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Milk-clotting proteases from *Pleurotus albidus*: an innovative alternative for the production of Minas frescal cheese

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ABSTRACT. *Pleurotus albidus*, a naturally growing species in the Amazon region, has been considered a promising source of milk-clotting proteases. The production of such enzymes using lignocellulosic residues is a sustainable alternative to replace mammalian rennet. The application of *P. albidus* milk-clotting proteases in cheese making has not yet been reported in the scientific literature. The aim of this study was to characterize the milk-clotting proteases of *P. albidus* and use these enzymes in the production of Minas frescal cheese. For the production of coagulating proteases, the mushroom was grown in açai seeds supplemented with rice bran (10%, w/w). The parameters affecting the production of coagulant, such as inoculum size, fermentation time, initial pH of cultivation medium and age of the inoculum were evaluated. The coagulant extract obtained under optimal production conditions was evaluated for optimal pH and temperature, pH and temperature stability, effect of ions and inhibitors. Significant production of coagulating proteases was obtained under the following conditions: inoculum size (2.5%), fermentation time (10 days), initial pH of the cultivation medium (6), and inoculum age (10 days). The coagulant exhibited significant catalytic activity in pH 5.0 at 55°C, with stability at 45°C and was completely inhibited by iodoacetic acid. The milk-clotting proteases of *P. albidus* were efficient for making Minas frescal cheese that presented 55.0% of moisture, 20.0% of lipids and 17.20% of protein. *Pleurotus albidus* is a potential source of milk-clotting proteases that can be applied in dairy industry for production of fresh Minas frescal cheese.

Keyword: Amazon; coagulant; edible mushroom; proteolytic enzymes; solid state fermentation.

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Introduction

Rennet is an enzymatic extract obtained from the stomach of ruminant animals, such as calf, camel and buffalo (Bezie & Regasa, 2019). Chymosin (EC3.4.23.4) is the main protease responsible for the coagulation of milk during cheese making (Hang et al., 2016). Due to biochemical characteristics such as specificity to cleave the peptide bond Phe105-Met106 in the k-casein chain, high coagulant activity, low proteolytic activity, high coagulant ratio (coagulant activity/proteolytic activity) and thermal lability, chymosin is considered the enzyme standard for the manufacture of different types of cheese (Lebedev et al., 2016).

However, due to the increase in cheese production, shortage of animal raw material, high price of curds, religious and food motivations or the ban on the use of recombinant animal rennet, researches have been carried out to find alternative sources of milk-clotting proteases to use in dairy industry (Narwal, Bhushan, Pal, & Malhotra, 2017; Liburdi, Boselli, Giangolini, Amatiste, & Esti, 2019).

Microbial sources are an option between non-animal sources of milk-clotting enzymes. Among the microbial sources, the fungi are considered efficient and renewable due to their biochemical diversity, easy genetic manipulation, and great recovery of bioactive compounds at the end of the process (Ayana, Ibrahim, & Saber, 2015; Daudi, Mukhtar, Rehman, & Haq, 2015). In this context, edible mushrooms are promising coagulant sources with appropriate characteristics to application on cheese production. They have high milk-clotting activity and low proteolytic activity (Shamtsyan, 2016).

Coprinus lagopides (Shamtsyan, Dmitriyeva, Kolesnikov, & Denisova, 2014), *Hericium erinaceum* (Nakamura, Kobayashi, & Tanimoto, 2014) and *Termitomyces clypeatus* (Majumder, Banik, & Khowala, 2015) are species of mushrooms that are commonly used in bioprocesses with low costs and reduced time of

production. Some studies reported the milk-clotting enzymes production by edible mushrooms and their use in cheese manufacturing (Sathya, Pradeep, Angayarkanni, & Palaniswamy, 2009; Alves, Merheb-Dini, Gomes, Silva, & Gigante, 2013; Bensmail, Mechakra, & Fazouane-Naimi, 2015).

The price of the milk-clotting enzymes affects the cheese manufacturing cost. As an alternative, solid-state fermentation process (SSF) promotes the production of milk-clotting protease by fungi in high concentration with low energetic consumption (Benlucvankar, Jebapriya, & Gnanadoss, 2015; Sindhu, Pandey, & Binod, 2015). The SSF provides desirable growth conditions in lignocellulosic residues which reduce the final cost and minimize the environmental impact (Manan & Webb, 2017). Lignocellulosic residues available in the Amazon as rice bran (*Oryza sativa* L.), cupuaçu exocarp (*Theobroma grandiflorum* (Willd. ex Spreng.) Schum.) and açai seeds (*Euterpe oleracea* Mart.) are used to edible mushrooms growth and milk-clotting protease production (Alecim, Palheta, Teixeira, & Oliveira, 2015).

