2021).

In this study, two *Ascomycetes* and two *Basidiomycetes* were selected for protease production and application in coagulation processes, using solid-state fermentation. Therefore, the present study had as an innovative nature of characterizing the proteases produced by solid-state fermentation and the application of crude extracts in milk coagulation.

Material and methods

Fungus

The fungi, previously selected by the cup plate method, were the isolates *Pleurotus pulmonarius* CCB20 (Collection of the Botanical Institute of São Paulo), *Pleurotus albidus* 018 (EMBRAPA Florestas - Colombo - PR), *Aspergillus* sp. 125, *Fusarium* sp. 132 and *Fusarium* sp. 206 (EMBRAPA Brasília - DF). The inoculum were obtained by cultivating them in Petri dishes and tubes inclined at 28°C for 7 days containing pure agar medium and wheat bran agar (Contato et al., 2020b), for *Ascomycetes* and *Basidiomycetes*, respectively.

Obtaining crude extracts

Solid-state fermentation was promoted in Erlenmeyer flasks (125 mL) containing 5 g of wheat bran and Vogel mineral medium (Vogel, 1956) to obtain an initial humidity of 60%. For *Basidiomycetes*, three plugs of 10 mm diameter of mycelium, obtained from fully colonized Petri dishes was used. For *Ascomycetes*, 1.2 mL of a solution of 10^7 spores was used as the inoculum. The flasks were incubated at 28°C for 10 days, then they were interrupted through the addition of 20 mL of distilled water and filtration. The filtrates were considered crude enzymatic extracts.

Determination of protease activity

The protease activity was analyzed according to Sarath, De La Motte, and Wagner (1989) with modifications. As substrate 1% azocasein was used, prepared in 100 mM Tris-HCl buffer, pH 7.0. A sample (0.150 mL) of the extract was mixed with the substrate (0.250 mL). The mixture was incubated in a water bath at 37°C for 20 min. Then, 1.2 mL of 0.6 M trichloroacetic acid (TCA) was added to the reaction tube. After 15 min. at room temperature, the samples were centrifuged at 4,000g for 10 min. Then, 1.2 mL of the supernatant was homogenized with 1.4 mL of NaOH and readings were performed at 440 nm. An unit of protease activity (U) was defined as the amount of enzyme required to change the absorbance by 1.0 in the 1 cm of cuvette.

Determination of amino acids and total proteins

The amino acids were determined using the ninhydrin method (Yemm & Cocking, 1955) and the total proteins were determined by Bradford method (Bradford, 1976).

Enzyme characterization

Optimum pH

The protease activity was determined using a wide pH range. The pHs of the reactions were adjusted using buffers (100 mM): sodium citrate (pH 5.0-6.0), Tris-HCl (pH 7.0-9.0) and glycine (pH 10.0-11.0).

Optimal temperature and thermostability

Optimal temperature was determined using a wide temperature range. The crude extract was incubated at each temperature (20-70°C) according to the standard methodology for measurement of protease activity. For thermostability the enzymatic extract was incubated at different temperatures (20-70°C), for several time intervals (20-60 min.).

Effect of salts

The effect of salts was determined with different salt solutions (CaCl₂, MgCl₂, MnCl₂, CuSO₄, FeSO₄ and ZnSO₄). They were added to the reaction tube at a final concentration of 1 mM, with the enzymatic activity of the crude extract being considered as control without adding any ions.

Effect of inhibitors on enzyme activity

The protease activity was determined after incubation of the enzyme extract with different enzyme inhibitors (1 mM): phenylmethylsulfonyl fluoride (PMSF), ethylenediamine tetraacetic acid (EDTA), pepstatin and iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA). The enzymatic activity of the crude extract without the presence of inhibitor was used as a control.

Polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic profile of the proteins obtained from the fungal cultures was performed by electrophoresis in a non-denaturing polyacrylamide gel (SDS-PAGE), described by Laemmli (1970), using a Mini-PROTEAN system (BIO-RAD). After electrophoresis the gel was stained with silver. The analyses of the hydrolysed proteins of casein and whey were also performed using the same methodology.

Zimogram

After electrophoresis, the gel was washed for 30 min. with 25% isopropanol in Tris-HCl buffer, and again for the same timespan only with the buffer. A gel was then prepared with 0.1 g of the substrate (azocasein), 1 g of agar and 50 mL of distilled water. The two gels were overlapped and incubated at 37°C for 30 min. The clear bands on the azocasein gel showed the presence of the proteases.

