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Original Article

Production of mycelial biomass, proteases and protease inhibitors by *Ganoderma lucidum* under different submerged fermentation conditions

Produção de biomassa micelial, proteases e inibidores de proteases por *Ganoderma lucidum* cultivado sob diferentes condições de fermentação submersa

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Abstract

Ganoderma lucidum is a medicinal mushroom widely recognized as a source of biomolecules with pharmacological properties, however, little is known about the factors that influence the synthesis of bioactive proteins by this fungus when cultivated under submerged fermentation. The objective of this work was to evaluate the production of mycelial biomass and intracellular proteases and protease inhibitors by *G. lucidum* cultivated under different submerged fermentation conditions. The cultivation was carried out in a medium composed of glucose (10 or 20 $g.L^{-1}$), soy peptone (2.5 or 5 $g.L^{-1}$) and yeast extract (5 $g.L^{-1}$), with incubation under agitation (120 rpm) and non-agitation, totaling 8 experimental conditions. Biomass production was determined from the dry weight, while glucose consumption was estimated by quantification of reducing sugars. The proteins were extracted in NaCl (0.15 M), and the protein extracts were submitted to protein quantification by the Bradford method, total proteolytic activity using azocasein, caseinolytic and fibrinolytic activity in Petri dishes, activity of serine (trypsin and chymotrypsin) and cysteine (papain) protease inhibitors. Cultivation in agitated condition showed higher biomass production with a maximum value of 7 $g.L^{-1}$, in addition to higher activities of trypsin, chymotrypsin and papain inhibitors, with 154 $IU.mg^{-1}$, 153 $IU.mg^{-1}$ e 343 $IU.mg^{-1}$ of protein, respectively. The non-agitated condition showed a greater potential for obtaining proteins, total proteases, caseinolytic and fibrinolytic enzymes, with maximum values of 433 $mg.g^{-1}$ of extract, 71 $U.mL^{-1}$ of extract, 63.62 mm^2 and 50.27 mm^2, respectively. Thus, a medium composed of soy peptone, yest extract and glucose in a 1:2:4 proportion is recommended, under agitation to produce protease inhibitors, and the non-agitated condition when the target is, mainly caseinolytic and fibrinolytic enzymes.

Keywords: medicinal mushroom, liquid fermentation, bioactive proteins, peptidases, anti-proteolytics.

Resumo

Ganoderma lucidum é um cogumelo medicinal amplamente reconhecido como fonte de biomoléculas com propriedades farmacológicas, entretanto, pouco se conhece acerca dos fatores que influenciam a síntese de proteínas bioativas por esse fungo, quando cultivado sob fermentação submersa. O objetivo deste trabalho foi avaliar a produção de biomassa micelial e de proteases e inibidores de proteases intracelulares por *G. lucidum* cultivado em diferentes condições de fermentação submersa. O cultivo foi realizado em meio contendo glicose (10 ou 20 $g.L^{-1}$), peptona de soja (2,5 ou 5 $g.L^{-1}$) e extrato de levedura (5 $g.L^{-1}$), com incubação sob agitação (120 rpm) ou não-agitado, totalizando 8 condições experimentais. A produção de biomassa foi determinada a partir do peso seco e o consumo de glicose a partir da quantificação de açúcares redutores. As proteínas foram extraídas em NaCl (0,15 M) e os extratos proteicos foram submetidos à quantificação de proteínas pelo método de Bradford, atividade proteolítica total usando azocaseína, atividade caseinolítica e fibrinolítica em placa de Petri, atividade de inibidores de serino-proteases (tripsina e quimotripsina) e cisteíno-protease (papaína). O cultivo em condição agitada apresentou maior produção de biomassa com valor máximo de 7$g.L^{-1}$, além de maiores atividades de inibidores de tripsina, quimotripsina e papaína, com 154 $UI.mg^{-1}$, 153 $UI.mg^{-1}$ e 343 $UI.mg^{-1}$ de proteína, respectivamente. A condição não-agitada demonstrou maior potencial para a obtenção de proteínas, proteases totais, enzimas caseinolíticas e fibrinolíticas, com valores máximos de 433 $mg.g^{-1}$ de extrato, 71 $U.mL^{-1}$ de extrato, 63,62 mm^2 e 50,27 mm^2, respectivamente. Assim, recomenda-se o meio composto de peptona de soja, extrato de levedura e glicose na proporção 1:2:4, em condição agitada para a produção de inibidores de proteases, e a condição não-agitada para a síntese de proteases, principalmente enzimas caseinolíticas e fibrinolíticas.

Palavras-chave: cogumelos medicinais, fermentação líquida, proteínas bioativas, peptidases, antiproteolíticos.

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1. Introduction

Ganoderma lucidum, known as Lingzhi or Reishi, is one of the most used medicinal mushrooms in East Asia for centuries, being used in formulations that aim to improve health, increase vitality and prolong life (Seweryn et al., 2021). Currently, it is known that the great potential of this mushrooms is associated with its composition of biomolecules, which include vitamins, minerals, phenolic compounds, alkaloids, terpenoids, sterols, polysaccharides, and proteins (consisting of essential amino acids such as leucine and lysine) (Ahmad et al., 2021).

