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Production of milk coagulating proteases by amazonian edible mushroom of the Polyporaceae family (Basidiomycota)

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ABSTRACT. Cheese is a dairy product that plays a fundamental role in the global economy. However, factors such as high rennet prices and religious and ethnic motivations against the use of animal-derived enzymes encourage the search for new coagulation agents. In this context, edible mushrooms have been emerging as promising sources of coagulant proteases for cheese production. The aim of this research was to evaluate the production of coagulant proteases and determine the parameters that influence the synthesis of these biocatalysts by *Lentinus crinitus* DPUA 1693. For enzyme production in liquid medium, the mushroom was cultivated in Potato Extract (PDA), Sabouraud Extract (SAB), Corn Meal (CMA), supplemented with yeast extract (YE) 0.5% (w/v) and Glucose, Yeast Extract, and Peptone (MGYP+YE). The proteolytic and coagulant activities were determined using 1% (w/v) azocasein solution and 10% (w/v) skimmed milk, respectively, as substrate. The parameters that interfere in the coagulant production were evaluated, such as inoculum size, fermentation time, and inoculum age. The protein profile of the crude extract and the zymogram to confirm the proteolytic action of the extract were determined by non-denaturing polyacrylamide gel electrophoresis (SDS-PAGE). The significant values of coagulant activity (18.21 U mL^{-1}) in SAB and MGYP, as well as protease activity (1.64 U mL^{-1} and 1.66 U mL^{-1}), respectively, with the ratio and strong clot. The significant coagulant production was verified under the following conditions: inoculum size (20%), fermentation time (10 days), and inoculum age (16 days). The significant values of activity and coagulant ratio, as well as the evidence of proteolytic action in zymograms, indicate the technological quality of the application of enzymes in the industrial sector. *Lentinus crinitus* DPUA 1693 is a natural source of coagulant with characteristics for the application of enzymes in the dairy sector.

Keywords: proteases, coagulation, dairy, *Lentinus crinitus*.

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Introduction

Proteases are hydrolases that act in the degradation of proteins into peptides and amino acids, playing an important role in the physiological conditions of living beings. These enzymes are widely used in industrial processes for the production of food, detergents, medicines, biofuels, and in the textile sector (Song et al., 2023).

In the food industry, coagulant proteases are used in the preparation of several dairy products (Pacifico et al., 2024). Rennet, an enzymatic preparation consisting of acid proteases (chymosin and trypsin) secreted by lactating animals, is the gold standard for cheese production due to its high degree of specificity in cleaving the Phe^{105}-Met^{106} peptide bond of κ-casein during milk coagulation (Zhang et al., 2023).

However, due to increased cheese production, high rennet prices, and religious and ethnic issues against the use of animal-derived enzymes, research has been carried out to find alternative sources of milk coagulant proteases for use in the dairy industry (Martim et al., 2021).

Fungi classified as Generally Recognized as Safe (GRAS) are becoming a biotechnological option for obtaining coagulants (Qasim et al., 2022). The application of these organisms in bioprocesses for the large-scale production of proteolytic enzymes is advantageous, as they are efficient and renewable, demonstrate biochemical diversity, and are easy to manipulate genetically (Martim et al., 2021).

Among fungi, mushroom species have been standing out as a source of proteolytic enzymes with significant milk coagulant activity. Under *in vitro* conditions, proteases from *Pleurotus albidus* (Martim et al., 2021), *Pleurotus florida* (Bakr et al., 2022), *Pleurotus ostreatus* (Lebedeva & Proskuryakov, 2009) and *Piptoporus soloniensis* (El-Baky et al., 2011) demonstrated suitable characteristics for use in cheese production (Martim et al., 2021). In this context, the objective of the research was to evaluate the production of coagulant proteases and determine the parameters that influence the synthesis of these biocatalysts by *Lentinus crinitus* DPUA 1693, a species isolated from the Amazon biome.