Application of crude extract to milk coagulation

The coagulant activity of the crude extracts obtained from the fungus cultures was evaluated according to the methodology described by Dahot, Khan, and Memon (1990) with some modifications. Briefly, 0.5 mL of the crude extract was added to 5 mL of a solution obtained from skimmed-milk powder Molico Nestlé® (10%, w v⁻¹) containing 0.05 M CaCl₂, pre-incubated at 37°C. The mixture was kept at the same temperature until the appearance of curds. The material was centrifuged at 4,000 g for 10 min. Commercial rennet (chymosin) was used to compare coagulation efficiency. The calculation of milk clotting activity was performed using the following equation:

$$CA = \frac{2400}{T} \cdot \frac{MV}{EV}$$

Where CA is the coagulant activity, *T* is the time (seconds) needed for milk to start coagulating after addition. From the crude enzyme extract, the *MV* is the milk volume in mL and the *EV* the extract volume (mL) containing the enzyme. CA was defined as the amount of enzyme that coagulates 1 mL of milk, at 37°C, per unit of time. Boiled crude extract was used as a control.

Hydrolysis of skimmed-milk powder casein

The casein was separated from a solution (10%) of skimmed-milk powder Molico Nestlé® through addition of HCl (2%) dropwise. The methodology was performed under constant agitation until the pH reached 4.6, in which casein precipitates. After settling the precipitate for 15 min., it was centrifuged at 4000 g for 10 min. The casein was frozen and then lyophilized. The lyophilisate was resuspended in 5 mL of Tris-HCl buffer, pH 7.0. The samples were incubated at 37°C for 5 min. and 0.5 mL of the enzyme solution was added. The samples were incubated for 20 min. in which aliquots were removed and boiled. The samples were submitted to the determination of total proteins by the method of Bradford (1976), total amino acids by the method of ninhydrin (Yemm & Cocking, 1955) and electrophoretic analysis (Laemmli, 1976).

Statistical analysis

Cultivations and analyses were performed in triplicate. The data were expressed as mean ± standard deviation. The t test was performed to determine if there was a significant difference between the means at the level of *p* < 0.05 using the GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Production of proteases in solid-state fermentation

The Figure 1 shows the protease activity from the crude extracts of the selected fungi. The biggest activity found was 9.279 ± 3.2 U.mL⁻¹ for *Aspergillus* sp. 125, followed by *P. albidus* 018, which presented an activity of

$5.029 \pm 2.0 \text{ U.mL}^{-1}$. The lowest activity values were obtained by fungi of the genus *Fusarium*, with no significant differences between them ($p > 0.05$). Few are the studies that use this genus for the production of proteases (Yang et al., 2016).

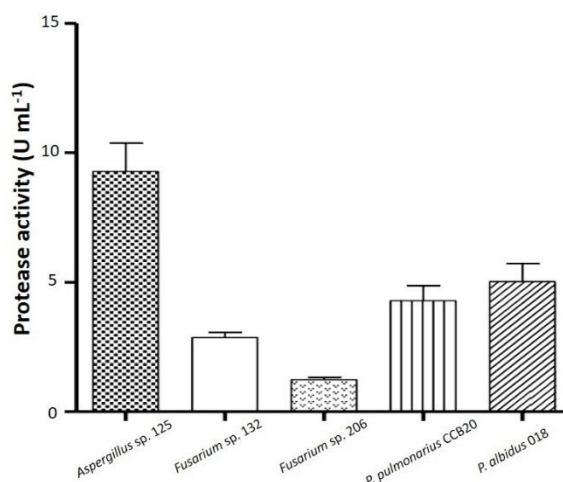


Figure 1. Protease activity (U mL^{-1}) of crude fungi extracts using wheat bran as a substrate.

The enzymatic profiles from extracts of the fungi *P. albidus*, *P. pulmonarius*, *Aspergillus* sp. and *Fusarium* sp. were analysed through electrophoresis and later the protease activity was revealed from a zymogram. Clear bands were observed in the gel corresponding to the proteases, but little was evidenced. The most apparent band was related to a protease from *P. albidus* with an estimated molecular weight of 36 kDa (Figure 2).