Pleurotus albidus is an edible mushroom that can adapt to different environmental conditions and grow in various lignocellulosic residues. These characteristics encourage the commercial cultivation of this species in many countries such as Argentina, Mexico and Brazil (Kirsch, Macedo, & Teixeira, 2016; Gambato et al., 2018). Studies have already shown that *P. albidus* synthesizes milk-clotting proteases in a liquid medium (Martim et al., 2017). However, the production of coagulant proteases by *P. albidus* grown in lignocellulosic residues and the application of these enzymes for the production of fresh cheeses has not yet been mentioned in the scientific literature.

In Brazil, the consumption and production of Minas frescal cheese has been growing annually. In 2017, the estimated production was 60,000 tons with per capita consumption of 0.33 kg/inhabitant/year (Bellini & Zocal, 2018). This type of cheese has high humidity content and must be eaten fresh (Melo et al., 2017). The process of Minas frescal cheese production is relatively simple and usually uses animal rennet and traditional equipment (Magenis et al., 2014). This work aimed to standardize the conditions of milk-clotting proteases production by *P. albidus*, determine their biochemical characterization and use them to produce Minas frescal cheese.

Material and methods

Materials

The açai seeds were obtained from Walter Rayol Municipal Market, Manaus, Amazon, Brazil; and the rice bran was purchased at a local store in the same city. Azocasein, Iodoacetic acid, Phenylmethylsulfonyl fluorides (PMSF), and Pepstatin were obtained from Sigma-Aldrich, São Paulo, Brazil. All other reagents were of analytical grade.

Mushroom and inoculum preparation

Pleurotus albidus DPUA 1692 was cultivated in Potato dextrose agar (PDA) with 0.5% (w/v) of yeast extract, in Petri dishes. The cultures were maintained at 25°C for 8 days, in the absence of light. Mycelial discs (8 mm in diameter) were removed from the border of the colony and used as inoculum for the production of milk-clotting proteases (Machado, Teixeira, Kirsch, Campelo, & Oliveira, 2016).

Solid-state fermentation

Açai seeds supplemented with rice bran were used for the cultivation and production of milk-clotting proteases by *P. albidus*, according to the document BR 102017014672-3 A2 (Teixeira & Martim, 2017). All the experiments were evaluated in triplicate.

Milk-clotting proteases extraction

The enzymes were extracted with distilled water in 1:5 proportion (substrate:water; w/v) at 30°C, 150 rpm. After 30 minutes, the crude extract was recovered by vacuum filtration using paper filter Whatman number 1 and using membranes of 0.45 µm and 0.22 µm, respectively (Machado et al., 2016).

Standardization of production conditions for milk-clotting proteases

The milk-clotting proteases production by *P. albidus* was evaluated by using four parameters: inoculum size (0.5%, 1.0%, 2.5%, 5.0%, 7.5% and 10%, w/v), fermentation time (6, 8, 10, 12, 14 and 16 days), initial media pH (5, 6, 7, 8, 9 and 10) and inoculum age (4, 7, 10, 13 and 16 days of cultivation).

Proteolytic activity

The proteolytic activity was determined according to Leighton, Doi, Warren, & Kelln (1973). Protease activity was determined in the crude extracts (150 µL) using 1.0% (w/v) azocasein (250 µL) in 0.2 M Tris-HCl buffer, pH 7.2. The reaction, including the standard, was incubated at 25 °C, in the absence of light. After one hour, the reaction was stopped adding 1200 µL of 10% (w/v) trichloride acetic acid. The mixture was centrifuged at 4 °C (8.000 x g / 5 minutes). The supernatant (800 µL) was added to 1400 µL of 1 M NaOH. One unit of proteolytic enzyme was defined as the amount of enzyme that produces a 0.1 increase of absorbance in 1 hour at 440 nm. All samples were prepared in triplicate.