Material and methods

Mushroom

In this research, *Lentinus crinitus* DPUA 1693 in the mycelial phase, provided by the DPUA Culture Collection, *Universidade Federal do Amazonas*, Manaus-AM, was used. To obtain a pure and viable culture, an 8 mm mycelial disc was seeded centrally on the surface of Potato Dextrose Agar (PDA) with 0.5% (w/v) yeast extract (YE) in Petri dishes. The cultures were maintained at 25ºC, in the absence of light, for twelve days (Batista et al., 2021).

Protease and coagulant production Submerged Fermentation

The fermentation media used for the production of proteases and coagulants by *L. crinitus* DPUA 1693 were the following media supplemented with yeast extract (YE 0.5% (w/w): potato extract (PDA+YE), Sabouraud extract (SAB+YE), corn meal (CMA+YE), and Malt Extract, Glucose, Yeast Extract and Peptone (MGYP+YE). The bioprocess was carried out in 125 ml Erlenmeyer flasks containing 50 ml of sterilized medium at 121°C for 15 min. After cooling the medium, 5 mycelial discs of 8 mm in diameter were inoculated in each flask. Fermentation was carried out at 150 rpm, 30°C for 8 days (Martim et al., 2021).

Extraction of proteases synthesized by *Lentinus crinitus* DPUA 1693

At the end of fermentation, the crude extract was separated from the biomass by vacuum filtration through Whatman No. 1 filter paper. Subsequently, the extract samples were filtered again using a cellulose ester membrane (0.45 µm), stored in a glass bottle, and kept at 4°C until enzymatic determination (Martim et al., 2017).

Determination of quantitative proteolytic activity

The determination of proteolytic activity was performed according to Leighton et al. (1973), using a 1% (w/v) azocasein solution in Tris-HCl buffer, pH 7.2, as substrate. 250 µL of azocasein and 150 µL of the crude enzyme extract were added to test tubes. The reaction mixture was incubated in the absence of light at 25°C for 60 minutes. After this period, the reaction was stopped by the 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, 800 µL was removed for homogenization in tubes containing 1.4 mL of 1 M NaOH. One unit (U) of protease activity was defined as the amount of enzyme required to produce a change in absorbance equal to 0.1 in 60 minutes at 440 nm. All experiments were performed in triplicate.

Determination of coagulant activity

The coagulant activity was determined according to the methodology described by Arima and Iwasaki (1970) a 10% (w/v) skim milk solution was prepared with 0.05 M $CaCl_2$ and homogenized on a magnetic stirrer until the milk was completely dissolved. Subsequently, the pH was adjusted to 5.8 with 0.1 M NaOH or HCl. A 5 mL aliquot of the milk solution was added to test tubes and kept at 40°C in a water bath with water circulation. After 15 minutes, 0.5 mL of the crude enzyme extract was added. The coagulant activity was evaluated by visualizing the formation of clots on the wall of the test tube within 40 minutes. The final coagulation time was determined with a stopwatch. All experiments were performed in triplicate. The qualitative result of the coagulant activity was expressed based on the formation of clots and the form of separation of the whey visualized in the test tubes as: strong coagulation (distinct clot and abundant whey) and weak coagulation (coagulation without visual separation of whey). One unit of milk coagulant activity (U) was defined as the amount of enzyme required to coagulate 1 mL of substrate in 40 minutes at 40°C. The milk coagulant activity was calculated, according to Shata (2005) and according to Equation 1:

$$U = \frac{2400}{T} x \frac{S}{E}$$

where: T = time required for clot formation; S = volume of milk (mL); E = volume of crude extract (mL).
The coagulant ratio (R) was calculated by the ratio between the values of the milk coagulant activity and the proteolytic activity, according to equation 2.

$$R = \frac{\text{Atividade coagulante}}{\text{Atividade proteolítica}}$$

Standardization of coagulant protease production conditions by *Lentinus crinitus* DPUA 1693

Protease production by *L. crinitus* DPUA 1693 was evaluated using the following parameters: inoculum size (6, 10, 16, and 20%), inoculum age (10, 12, 14, and 16 days), and fermentation time (8, 10, 12, and 14 days) (Martim et al., 2021). All experiments were performed in triplicate.

Polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic profile of proteins obtained from *L. crinitus* from extracts concentrated with 80% acetone was performed by non-denaturing polyacrylamide gel electrophoresis (SDS-PAGE); in all samples, 5% stacking and 12% separating gels were used. Proteins were then stained for 90 min. in a 0.1% Coomassie Brilliant Blue R-250. Analyses of hydrolyzed casein and whey proteins were also performed using the same methodology (Ning et al., 2021).

Substrate immersion zymography

After electrophoresis, the gels were washed three times in 67 mM glycine-NaOH buffer (pH 8.0) containing 10% methanol. The gels were rinsed for 5 min. in the same buffer without methanol and then incubated in glycine-NaOH (pH 8.0) containing 0.8% casein for 90 min. After discarding the substrate solution, the gels were rinsed with the same buffer (pH 7.0) without casein for 30 min. Proteins were then stained for 90 min in a 0.1% Coomassie Brilliant Blue R-250. After bleaching, the unstained bright bands of the gel indicated proteolytic activity (Ning et al., 2021).

Statistical analysis

In all experiments, data were subjected to statistical analysis of variance, and means were compared by Tukey's test ($p < 0.05$) using the Minitab program, version 18.0. (Minitab, 2017).

Results and discussion

Production of coagulant proteases by submerged fermentation

The production of enzymes by filamentous fungi is influenced by several factors, such as the nature of fermentation, environmental conditions of the bioprocess, and the nutritional characteristics of the culture medium. Under the conditions evaluated, it was verified that the synthesis of proteases by *L. crinitus* DPUA 1693 was influenced by the fermentation medium. The values of proteolytic activity, coagulant activity, coagulant ratio, and clot strength are shown in Table 1. Regarding proteases, significant activity was determined in the cultures in MGYP+YE (1.67 U mL^{-1}) and SAB+YE (1.64 U mL^{-1}). Magalhães et al. (2019) and Braga et al. (2020) evaluated the protease activity of *L. crinitus* in GGE (glucose, gelatin, and yeast extract) and GYPG (glucose, yeast extract, peptone, and gelatin) medium and found significant values of 1.90 U mL^{-1} and 69.0 U mL^{-1}, respectively. Batista et al. (2021) cultivated *L. crinitus* in cupuaçu exocarp and found proteolytic activity of 6.71 U mL^{-1}.

In the scientific literature, there is a lack of studies reporting the production of coagulant proteases by *Lentinus* species. Coagulant activity was observed in all tested media, with significant values in SAB+YE and MGYP+YE (18.21 U mL^{-1}). In CMA+YE and PDA+YE, coagulant action values were 21.47 and 31.30% lower, respectively, when compared to the SAB+YE medium. Okamura-Matsui et al. (2001) found lower results of coagulant activity of *Schizophyllum commune* proteases (17.4 U mL^{-1}). Martim et al. (2017) and Bakr et al. (2022) detected values of 73.39 and 75.49 U mL^{-1} of coagulation activity of proteases synthesized by *P. albidus* and *P. florida*, respectively. Studies have shown that basidiomycetes produce coagulants with catalytic activity

and biochemical characteristics similar to commercial enzymes. These biocatalysts facilitate milk coagulation and also control cheese yield, texture, and flavor (Nolli et al.,2022; Roohi et al., 2019).

Table 1. Proteolytic activity, coagulant, coagulant ratio, and clot strength of extracts of *Lentinus crinitus* DPUA 1693 grown in submerged fermentation.

Fermentation médium	Proteolytic activity (U mL^{-1})	Coagulant activity (U mL^{-1})	Coagulant ratio	Clot strength
BDA+YE	1.37 ± 0.09^{b}	12.51 ± 0.39^{c}	9.19 ± 0.54^{b}	Strong
MGYP+YE	1.67 ± 0.02^{a}	18.21 ± 0.82^{a}	10.93 ± 0.51^{a}	Strong
CMA+YE	1.33 ± 0.04^{b}	14.30 ± 0.51^{b}	10.72 ± 0.15^{ab}	Weak
SAB+YE	1.64 ± 0.12^{a}	18.21 ± 0.82^{a}	11.12 ± 0.97^{a}	Strong

(SAB) Sabouraud Extract; (BDA) Potato Extract; (MEA) Malt Extract; (MGYP) Malt Extract, Glucose, Yeast Extract and Peptone; (CMA) Corn Meal; (YE) Yeast Extract. Equal letters in the column do not differ statistically by Tukey's test ($p < 0,05$).

