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Chemistry

Production and partial characterization of proteases from *Pleurotus ostreatus* (Jacq.) P. Kumm. in submerged cultivation

Produção e caracterização parcial de proteases de *Pleurotus ostreatus* (Jacq.) P. Kumm. sob cultivo submerso

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ABSTRACT

Proteases, including those obtained from edible mushrooms, are very important in the world market because they are used in numerous industrial processes. The objective of this research was to investigate the production of mycelial biomass and protease activity using *Pleurotus* species in submerged culture, and their characterization and application as a milk coagulating agent. Of the six strains evaluated, *Pleurotus ostreatus* CMINPA 544 expressed a higher production of mycelial biomass and proteolytic activity in 15 days of culture. Lactose was the carbon source that favored the higher biomass production; however, proteolytic activity was favored in the presence of glucose. Gelatin and malt extract promoted mycelial biomass production, 8.28 g/L and 7.85 g/L, respectively, while the base medium expressed higher enzymatic activity. The proteases from *P. ostreatus* CMINPA 544 showed optimal activity at pH 6 at 60 °C and exhibited high stability in the range of 25 to 60 °C and at pH 7 to 9 and a weak coagulation of the milk.

Keywords: Mushrooms; Proteolytic activity; Milk coagulation

RESUMO

Proteases, incluindo aquelas obtidas de cogumelos comestíveis, são muito importantes no mundo porque são utilizadas em inúmeros processos industriais. O objetivo desta pesquisa foi investigar a produção de biomassa micelial e atividade de proteases utilizando espécies de *Pleurotus* em cultivo submerso, e sua caracterização e aplicação como agente coagulante do leite. Das seis linhagens

avaliadas, *Pleurotus ostreatus* CMINPA 544 expressou maior produção de biomassa micelial e atividade proteolítica em 15 dias de cultivo. Lactose foi a fonte de carbono que favoreceu a maior produção de biomassa; entretanto, a atividade proteolítica foi favorecida na presença de glicose. Gelatina e extrato de malte promoveram produção de biomassa micelial, 8,28 g/L e 7,85 g/L, respectivamente, enquanto no meio basal expressou maior atividade enzimática. As proteases de *P. ostreatus* CMINPA 544 apresentaram atividade ótima em pH 6 a 60 ºC e exibiram alta estabilidade na faixa de 25 a 60 ºC em pH 7 a 9 e fraca coagulação do leite.

Palavras-chave: Cogumelos; Atividade proteolítica; Coagulação do leite

1 INTRODUCTION

Edible mushrooms are highly appreciated in gastronomy due to their pleasant taste, high content of proteins, vitamins and minerals and low levels of fats and carbohydrates. In addition to their nutritional value, these macrofungi produce metabolites such as lectins, alkaloids, terpenoids, phenolic compounds and polysaccharides that give them therapeutic bioactivities with antiviral, antioxidant, anti-inflammatory, hepatoprotective, immunomodulatory, antioxidant, anticancer, antimicrobial, and other important effects for medicine and the pharmaceutical industry (Hamza et al., 2024).

These organisms have also gained notoriety for their ability to synthesize other molecules with known physiological and biotechnological properties, such as proteases, which break the peptide bonds present in proteins, transforming them into smaller units of peptides and amino acids (Chandrawanshi et al., 2022). Among the species of edible mushrooms, some species of the genus *Pleurotus* have already been investigated in relation to the production of proteases, as is the case of *P. albidus* (Martim et al., 2017), *P. citrinopileatus* (Cui et al., 2007), *P. eryngii* (Cha et al., 2010), *P. ostreatoroseus* (Machado et al., 2017), *P. sajor-caju* (Benmrad et al., 2019), *P. ostreatus* (Hu et al., 2023), and *P. pulmonarius* (Contato et al., 2022).

Proteases belong to class 3 of the hydrolases, subclass 4 (peptide hydrolases) and are grouped according to their catalytic action on endopeptidases and exopeptidases. They are also subclassified as to the chemical nature of the catalytic

site in serine, aspartic, cysteine and metalloproteases and as to the optimal pH range of action into alkaline, neutral or acidic proteases (Chandrawanshi et al., 2022). Due to their versatility and use in various industrial processes, proteases represent about 65% of the global enzyme market (Song et al., 2023), with applications in leather tanning, bioremediation, the textile industry and drug production (Razzaq et al., 2019; Chandrawanshi et al., 2022). Alkaline proteases are widely used in the detergent industry for the purpose of removing stains and dirt; they are efficient in food production processes such as meat tenderizing, baking, preparation of protein hydrolysates, and in cheese making, they are fundamental in milk coagulation due to their destabilization of casein micelles (Song et al., 2023).

