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Cultivation conditions and biochemical characterization of the proteolytic enzymes with fibrinolytic action obtained from mushrooms in the last ten years

Condições de cultivo e caracterização bioquímica das enzimas proteolíticas com ação fibrinolítica obtidas a partir de cogumelos nos últimos dez anos

Condiciones de cultivo y caracterización bioquímica de las enzimas proteolíticas con acción fibrinolítica obtenidas a partir de hongos de los últimos diez años

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Abstract
Fibrinolytic enzymes are proteases that are capable of degrading the fibrin mesh present in blood clots and can be obtained through fungal extracts. Thus, the objective of this paper was to seek out and compare all the information regarding the production and biochemical characterization of fibrinolytic enzymes of mushroom species published in papers in the last ten years. The research was carried out in eight national and international electronic databases using the terms: "fungal enzymes", "fibrinolytic Proteases", "thrombosis" and "mushrooms". The results obtained were analyzed according to each step. In the first stage, the titles and abstracts of all papers were analyzed independently; in the second stage, all papers were read and those that did not meet the inclusion criteria were excluded. Then, the remaining papers were reviewed and the data were tabulated and compared. As a result, it was found that enzymes with fibrinolytic action were obtained from eight species of mushrooms in the period of this research: *P. sajor-cashew, P. ferulae, P. ostreatus, L. shimeji, A. polytricha, H. erinaceum, C. comatus* and *C. militaris*. The enzymes were identified as protease SPPs, metalloproteases, serine proteases and serine metalloproteases. The methods used to evaluate fibrinolytic activity were the fibrin plate method and/or the formation of artificial thrombus through thrombin and fibrinogen. The molecular mass of these enzymes ranged from ~18 to 66 kDa and, for the biochemical characterization of the extracts, the pH ranged from 4.0 to 9.5 and the optimum temperature from 25 to 70 ºC. Via the review, it was observed that few articles were published on the subject in question, which made it necessary to carry out more studies to discover the biotechnological properties of mushrooms and define their applications in the most diverse sectors of industries, not only in commercial species of mushrooms, but also in native species.
Keywords: Fibrinolytic proteases; Mushroom; Thrombolytic therapy; Fibrin.

Resumo

Enzimas fibrinolíticas são proteases que degradam a fibrina presente em coágulos sanguíneos e podem ser obtidas através de extratos fúngicos. Assim, o objetivo do estudo foi analisar todas as informações referentes a produção e caracterização de proteases fibrinolíticas de espécies de cogumelos publicados em plataformas cientificas dos últimos dez anos. A busca foi realizada em oito bases de dados eletrônicos nacionais e internacionais utilizando diversos palavras-chave. Os resultados obtidos foram analisados de acordo com cada etapa: na primeira etapa, os títulos e resumos de todos os artigos foram analisados de forma independente; na segunda etapa todos os artigos foram lidos e aqueles que não atendiam aos critérios de inclusão, foram excluídos. Em seguida foram revisados os artigos restantes e os dados tabulados e comparados. Foi verificado que enzimas com ação fibrinolíticas foram obtidas de oito espécies de cogumelos: *P. sajor-caju, P. ferulae, P. ostreatus, L. shimeji, A. polytricha, H. erinaceum, C. comatus* e *C. militaris. As enzimas foram identificadas como protease SPPS, Metaloprotease, Serina Aspártica e Serina Metaloprotease*. Além disso, dois métodos de determinação da atividade fibrinolítica foram identificados, o método da placa de fibrina e por formação do trombo artificial. A massa molecular das enzimas variou entre ~18 a 66 kDa, a caracterização bioquímica dos extratos teve o pH de 4,0 a 9,5 com temperatura ótima de 25 a 70 ºC. Dessa forma, foi possível concluir que os cogumelos são fontes de proteases fibrinolíticas e que podem ser indicados no tratamento de trombose.

Palavras-chave: Proteases fibrinolítica; Cogumelos; Terapia trombolítica; Fibrina.