In the selection of new enzyme sources for use in the dairy industry, the coagulant ratio influences the sensory characteristics and technological properties of the cheese (Martim et al., 2017). In the present study, the significant coagulant ratio was determined in SAB+YE (11.12). Majumder et al. (2015) found that proteases from *Termitomyces clypeatus* had a coagulant ratio of 64.00.

Regarding clot strength, the extracts obtained from the SAB+YE, PDA+YE, and MGYP+YE cultures showed strong clot formation and abundant serum. Only in MEA+YE and CMA+YE was weak coagulation evidenced. Martim et al. (2021) also found strong milk coagulation when analyzing *P. albidus* proteases. The process to replace rennet is rigorous and specific; its enzymatic properties must be as close as possible to enzymes such as rennin, that is, they must present high milk coagulation activity (Shamtsyan et al., 2014).

The results obtained in the present study indicate that the coagulant proteases of *L. crinitus* DPUA 1693 that demonstrate strong coagulation have potential for use in cheese production (Figure 1).

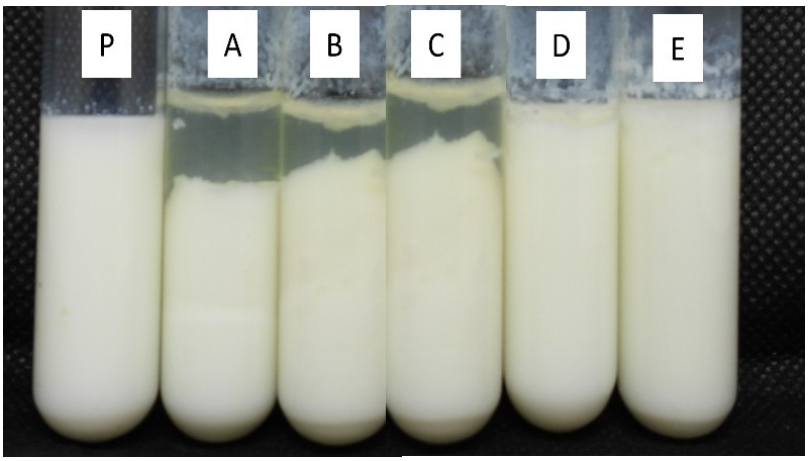

Figure 1. Coagulant of *Lentinus crinitus*: Milk clot strength characteristics: (P) Standard; (A) SAB+YE- Strong; (B) MGYP- Strong; (C) BDA+YE- Strong; (D) CMA+YE- Weak; and (E)MEA+YE- Weak.

Standardization of coagulant protease production conditions

The evaluation of parameters such as temperature, humidity, pH, inoculum size, inoculum age, and carbon and nitrogen sources is essential to verifying the appropriate conditions for the production of milk coagulant enzymes by edible mushrooms. However, few studies evaluate these environmental conditions, which are essential for the incorporation of a new milk coagulant agent on a commercial scale (Martim et al., 2021).

The influence of inoculum size on the synthesis of coagulant proteases by *L. crinitus* DPUA 1693 is shown in Figure 2. Under the conditions evaluated, significant coagulant production (40.27 U mL^{-1}) and strong coagulation were observed in the bioprocess in which an inoculum size of 20% (w/w) was used. Martim et al. (2021) found that the 2.5% (w/w) inoculum favored the production of coagulant by *Pleutotus albidus* with significant activity (42.60 U mL^{-1}) and strong coagulation. Sathya et al. (2009) found that the 30% inoculum promoted the production of coagulant proteases by *Mucor circinelloides* grown in Indian grain husks. The inoculum size interferes with the production of biomass and primary and secondary metabolites. At high concentrations, it negatively affects the production of biocatalysts, and at low concentrations, it reduces the catalytic activity of enzymes (Martim et al., 2021; Bensmail et al., 2015).