These enzymes are obtained via semi-solid or submerged culture. Although semi-solid cultivation has a low production cost and a low risk of contamination due to the reduced moisture content and the simplicity in cultivation, it can limit the determination of parameters such as aeration, temperature, pH, low homogeneity of the medium and moisture (Aguilar & Sato, 2018). Meanwhile, submerged cultivation is extensively exploited in the enzyme market and accounts for about 90% of production in the industry. Some of the advantages include the homogeneous distribution of nutrients in the broth, which favors absorption by the fungus, control of physicochemical parameters and ease in extracellular recovery of the enzymes (Gimenes et al., 2019).

Research involving submerged cultivation has already been carried out for the species *P. sajor-caju* (Benmrad et al., 2019), *P. ostreatus* (Sakovich et al., 2019) and *P. ostreatoroseus* (Barbosa et al., 2020). In the present study, different nutritional sources were evaluated in submerged culture for the production of biomass and proteases from *Pleurotus* spp., the biochemical characteristics and efficiency in enzymatic coagulation of milk.

2 MATERIAL AND METHODS

2.1 Macrofungi

P. ostreatus CMINPA 474, *P. ostreatus* CMINPA 542, *P. ostreatus* CMINPA 544 and *Pleurotus* sp. CMINPA 589 were obtained from the Collection of Microorganisms of Agrosilvicultural Interest of the Instituto Nacional de Pesquisas da Amazônia (INPA) and two commercial strains were obtained from Funghi and Flora: *P. djamor* FF0137, *P. ostreatus* FF0152. The macrofungi were reactivated on potato dextrose agar (PDA) with 0.5% yeast extract (YE) and kept at 25 ºC in the absence of light for up to 10 days (Kirsch et al., 2011).

2.2 Submerged cultivation

After 10 days of growth, three mycelial discs (Ø= 1 cm) of *Pleurotus* spp. were inoculated in 50 mL of base medium (20 g/L glucose, 5 g/L peptone, 0.5 g/L KH_2PO_4 and 0.5 g/L $MgSO_4$, pH 6.5) for 15 days at 25 °C, 150 rpm, in the absence of light.

The selected *Pleurotus* strain was grown in different carbon and nitrogen sources, replacing glucose and peptone in the base medium. The sources that favored better biomass production were tested at different concentrations, under the same growing conditions previously mentioned.

2.3 Estimation of mycelial growth

The mycelial biomass obtained was separated via vacuum filtration on filter paper of known weight, washed with sterilized distilled water and dehydrated at 50 °C in a heating chamber until reaching constant weight (Kirsch et al., 2016). The fermented broth was kept at 4 ºC for quantification of the proteases.

2.4 Protease activity

Protease activity was determined using 250 µL of azocasein [Sigma Chemical (St. Louis, MO, USA)] at 1% (w/v) in Tris-HCl buffer (0.2 M, pH 7.2) and 150 µL of the

enzyme broth (Leighton et al., 1973), incubated for 1 hour in the absence of light at 25 °C. The reaction was stopped with trichloroacetic acid (TCA) 10% (w/v), the samples were centrifuged, and the supernatant was added to 1 M NaOH. Absorbance readings were performed using a microplate reader (SpectraMax® Plus384, San Jose, CA, USA) at 440 nm. One unit of the proteolytic enzyme was defined as the amount of enzyme needed to produce a variation in absorbance equal to 0.01 in 1 hour. All experiments were performed in triplicate.

2.5 Effect of pH and temperature on protease activity

The effect of pH on protease activity was evaluated in 0.2 M citrate-phosphate buffer (pH 6.0), 0.2 M Tris-HCl (pH 7.0-8.0) and 0.2 M glycine-NaOH (pH 9.0-10.0) and the effect of temperature by incubating the enzyme broth at the optimal pH at the following temperatures: 25, 30, 40, 50, 60, and 70 ° C (Kirsch et al., 2013). The reaction took place for 1 hour and the protease activity was determined as previously described.

2.6 Effect of pH and temperature on protease stability

The effect of pH on protease stability was determined every 30 minutes for 90 minutes at 25 ºC in 0.2 M citrate-phosphate (pH 6.0), 0.2 M Tris-HCl (pH 7.0-8.0) and 0.2 M glycine-NaOH (pH 9.0-10.0) buffers. Thermal stability was evaluated for 90 minutes at different temperatures (25, 30, 40, 50, 60 and 70 ºC) in 0.2 M Tris-HCl buffer (pH 7.2). Enzyme activity was determined every 30 minutes (Kirsch et al., 2013).

2.7 Protease activity in milk coagulation

The coagulant activity of milk was evaluated according to Arima et al. (1970) in a solution of 10% (w/v) skimmed milk in 0.05 M $CaCl_2$. Aliquots of the solution were incubated at 40 ºC in a microprocessor-controlled water bath (Quimis Q214-1, Diadema, SP, Brazil) for 15 minutes and the enzymatic broth was added to the substrate for observation of coagulation by manual rotation of the tubes for up to 40 minutes.