Resumen

Las enzimas fibrinolíticas son proteasas que degradan la fibrina presente en los coágulos sanguíneos y pueden obtenerse a través de extractos fúngicos. Por tanto, el objetivo de este trabajo fue analizar toda la información sobre la producción y caracterización bioquímica de proteasas fibrinolíticas de especies de hongos publicada en artículos de los últimos diez años. La investigación se realizó en ocho bases de datos electrónicas nacionales e internacionales utilizando varios términos clave. Los resultados obtenidos se analizaron de acuerdo a cada paso. En la primera etapa, los títulos y resúmenes de todos los trabajos fueron analizados de forma independiente; en la segunda etapa se leyeron todos los trabajos y se excluyeron aquellos que no cumplían con los criterios de inclusión. Se revisaron los documentos restantes y se tabularon y compararon los datos. Como resultado se encontró que se obtuvieron enzimas con acción fibrinolítica de ocho especies de hongos en el periodo de esta investigación: P. sajor-cashew, P. ferulae, P. ostreatus, L. shimeji, A. polytricha, H. erinaceum, C. comatus y C. militaris. Las enzimas se identificaron como proteasas SPPs, metaloproteasas, serina proteasas y serina metaloproteasas. Además, Se han identificado dos métodos para determinar la actividad fibrinolítica, el método de placas de fibrina y/o la formación de trombos artificiales. La masa molecular de estas enzimas osciló entre ~18 y 66 kDa y, para la caracterización bioquímica de los extractos, el pH osciló entre 4,0 y 9,5 y la temperatura óptima entre 25 y 70 ºC. A través de la revisión se constató que los hongos son fuente de proteasas fibrinoliticas e que estos pueden actuar para el tratamiento de la trombosis.

Palabras clave: Proteasas fibrinoliticas; Hongos, Terapia trombolítica; Fibrina.

1. Introduction

Enzymes are biocatalysts that accelerate the speed of specific chemical reactions. Biotechnology has revolutionized the use of traditional enzymes so that they can be applied in commercial and industrial fields (Wu et al., 2021). Global demand and trade for industrial enzymes is growing steadily and is estimated to reach $7 billion by 2023 (Fasim et al., 2021).

Proteases are enzymes that catalyze the hydrolysis (covalent bond breaking with the participation of a water molecule) of peptide bonds of other proteins, cleaving them into amino acid fragments. Among the various applications of proteases, fibrinolytics stand out, since they are able to degrade fibrin, the most abundant protein in blood clots (Ali et al., 2022; Li et al., 2021). Clot formation is usually regulated by the biological system; but, when fibrin is not hydrolyzed, thrombosis and other cardiovascular diseases can occur (Sharma et al., 2021).

Mushroom extracts are widely used as nutritional supplements and medicines, and present benefits for human health. Most fibrinolytic enzymes (FEs) used industrially are of microbial origin and especially of fungal origin (Altaf et al., 2021). The main advantages with fibrinolytic agents of microbial origin are that they have high yields in activity, reproducibility, economic production, exponential growth, easy optimization and mass production of FEs. These advantages are the main criteria for meeting global market demand on an industrial scale (Katrolia et al., 2020; Acosta et al., 2022).

Proteases have been purified and characterized from reproductive structures (basidiome or ascoma) of many species of medicinal or edible mushrooms, including *Pleurotus ostreatoroseus*, *Pleurotus albidus*, *Pleurotus djamor*, *Pleurotus florida*,

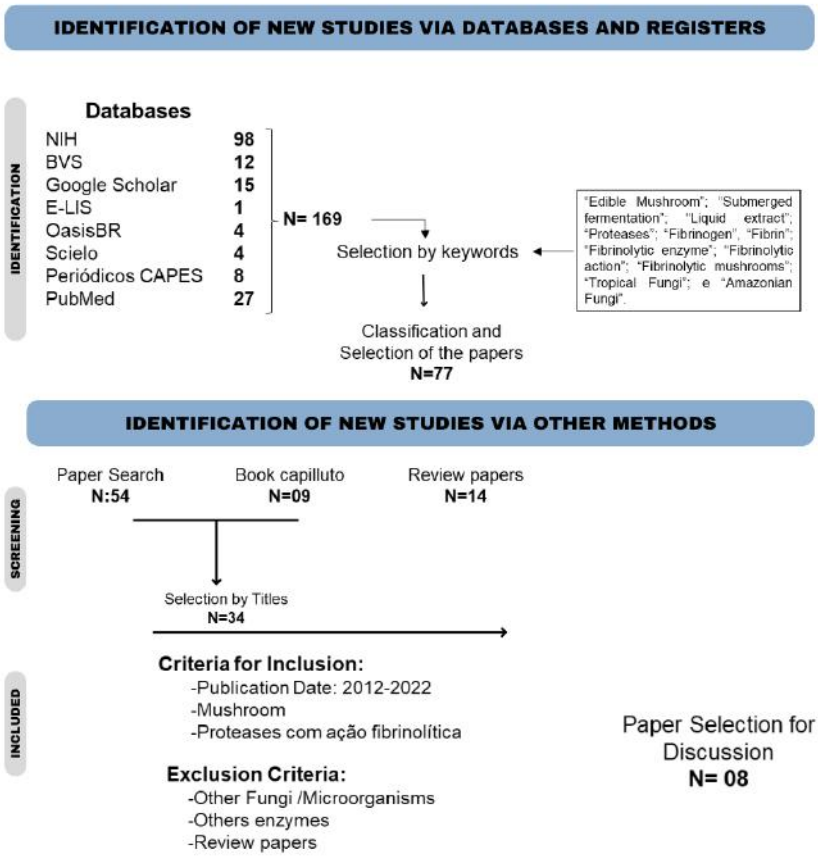
Pleurotus ostreatus, Coprinus Comatus, Hericium erinaceum, Auricularia polytricha, Lyophyllum shimeji, Cordyceps militaris and *Lentino citrinos* (Fonseca, 2013; Machado et al., 2016; Brabosa et al., 2020; Pimenta et al., 2021; Brito et al., 2021; Coelho et al., 2022). Although mushrooms are sources of proteases, there are little data available in the literature on their fibrinolytic action. The objective of this review was to present an overview of the features, production and biochemical characterization mechanisms of fibrinolytic protease from mushroom species.