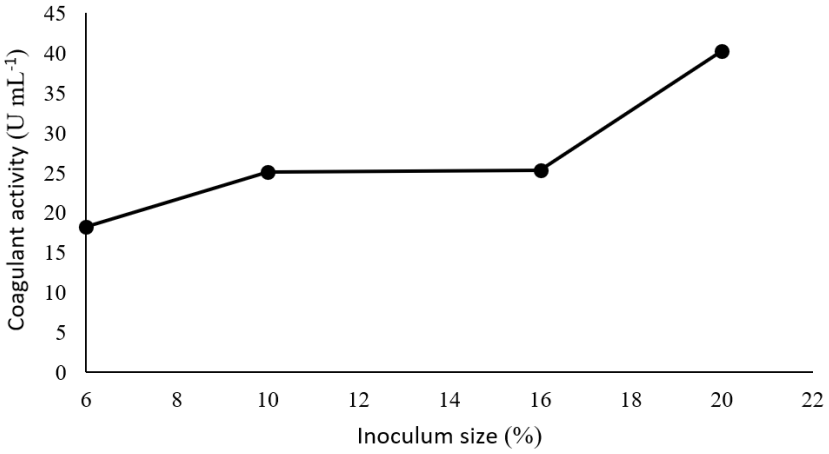

Figure 2. Effect of inoculum size on protease activity and milk coagulation of *Lentinus crinitus*.

Lentinus crinitus DPUA 1693 synthesized milk coagulants at all fermentation times (Figure 3). However, the significant value was verified at 10 days of fermentation (21.09 U mL^{-1}). In 14 days of culture, the coagulant action was 13.6% lower when compared to 10 days of fermentation, and the least expressive value was determined in eight days of submerged culture (9.76 U mL^{-1}). Similar results were described by (Martim et al., 2021) for *Pleurotus albidus* proteases. Ravikumar et al. (2012) and Aljammas et al. (2022) cited that *Pleurotus sajor-caju* and *Rhizomucor miehei* proteases produced coagulant with significant action in four and three days of fermentation, respectively. According to Mamo et al. (2020) the reduction in coagulant activity after the ideal period possibly occurs due to the depletion of nutrients available for mycelial growth.

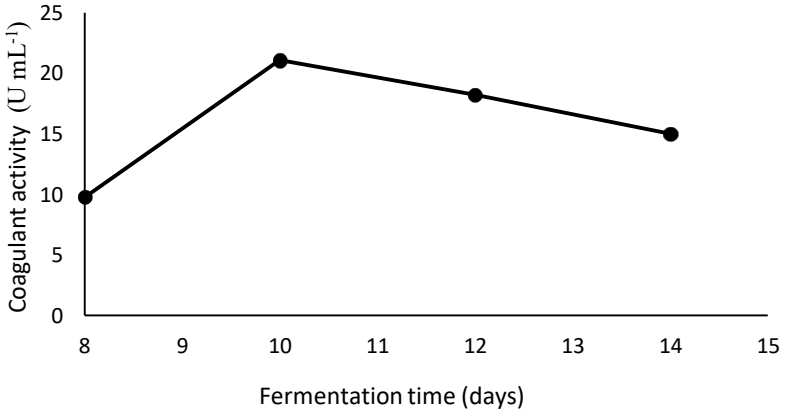

Figure 3. Effect of fermentation time on proteolytic activity and milk coagulation of *Lentinus crinitus*.

The influence of inoculum age on coagulant production by *L. crinitus* DPUA 1693 is shown in Figure 4. There was an increase in activity when increasing inoculum age, with a significant value obtained with a 16-day inoculum (33.49 U mL^{-1}). This result differs from those reported by (Martim et al., 2021) who found significant synthesis of coagulant proteases by *Pleurotus albidus* when using a 10-day inoculum. According to (Martim et al., 2021), inoculum age is a biological factor that affects coagulant production by edible mushrooms.

Casein hydrolysis by *Lentinus crinitus*

The protein profiles of *L. crinitus* were analyzed by electrophoresis, and subsequently the protease activity was revealed. Clear bands were observed in the gel corresponding to a variety of proteins found in the *L. crinitus* extract (Figure 5).