As it is a species that is rarely found in nature, *G. lucidum* needs to be cultivated under controlled conditions to supply the demand of the international market, being quite common the cultivation in wood logs and plastic bags containing sawdust (Bulam et al., 2019). However, these cultivation methods can have disadvantages that include long production cycles, low yields and high cost. Therefore, submerged fermentation has received attention, as it is a promising alternative from an industrial point of view, with a short production cycle and higher productivity (Sun et al., 2021).

Among the classes of biomolecules produced by *G. lucidum,* proteases represent a group of enzymes capable of hydrolyzing proteins and peptides by breaking peptides bonds between amino acid residues, leading to the release of peptides and amino acids (Scott et al., 2021). These enzymes can be found in all living beings, since they are necessary in several biological processes, playing key roles in the degradation of specific proteins. Furthermore, proteolytic enzymes can be applied in different industrial processes, such as in the detergent, food and pharmaceutical industries (Naureen et al., 2022; Solanki et al., 2021).

Fibrinolytic enzymes, a group of proteases, are of great interest to the pharmaceutical industry, since they are involved in fibrinolysis, by the lysis of fibrin clots formed from the conversion of fibrinogen to fibrin by thrombin during the blood clotting process (Altaf et al., 2021). In this context, its application occurs when there is an imbalance between physiological fibrin formation and degradation, resulting in the formation of thrombus, causing the disease known as thrombosis. This pathological condition is the main cause of cardiovascular diseases, causing millions of deaths worldwide (Barzkar et al., 2022).

Despite their important physiological role, proteases have been correlated with the progression of several pathologies, such as bleeding disorders, hypertension, cancer, and neurodegenerative process (Rauf et al., 2021). In this context, protease inhibitors (PIs) correspond to proteins capable of interacting with proteases and restricting their catalytic activity on other proteins. As a result, PIs may have several beneficial health effects, including antibacterial, antioxidant, anti-obesity, anti-inflammatory, antifungal, hypotensive, antiproliferative and suppressive of oncogene expression activities (Cid-Gallegos et al., 2022).

In this scenario, there are already reports of proteases with fibrinolytic and antithrombotic activity, as well as protease inhibitors for *G. lucidum* (Choi and Sa, 2000; Kumaran et al., 2011a; Tian and Zhang, 2005). Thus, considering the potential application of these proteins and the little knowledge about the influence of different submerged fermentation conditions on the production of proteins by *G. lucidum*, the present work aimed to detect proteases and protease inhibitors in the mycelial biomass of *G. lucidum*, cultivated under different submerged fermentation conditions.

2. Material and Methods

2.1. Submerged fermentation

The fungus *Ganoderma lucidum* (CC22), provided by the company Funghi Flora and maintained at the Edible Fungi Cultivation Laboratory, belonging to the Technology and Innovation Coordination, of the Instituto Nacional de Pesquisas da Amazônia (COTEI-INPA), Manaus, Amazonas, Brazil. The fungus was initially reactivated in a Petri dish, containing PDA medium (potato dextrose agar) and incubated in BOD (Eletrolab, EL 101/3) at 25 °C. After growth, 7 mycelium discs (Ø = 7 mm) were inoculated into 250 mL erlenmeyes containing 125 mL of liquid medium, with an initial pH adjusted to 6.0 and composition described in Table 1.

The Erlenmeyers were incubated in a shaker for 12 days, at 25 °C, under agitated (120 rpm) and non-agitated conditions. After the fermentation period, the contents of the erlenmeyers were centrifuged (10 minutes, 10,000 rpm, 20 °C) to separate the mycelium from the fermented broth. The dry weight was determined from drying the micelial biomass in an oven at 60 °C, until constant mass was obtained.

2.2. Glucose consumption

Glucose consumption was determined by the DNS method (3,5-dinitrosalicylic acid), according to

Table 1. Composition of submerged fermentation culture media.

Medium components	Concentration ($g.L^{-1}$)			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Glucose	10	20	10	20
Yeast extract	5	5	5	5
Soy peptone	2.5	2.5	5	5

Vasconcelos et al. (2013). Samples of the fermented broth (0.5 mL) were incubated with 0.5 mL of DNS reagent (50 $g.L^{-1}$) at boiling temperature (~100 ºC) for 15 minutes. Subsequently, the reaction was stopped in cold-water bath, followed by the addition of distilled water (4 mL). Spectrophotometric readings were obtained at 540 nm and the concentration of reducing sugars was determined using a glucose standard curve.

2.3. Protein extraction

The mycelial biomasses (5 g) were subjected to protein extraction in 0.15 M sodium chloride (1:20 w/v), under agitation (2 hours, 120 rpm, 10 °C). After this period, the material was centrifuged (20 minutes, at 10,000 x*g*, 4 °C). The supernatant was dialyzed against distilled water for 48 hours under refrigeration (4 °C), then centrifuged (20 minutes, 5,500 rpm, 4 °C) and the supernatant was lyophilized, resulting in protein extracts.