Coagulant activity was calculated according to Shata (2005): U = 2,400/T x S/E, where T is the time required for clot formation, S is the volume of milk (mL) and E is the volume of enzyme broth used (mL). The coagulant ratio was calculated through the relationship between milk coagulant activity and protease activity. The clot was classified as based on clot formation and the form of whey separation observed in the test tubes as: strong clotting (distinct clot and abundant whey) or weak clotting (clotting without visual whey separation) (Alecrim et al., 2015). Commercial rennin was used as a positive control. The assay was performed in triplicate.

2.8 Statistical analyses

The results were submitted to a test of variance (ANOVA) and the means compared using the Tukey test ($p \leq 0.05$) in the Minitab software, version 17.1.0. (Minitab, 2013).

3 RESULTS AND DISCUSSION

3.1 Selection of mycelial biomass and protease producing macrofungi

Mycelial growth was observed in all the macrofungi, principally *P. ostreatus* CMINPA 544, on the 15th day of cultivation (10.37 g/L). All the fungi showed an increase in production of mycelial biomass between the 10th and 15th day of cultivation (Table 1), with the exception of *P. djamor* FF0137, possibly because it had already reached the phase of decline or cell death.

Previous research has evaluated the influence of cultivation time on mycelial biomass production by macrofungal species in liquid media and observed that the highest yield was 11.2 g/L for *P. ostreatus* on the 15th day of cultivation (Özdal et al., 2019), which is similar to what was found in this work. However, it was reported that *P. ostreatus* and *P. eryngii* had the highest biomass production, 16.75 g/L and 11.58 g/L, respectively, on the 26th day of cultivation (Argyropoulos et al., 2022).

The highest proteases values were obtained by *P. ostreatus* CMINPA 544 in all the periods evaluated, reaching 23.22 U/mL on the 15th day, but without statistical difference between it and *P. djamor* FF0137 on the 10th day. *P. ostreatus* PLO showed better protease activity after 5 days of culture (Genier et al., 2015) and *Lentinus villosus* AM 169 after 12 days of cultivation (Braga et al., 2020). *P. ostreatus* CMINPA 544 expressed higher production values for mycelial biomass and protease and was selected for the following experiments.

Table 1 – Production of mycelial biomass (g/L) and protease activity (U/mL) by *Pleurotus* spp. in different cultivation times

Pleurotus	**Mycelial Biomass (g/L)**			**Protease activity (U/mL)**		
	Growing Days			**Growing Days**		
spp.	**5**	**10**	**15**	**5**	**10**	**15**
P. djamor FF0137	1.42±0.16^{cD}	3.69±0.07^{aD}	2.30±0.10^{bE}	8.55±0.50^{bBC}	15.67±3.00^{aA}	6.11±2.12^{bC}
P. ostreatus FF0152	1.20±0.09^{bD}	1.63±0.39^{abE}	2.10±0.07^{aE}	9.00±0.69^{aB}	8.33±0.81^{aB}	6.94±2.72^{aC}
P. ostreatus CMINPA 474	3.36±0.33^{cC}	4.74±0.19^{bC}	7.12±0.23^{aD}	4.55±0.77^{bE}	6.00±0.42^{aB}	6.00±0.84^{aC}
P. ostreatus CMINPA 542	2.88±0.04^{cC}	7.12±0.18^{bB}	9.24±0.40^{aB}	7.33±0.42^{bD}	6.80±0.88^{bB}	14.44±1.16^{aB}
P. ostreatus CMINPA 544	5.75±0.31^{cA}	8.67±0.59^{bA}	10.37±0.52^{aA}	15.22±0.27^{cA}	17.55±1.08^{bA}	23.22±1.42^{aA}
Pleurotus sp. CMINPA 589	4.23±0.42^{bB}	7.87±0.09^{aAB}	8.19±0.42^{aC}	7.66±0.36^{bCD}	7.77±0.54^{bB}	17.33±1.19^{aB}