2. Methodology

2.1 Included, screening, and identification of new studies via databases and registers

This bibliographic review was conducted following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) checklist, which is a method also used by (Bezerra et al., 2021). For data collection, a survey was conducted in international electronic databases (Web of Science (WoS) Core Collection electronic database, National Center for Biotechnology Information, E-prints in Library and Information Science and Scientific Electronic Library Online, and Brazilian Open Access Publications and Scientific Data Portal, Regional Portal of the Virtual Health Library-VHL, CAPES Journal Portal, as shown in Figure 1.

Figure 1 - Flow diagram of literature search and selection criteria.



Source: Authors (2022).

The results of the literature (research papers, reviews, and book chapters) survey were filtered using terms: “Edible Mushroom”; “Submerged fermentation”; “Liquid extract”; “Proteases”; “Fibrinogen”, “Fibrin”; “Fibrinolytic enzyme”;

"Fibrinolytic action"; "Fibrinolytic mushrooms"; "Tropical Fungi"; and "Amazonian Fungi" in the topic item that includes the title, summary, and keywords. Additionally, we restricted our search to reports published from January 1, 2012 to January 30, 2022.

2.2 The selection of the papers was conducted in two stages.

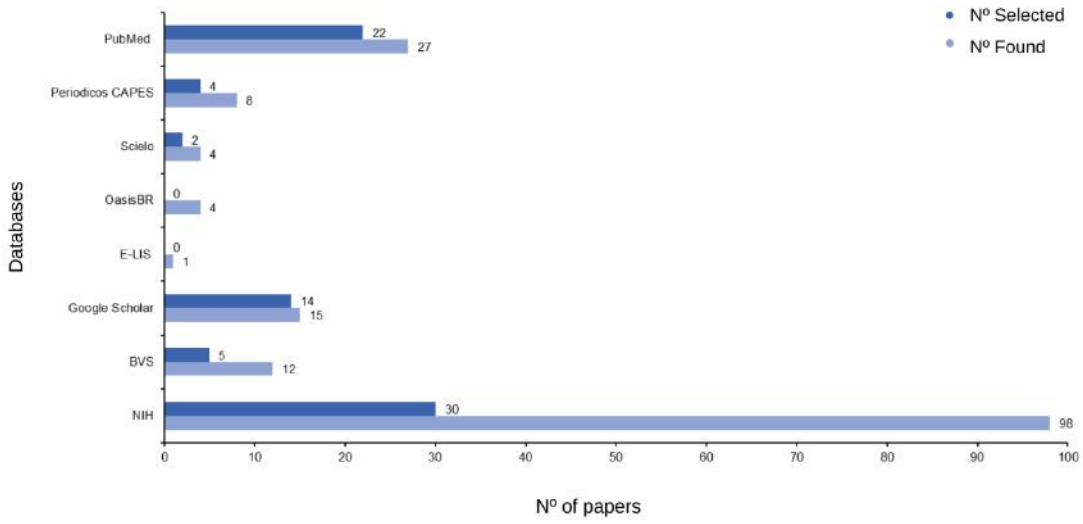
In stage one, the titles and abstracts of all the papers were analyzed independently. This first review selected papers that seemed to meet the inclusion criteria based on the title and abstract. Studies that did not meet any of the inclusion criteria or that were not related to the topic of this review were excluded. In stage two, the papers were read and those that did not meet the inclusion criteria were excluded. Finally, the studies were reviewed and selected for analysis and discussion of the data.