Gel zymography is of great importance because it assesses the enzymatic activity of enzymes produced by *L. crinitus*. Figure 4 shows the electrophoretic profile of caseinase action of proteases from *L. crinitus* extracts. Proteases in the 48-245 kDa range were able to hydrolyze casein, detected in the clear area on the gel, therefore evidencing the ability to degrade milk casein. Qasim et al. (2022) evaluated the proteinase activity of *Mucor racemosus* species in bands approximately 30 kDa and in the range of 80 kDa and 100 kDa. (Ning

et al., 2021) detected in the *Bacillus velezensis* extract the presence of 14 substrate hydrolysis bands in the range of 25 kDa to 180 kDa and bands below 11 kDa. The molecular weights of the proteases with caseinase action produced by *L. crinitus* found in this work are in agreement with the literature and show the presence of a diversity of enzymes with coagulant potential for application in cheese production.

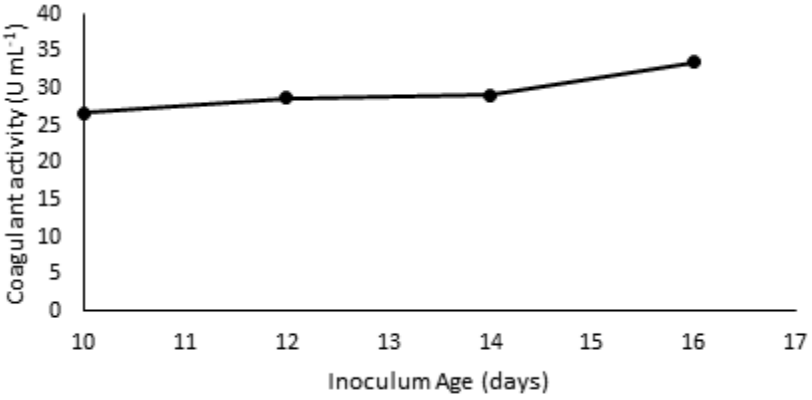

Figure 4. Effect of inoculum age (days) on proteolytic activity and milk coagulation of *Lentinus crinitus.*

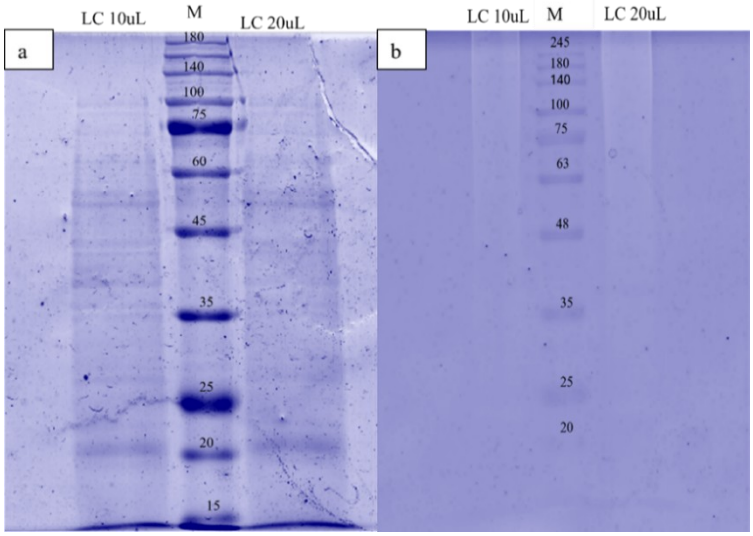

Figure 5. Protein enzymatic profile of *Lentinus crinitus* extract. (a) Zymogram indicating casein hydrolysis from *L. crinitus* extracts. (b). (LC) *L. crinitus* extract. (M) Molecular weight marker.

Conclusion

Lentinus crinitus DPUA 1693 is an edible mushroom species efficient for the production of bovine milk coagulant proteases and demonstrates economically viable nutrient selectivity for use in industrial bioprocesses. The effective parameters for coagulant production are as follows: inoculum size 20.0%, 10 days of fermentation, and inoculum with 16 days. The coagulant synthesized by *L. crinitus* DPUA 1693 is an innovative product that has desirable characteristics for use in cheese production.

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