2.4. Protein quantification

Protein concentration in the extracts was determined using the Bradford (1976) method, adapted for 96-well microplates. Samples (5 µL) were incubated with 250 µL of Bradford reagent (Sigma-Aldrich) for 10 minutes at 25 ± 2 °C. The absorbances were obtained from spectrophotometric readings at 595 nm and the protein concentration determined based on a standard curve of bovine serum albumin – BSA (Sigma-Aldrich).

2.5. Total proteolytic activity

The determination of the total proteolytic activity in the protein extracts was performed according to the methodology of Hamada et al. (2017), with adaptations, using the non-specific substrate azocasein. Initially, the samples were incubated with 12 µL of DTT (dithiothreitol, 3mM, Sigma-Aldrich) and EDTA (ethylenediaminetetraacetic acid, 2mM, Sigma-Aldrich), and 108 µL of sodium acetate buffer (50 mM, pH 5.0) for 10 minutes at room temperature (24 ± 2 °C). Subsequently, 60 µL of azocasein (1% w:v) were added, continuing the incubation for 1 hour at 37 °C. After this period, the reaction was stopped by the addition of 90 µL of TCA (trichloroacetic acid, 20%). The reaction product was centrifuged at 8,680 x*g* for 10 minutes. The supernatants were basified with NaOH (sodium hydroxide, 2M) and read spectrophotometrically at 420 nm. The results were expressed in units of activity per mL of extract ($U.mL^{-1}$), where U was defined as the amount of enzyme capable of increasing the absorbance by 0.01 units.

2.6. Caseinolytic and fibrinolytic activity

The determination of caseinolytic and fibrinolytic activities were performed according to Rovati et al. (2010). For the caseinolytic assay, a culture medium was prepared with agarose (1%) and skim milk (1%, Molico®, Nestlé, Brazil) dissolved in Tris-HCl buffer (50 mM, pH 7.8) and autoclaved at 1 atm, 15 minutes. The medium was transferred to a Petri dish (~20 mL), left to rest until solidification, where circular holes were made (Ø = 7 mm) and 10 µL of the extracts were added. The plates were incubated for 24 hours at 37 °C and the proteolytic activity was expressed in mm^2, calculated from the area of translucent halos produced by casein degradation.

To determine the fibrinolytic activity, 5 mL of a fibrinogen solution (0.5% w:v), 5 mL of agarose (5%) and 0.1 mL of thrombin (100 NIH $U.mL^{-1}$) were added to Petri dishes and left to rest for 30 minutes. Subsequently, circular holes were made (Ø = 7 mm) and 10 µL of extracts were added. The plates were incubated for 24 hours at 37 °C and the activity was determined from the area (mm^2) of the halos formed by the degradation of fibrin into soluble low molecular weight peptides.

2.7. Serine proteases inhibitors activities

The activities of serine protease inhibitors were determined with chromogenic substrates BAPNA (*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride) and BTPNA (Benzoyl-*L*-tyrosine-*p*-nitroanilide), specific for trypsin and chymotrypsin, respectively, according to the methodologies adapted from Menon and Rao (2012). Initially, the samples (35.3 µL) were pre-incubated with Tris-HCl (50 mM, pH 7.5) and the enzyme (trypsin or chymotrypsin, Sigma-Aldrich, 0.2 $mg.mL^{-1}$) at 37 °C for 30 minutes. Subsequently, the substrate (BAPNA or BTPNA, 70.6 µL, 1.25 mM) was added, continuing the incubation at 37 °C for 30 minutes. Finally, the enzymatic reaction was stopped by the addition of acetic acid (53 µL, 30% v:v) and the reaction product was read in a spectrophotometer at 405 nm. The results were expressed in units of inhibition per mg of protein ($IU.mL^{-1}$), where IU was defined as the amount of inhibitor capable of inhibiting one unit of protease activity.

2.8. Cysteine proteases inhibitors activity

The activity of cysteine protease inhibitors was determined using the synthetic substrate BANA (*N*-benzoyl-DL-arginine β-naphthylamide hydrochloride) and the dye DMACA (*p*-dimethylaminocinnamaldehyde), according to the methodology adapted from Cruz et al. (2013). Initially, 5.3 µL of papain (0.1 $mg.mL^{-1}$) was pre-incubated with 7.06 µL of activation solution (2 mM EDTA and 3 mM DTT) and 58.2 µL of sodium acetate buffer (50 mM, pH 6.0) for 10 minutes at room temperature, with subsequent addition of 17.65 µL of sample and incubation for 30 minutes at 37 °C. At the end of this period, the substrate BANA (1 mM) was added, and the enzymatic reaction was incubated for another 30 minutes at 37 °C. The reaction was stopped by adding 88.23 µL of hydrochloric acid (2% in ethanol), followed by the addition of 88.23 µL of DMACA (0.06% in ethanol) and rest for 40 minutes, before reading absorbance at 540 nm.