3. Results and Discussion

3.1 Study selection and characteristics

After applying the search procedures in the electronic platforms established in the methodology, initially, twelve keywords were used to elaborate and contextualize this study. However, for the discussion of this paper, only four relevant keywords were needed: fibrinolytic proteases, mushroom, thrombolytic therapy, fibrin. More than 50% were found in the National Center for Biotechnology Information – NIH platform. Of these, few references were obtained in the databases E-LIS, OasisBR, Scielo and periódicos CAPES (Figure 2).

Figure 2 - Graph illustrating numbers of papers selected from the databases, according to the criteria.



Source: Authors (2022).

Of the 34 papers were found involving fibrinolytic protease enzymes produced by mushrooms. Subsequently, after applying the inclusion and exclusion criteria, a review of the full texts was completed, and eight papers were selected for this review. Following the selection criteria according to title and abstract, 46% of the papers were analyzed, all review papers were excluded, among the other criteria already established in the methodology. All eight papers were read and the main characteristics of the studies, including authorship, impact factor, number of citations, title and most relevant considerations of the study were summarized (Table 1).

Table 1 - Characterization of the main characteristics of the eight selected papers: a descriptive analysis.

Author(s)/Year/Journal	Impact factor/number of citations	Title	Study considerations
Xiao-lan Liu, Xiqun Zheng,Juan-kun Zhang (2012) Applied Mechanics and Materials	5.587**-5.246* 7	Production of a Fibrinolytic Enzyme from *Coprinus comatus* YY-20	The enzyme of the species under study can be applied as a natural agent in the form of oral anticoagulants, in the therapy or prevention of thrombosis.
Bong-Suk Choi, Kumar Sapkota, Jun-Hui Choi, et al., (2013) Appl Biochem Biotechnol	3.014**-2.431* 44	Herinase: A Novel Bi-functional Fibrinolytic Protease from the Monkey Head Mushroom, *Hericium erinaceum*	Enzyme isolation was performed and the biochemical properties of herinase appear to be a promising for further studies in thrombolytic therapy.
Sharjahan Mohamed Ali, Tau Chuan Ling, et al., (2014) Separation and Purification Technology	8.425**-7.312* 46	Recovery and partial purification of fibrinolytic enzymes of *Auricularia polytricha* (Mont.) Sacc by an aqueous two-phase system	The crude lyophilized extract showed significant fibrinolytic activity (10.83 U/mg). This was purified (ATPS) and its enzyme characterized for which the molecular weight of the enzyme is 66 kDa.
Xiao-lan Liu, Xi-qun Zheng, Peng-zhi Qian, et al., (2014) J. Microbiol. Biotechnol	3.45**-2.351* 50	Purification and Characterization of a Novel Fibrinolytic Enzyme from Culture Supernatant of *Pleurotus ostreatus*	The fibrinolytic enzyme of *Pleurotus ostreatus* can also be applied as a natural agent for oral fibrinolytic therapy or thrombosis prevention.
Sung-Min Moon, Jae-Sung Kim, et al., (2014) Journal of Bioscience and Bioengineering	3.313**-2.894* 43	Purification and characterization of a novel fibrinolytic, a chymotrypsin like serine metalloprotease from the edible mushroom *Lyophyllum shimeji*	Chymotrypsin as purified serine-metalloprotease may be useful for thrombolytics, similar to the known fibrinolytic enzymes such as lumbrokinase and nattokinase.
Xiaolan Liu, Narasimha kumar Kopparapu, et al., (2015) Journal of Agricultural and Food Chemistry	5.504 **- 5.279 * 22	Purification and Biochemical Characterization of a Novel Fibrinolytic Enzyme from Culture Supernatant of *Cordyceps militaris*	The enzyme in this research may act as an anticoagulant and prevent thrombosis. Therefore, the enzyme can be used as a therapeutic agent for the treatment of thrombosis.
Jun-Hui Choi, Dae-Won Kim, Seung Kim, Sung-Jun Kim (2017) Preparative Biochemistry and Biotechnology	2.358** 17	Purification and partial characterization of a fibrinolytic enzyme from the fruiting body of the medicinal and edible mushroom *Pleurotus ferulae*	Evidence of antithrombotic potential by the action of the metalloprotease enzyme isolated from the mushroom, showing effective activity against thrombin and fibrinogen, necessary for the formation of the fibrin clot.
Maroua Omrane Benmrad, Sondes Mechri, et al. (2019) BMC Biotechnology	3.252**-2.563* 38	Purification and biochemical characterization of a novel thermostable protease from the oyster mushroom *Pleurotus sajor-caju* strain CTM10057 with industrial interest	This mushroom constitutes a new source of proteolytic activity (SPPS) for the applications, showing a high degree of hydrolysis and catalytic efficiency when compared to SPTC and the commercial proteases Flavourzyme® 500 L and Thermolysin type X.