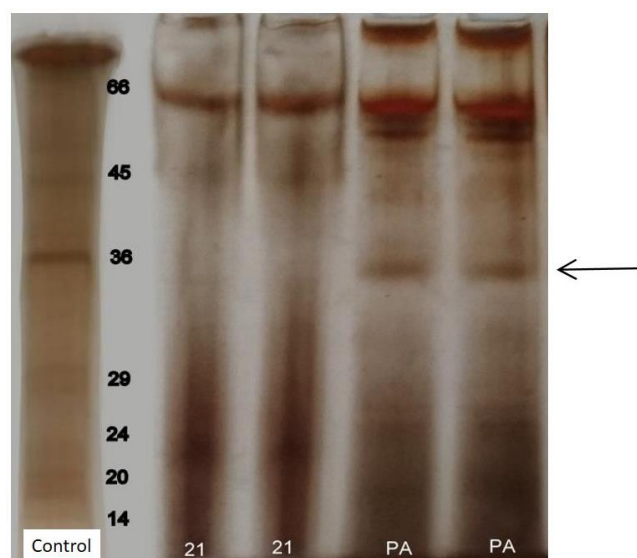


Figure 2. Electrophoresis of the crude extracts of the evaluated isolates: *Aspergillus* sp. (21); *P. albidus* (PA). The proteins present in the standard were: bovine albumin (66kDa), ovoalbumin (46 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α -lactalbumin (14 kDa).

Characterization of the enzymatic crude extract

Optimal pH

As *P. albidus* and *Aspergillus* sp. presented the best results, the study followed up with these two microorganisms. The Figure 3a shows that the optimal pH of the protease activity of the crude extract of *P. albidus* was 7.0, but with relative activity above 60% at pH 5.0 and greater than 80% at pH 6.0. At more alkaline pH values (8.0 and 9.0), a relative activity above 80% is still possible. At pH 10.0, a sharp drop in activity can be observed.

The optimal pH of *Aspergillus* sp. 125 was 8.0 (Figure 3b), with greater relative activity, at pH values higher (9.0-11.0) than its optimal pH, when compared to *P. albidus*. At pH 5.0, the enzymatic activity was greater than 40% for both fungi.

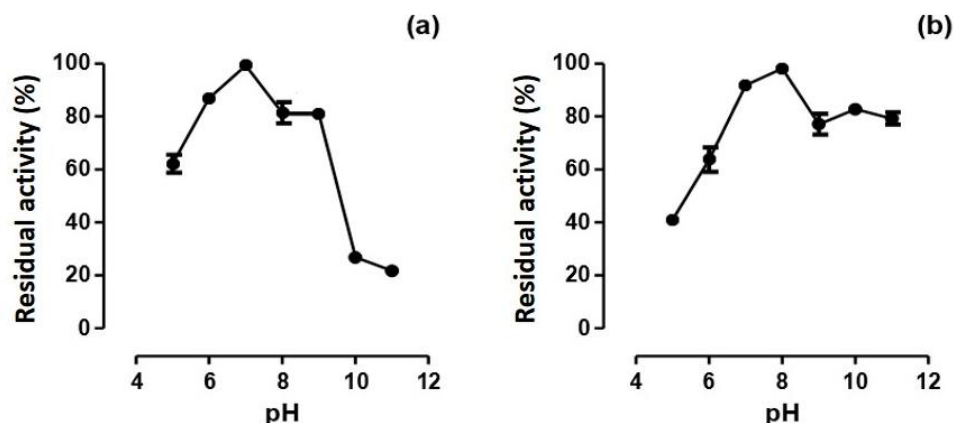


Figure 3. Effect of pH on the protease activity of (a) *P. albidus* and (b) *Aspergillus* sp.

Optimal temperature and thermostability

The optimal temperature found for both the isolates was 50°C (Figure 4). The thermal stability of the proteases from the crude extract of *P. albidus* is shown in Figure 5a. At temperatures of 40 and 50°C the relative activities are maintained at up to 60% for up to 40 min. of reaction, however in 20 min. of reaction at 40°C the activity is reduced by up to 30%. At 70°C, proteases show more than 50% of the activity in the first 10 min. of the reaction, but after 1h of incubation the activity is just over 10%. The average half-life of the crude extract proteases was 40 minutes at 60°C and 20 minutes at the same temperature for *P. albidus* and *Aspergillus* sp. respectively (Figure 5b). Therefore, the thermal stability of the proteases from *Aspergillus* sp. it is quite reduced at higher temperatures although it presents activity above 50% at the end of 1h at 50°C. Thus, we can assume that this enzyme is quite unstable if kept at high temperatures for long periods.

