Lentinus villosus Klotzsch (1833) AM 169: a natural and renewable source of alkaline protease

Lentinus villosus Klotzsch (1833) AM 169: uma fonte natural e renovável de protease alcalina

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ABSTRACT
Mushrooms have stood out as sources of proteases with industrial applicability. The objective of this work was to evaluate the parameters that influence the synthesis of proteases by *Lentinus villosus* Klotzsch (1833) AM 169 and to characterize these enzymes. The matrix culture, prepared on potato dextrose agar supplemented with yeast extract, was maintained for 8 days at 25 °C. Submerged fermentation was carried out in GYPG (glucose, yeast extract, peptone and gelatin). Azocasein solution (1%, w/v) was used as an enzyme substrate. The best conditions for protease production were: initial pH of the culture medium (6), fermentation time (12 days), age of the inoculum (6 days) and size of the inoculum (10%). The proteases were active at pH 8, at 60 °C, stable at pH (5 to 9) and temperature (30 °C to 60 °C). These enzymes were classified as cysteine proteases, stimulated by Fe$^{2+}$ and Mn$^{2+}$. *Lentinus villosus* synthesizes proteases with potential for application in the detergent, textile, leather processing and bioremediation processes.

Keywords: basidiomycetes, peptidases, submerged fermentation.

RESUMO
Os cogumelos têm se destacado como fontes de proteases com aplicabilidade industrial. O objetivo desse trabalho foi avaliar os parâmetros que influenciam na síntese de proteases por *Lentinus villosus* Klotzsch (1833) AM 169 e caracterizar essas enzimas. A cultura matriz, preparada em ágar batata dextrose suplementado com extrato de levedura, foi mantida por 8 dias a 25 °C. A fermentação submersa foi realizada em GYPG (glicose, extrato de levedura, peptona e gelatina). Solução de azocaseína (1%, p/v) foi utilizada como substrato enzimático. As melhores condições para a produção de protease foram: pH inicial do meio de cultura (6), tempo de fermentação (12 dias), idade do inóculo (6 dias) e tamanho do inóculo (10%). As proteases foram ativas em pH 8, a 60 °C, estáveis ao pH (5 a 9) e à temperatura (30 °C a 60 °C). Estas enzimas foram classificadas como cisteíno proteases, estimuladas por Fe$^{2+}$ e Mn$^{2+}$. *Lentinus villosus* sintetiza proteases com potencial para aplicação nas indústrias de detergente, têxtil, no beneficiamento do couro e em processos de biorremediação.

Palavras-chave: basidiomicetos, peptidases, fermentação submersa.
1 INTRODUCTION

Proteases, peptidases, proteinases or proteolytic enzymes are hydrolases that catalyze the breakdown of peptide bonds in proteins (Gurumallesh et al., 2019). These biocatalysts play physiological functions essential for the maintenance of the life of living beings, such as cell signaling, inflammation, apoptosis, blood coagulation and hormone processing (Bond, 2019; Boon et al., 2020).

Due to broad biotechnological applications, proteases correspond to 65% of the enzyme market (Kummari & Prasad, 2015; Lanka et al., 2017). In the industrial sector, the peptidases are used in the manufacture of cheeses, beverages, softening of meats and bakery products. They are also used in wastewater treatment, bioremediation processes and medical diagnostics (Ahmed et al., 2017).

The alkaline proteases are a group of proteolytic enzymes with significant catalytic activity at pH > 8.0 (Srilakshmi et al., 2014). These biocatalysts have outstanding economic importance in the production of detergents, but can also be used in the textile and chemical industries, leather processing and bioremediation (Sharma et al., 2017; Zhou et al., 2020).

In nature, vegetables, animals and microorganisms, including bacteria and filamentous fungi are sources of proteolytic enzymes (Aljammas et al., 2018; Souza et al., 2019). The production of enzymes synthesized by microorganisms has acquired space in the market and industrial relevance, since proteases extracted from plants and animals are not supplying the commercial demand (Razzaq et al., 2019). In addition, proteases of microbial origin are Generally Recognized as Safe (GRAS) according to the US Food and Drug Administration (Tavano et al., 2018).