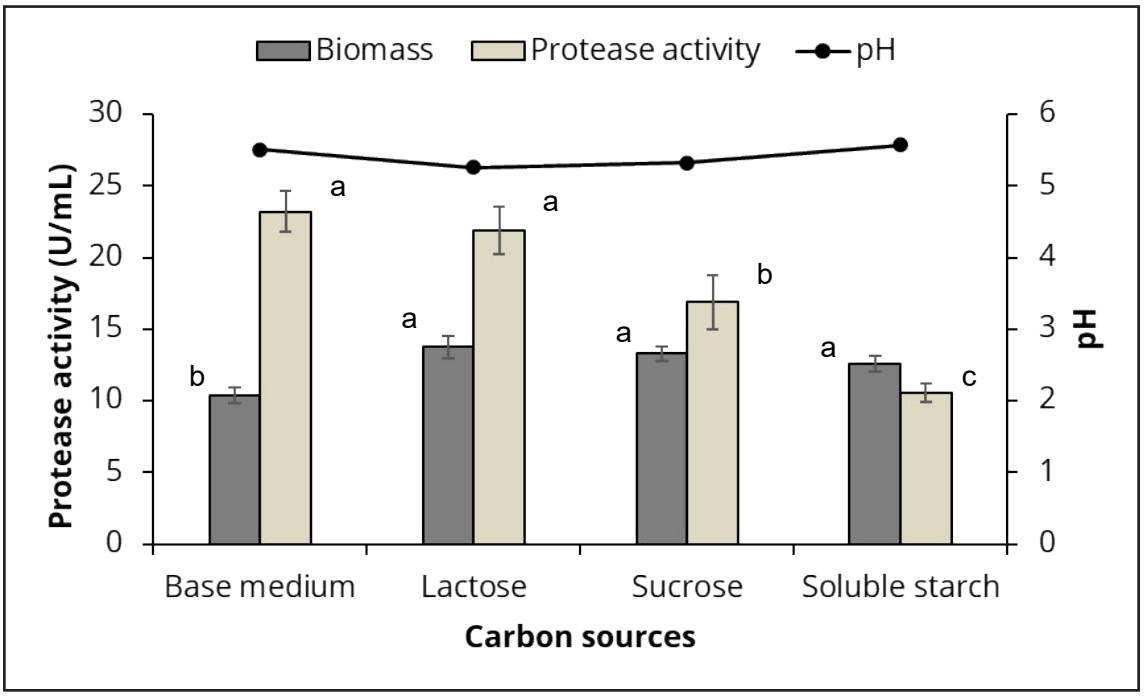
*Means followed by equal letters, lowercase in the rows and uppercase in the columns, do not differ from each other according to the Tukey test (p<0.05).
Source: Authors (2024)

3.2 Effect of carbon and nitrogen sources on mycelial biomass production and protease activity

All the carbon sources promoted mycelial growth of *P. ostreatus* CMINPA 544, but without significant difference between them, with means of 13.74 g/L,

13.30 g/L and 12.56 g/L for lactose, sucrose and soluble starch, respectively; thus, higher than the base medium (Figure 1). Although it has already been reported that monosaccharides are preferable in the production of mycelial biomass by *Pleurotus* species (Hassan & Medany, 2012; Barakat & Sadik, 2014), as they are sugars that are easily metabolized by macrofungi. There are reports of the use of disaccharides and polysaccharides, such as lactose, sucrose and starch, which promote better mycelial growth for *P. albidus* (Kirsch et al., 2016), *P. ostreatus* (Barakat & Sakid, 2014) and species of *Cordyceps* (Park et al., 2001; Xiao et al., 2006).

Figure 1 – Influence of carbon sources on production of mycelial biomass (g/L) and protease activity (U/mL) by *P. ostreatus* CMINPA 544



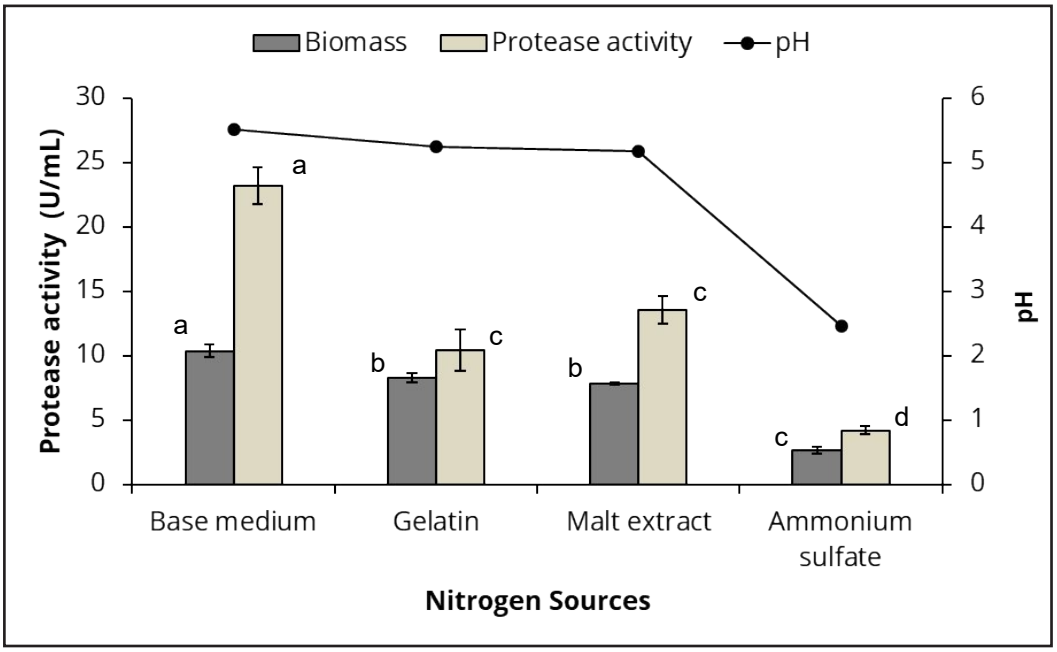
Source: Authors (2024)