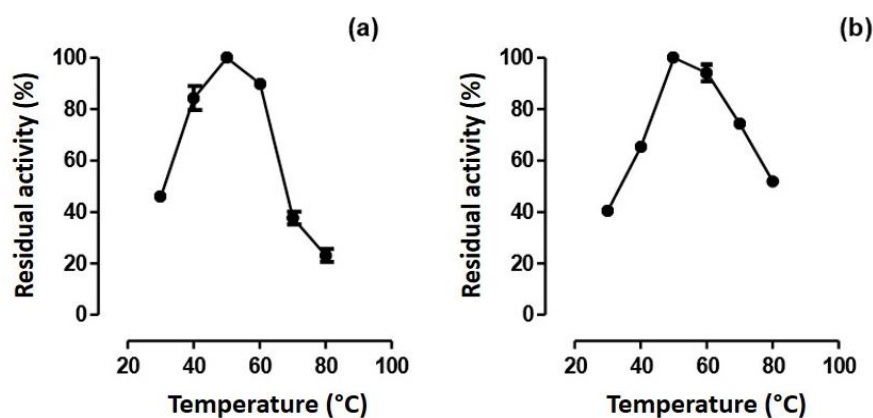


Figure 4. Effect of temperature on protease activity of: (a) *P. albidus* and (b) *Aspergillus* sp.

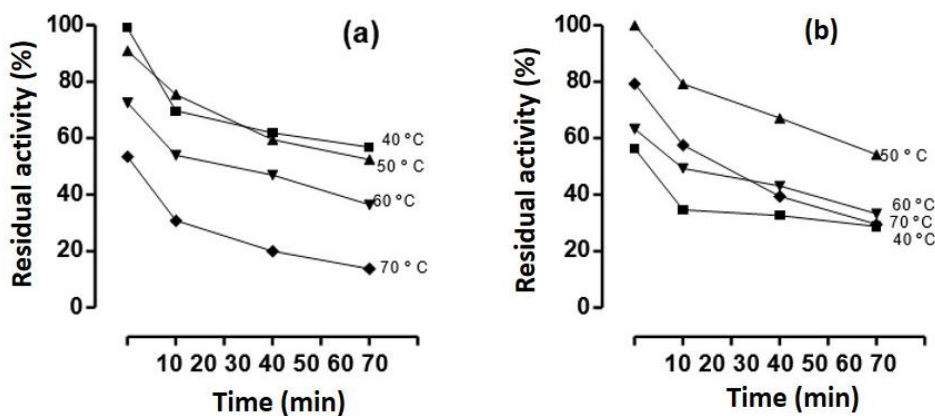


Figure 5. Effect of temperature on the stability of protease activity in (a) *P. albidus* and (b) *Aspergillus* sp.

Effect of salts

The Table 1 shows the effect of salts on the protease activity of the studied fungi. The activity was not affected by CaCl_2 . The salt that most inhibited the enzymatic activity was ZnSO_4 , maintaining activity of 64.3% (*P. albidus*) and 48.9% (*Aspergillus* sp.).

Table 1. Effect of salts on the protease activity of the crude extract.

Salt (1 mM)	Residual activity (%) <i>P. albidus</i>	Salt (1 mM)	Residual activity (%) <i>Aspergillus</i> sp.
Controle	100 ± 0	Controle	100 ± 0
CaCl_2	88.76 ± 1.89	CaCl_2	81.91 ± 0.92
MgCl_2	87.14 ± 0.52	MgCl_2	80.59 ± 1.04
MnCl_2	84.97 ± 0.95	MnCl_2	79.14 ± 2.00
CuSO_4	74.64 ± 5.32	CuSO_4	49.54 ± 2.19
FeSO_4	82.79 ± 1.02	FeSO_4	67.29 ± 1.68
ZnSO_4	64.31 ± 1.39	ZnSO_4	48.91 ± 0.99

CaCl_2 : calcium chloride; MgCl_2 : magnesium chloride; MnCl_2 : manganese chloride; CuSO_4 : copper sulfate; FeSO_4 : iron (II) sulfate; ZnSO_4 : zinc sulfate.