Species of filamentous fungi are considered excellent sources of proteases because they are easy to culture and secrete extracellular enzymes, conditions that facilitate the production of proteolytic biocatalysts on a large scale (Inácio et al., 2015). Among the filamentous fungi, mushrooms have been highlighted as sources of proteases with potential for industrial application (Martim et al., 2017a).

The submerged fermentation technology has been used for the production of proteases by different species of mushrooms: Lentinus citrinus Walleyn & Rammeloo (1994) (Kirsch et al., 2013), L. crinitus (L.) Fr. 1825 (Magalhães et al., 2019), Pleurotus albidus (Berk.) Pegler 1983 (Martim et al., 2017b) and Termitomyces clypeatus R. Heim 1951 (Majumder et al., 2014). This work aimed to evaluate the parameters that influence the synthesis of proteases by Lentinus villosus Klotzsch (1833) and to characterize these biocatalysts of industrial importance.
2 MATERIAL AND METHODS

2.1 MUSHROOM

_Lentinus villosus_ AM 169, from the Cultures Collection of the Parasitology Department of Federal University of Amazonas, was cultivated in Petri dishes containing PDA culture medium [potato dextrose agar supplemented with 0.5% (w/v) yeast extract]. The cultures were kept at 25 °C for eight days.

2.2 PROTEASES PRODUCTION BY SUBMERGED FERMENTATION

For the proteases production in liquid medium, three 80mm (Ø) mycelial fragments were inoculated in 125 mL Erlenmeyer flasks containing 50 mL of GYPG liquid medium (20 g of glucose, 5 g of yeast extract, 5 g of peptone and 5 g of gelatin per 1000 ml of water). The liquid medium, pH 5.6, was sterilized at 121 °C for 15 minutes. The fermentation was conducted at 30 °C, 150 rpm. After five days, the biomass was filtered off under vacuum on Whatman number 1 filter paper, followed by centrifugation at 4 °C (8,000 x g / 5 minutes). The proteolytic activity was determined in the crude extract.

2.3 DETERMINATION OF PROTEOLYTIC ACTIVITY

Proteolytic activity was determined according to Leighton _et al._ (1973). As a substrate, 250 µl of 1% (w/v) azocasein in 150 µL Tris-HCl buffer of the crude enzyme extract was used. The reaction mixture was maintained for 60 minutes at 25 °C and the reaction was interrupted by addition of 1.2 mL of 10% (w/v) trichloroacetic acid followed by centrifugation at 4 °C (8,000 x g / 5 minutes). From the recovered supernatant was removed 0.8 mL for homogenization with 1.4 mL of 1M NaOH. The blank was prepared maintaining the same conditions as the sample. One unit of proteolytic activity was defined as the amount of enzyme required to produce an absorbance change of 0.01 in one hour.

2.4 STANDARDIZATION OF FERMENTATION PARAMETERS FOR PROTEASE PRODUCTION

In order to evaluate the optimal conditions of proteases production by _L. villosus_, the initial pH of the culture medium (4, 5, 6, 7 and 8), influence of inoculum age (6, 8, 10, 12 and 14 days), fermentation time (2, 4, 6, 8, 10, 12 and 14 days) and inoculum size (2%, 4%, 8%, 10% and 20%) were analyzed.
2.5 PARTIAL CHARACTERIZATION OF PROTEASES

The proteases obtained under the optimum conditions of fermentation were characterized for optimum pH and temperature, pH and temperature stability and effects of ions and inhibitors as described by Martim et al. (2017b).

For optimal pH testing, proteolytic activity was determined at 25 °C at different pH values using the following buffer solutions (0.1 M): sodium acetate (pH 5 and 6), Tris-HCl (pH 7 and 8) and Glycine-NaOH (pH 9 and 10). The optimum temperature was determined by incubating the crude extract at different temperatures (30, 40, 50, 60, 70 and 80 °C).