In the base medium and in the presence of lactose (Figure 1), the value of protease activity did not differ from each other, while a low activity was observed in soluble starch (10.55 U/mL), which is different from the research who evaluated starch, sucrose, fructose and galactose as carbon sources and observed that sucrose promoted higher protease production in *P. eryngii* culture after 96 hours (Bano et al., 2016).

Biomass production in the base medium (Figure 2) was higher (10.37 g/L) compared to the use of gelatin (8.28 g/L) and malt extract (7.85 g/L), which is similar to what was observed with *Macrolepiota procera* (Pekşen & Kibar, 2016), whose mycelial biomass was 9.40 g/L in peptone. However, the addition of ammonium sulfate in the culture medium did not favor the growth of the fungus, with biomass production four times lower than that of the control medium.

This inorganic source does not seem to be suitable for biomass production, since it was reported that the release of H^+ ions reduces the pH of the culture medium, leading to inhibition of fungal growth (Sharma et al., 2019). This is evidenced in Figure 2, since, at the end of the culture, the pH of the medium was below 3.0. In mycelial growth, organic nitrogen sources stand out when compared to inorganic compounds, since certain essential amino acids are only synthesized by those sources (Singh et al., 2020). Data in the literature show that organic sources of nitrogen are preferred for growth of macrofungi, such as *P. ostreatus* (Almeida et al., 2015) and *P. eryngii* (Singh et al., 2020) and *P. citrinopileatus* (Wu et al., 2008).

Figure 2 – Influence of nitrogen sources on the production of mycelial biomass (g/L) and protease activity (U/mL) by *P. ostreatus* CMINPA 544



Source: Authors (2024)

Of the nitrogen sources, malt extract favored the second-best production, with 13.55 U/mL, after the base medium (23.22 U/mL), while ammonium sulfate showed low enzymatic activity during submerged cultivation (Figure 2).

The success of protease production is significantly influenced by the availability of carbon and nitrogen sources, as they play a regulatory role in enzyme synthesis and more complex sources of carbon and nitrogen are promising substrates. In addition to nutritional factors, the synthesis of these enzymes is linked to physical parameters, such as the pH of the culture medium, cultivation time, aeration and inoculum size (Bellettini et al., 2019).

3.3 Influence of glucose and peptone concentrations on mycelial biomass production and protease activity

The increase in glucose concentration in the culture medium was proportional to the increase in biomass production, with 30 g/L and 40 g/L being the most promising concentration, followed by 20 g/L (Table 2). Research with *P. ostreatus* LGAM 1123 expressed maximum biomass yield at 40 g/L and 50 g/L concentration, with no statistical difference between them (Bakratsas et al., 2024). The same pattern was observed for protease activity; when increasing the concentration from 0 g/L to 20 g/L, this promoted an activity of 23.22 U/mL. The enzyme activity was almost halved at concentrations of 30 g/L and 40 g/L, not differing statistically from each other. It was observed that the concentration of 20 g/L of glucose favored the production of proteases by *L. citrinus* DPUA 1694 (da Silva Magalhães et al., 2019).

The concentration of 10 g/L of peptone was ideal for promoting the best mycelial biomass production (14.34 g/L). The increase in peptone concentration from 0 g/L to 5 g/L favored protease activity, with production of 23.22 U/mL, but remained similar for the other concentrations tested in this assay. Although the highest biomass values were obtained at 10 g/L, there was no significant difference between concentrations of 5 to 10 g/L for protease activity (Table 3).