Impact factor according to the Web of Science (*) and Scopus (**) databases. **Source:** Authors (2022).

In this context, the impact factor (IF) is one of the criteria for confirming the quality of journals and also the technical resource of evaluation in journals (Strehl, 2005; Ruiz et al., 2009). It was observed that more than 55% of the selected papers were published in different journals, with an impact factor ranging from 0.738 to 7.312 in the Web of Science collection data, and from 0.494 to 8.425 in the Scopus data.

Of these, the article by Ali et al. (2014) was published in the Journal Separation and Purification Technology, which presented the highest impact factor (8.425), and the article by Liu et al. (2014) was published in the Journal Microbiology Biotechnology, which presented the lowest impact factor (2.351).

Another factor analyzed in this study was how often the selected papers were cited and referenced by other authors. The paper authored by Liu et al. (2014), published in the Journal of Microbiology and Biotechnology, had the highest number of citations (50), and the paper by Liu et al. (2012), published in the journal Applied Mechanics and Materials, had the lowest, with seven citations. Finally, all the studies evaluated had as their main objective to verify the potential of proteolytic enzymes with fibrinolytic action produced by mushrooms in their most varied forms of cultivation.

3.2 Review and analysis of papers

Among the fungi found in this study, it can be seen that mushrooms produce a variety of enzymes that meet the nutritional and pharmaceutical requirements for development and improvement in the human physiological process. They present mild processing conditions, catalyze reactions and are chemically and environmentally sustainable and less toxic (Sharma; Bajaj, 2017).

Liu et al. (2012) studied the mushroom *C. comatus* and the enzyme extracted from the submerged culture, which was not characterized, but showed catalytic activity of 164.49 U/mg, under conditions of pH 5.0 at 25 ºC. Choi et al. (2013) analyzed and characterized the enzyme extracted from *H. erinaceum*, which was a metalloprotease type, with reaction at 220.65 U/mg, at a slightly acidic pH (5.5) and at 30 ºC.

Ali et al. (2014) demonstrated that *A. polycrita* had significant fibrinolytic activity at 10.83 U/mg, when extracted from the lyophilized basidiome, under conditions of neutral pH (7.0) at 37 ºC. Moon et al. (2014) concluded that the mushroom *L. shimeji* has an enzyme of serine metalloprotease type with the same action of lumbrokinase and nattokinase. The catalytic reaction of the enzyme was determined as 178.9 U/mg under alkaline conditions (pH 8.0) at 37 ºC.

Choi et al. (2016) disccovered that metalloproteases obtained from the *P. ferulae* basidiome have antithrombotic potential. The catalytic reaction, effect and stability of the enzyme was estimated at 1253.33 U/mg under acidic conditions (pH 4.0) at 50 ºC. Benmrad et al. (2019) found that *P. sajor-caju* proteases obtained in a submerged bioprocess demonstrate fibrinolytic activity similar to commercial drugs. The enzyme showed catalytic action at 79,000 U/mg under alkaline conditions (pH 9.5) at 70 ºC.

3.3 Biochemical characterization of fibrinolytic protease

Chemically, enzymes are defined as proteins built from long polymers and chains of amino acids, which can play a very effective catalytic role in the cellular system. Fibrin, an insoluble protein derived from a soluble precursor, fibrinogen, consists of three combinations of polypeptide chains, which are designated Aα, Bβ and γ, which are arranged in an elongated structure of 45 nm in length and 2-5 nm in diameter (Kollman et al., 2009; Pechik et al., 2006). This protein is a component of the blood that plays an important role in hemostasis; as a binding and healing agent in inflammation.