For pH stability, the crude extract was diluted (1:1) in the following 0.1 M buffer solutions: sodium acetate (pH 5 and 6), Tris-HCl (pH 7 and 8) and Glycine-NaOH (pH 9 and 10) and maintained at 25 °C for 24 hours. At thermal stability, the extract was incubated at different temperatures (30, 40, 50, 60, 70 and 80 °C) for 1 hour. Azocasein 1% (w/v) dissolved in Tris-HCl buffer was used as substrate. Relative activities were determined under optimum pH and temperature conditions.

The effect of inhibitors on enzyme activity was investigated using 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM iodoacetic acid and 0.1 mM pepstatin in the reaction mixture. The metal ions used in the 10 mM concentration in the reaction mixture were CaCl₂, CuSO₄, FeSO₄, MgSO₄, MnSO₄, ZnSO₄, NaCl and KCl. The samples were incubated under optimum pH and temperature conditions. The residual enzyme activity was compared to the control (incubated without inhibitors and metal ions) and corresponded to 100% activity.

3 STATISTICAL ANALYSIS

In all experiments the data were submitted to analysis of variance and the means were compared by the Tukey method (*p* < 0.05) using Minitab program, version 18.0 (Minitab, 2017). All analyzes were performed in triplicate.

4 RESULTS AND DISCUSSION

4.1 STANDARDIZATION OF FERMENTATION PARAMETERS FOR PROTEASE PRODUCTION

The synthesis of proteases by microorganisms using the submerged fermentation technology is influenced by environmental factors and nutritional characteristics of the culture medium (Ibrahim *et al.*, 2015). The influence of the initial pH of the culture medium on the production of proteases by *L.*
villosus is shown in figure 1. Lentinus villosus synthesized significant quantitative of proteases at pH 6 (69.0 U/mL). There was a decrease in proteolytic activity at pH 7 and 8, as shown in figure 2. This same pH value was considered the optimal pH for protease production by Rhizopus stolonifer (Ehrenb.) Vuill. (1902) (Gais et al., 2009). Daudi et al. (2015) and Bano et al. (2016) reported that at pH 5.5 and 6.5 there was maximum protease production by Mucor pusillus Lindt (1886) and P. eryngii (DC.) Quél. 1872, respectively. The pH of the culture medium interferes with enzyme production, enzyme activity and nutrient transport across the cell membrane (Sharma et al., 2017).

Under the conditions evaluated, the maximum protease production (130.0 U/mL) by L. villosus was verified with six-day inoculum cultured in PDA (Figure 2). Boukhalfa-Lezzar et al. (2014) and Martim et al. (2017b) observed that the five-day inoculum favored the production of proteases by Aspergillus oryzae (Ahlb.) (1884) 2220 and P. albidus, respectively. Irfan et al. (2011) reported that R. oligosporus Saito (1905) M-30 produced maximum quantitative of proteolytic enzymes when using 7-day old inoculant. The optimal age of the inoculum to the production of enzymes varies according to the physiological characteristics of the microorganism and the environmental conditions of the bioprocess (Maldonado et al., 2014).
Fermentation time is a parameter that significantly influences the protease production by filamentous fungi. In the present study, it was observed that *L. villosus* synthesized and excreted proteases in all conditions evaluated. However, maximum peptidase production was verified at 12 days of fermentation (172.88 U/mL), as shown in figure 3. Brito *et al.* (2019) when evaluating the production of proteases by *L. crinitus* DPUA 1693 also reported a similar result. The significant protease production by *P. ostreatus* (Jacq.) P. Kumm. (1871) and *P. eryngii* in liquid medium was verified at 14 and 4 days, as reported by Sales-Campos *et al.* (2010) and Bano *et al.* (2016), respectively. Protease production increases with incubation time to some extent. Then, there is a reduction in the synthesis of proteolytic enzymes due to the insufficient availability of some nutrients in the growth medium or decomposition of the protease (Niyonzima & More, 2013).
Figure 3. Influence of fermentation time on the protease production by Lentinus villosus.