Table 2 – Influence of different glucose concentrations (g/L) on the production of mycelial biomass and protease activity by *P. ostreatus* CMINPA 544

Glucose concentration (g/L)	Mycelial biomass (g/L)	Protease activity (U/mL)
0	1.01±0.41^{d}	5.33±0.00^{d}
10	5.26±0.44^{c}	10.33±0.55^{c}
20	10.37±0.52^{b}	23.22±1.42^{a}
30	12.71±0.41^{a}	12.33±0.81^{b}
40	13.73±0.91^{a}	12.66±0.70^{b}

*Columns with equal letters do not differ from each other according to the Tukey test (<0.05) and confidence of 95%.
Source: Authors (2024)

Table 3 – Influence of different concentrations of peptone (g/L) on the production of mycelial biomass and protease activity by *P. ostreatus* CMINPA 544

Peptone concentration (g/L)	Mycelial biomass (g/L)	Protease activity (U/mL)
0	2.46±0.10^{d}	3.33±0.00^{c}
2,5	9.95±0.31^{c}	10.55±0.50^{b}
5	10.37±0.52^{c}	23.22±1.42^{a}
7,5	13.30±0.38^{b}	22.16±1.14^{a}
10	14.34±0.21^{a}	22.33±0.51^{a}

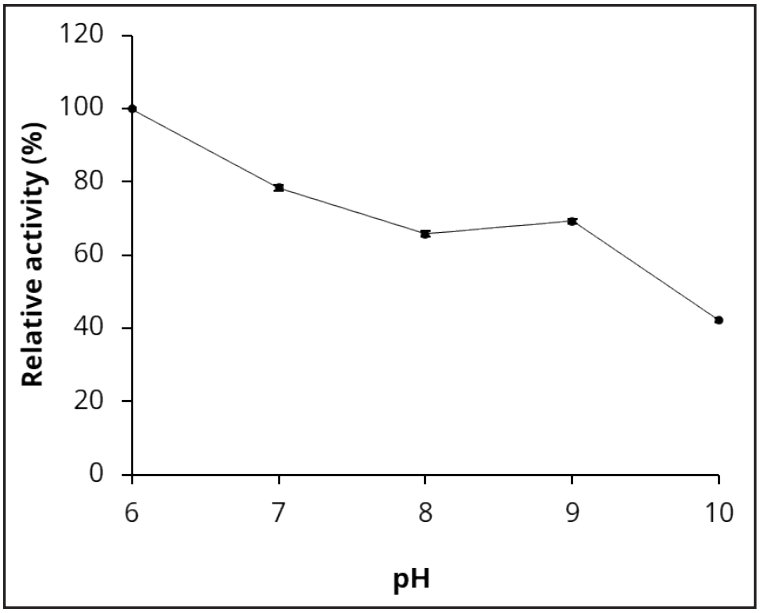
*Columns with equal letters do not differ from each other according to the Tukey test (p<0.05) and confidence level of 95%.
Source: Authors (2024)

3.4 Effect of pH and temperature on proteolytic activity

The optimal protease activity of *P. ostreatus* CMINPA 544 was observed at pH 6 at 25 ºC. This was followed by a decline with increasing pH, but without enzyme inactivation, although at pH 10 there was a 57% reduction in enzyme activity (Figure 3). This result is similar to that of *P. ostreatoroseus*, grown in the absence of light on a solid medium in the presence of Amazonian substrates (cupuaçu exocarp, acai seed and sawdust) (Fonseca et al., 2014). The same was observed with proteases produced by *L. citrinus* (Brito et al., 2019). In conditions outside the optimal pH, loss

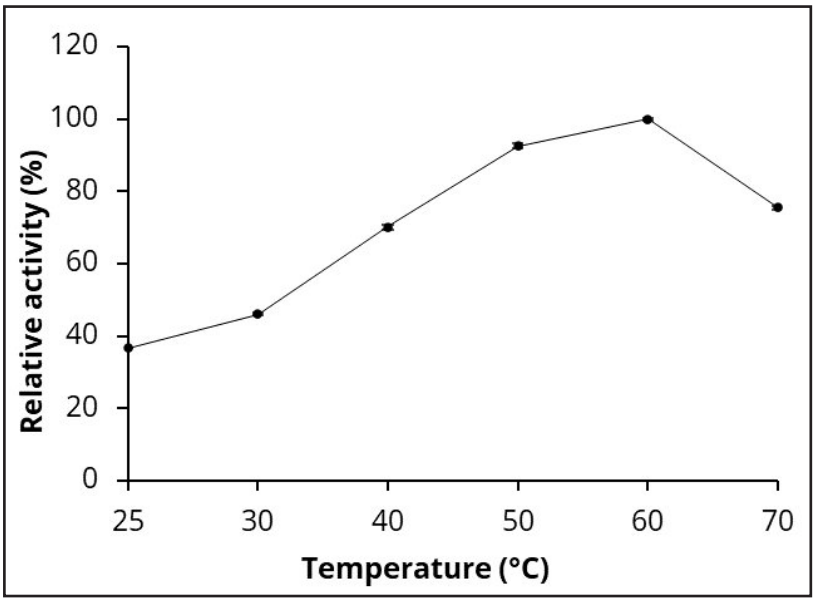
of enzyme activity can occur due to changes in protein conformation, which reduces the interaction between the enzyme and the substrate (Salwanee et al., 2013). Fungi produce acidic, neutral and alkaline proteases and it is known that a species has the ability to produce several types of these enzymes whose optimal activity varies over a wide pH range (4 to 11) (Song et al., 2023).