2.9. Statistical analysis

The experiments were arranged in a completely randomized design, consisting of 4 culture media and 2 incubation conditions (agitated and non-agitated), totaling 8 submerged fermentations. The biochemical assays were performed in triplicate and the statistical analysis was performed using Sisvar software (Ferreira, 2019). The data

obtained were subjected to analysis of variance (ANOVA), and the means compared by Tukey's test, with a significance level of 1% ($p \leq 0.01$). Data on proteolytic activity and protease inhibition were subjected to Pearson's Linear Correlation Analysis and to statistical comparison based on two-way ANOVA, using Prism software version 8.0.1.

3. Results

3.1. Micelial biomass production and glucose consumption

Among the culture conditions evaluated, experiment 2 under agitation (E2A) showed the highest production of mycelial biomass (7.02 ± 0.82 $g.L^{-1}$), differing statistically from the other experiments, with production about 6.6 times higher than experiment 3 in non-agitated (E3NA) condition, which presented the lowest mycelial biomass production. In experiments 1, 2 and 4, under non-agitated conditions (E1NA, E2NA and E4NA) there was no statistical difference in the value of biomass production (Figure 1-A).

Regarding the consumption of glucose (carbon source), obtained from the difference between the initial concentration (day 0) and the final cultivation (day 12), it was observed, in general, higher consumption of this sugar in the non-agitated condition, with highlight for E4NA (Figure 1-B). However, in E3NA, the lowest glucose consumption was observed.

3.2. Protein content

Protein contents in the extracts varied according to the agitation conditions, with the highest levels observed in the non-agitated experiments, with values in the order of 400 $mg.g^{-1}$. In general, the protein contents observed in E1NA and E2NA were about four times higher, compared to the agitated condition of these experiments. Whereas in E3NA and E4NA, the protein values were about twice as high compared to their respective experiments under agitation (Table 2).

3.3. Total proteolytic, caseinolytic and fibrinolytic activity

The total proteolytic activity was higher in protein extracts from mycelial biomass from fermentations under non-agitated conditions, being about 6 times higher in relation to the agitated conditions. Additionally, among the evaluated experiments, E3 showed the lowest proteases activities, both under agitated and non-agitated conditions. Regarding the components of the fermentation media, the activity of proteases was not influenced by the amount of carbon (10 and 20 $g.L^{-1}$ of glucose), while the highest concentrations of nitrogen (5 $g.L^{-1}$ of soy peptone) reduced the enzymatic activity (Table 3).

The casein and fibrin degradation activity were verified only under non-agitated conditions, with emphasis on experiment 2, which present the highest values for both activities. In contrast, experiment 3 did not show caseinolytic and fibrinolytic activity for the two conditions evaluated (Table 3). It is important to emphasize that the samples with caseinolytic and fibrinolytic activity correspond to those with higher protease activity, especially E1NA and E2NA, which did not show significant statistical differences.

Table 2. Protein content of extract obtained from the mycelial biomass of *Ganoderma lucidum* cultivated under different submerged fermentation conditions.

Fermentative conditions		Protein soluble ($mg.g^{-1}$ of extract)*
Experiment 1	Agitated	111.92 ± 6.2Ce
	Non-Agitated	420.17 ± 7.95Aa
Experiment 2	Agitated	122.28 ± 4.86Cde
	Non-Agitated	433.42 ± 2.12Aa
Experiment 3	Agitated	151.14 ± 5.84Bd
	Non-Agitated	242.64 ± 18.02Bb
Experiment 4	Agitated	203.78 ± 3.41Ac
	Non-Agitated	413.99 ± 10.88Aa

Data presented as mean ± standard deviation. *Capital letters compare the means of samples from the same incubation condition (agitated or non-agitated) and lowercase letters compare the means of all experimental treatments.

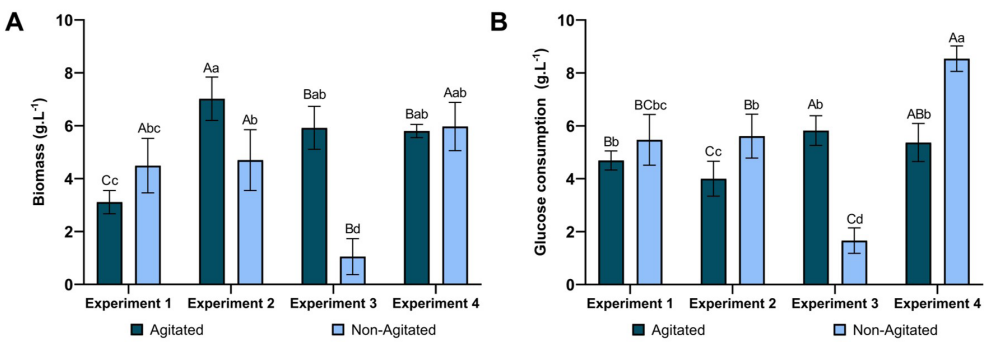

Figure 1. Mycelial biomass production (A) and glucose consumption (B) by *G. lucidum* under different conditions of submerged fermentation. Capital letters compare the means of samples from the same incubation condition (agitated or non-agitated) and lowercase letters compare the means of all experimental treatments.