The influence of inoculum size on the protease production by *L. villosus* is shown in figure 4. In the conditions evaluated, it was observed that 10% inoculum (w/v) stimulated the maximum protease production (237.33 U/mL) by *L. villosus*. However, the use of 20% inoculum decreased the protease production by 8.65%. Martim *et al.* (2017b) and Brito *et al.* (2019) also observed that the synthesis of proteases by *P. albidus* and *L. crinitus* was stimulated with the use of 10% inoculum. On the other hand, Benluvankar *et al.* (2015) reported that the 5% inoculum stimulated the protease production of *Penicillium* sp. Link (1809) LCJ228. The use of high concentrations of inoculum favors the rapid consumption of nutrients, resulting in a reduction in the synthesis of alkaline proteases by filamentous fungi (Niyonzima & More, 2013).
4.2 PARTIAL CHARACTERIZATION OF PROTEASES

In this study, the proteases of *L. villosus* had the optimal activity at pH 8.0, therefore classified as alkaline. There was an activity reduction of 23.64% and 18.9% at pH 9 and 10, respectively, as shown in Figure 5. Sun *et al.* (2011) and Ravikumar *et al.* (2012) reported that the proteases of *Amanita farinosa* Schwein. (1822) and *P. sajor-caju* (Fr.) Singer (1951) also demonstrate maximum catalytic activity at pH 8.0, respectively. Zhang *et al.* (2011) reported that the peptidases of *T. albuminosus* (Berk.) R. Heim (1941) have significant protease activity at pH 10.6. A change in the hydrogen potential value, in addition to the ideal pH, can lead to protonation or deprotonation of enzyme side groups. Under these conditions, there may be structural changes in the enzymes, resulting in decreased enzyme activity (Chittoor *et al*., 2016).
Temperature influences the growth and production of biocomposites by different species of microorganisms. In this study it was observed that the proteases of *L. villosus* had maximum catalytic activity at 60 °C. Relative activities of 95.98%, 96.43% and 96.61% were observed at temperatures of 30 °C, 40 °C and 50 °C, respectively. A lower relative activity (12.19%) was observed at 70 °C, as shown in Figure 6. Similar results were verified by Ravikumar *et al.* (2012) and Genier *et al.* (2015), which reported the maximum catalytic activity of *Pleurotus sajor-caju* and *P. ostreatus* proteases at 60 °C, respectively. Brito *et al.* (2019) reported that *L. crinitus* proteases demonstrated significant activity at 50 °C. At high temperatures, weak hydrogen and hydrophobic bonds break, which maintain the structure protease. Under these temperature conditions, the enzyme is denatured, resulting in loss of enzyme activity (Oueslati & Mounirhaouala, 2014; Martim *et al.*, 2017b).
The proteases of *L. villosus* demonstrated stability in the range of pH 5 to 8, with values of proteolytic activity higher than 90%. The enzyme presented 86.78% of activity at pH 10 (Figure 7). Brito *et al*. (2019) found that *L. crinitus* proteases maintained stability in the range of 30 °C to 60 °C, with relative activities greater than 80%. El-Baky *et al*. (2011) reported that proteases of *Piptoporus soloniensis* (Dubois) Pilát (1937) retained 70% activity in the range of pH 3 to 5. Silva *et al*. (2017) report that the proteases of *Phanerochaete chrysosporium* Burds. (1974) were stable in the range of pH 3 to 8.
The effect of temperature on the enzymatic stability of *L. villosus* is shown in figure 8. Under the conditions evaluated, the proteases of *L. villosus* maintained stability greater than 90% in the range of 30 °C to 60 °C. However, at the temperature of 70 °C there was a decrease in activity, with retention of only 23.14% of catalytic activity (Figure 8). Martim *et al.* (2017b) and Brito *et al.* (2019) found similar results for *P. albidus* and *L. crinitus* proteases, respectively. Leonhardt *et al.* (2016) observed that proteases of *P. pulmonarius* (Fr.) Quél. (1872) maintained stability greater than 80% in the temperature range of 20 °C to 40 °C.