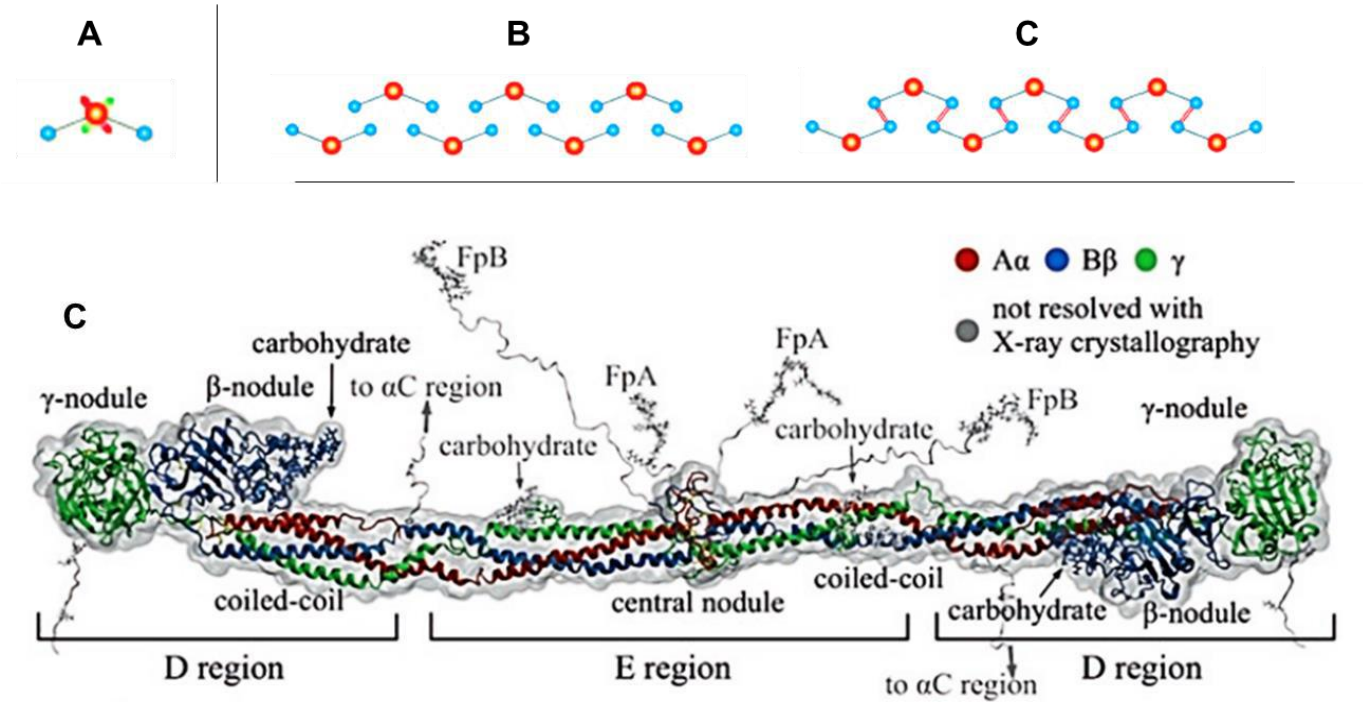
The accumulation and formation of inadequate clots in the vessels or arteries, under certain pathophysiological conditions of the organism, is an important risk factor for heart disease (Mander et al., 2011; Bajaj et al., 2014), especially in the complications of acute myocardial infarction, ischemic heart disease, valvular diseases, peripheral vascular diseases,

arrhythmias, high blood pressure and stroke (Mine et al., 2005).

Fibrinolytic proteases act as biocatalysts, in which they degrade fibrin clots, and thus gain an important role in the pharmaceutical industry as chemotherapeutic agents in the treatment of cardiovascular diseases (Cho et al., 2004; Das & Prasad, 2010; Feijoo-Siota &Villa, 2011).

The protein catalytic reaction is formed by proteolytically converting soluble fibrinogen molecules into fibrin (polymeric) monomers that spontaneously polymerize and create a fibrin gel network, as illustrated in Figure 3 (Anna et al., 2017; Buba, 2018).

Figure 3 - Representation with schematic edits adapted to better understand the fibrinogen molecule (A), the interaction of soluble (B) and insoluble fibrin polymers (C). Scheme C illustrates the three-dimensional structure of the fibrinogen molecule with its D domains, at the ends, and E domain in the center interacting with the Aα and Bβ chains with FpA and FpB in the central node and the beginning of the αC regions.