Figure 3 – Effect of pH on the protease activity of *P. ostreatus* CMINPA 544



Source: Authors (2024)

Figure 4 – Effect of temperature on the protease activity of *P. ostreatus* CMINPA 544



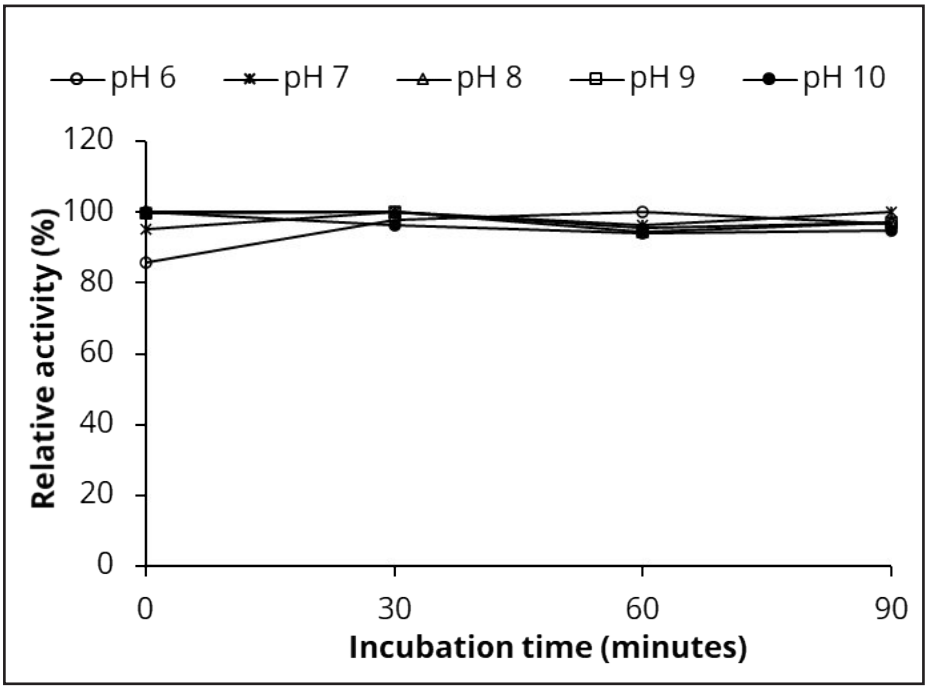
Source: Authors (2024)

The effect of temperature on protease activity was determined using pH 6 at temperatures in the range of 25 ºC to 70 ºC. Under these conditions, there was a gradual increase in enzyme activity. At 50 ºC, the relative activity was 92% followed by 100% activity at 60 ºC; however, there was a 25% reduction in protease activity at 70 ºC (Figure 4). Similar results with significant protease activity of *P. albidus* at 60 ºC were observed (Pimenta et al., 2021; Martim et al., 2017), while optimal temperature for proteases of *P. ostreatoroseus* was 40 ºC and 50 ºC, respectively (Barbosa et al., 2020; Coelho et al., 2021).

The results obtained indicate the presence of acid proteases with optimal activity in the range of 60 ºC in *P. ostreatus* CMINPA 544, characteristics widely applied in the food sector such as beverage clarification, cheese production, meat processing, protein hydrolysates and soy sauce manufacturing (Razzaq et al., 2019). Knowledge about the biochemical characteristics of microbial proteases is essential, especially when the objective is an enzyme with production and commercialization potential, and these are parameters that signal the viability of its insertion in the market (Barbosa et al., 2020).