Effect of inhibitors on protease activity

Protease inhibitors can be grouped according to their mode of action or even structural characteristics. They can belong to different classes, being of synthetic or natural origin. Site-specific inhibitors work by modifying an amino acid from the active protease site. Table 2 shows the results of the effect of the following inhibitors: iodoacetamide, cysteine protease inhibitor, pepstatin, which inhibits aspartic or acid proteases, EDTA, a metalloprotease inhibitor and finally, the PMSF which inhibits serine proteases.

Table 2. Effect of inhibitors on the protease activity.

Inhibitors (1 mM)	Type of protease inhibited	<i>P. albidus</i> Residual activity (%)	<i>Aspergillus</i> sp. Residual activity (%)
Control	-----	100 ± 0	100 ± 0
Iodoacetamide	Cysteine protease	69.37 ± 0.26	73.88 ± 0.16
Pepstatine	Aspartic protease	77.92 ± 0.08	73.16 ± 0.96
EDTA	Metalloprotease	15.16 ± 0.08	68.10 ± 1.99
PMSF	Serine proteases	49.50 ± 1.51	55.93 ± 0.37

EDTA: ethylenediamine tetraacetic acid; PMSF: phenylmethylsulfonyl fluoride.

Application of crude extract to milk coagulation

The Table 3 shows the data regarding milk coagulation. A CA value of 240 U mL⁻¹ was found for the *Ascomycete* and 153 U mL⁻¹ for the *Basidiomycete*.

Table 3. Total protein, milk clotting activity (CA)*, specific activity (EA), protein activity (PA) and ratio between coagulation activity and protein activity (R) of the evaluated crude extracts.

Fungi	Crude extract protein (mg mL ⁻¹)	PA (U mL ⁻¹)	EA (U mg ⁻¹)	CA (U mL ⁻¹)	R
<i>Aspergillus</i> sp.	0.75 ± 0.01	13.62 ± 0.13	18.16 ± 0.03	240	17.62 ± 0.17
<i>P. albidus</i>	1.54 ± 0.09	2.33 ± 0.01	151 ± 0.01	153	57.20 ± 0.29
Chymosin	0.15 ± 0.01	3.95 ± 0.02	26.33 ± 0.02	480	121.51 ± 4.01

*CA = (2400 / T) * (MV/EV), T = clotting time in seconds, MV = milk volume, EV = enzyme volume.

Hydrolysis of skimmed-milk powder casein

The Table 4 shows the quantification of proteins and amino acids present in casein after lactic coagulation by the addition of HCl and results obtained after hydrolysis of 100 mg mL⁻¹ of casein suspended in buffer. There was an increase in amino acids as result of the action of the proteases contained in the extracts.

The Figure 6 shows the electrophoretic profile of a casein sample hydrolysed by proteases from the crude extracts of the two selected isolates. The proteases were able to hydrolyse casein by separating it into its main fractions, known as α -casein, β -casein and κ -casein. Although the data have shown that crude extracts obtained from the cultivation of fungi with caseinolytic action have satisfactory coagulation activity (R above 1).

Table 4. Protein and total amino acids in the casein sample after hydrolysis with the crude fungal extract.

Fungi	Protein (mg mL ⁻¹)	Amino acids (ug mL ⁻¹)
<i>Aspergillus sp. 125</i>	126.2 ± 4.06	46.46 ± 1.90
<i>P. albidus</i>	132.0 ± 3.22	64.54 ± 5.07
Control*	115.5 ± 0.06	15.8 ± 1.03

*Casein (100 mg mL⁻¹) obtained from milk clotting by HCl.

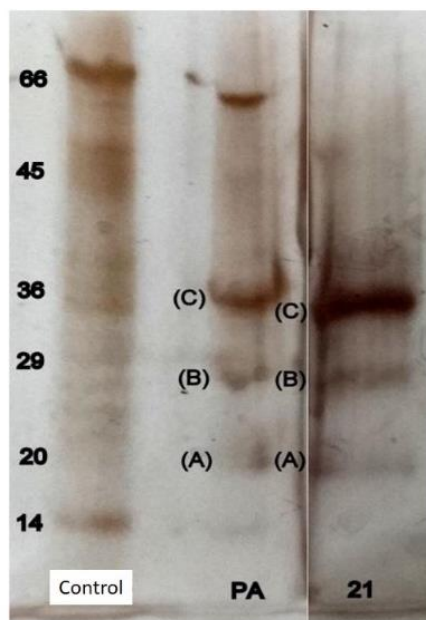


Figure 6. Electrophoresis of the casein sample hydrolyzed by proteases from crude extracts of *Aspergillus sp.* (21) and *P. albidus* (PA). (A): α-casein; (B): β-casein and (C): κ-casein.