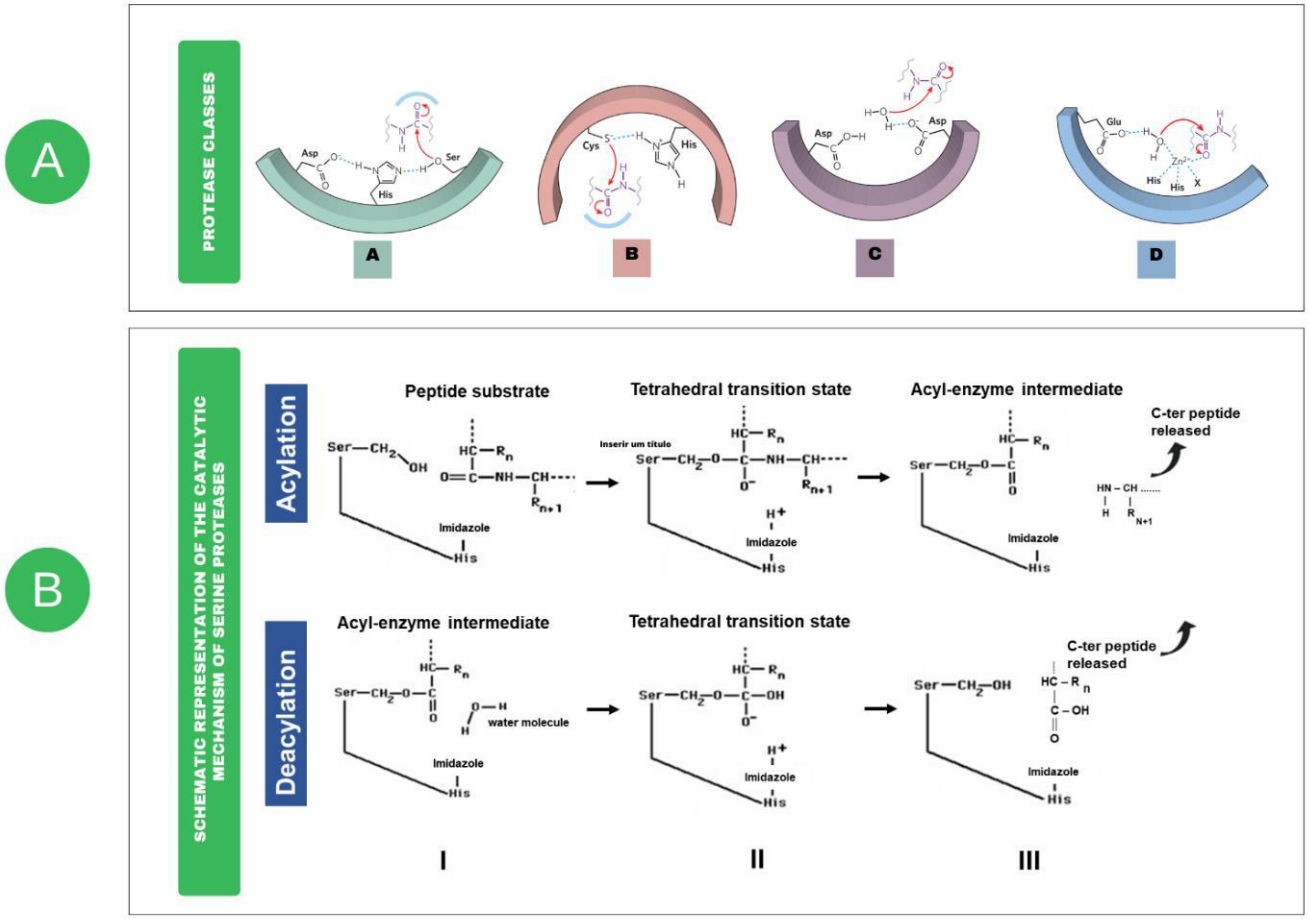


Source: Weisel; Litvinov (2017).

For fungal proteases, four classes have been recognized by the International Union of Biochemistry (Figure 4A): serine endopeptidases (EC 3.4.21.-), cysteine-endopeptidases (EC 3.4.22.-), aspartic endopeptidases (EC 3.4.23.-), and metallo-endopeptidases (EC 3.4.24.-) (Angelo et al., 2010).

The enzyme families are determined by the mechanism of action and the presence of amino acid residues (Figure 4B) to form an acyl-enzyme intermediate that covalently binds to the N-terminus of the enzyme substrate (Erez et al., 2009; Shafee, 2014; Naeem et al., 2022).

Figure 4 - Schematic representation of enzyme classes and the catalytic mechanism. A) Soluble serine proteases (EC 3.4.21; A in the image)), the mechanism of cysteine proteases (EC3.4.22; B in the image), the aspartyl proteases (EC 3.4.23; C in the image) Metalloproteases (EC 3.4.24; D in the image). B) Schematic representation of the catalytic mechanism of serine proteases. http://delphi.phys.univ-tours.fr/Prolysis/.