Milk-clotting activity

Milk-clotting activity was determined according to Arima, Yu, & Iwasaki (1970) using 10% (w/v) skimmed milk powder in 0.05 M CaCl₂ as substrate. Briefly, 5 mL of milk solution were distributed in test tubes and pre-incubated in water bath (Gant, model 179, Cambridge, England) at 40°C for 15 minutes. The enzyme extract (0.5 mL) was added to the milk and counting time started. Clot formation was observed while manually rotating the test tube. The time at which the first particles were formed was measured. All samples were prepared in triplicate. A unit of milk-clotting activity (U) was defined as the amount of enzyme required to coagulate 1 mL of substrate in 40 minutes at 40°C. Milk-clotting activity was calculated according to Shata (2005): $U = 2400/T \times S/E$, where T is the necessary time to clot formation, S is the volume of milk (mL) and E is the volume of crude extract used (mL).

Biochemical characterization of milk-clotting proteases

Effect of pH and temperature on activity and stability of milk-clotting enzymes

To assay optimum pH, proteolytic activity was determined at 40 °C at different pH values using the following 0.1 M buffer solutions: sodium acetate (5.0 and 6.0), Tris-HCl (7.0 and 8.0) and Glycine-NaOH (9.0 and 10.0). For the pH stability, the crude extract was dispersed (1:1, v/v) in the following 0.1 M buffer solutions: sodium acetate (5.0 and 6.0), Tris-HCl (7.0 and 8.0) and Glycine-NaOH (9.0 and 10.0) and maintained at 25°C for 24 hours (Martim et al., 2017).

Optimum temperature was determined by incubating the enzyme extract at different temperatures ranging from 30 °C to 80 °C and assaying the activity at the pH determined as optimum. In thermal stability, the extracts were incubated at different temperatures ranging from 30 to 80°C 1 hour. The solution of 10% (w/v) skimmed milk powder in 0.05 M CaCl₂ was used as substrate. All samples were prepared in triplicate. Relative enzyme activities were determined according to the optimal conditions of pH and temperature (Martim et al., 2017).

Effect of protease inhibitors and metal ions on milk-clotting activity

The effect of inhibitors and metal ions on enzyme activity was investigated by using 10 mM of phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), pepstatin A (0.1mM), iodoacetic acid, CaCl₂, CuSO₄, KCl, FeSO₄, MgSO₄, MnSO₄, NaCl and ZnSO₄. Samples were incubated at 37 °C for 60 minutes and residual enzyme activities were determined and compared to the control, which was incubated without the inhibitors and metal ions and corresponds to 100% of activity. All samples were prepared in triplicate (Martim et al., 2017).

Production and Proximate Analysis of Minas frescal cheese

Minas frescal cheese was prepared according to the methodology described by Silva (2005), replacing bovine chymosin for the milk-clotting protease of *P. albidus* (Teixeira & Martim, 2017). The cheese was produced in semi-industrial scale at Dairy Factory Uirapuru, located at Km 24 of road BR 174.

In the analysis of the proximate composition, moisture, ash, crude protein, crude fat and carbohydrates were determined, according to AOAC (2005). Carbohydrates were estimated by difference (100 g - total moisture, ash, proteins and lipids) and total energy value was calculated using the Atwater factor (NEPA, 2006).

Statistical analysis

In the present study, all tests were performed in triplicate. The data obtained in all experiments were subjected to analysis of variance and the means compared by the Tukey test ($p < 0.05$) using the Minitab program, version 18.0 (MINITAB, 2017).

Results and discussion

Effect of different parameters in the milk-clotting proteases production

There are few studies reporting the optimum environmental conditions for the production of milk-clotting enzymes by edible mushrooms. However, it is known that parameters such as temperature, humidity, pH, inoculum size, carbon and nitrogen sources affect the production of enzymes by micro-organisms. For that reason, it is very important to verify the best conditions of maximum production of enzymes with commercial interest and promote the same results in industrial scale (Gais, Fazouane, & Mechakra, 2009; Narwal et al., 2017).

The inoculum size is a biological factor which is associated with biomass and enzymes production in fermentative processes. Inoculums with high concentration favor the biomass production in excess (Ravikumar, Gomathi, Kalaiselvi, & Uma, 2012; Vijayaraghavan, Lazarus, & Vincent, 2014). In this context, the nutrients present in culture media are fast consumed which affect the production of metabolites. On the other hand, the use of inoculums with low concentration can hinder the microbial growth and also the production of proteolytic enzymes (Sher, Nadeem, Syed, Irfan, & Baig, 2012; Bensmail et al., 2015).