3.4. Protease inhibitor activity

Protein extracts from the mycelial biomass of *G. lucidum*, in general, showed higher inhibitory activities against trypsin when cultivated under submerged fermentation in non-agitated condition, except for E1A, which showed activity in the order of 154 IU.mg^{-1} of protein in agitated condition. E3NA and E4NA exhibited the highest values of trypsin inhibition, with about 108 IU.mg^{-1} of protein, in non-agitated condition (Figure 2-A).

Regarding the inhibition of the chymotrypsin enzyme, only E1A and E2NA showed inhibitory activity. Among these extracts, chymotrypsin inhibition activity in E1A was about 14 times higher compared to E2NA (Figure 2-B). It is important to highlight that the E1A showed high

Table 3. Total proteolytic, caseinolytic and fibrinolytic activity of protein extracts from the mycelial biomass of *G. lucidum* cultivated under different conditions of submerged fermentation.

Fermentative conditions		Proteolytic activity (U.mL^{-1})*	Caseinolytic activity (mm^2)**	Fibrinolytic activity (mm^2)**
Experiment 1	Agitated	9 ± 1.2Bd	-	-
	Non-Agitated	70.9 ± 6.2Aa	44.38 ± 5.90B	50.27 ± 0.00A
Experiment 2	Agitated	9.1 ± 1.2Bd	-	-
	Non-Agitated	67.8 ± 5.5Aab	63.62 ± 0.00A	50.27 ± 0.00A
Experiment 3	Agitated	17,8 ± 3.5Ad	-	-
	Non-Agitated	35.8 ± 4.2Bc	-	-
Experiment 4	Agitated	16.1 ± 1.9Ad	-	-
	Non-Agitated	57.7 ± 2.2Ad	20.42 ± 7.85C	23.95 ± 4.32B

Data presented as mean ± standard deviation. *Capital letters compare the means of samples from the same incubation condition (agitated or non-agitated) and lowercase letters compare the means of all experimental treatments. **Capital letters compare the means for each enzyme.

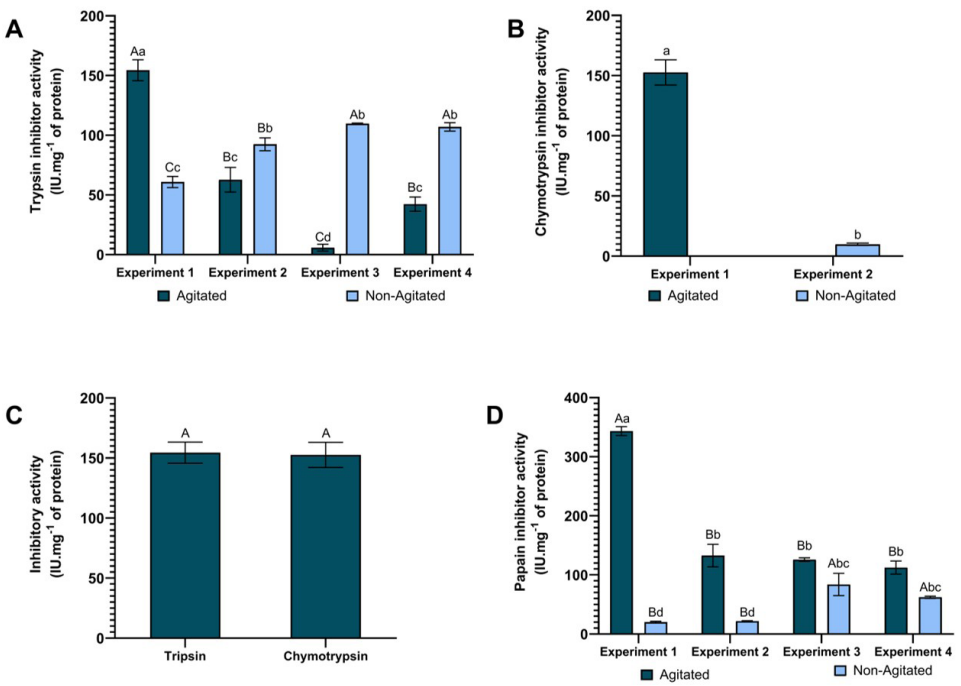

Figure 2. Activity of trypsin inhibitors (A), chymotrypsin inhibitors (B), comparison of trypsin and chymotrypsin inhibitory activity by E1A (C) and activity of papain inhibitors (D) in protein extracts from the mycelial biomass of *G. lucidum* cultivated under different conditions of submerged fermentation. Capital letters compare the means of samples from the same incubation condition (agitated or non-agitated) and lowercase letters compare the means of all experimental treatments.

inhibitory activity of trypsin and chymotrypsin, with no statistically significant differences in the inhibition of these enzymes (Figure 2-C).