Discussion

Characterization of the enzymatic crude extract

Optimal pH

Similar results to optimal pH of *P. albidus* were found by Shaba and Baba (2012) in a study carried out with the crude extract of *Pleurotus ostreatus*.

Alkaline protease-producing fungi are more easily found (Salihi, Asoodeh, & Aliabadian, 2017) but it is also the fungi that produce the majority of acid proteases (Gutiérrez-Méndez et al., 2019). These and neutral proteases are important in cheese, meat, cereal and beverage industry. The wide range of activity of the proteases of the isolates studied, mainly alkaline, makes them promising for the production of proteases for application in the food industry. Both neutral and alkaline proteases can be used in the cheese industry (Leya, Parameswaran, & Pandey, 2016).

Optimal temperature and thermostability

The optimal temperature found for the two isolates was 50°C (Figure 4). Studies with *Aspergillus* of various species show activity of their enzymes, not only of proteases, with an optimal temperature also around 45-50°C (Ma et al., 2016).

Effect of salts

The activity was not affected by CaCl₂. The proteases resistant to this salt are important in the lactic industry where calcium chloride, when used, favors the coagulation of milk and makes the rennet more firm and with better adhesion (Vishwanatha et al., 2010).

Effect of inhibitors on protease activity

The results show that there was a partial inhibition of the *P. albidus* protease when each of the inhibitors was used, with the exception of EDTA. It is possible that because it is a crude extract, a metal-type protease

is present in greater quantity, since the inhibition by EDTA resulted in only 15.2% of the relative activity. Similar results for the *Aspergillus* sp. extract were obtained, but not for inhibition by EDTA. In the presence of this inhibitor the extract still maintained its activity at 55.9%. The samples of the two isolates can contain proteases of different classes. Variation in the pattern of inhibition can also occur, for example, serine proteases that are not strongly inhibited by PMSF (McSweeney, 2004; Kumura, Ishido, & Shimazaki, 2011).

Application of crude extract to milk coagulation

A CA value of 240 U mL⁻¹ was found for the Ascomycete and 153 U mL⁻¹ for the Basidiomycete. However, what determines the potential in the use of cheese making by these enzymes is the calculated ratio between clotting activity and protease activity, so that it should not be less than 1, as well as the proteolytic activity being greater than the coagulant, the cheese could have a bitter taste. Through this calculated index (Table 2) it is possible to perceive that the two studied enzymes have potential for application in the coagulation of milk, although it is noticeable that chymosin has a much higher R than the studied enzymes, since it has specific action. It is also necessary to take into account that studies to optimize the production of proteases are important, especially regarding isolates of Basidiomycetes that are gaining more prominence in the research of their enzymes.

Hydrolysis of skimmed-milk powder casein

The proteolysis that occurs in milk, both in enzyme-induced coagulation and in maturation, degrades proteins into intermediate, short chain peptides and amino acids and in maturation plays an important role since it determines texture. In this context, proteolysis with production of short-chain peptides and amino acids can give to the product an unpleasant taste (bitterness). Hydrophobic peptides, of low molecular weight, formed during the maturation of the cheese are responsible for the bitter taste, but works carried out with Prato cheese allow us to conclude that the intensity of the bitter taste depends on the coagulant and the time of maturation of the cheese (Baptista, Araújo, Eberlin, & Gigante, 2017). Although several enzymes participate in the protease activity in cheeses, the main source of proteases is the residual rennet and its action depends on some factors such as the amount of the coagulant, pH of the cheese and temperature during processing. These factors influence the activity of chymosin (rennet), an enzyme commonly used in cheese making (Fox, McSweeney, Cogan, & Guinee, 2004).

Conclusion

In conclusion, the solid-state fermentation showed efficiency in the production of proteases by the chosen fungi through an initial survey using a simple method of enzyme production evaluation. The enzymes with caseinolytic activity present in the crude extracts showed interesting characteristics regarding their optimal pH and temperature, thermal stability and calculated ratio between the coagulation activity and the protein activity, which shows potential for use in the industrial conditions of dairy products, especially cheeses.

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