In this study, the influence of inoculum size in the production of milk-clotting enzymes by *P. albidus* is demonstrated in Figure 1. The edible mushroom produced milk-clotting enzymes using all inoculum sizes tested. The significant result was observed in the inoculum with size of 2.5% (w/w) resulting in milk-clotting activity of 42.60 U and strong coagulation. The inoculum of 30% promoted milk-clotting enzymes production by *M. circinelloides* cultivated in Indian grain bark (Sathya et al., 2009). The inoculums of 5% and 10% were considered as optimum for proteases production by *R. arrhizus*-M26 and *R. oligosporus*-M30, respectively (Irfan, Rauf, Syed, Nadeem, & Baig, 2011). A mutant species of *Aspergillus flavus* AS2 produced high quantitative of alkaline proteases using an inoculum size of 10% (Rani, Prasad, & Sambasivarao, 2012).

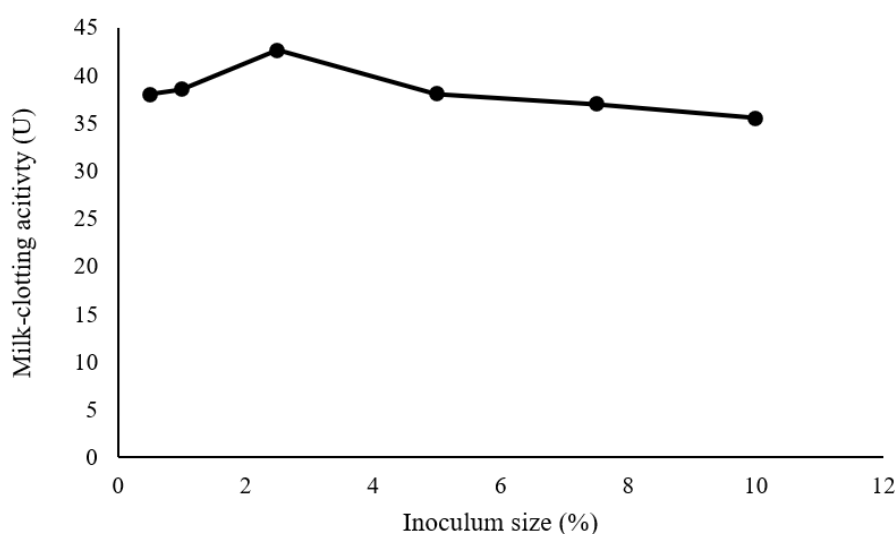


Figure 1. Effect of inoculum size on the milk-clotting activity of *Pleurotus albidus*.

Pleurotus albidus produced milk-clotting proteases in all fermentation times (Figure 2). The highest production was observed at the tenth day of fermentation (35.10 U) and the lowest production at the sixth day (19.00 U). *Pleurotus sajor-caju* cultivated in corn flour produced milk-clotting proteases at the fourth day of cultivation (Ravikumar et al., 2012) and *A. oryzae* NCIM 1032 at the fifth day of fermentation in a mixture of rice bran and wheat bran (Patil, Kulkarni, & Kininge, 2012). The micro-organism and cultivation conditions used in the bioprocess can promote peptidases production in a time ranging from 1 day to weeks (Sharma, Kumar, Panwar, & Kumar, 2017). The decrease of milk-clotting activity by fungi enzymes after the optimum fermentation time would be associated with the reduction of nutritional sources in the culture media. The production of toxic compounds can influence on the enzyme production and on the fungi growth (Bensmail et al., 2015).

The pH of culture media is another parameter that influences in the production of proteases by different fungi species. In the evaluated conditions, *P. albidus* produced milk-clotting enzymes with the highest activity

at pH 6.0 (49 U) (Figure 3). *Rhizopus stolonifer* also produced enzymes at this pH range when cultivated in solid matrix (Gais et al., 2009). Bano, Dahot, and Naqvi (2016) showed that *P. eryngii* produced a high amount of proteases at pH 6.5. Santhi (2014) reported that *A. niger* MTCC 281 produced a significant amount of protease when grown in *Punica granatum* residue, pH 5.0. Bensmail et al. (2015) cited that variations of pH during the bioprocess have interfered in the growth of microorganisms and also in the enzyme production. This occurs due to the modification in the catalytic process and in the transport of nutrients through cell membrane.