![Figure 8. Effect of temperature on the enzymatic stability of *Lentinus villosus*.](image)

The effect of inhibitors and metal ions on the proteolytic activity of *L. villosus* is shown in Table 1. Iodoacetic acid caused a reduction of 20.29% in the proteolytic activity of *L. villosus*, suggesting the presence of a greater amount of cysteine protease in the enzymatic extract of this mushroom. In the presence of EDTA, PMSF and Pepstatin the proteases maintained catalytic activity of 100%, 99.58% and 99.47%, respectively. Machado *et al.* (2017), Martim *et al.* (2017a) and Brito *et al.* (2019) reported inhibition of proteolytic activity of *P. ostreatoroseus* Singer (1961), *P. albidus* and *L. crinitus* in 95%, 62% and 11%, respectively, in the presence of iodoacetic acid.

In the present study, ions Fe$^{2+}$ and Mn$^{2+}$ stimulated *L. villosus* protease activity in 77.25% and 45.75% respectively. On the other hand, the ions Zn$^{2+}$, Ca$^{2+}$, K$^+$ and Na$^+$ promoted reduction of the enzymatic activity in 9.23%, 8.77%, 6.05% and 5.21%, respectively. The ion Cu$^{2+}$ caused greater inhibition in the enzymatic activity, reducing the catalytic activity in 53.68%. Zhang *et al.* (2010) also reported that protease catalytic activity of the *Helvella lacunosa* Afzel. (1783) was stimulated in the
presence of Fe\(^{2+}\) and Mn\(^{2+}\), but in the presence of Cu\(^{2+}\), Hg\(^{2+}\) and Fe\(^{3+}\) there was a reduction of protease activity. Magalhães et al. (2019) observed a 29.43% increase in the proteolytic activity of *L. crinitus* in the presence of Mn\(^{2+}\). On the other hand Shivashankar and Premkumari (2014) observed that Fe\(^{2+}\) ions did not influence the proteolytic activity of *Hypsigygus ulmarius* (Bull.) (1984), whereas Zn\(^{2+}\), Ca\(^{2+}\) and Cu\(^{2+}\) completely inhibited the proteolytic activity.

### Table 1. Effect of inhibitors and metal ions on the protease activity of *Lentinus villosus*.

| Chemical products | Concentration (mM) | Relative activity (%) |
|-------------------|--------------------|-----------------------|
| Control           | 10                 | 100                   |
| EDTA              | 10                 | 94.79 ± 2.55\(^d\)    |
| PMSF              | 10                 | 93.63 ± 0.38\(^d\)    |
| Pepstatin         | 1                  | 93.44 ± 1.45\(^d\)    |
| Iodoacetic acid   | 10                 | 79.71 ± 0.38\(^d\)    |
| FeSO\(_4\)        | 10                 | 177.25 ± 1.92\(^a\)   |
| MnSO\(_4\)        | 10                 | 145.75 ± 1.38\(^b\)   |
| MgSO\(_4\)        | 10                 | 100.00 ± 2.14\(^c\)   |
| KCl               | 10                 | 94.79 ± 2.55\(^d\)    |
| NaCl              | 10                 | 93.95 ± 1.072\(^d\)   |
| CaCl\(_2\)        | 10                 | 91.23 ± 2.14\(^e\)    |
| ZnSO\(_4\)        | 10                 | 90.77 ± 1.33\(^e\)    |
| CuSO\(_4\)        | 10                 | 46.32 ± 0.66\(^f\)    |

### 5 CONCLUSION

*Lentinus villosus* produces predominantly cysteine proteases in liquid medium. The synthesis of these proteolytic enzymes is influenced by the initial pH of the culture medium, fermentation time, age and size of the inoculum. Proteases have maximum catalytic activity at pH 8 to 60 °C, stability at pH and temperature. These biocatalysts have the potential for application in detergents, textiles, leather production and bioremediation processes.

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