All protein extracts showed inhibition of papain, with the highest values observed in fermentations under agitation, with emphasis on E1A, which was about 3 times higher compared to the agitated condition of the other experiments. Furthermore, it is possible to observe that in the non-agitated condition, the increase in the concentration of carbon source (10 to 20 $g.L^{-1}$) did not influence the activity of these inhibitors, while the increase in the concentration of the organic nitrogen (2.5 to 5 $g.L^{-1}$ of soy peptone) increased anti-papain activity by about 3 to 4-fold (Figure 2-D).

3.5. Correlation analysis

Pearson's linear correlation analysis was performed to verify relationship between proteolytic and protease inhibitor activity in different protein extracts. From the diagram obtained, it is possible to observe a strong correlation between the assays of fibrinolytic, caseinolytic and total proteases, showing a directly proportional relationship. Likewise, there is a direct correlation, with lesser intensity, between the results obtained for the protease inhibition assays. However, between the proteolytic and inhibitory activities, there is already a tendency of absence or inversely proportional correlation (Figure 3). Thus, it is possible to infer that the sample with activity of proteolytic inhibitors has a low chance of presenting a high enzymatic activity and vice versa.

The analysis of means based on two-way ANOVA was performed to statistically demonstrate the influence of the fermentation condition on the activity of proteases and proteolytic inhibitors, from the grouping of variables of each condition. In this case, the analysis is based on the staining intensity, determined by the score obtained for each sample in the activities (proteases in column A or inhibitors in column B), where a higher intensity corresponds to a higher value for each activity.

Regarding proteolytic activity (column A), the protein extracts from experiments 1 and 2 in non-agitated conditions (E1NA and E2NA), A2 and A4 in diagram, respectively, did not show a significant difference between their results. Thus, it can be inferred that the E1NA sample has a greater potential for application, as a lower concentration of carbon (glucose) was used during the submerged fermentation, representing a positive factor in reducing the cost of the process (Figure 4).

The intensity close to zero (white color), obtained for the proteolytic activity in some extracts, may be due to the high number of outliers (presence of proteolytic activity, but with absence of caseinolytic and fibrinolytic activities) (Figure 4). As for the activity of protease inhibitors (column B), it is confirmed that E1A presents higher activities of protease inhibitors, demonstrated by the highest intensity of staining compared to the other extracts. Furthermore, this analysis showed a value of $p < 0.05$, indicating that there is a significant variation between proteolytic and inhibitory activity, depending on the culture condition (Figure 4).

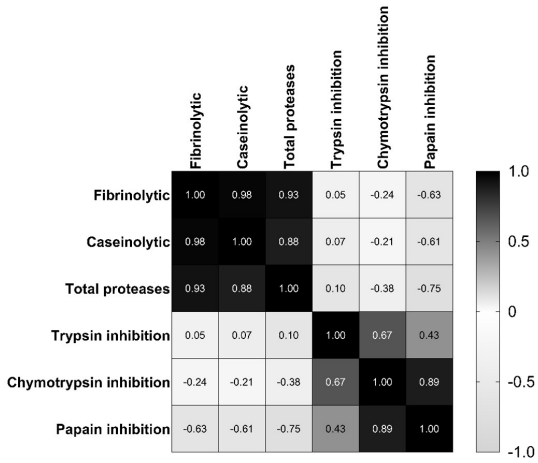

Figure 3. Pearson correlation coefficient matrix color block diagram between the protease and protease inhibitor activities.

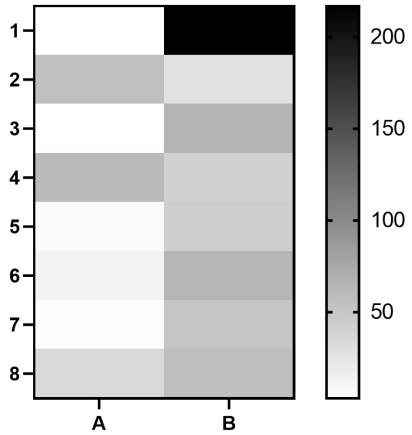

Figure 4. Two-Way ANOVA comparation diagram between the scores obtained from the means of protease and protease inhibitor activity. (A) Score of proteolytic activity; (B) Score of protease inhibitor activity. 1 – E1A; 2 – E1NA; 3 – E2A; 4 – E2NA; 5 – E3A; 6 – E3NA; 7 – E4A; 8 – E4NA.

4. Discussion

Wagner et al. (2004) when evaluating the submerged cultivation of *G. lucidum*, obtained a biomass production value of 7.4 $g.L^{-1}$ on the 13th day of a pre-culture in a medium composed of glucose, peptone, yeast extract and mineral salts, corroborating with the values obtained in the E2A of the present work. Fang and Zhong (2002), when evaluating the influence of the use of different concentration of glucose on the production of mycelial biomass of *G. lucidum,* under submerged fermentation, verified a greater accumulation of biomass in a medium containing 20 $g.L^{-1}$ of glucose, as well as verified for E2A in relation to E1A (10 and 20 $g.L^{-1}$, respectively).