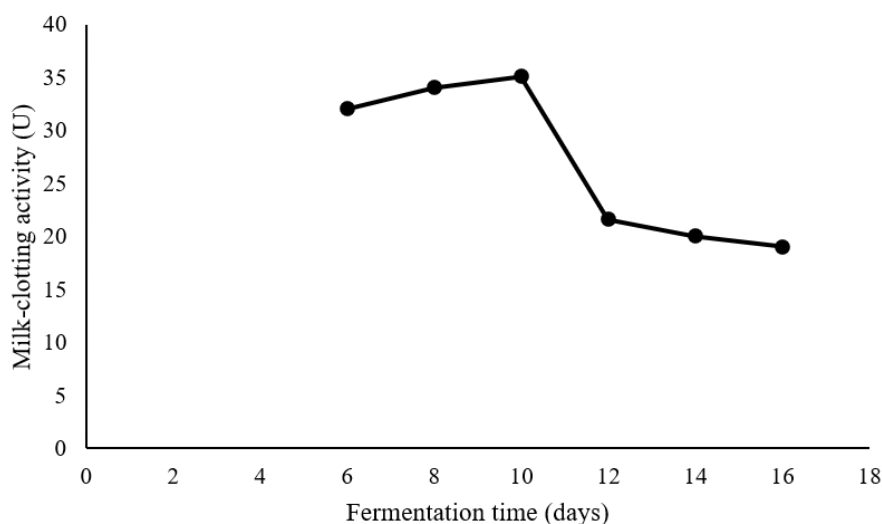


Figure 2. Effect of fermentation time on the milk-clotting activity of *Pleurotus albidus*.

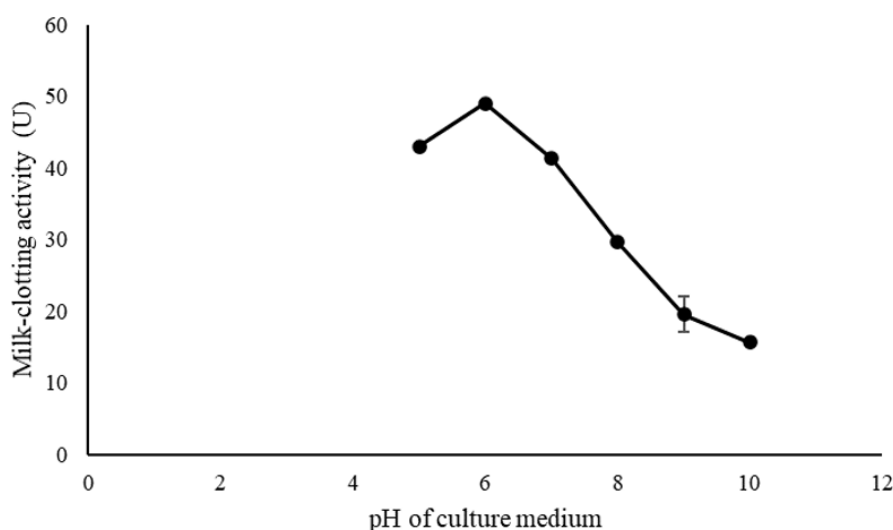


Figure 3. Effect of pH of culture medium on the milk-clotting activity of *Pleurotus albidus*.

The inoculum age is a biological factor that affects the production of proteases by filamentous fungi. In this research, the highest milk-clotting enzymes production by *P. albidus* was observed in the 10-day-age inoculum (74.0 U) (Figure 4). *Rhizopus arrhizus* M-26 produced enzymes at 8-day-age inoculum (Irfan et al., 2011) and *A. flavus* AS2 and *R. oligosporus* NCIM 1215 produced enzymes at 4-day-age inoculum (Prasad & Raju, 2013).

The effect of pH on the activity and stability of the milk-clotting activity of proteases from *P. albidus* is shown in Figure 5. The pH-optimum of activity of the milk-clotting enzymes produced by *P. albidus* was at 5.0. The activity decreased according to the increasing of pH ranges. At pH 9.0 the enzymes maintained 39% of their activity and at pH 10.0 the enzymes were completely inhibited. Lebedeva & Proskuryakov (2009), Yegin, Goksungur, & Fernandez-Lahore (2012) and Majumder et al. (2015) reported that the milk-clotting enzymes from *P. ostreatus*, *M. mucedo* DSM 809 and *T. clypeatus* MTCC 5091, respectively, also were pH-optimum at 5.0.