3.5 Effect of pH and temperature on the stability of proteolytic activity

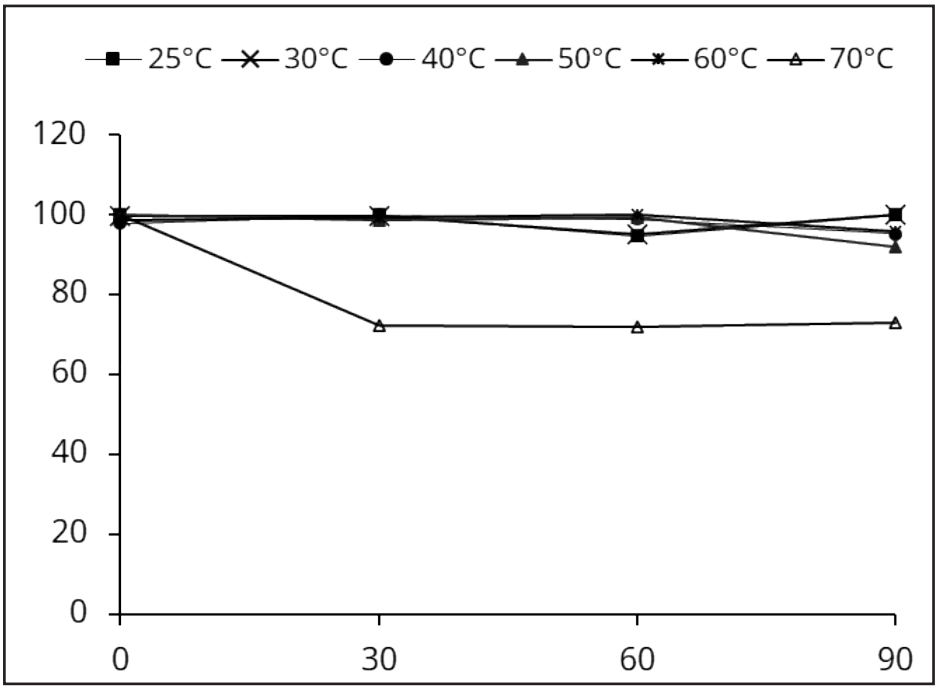
Figure 5 – Effect of PH on the stability of the proteases from *P. ostreatus* CMINPA 544



Source: Authors (2024)

The proteases of *P. ostreatus* CMINPA 544 remained stable between pH 7 to 9 with relative activity over 99% for up to 90 minutes. A reduction in stability was observed at pH 10 (Figure 5). The proteases maintained their stability at temperatures from 25 to 60 ºC, exhibiting 100% and 99% relative activity, respectively (Figure 6). The knowledge about enzyme's stability is fundamental, as this is a parameter required in industrial processes regarding the application of proteases (Moretti et al., 2012).

Figure 6 – Effect of temperature on the stability of the proteases from *P. ostreatus* CMINPA 544



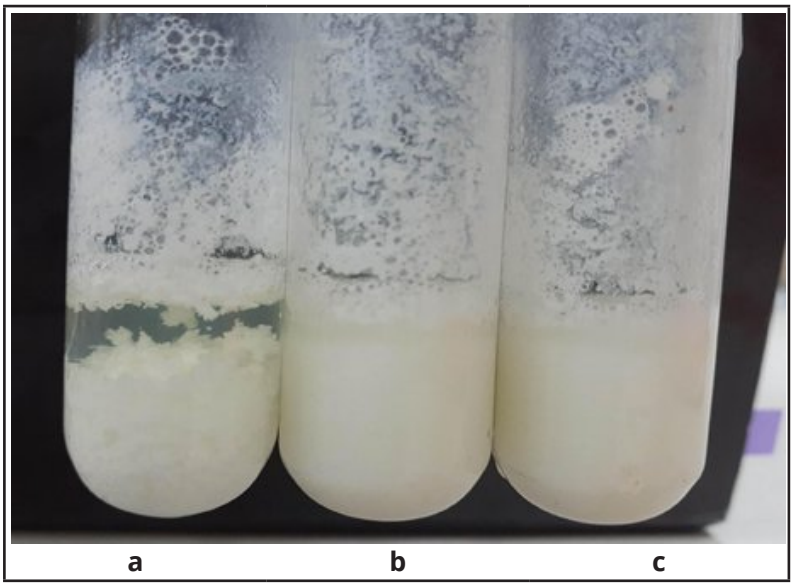
Source: Authors (2024)

3.6 Activity of proteases in milk coagulation

P. ostreatus CMINPA 544 expressed 13.7 U/mL of coagulant activity and a 0.49 coagulant ratio at 40 ºC, but there was no distinction between the coagulation and the whey. These data show a weak coagulation compared to commercial rennin (133.3 U/mL) when used as a positive control, whose visual classification was considered strong (Figure 7). Similar coagulation was observed in a study with *Coprinus lagopides* (Shamtsyan et al., 2014) and *Funalia trogii* (Gannochka et al., 2017) with coagulating enzymes of 13.30

U/mL and 9.02 U/mL, respectively. While studying coagulating proteases from *P. albidus*, Martim et al. (2017) obtained a coagulant activity of 73.39 U/mL.

Figure 7 – Coagulant activity. a - Positive control (ennin); b and c - Determination of coagulant activity of *P. ostreatus* CMINPA 544



Source: Authors (2024)

4 CONCLUSION

Of the mushroom strains evaluated, *P. ostreatus* CMINPA 544 was more efficient in the production of mycelial biomass and protease activity in submerged culture in the presence of glucose (20 g/L) and peptone (5 g/L). The proteases produced showed optimal activity in slightly acidic pHs and at 60 ºC, high stability up to this temperature and at pHs from 7 to 9. These biocatalysts provided a weak coagulation in milk; therefore, the activities tested in this research should be improved aiming at the possible use of these proteases in the enzyme market.

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