Regarding the E3NA fermentation, a hypothesis that may explain the lower biomass production is the

composition of the medium, since the yeast extract and soy peptone are sources of organic nitrogen, in addition to the carbon:nitrogen ratio (C:N) was close to 1:1, which may be unfavorable to mushroom growth. On the other hand, in the same experiment under agitated conditions, high biomass production was observed. Thus, a possible explanation is that the agitation process strongly influences the production of extracellular enzymes, directly affecting the nutrient viability of the culture medium, favoring the growth of the fungus in this microenvironment (Abusham et al., 2009; Purwanto et al., 2009).

The literature describes that the combination of yeast extract, peptone and glucose in a 1:1:4 ratio has a positive effect on the cell growth of the mushroom *G. lucidum* (Cheong et al., 2018). This proportion is similar to that used in experiment 4 (E4A and E4NA), which present one of the highest yields of mycelial biomass. However, considering the importance of reducing process costs, E2A is of greater interest, given the lower concentration of nitrogen used in this experiment.

The consumption of the carbon by a microorganism in a substrate is one of the most important variables to be analyzed during fermentation optimization processes, as it is directly related to cell growth and product formation, such as mycelial biomass or specific metabolites of interest and, therefore represent the efficiency of the bioprocess (Liu, 2016; Poznyak et al., 2018). In E3NA, low glucose consumption was observed, corroborating the hypothesis that the lower growth in this condition is associated with the carbon-nitrogen ratio. An interesting fact is that this problem is also reported in solid-state fermentation, where excess nitrogen in the substrates slows or inhibits the mycelial growth of *G. lucidum* (Rolim et al., 2014). Finally, in this study it can be inferred that in the E4NA condition, *G. lucidum* presents greater efficiency in the conversion of glucose into other products of its metabolism.

Slynko et al. (2017) detected a protein concentration of 2.03 $\mu g.mg^{-1}$ in aqueous extracts from the basidiocarps of *G. lucidum*, value below those of the present work. Furthermore, the difference in the protein content between the agitation and non-agitated condition may be due to the shear forces of the agitation process, damaging the mycelium structure and, consequently, causing the leakage of cellular content into the culture medium (Elisashvili 2012; Petre and Petre 2016). Thus, it can be inferred that the cultivation of *G. lucidum* under submerged fermentation in a non-agitated condition favors the process of protein recovery from mycelial biomass, resulting in protein extracts with a higher content of soluble proteins.

Martim et al. (2017) when working with submerged fermentation of *G. lucidum* at 150 rpm, in a culture medium similar to experiment 4 of the present work, but supplemented with gelatin, found a proteolytic activity of 23.5 $U.mL^{-1}$. Thus, it is suggested that the cultivation of *G. lucidum* in submerged fermentation in non-agitated condition is more efficient in the recovery of proteases. Additionally, Silva (2015) when studying the submerged culture broth of *G. lucidum*, composed of yeast extract, peptone and glucose (1:2:4), obtained proteolytic activity of 13.11 $U.mL^{-1}$. Thus, it is possible to suggest that a higher concentration of yeast extract, in relation to peptone, may be more efficient in the production of proteases.

Regarding the composition of the culture medium, the nitrogen concentration directly influences the production of these enzymes, where the reduction of available nitrogen in the culture medium is related to the synthesis of proteases, while the presence of easily metabolizable nitrogen sources is related to the inhibition/reduction of synthesis and enzymatic activity (Inácio et al., 2015). As previously discussed, considering that agitation in a fermentation process influences the occurrence of shear forces, causing damage to the fungal hyphae and leading to changes in the yield of its metabolism products (Kirsch et al., 2016), it is possible to infer that in the fermentation in non-agitated fermentation there is a higher concentration of these enzymes in the mycelial biomass, since shears do not occur and, therefore, there is no excretion of these biomolecules into the extracellular medium.

The milk casein degradation assay is widely performed in studies that aim to obtain fibrinolytic enzymes, being common for the screening of samples. The application of this pre-test is based on the linear relationship between caseinolytic and fibrinolytic activity with the enzyme plasmin (Cole and Mertz, 1961). This relationship was later confirmed by Moser and Frey (1966), who reported the possibility of using mathematical formulas for the conversion of caseinolytic activity to fibrinolytic activity of plasmin and other fibrinolytic agents.

Osmolovskiy et al. (2022) when working with several species of micromycetes cultivated under submerged fermentation, found a correlation between the results of caseinolytic and fibrinolytic activities. These authors also propose that proteases with higher casein degradation, such as E2NA in the present study, are likely to cleave globular proteins more effectively, whereas those with higher fibrin degradation, such as E1NA and E4NA, may be more active against fibrillar proteins.

The production of fibrinolytic enzymes has already been reported for different strains of the species *G. lucidum*, both in their basidiocarps (solid state fermentation), and in mycelial biomass (submerged fermentation), as well as in their mycelium obtained from Petri dish cultivation in agar-added media (Cha et al., 2011; Choi and Sa, 2000; Kumaran et al., 2011b). Kumaran et al. (2010), when testing different carbon sources in the induction of the synthesis of intracellular fibrinolytic enzymes by *G. lucidum*, found a greater potential for glucose, while among the nitrogen sources, peptone stood out, followed by yeast extract. Thus, it can be inferred that the cultivation conditions of the E1NA and E2NA samples are promising for the production of these proteases.