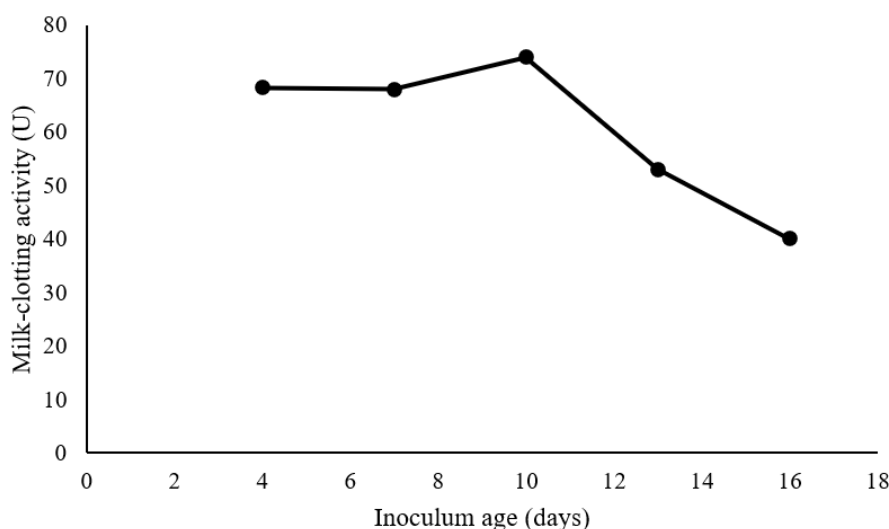


Figure 4. Effect of inoculum age on the milk-clotting activity of *Pleurotus albidus*.

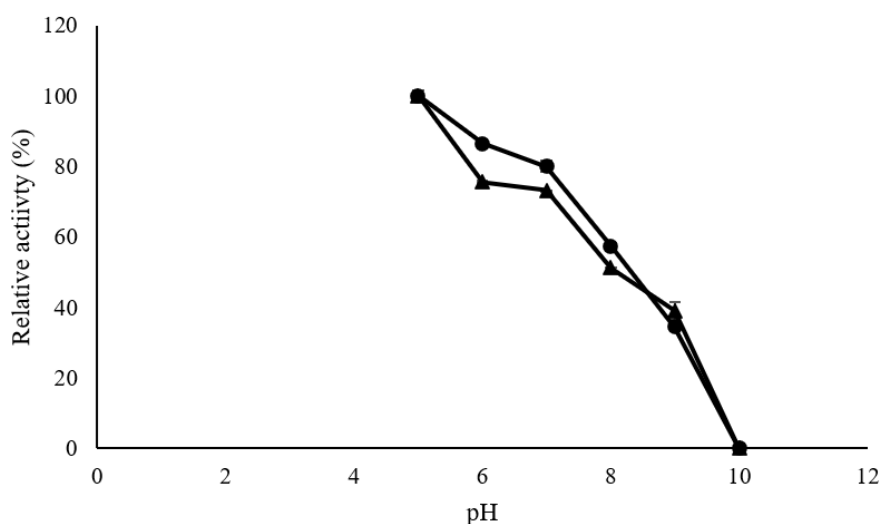


Figure 5. Effect of pH on the activity (▲) and stability (■) of the milk-clotting activity of proteases from *Pleurotus albidus*.

Biochemical characterization of milk-clotting proteases from *Pleurotus albidus*

According to the studied parameters, the enzymes from *P. albidus* were 100% of activity at pH 5.0. The enzymes maintained more than 50% of activity at pH 6.0, 7.0 and 8.0. At pH 10.0 the milk-clotting activity was not observed. Proteases from *R. microsporus* var. *rhizopodiformis* maintained enzymatic activity at pH 2.0–8.0 (Sun, Wang, Yan, Chen, & Jiang, 2014) and *A. flavo furcatis* enzymes maintained 76–88% of stability between pH 4.0 and 6.0 (Alecim et al., 2015).

According to Oueslati & Mounirhaouala (2014) and Ahmed, Wehaidy, Ibrahim, El Ghani, & El-Hofi (2016) it is known that pH modifications can change the electric charge of amino acids in the enzymatic active site or change the secondary and tertiary structures of enzymes. These modifications affect the link of substrate and enzyme which result in loss of catalytic activity.

Figure 6 shows the effect of temperature on the activity and stability of the milk-clotting activity of proteases from *P. albidus*. The milk-clotting activity was affected according to the temperature increase. At 50°C, the enzymes maintained 62.76% of its activity. The highest activity was observed at 55°C (222 U) and at 60°C, the enzymes were completely inhibited (Figure 6). The enzymes from *T. indicae-seudaticae* N31 showed activity until 70°C (Merheb-Dini, Gomes, Boscolo, & Silva, 2010). *Coprinus lagopides* showed optimum activity in the range of 34 to 37°C (Shamtsyan et al., 2014), *P. soloniensis* at 35 to 40°C (El-Baky, Linke, Nimtz, & Berger, 2011) and *T. clypeatus* MTCC 5091 at 45°C (Majumder et al., 2015).

Under the conditions analyzed, the milk-clotting proteases from *P. albidus* were stable in the range from 30 to 45°C with activity higher than 85%. After 1 hour of incubation at 50 and 55°C, the enzymes from *P.*

albidus lost 67 and 81% of activity, respectively. At 60°C the enzymes were completely inhibited. Majumder et al. (2015) reported that the milk-clotting enzymes from *T. clypeatus* MTCC 5091 were stable in the range from 35 to 50°C. In the research of Sun et al. (2014) and El-Baky et al. (2011) the proteases from *R. microsporus* var. *rhizopodiformis* and *P. soloniensis* maintained stability until 40°C for 30 minutes and were inactivated at 55 and 60°C, respectively.

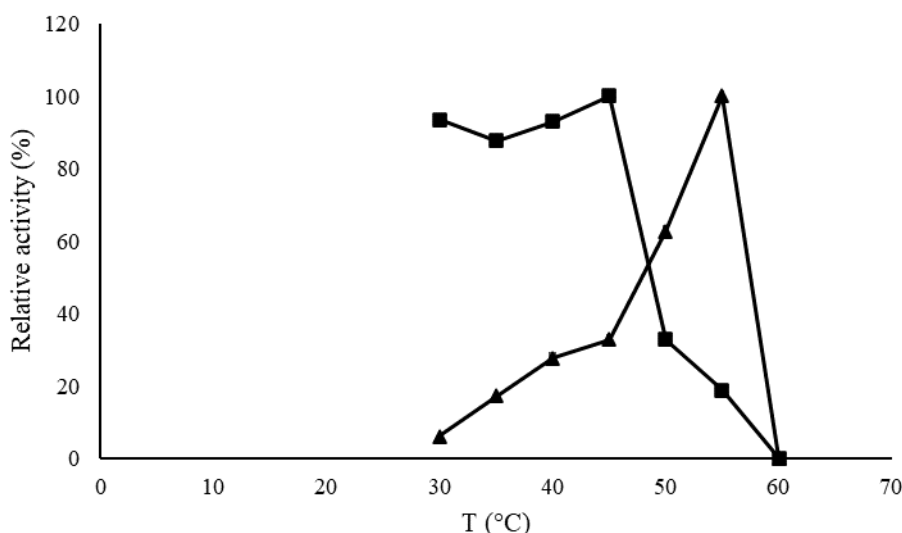


Figure 6. Effect of temperature on the activity (▲) and stability (■) of the milk-clotting activity of proteases from *Pleurotus albidus*.

The effect of metallic ions and inhibitors in the milk-clotting activity of enzymes from *P. albidus* are demonstrated in Table 1. The ions Zn^{2+} e K^+ increased the milk-clotting activity in 42 and 5%, respectively. Fe^{2+} e Mg^{2+} promoted the decreasing of activity in 32 and 11%, respectively. Ca^{2+} and Mn^{2+} did not present any effect in the enzymes activity. The enzymes from *Amylomyces rouxii* were also stimulated in the presence of Zn^{2+} and K^+ (Yu & Chou, 2005). The ion Zn^{2+} stimulated in 12% the milk-clotting activity of enzymes from *R. microsporus* var. *rhizopodiformis* (Sun et al., 2014). According to Merheb-Dini et al. (2010), the ions can connect amino acids residues and change the conformation of the protein. This can result in modifications of protease catalytic activity.

The proteases can be classified in groups based on its activity sites or the need for metallic ions: serine proteases, aspartic acid proteases, cysteine proteases, metalloproteases, threonine proteases and glutamic acid proteases (Li, Yi, Marek, & Iverson, 2013; Hsiao et al. 2014). Proteases inhibitors are used to check which group is present at the enzyme activity site. Iodoacetic acid was the substance tested between the proteases inhibitors able to inactivate the milk-clotting enzyme. This inhibition activity suggests that the protease activity site produced by *P. albidus* is a cysteine protease. When tested with EDTA, pepstatin A and PMSF the inhibition was respectively 13%, 38% and 20%.

Table 1. The effect of metallic ions and inhibitors on the milk-clotting activity of proteases from *Pleurotus albidus*.