Sales-Campos et al. (2021), when working with saline extracts from the basidiocarp of *Pleurotus ostreatus*, obtained a fibrinolytic activity of 3.14 ± 0.00 mm^2. Okamura-Matsui et al. (2003), when studying the mycelium of the mushrooms *Agaricus blazei*, *Grifola frondosa*, *Laetiporus sulphureus* e *Pleurotus eryngii*, found a fibrinolytic activity of 35 ± 1; 42 ± 1; 36 ± 1 and 63 ± 1 mm^2, respectively. Therefore, the samples of the present work may represent a high potential as a source of these enzymes, mainly E1NA.

Tian and Zhang (2005), when isolating a protein of 38 kDa from the mycelial biomass of *G. lucidum*, with specificity for proteinase A, also observed a half-affinity interaction for trypsin with a small inhibition activity. Trypsin inhibitors have also been reported in other mushroom species, such as *Armillaria mellea*, *Amanita Phalloides*, *Abortiporus biennis*, *Macrolepiota procera*, *Pleurotus floridanus* and *Pleurotus ostreatus* (Ali et al., 2014; Lukanc et al., 2017; Sales-Campos et al., 2021; Zuchowski et al., 2009).

Lim et al. (2003) when working with extract of the basidiocarp of *G. lucidum*, obtained a specific chymotrypsin inhibitory activity of 67.3 UI.mg^{-1} of protein, being identified four isoforms. Thus, comparing this specific activity obtained by the authors with that observed for E1A, it can be inferred that submerged fermentation represents a faster alternative for obtaining these proteins, in addition to providing a higher recovery yield of this inhibitor.

As for the low or absence of inhibition of the chymotrypsin enzyme by most protein extracts and the high potential of trypsin inhibition, the scientific literature cites inhibitors of serine proteases belonging to the I66 family, known as mycospins, which are non-secretable cytoplasmatic proteins, synthesized by macrofungi for the regulation of the activity of endogenous proteases, as well as for the defense against predators and pathogens. Among this class, inhibitors with high specificity for trypsin and low specificity for chymotrypsin can be found, as already reported in mushrooms such as *Lentinula edodes*, *Clitocyber nebularis* and *Coprinopsis cinerea* (Sabotič and Kos, 2016; Sabotič et al., 2019).

Dual inhibitory activity (trypsin and chymotrypsin) may be to modifications of amino acid residues at the P1 position in the loops of inhibitors of the mycospins family, which may result in the production of double-headed inhibitors capable of inhibiting trypsin and chymotrypsin (Sabotič et al., 2019). Thus, although the natural occurrence of this type of inhibitors has not yet been reported in macrofungi, it is suggested that the inhibitors present in the E1A extract correspond to a double-headed inhibitor, similar to Bowman-Birk family, commonly reported in plant species of the Fabaceae family, capable of to interact with two proteases of the same or different classes, simultaneously or independently, with specific reactive sites for trypsin and chymotrypsin (Rachel and Sirisha, 2014).

Although E1A showed inhibition potential for all classes of proteases evaluated in the present work, the highest inhibitory activity observed was against papain, indicating the occurrence of cysteine protease inhibitors. Sales-Campos et al. (2021) when working with saline extracts of *P. ostreatus* and testing the inhibition potential against trypsin and papain, obtained inhibitory values in the range of 240 IU.mg^{-1} of protein for serine protease, and of 150 IU.mg^{-1} of protein for cysteine proteases, which differs from that found in the present study, however, it is important to emphasize the possibility of variation in the specificity of inhibition for different proteases from the same protein extract of a mushroom.

Cysteine protease inhibitors from the mycocypins family are normally low molecular weight cytoplasmatic proteins that, like mycospins, act in the regulation of endogenous proteolysis, as well as in defense against predators and pathogens. In addition, these inhibitors can act on proteases of different origins and show strong inhibition against papain-like enzymes (Sabotič et al., 2019).

Obtaining these inhibitors become important since they are closely associated with the protection of the organism against parasitic microorganisms that use cysteine proteases to invade the host tissue (Tušar et al., 2021). Additionally, the inhibition of papain-like enzymes has gained notoriety recently due to the COVID-19 pandemic, where several studies have evaluated *in silico* the potential of natural products in inhibiting a SARS-CoV-2 papain-like protease (PLpro), seeking an alternative treatment for this infection, since this enzyme is essential for viral replication, being responsible for processing polyproteins, making them functional (Parmar et al., 2022).

5. Conclusion

G. lucidum when cultivated under different conditions of submerged fermentation showed variations in biomass production, protein content and bioactivity. Thus, experiment 1 non-agitated is recommended to produce proteases, including fibrinolytic enzymes, while the same experimental condition under agitation is more favorable for the synthesis of proteases inhibitors. Additionally, experiment 2 under agitation may be favorable to biomass production. Furthermore, the occurrence of double-headed inhibitors in *G. lucidum* is suggested, however further biochemical studies are strongly recommended to confirm this hypothesis.

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