Chemicals	Concentration (mM)	Relative activity (%)
Control	—	100 ± 0.01
Ca^{2+}	10	100 ± 0.03
Cu^{2+}	10	0.00 ± 0.00
Fe^{2+}	10	68 ± 0.01
K^+	10	105 ± 0.02
Mg^{2+}	10	89 ± 0.01
Mn^{2+}	10	100 ± 0.02
Na^+	10	95 ± 0.00
Zn^{2+}	10	142 ± 0.0
EDTA	10	87 ± 0.60
Iodoacetic acid	10	0.00 ± 0.00
PMSF	10	80 ± 0.90
Pepstatin A	1	62 ± 0.30

EDTA = Ethylenediaminetetraacetic acid; PMSF = Phenylmethylsulfonyl fluoride.

According to different studies, milk-clotting enzymes synthesized by mushroom can be different at their catalytic group formation. Knowing that, coagulant peptidases produced by *P. soloniensis* were inhibited by pepstain A and were classified as aspartic acid proteases (El-Balky et al., 2011). Coagulant peptidases produced by *T. clypeatus* reduced 11.9% of its activity by EDTA and were classified as metalloproteases by Majunder et al. (2015). Lebedeva & Proskuryakov (2009) cited the production of coagulant proteases by *P. ostreatus* and the classification of it as metalloproteases and serine proteases.

Proximate analysis of minas frescal cheese

The proximate analysis of cheeses made with commercial rennet and with the proteases of *P. albidus* are shown in Table 2. In the analyzed conditions, a statistical difference was verified in the contents of ash, carbohydrates and energy value. The cheese made with *P. albidus* coagulant showed 55% moisture, being classified as very high moisture. This result is in accordance with values established by the Brazilian legislation (Brasil, 2004). The values of the other centesimal fractions were, in decreasing order: lipids (20.0%), proteins (17.20%), ash (4.0%) and carbohydrates (3.80%), in addition to the energy value of 264 kcal 100g⁻¹.

Ricardo, Katsuda, Maia, Abrantes & Oshiro (2011) reported moisture contents of 68.96% to 34.66% in Minas frescal cheese marketed in the city of Londrina. Magenis et al. (2014) reported values of humidity (55.05%), lipids (22.27%) and proteins (16.79%) for commercial samples of Minas frescal cheese. Piazzon-Gomes, Prudêncio, & Silva (2010) found that Minas frescal cheeses made with commercial coagulant had the following proximate composition: moisture (59.57%), proteins (14.98%), lipids (18.10%), ashes (2.79%) and carbohydrates (4.57%). Oliveira, Silva, & Pascoal (2014) reported that the average energy value of Minas frescal cheese is 218.4 (Kcal 100g⁻¹), which is lower than that reported for the same product made with milk-clotting proteases from *P. albidus*.

Table 2. Proximate composition of Minas cheese type fresh produced with commercial rennet and with milk-clotting proteases from *Pleurotus albidus*.

Parameters	Minas frescal cheese made with commercial rennet (g 100 g ⁻¹) ± SD	Minas frescal cheese made with milk-clotting proteases from <i>P. albidus</i> (g 100 g ⁻¹) ± SD
Moisture (%)	55.00 ± 0.5 ^a	55.00 ± 0.2 ^a
Crude fat (%)	20.00 ± 0.8 ^a	20.00 ± 1.3 ^a
Crude protein	18.05 ± 0.9 ^a	17.20 ± 0.20 ^a
Ash (%)	4.20 ± 0.02 ^a	4.00 ± 0.05 ^b
Carbohydrates (%)	2.75 ± 0.08 ^b	3.80 ± 0.00 ^a
Total energy (Kcal 100 g ⁻¹)	263.20 ± 0.05 ^b	264.00 ± 0.08 ^a

Same letters on the same line do not differ statistically by Tukey's test ($p < 0.05$). SD = Standard deviation.

According to Brigido et al. (2004) the variations observed in the proximate composition of Minas frescal cheeses occur due to differences in industrial processing related to the non-standardization of coagulant, the use of different temperatures of milk coagulation and the use or not of pressing in the coagulated mass.

Conclusion

Pleurotus albidus synthesizes milk-clotting proteases appropriate for the production of Minas frescal cheese. The parameters of best enzyme production are: 10-day-old inoculum, initial medium pH 6.0, inoculum size 2.5%, during 10 days of fermentation. These proteases express optimal activity at pH 5.0, temperature of 55°C, stability up to 45°C and classified as cysteine proteases. *Pleurotus albidus* is an innovative source of milk clotting proteases that can replace commercial curds in the production of Minas frescal cheese.

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