Source: Erez et al., (2009).

3.4 Mushrooms as sources for the production of fibrinolytic enzymes

The scientific community already effectively utilizes all available information on fibrinolytic enzymes. As a future perspective, the community should focus on exploration edible mushrooms. Fibrinolytics can be obtained from several natural sources and are widely researched in food and non-food sources. In this context, there is a particular interest in fibrinolytic enzymes obtained from foods, especially those from fermented foods, which present a different perspective in relation to fibrinolytics from other sources (Ali & Bavisetty, 2020).

The emergence of an interest in the study of basidiomycetes as possible producers of proteolytic enzymes is a consequence of the lack of animal raw materials and the imperfection of microbial preparations (Ali et al., 2022). The search for enzymes for the food and medical industry among basidiomycetes is determined by two circumstances: the absence of sporulation in culture, which reduces the risk of occupational diseases in industrial conditions, and the presence of a large number of edible mushrooms (Katrolia et al., 2020; Li et al., 2021).

However, FEs of microbial origin have come into the spotlight due to their advantages such as ease of handling, substrate specificity, and ease of genetic manipulation for large-scale enzyme production. Several promising and potential strains have been isolated, especially from traditional fermented foods. In this context, this topic aims to throw a spotlight on the production of FEs through statistical optimization for fermentative bioprocessing, and genetic engineering approaches with

strains isolated from diverse sources (Fasim et al., 2021).

Eight species of mushrooms grouped in the phylum Basidiomycota were identified: *P. Sajor-cashew; P. ferulae; P. ostreatus; L. shimeji; A. polytricha; H. erinaceum;* and *C. comatus*; and, for Ascomycota: *C. militaris*. Regarding the biochemical characterization of the fungal extracts, for the effect on the activity and stability of the enzyme, the species showed significant action ranging in pH from 4.0 to 9.5 at temperatures from 25 to 70 ºC. These characteristics are considered crucial for assessing the conditions under which the enzyme reaches maximum activity and for it to be used in various industrial processes (Aguilar & Sato, 2018; Banerjee & Ray, 2017).

As shown in Table 2, mushrooms that are considered the most valuable resources for the production of fibrinolytic enzymes, while there are no studies that have evaluated the fibrinolytic potential of edible mushrooms-derived enzymes. It should be noted that the catalytic activity of fibrinolytic enzymes can be improved by chemical modification.

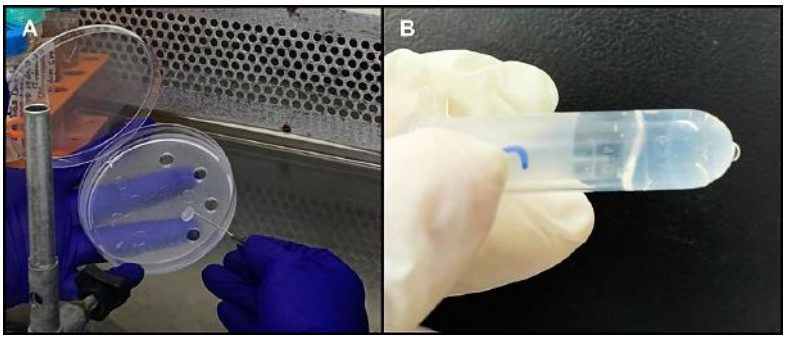
In this study, it was observed that the vast majority of the mushrooms studied synthesized fibrinolytic enzymes, which were identified as protease SPPS (Benmrad et al., 2019), metalloproteases (Choi et al., 2016; Liu al., 2014; Choi et al., 2013), serine proteases (Liu et al., 2015) and serine metalloproteases (Moon et al., 2-14).

The molecular mass of the enzymes identified in the selected papers range from ~18 to 66 kDa under different submerged culture conditions to favor the development and performance of the enzyme. These were analyzed in a short period of time and also from the direct extraction of the basidiome of the mushroom. According to Cardoso et al. (2022), even if it involves chemical or physical agents, enzymatic characterization is able to affect the native state of the enzyme, yet when intended for the pharmaceutical industry, it is essential.

3.5 Fibrinolytic tests

The fibrinolytic action of mushroom species can be determined in different methodologies, such as the fibrin plate method, which is a direct measure of qualitative activity, evidenced by the degradation halo that is formed around the wells (Figure 5A) and by dissolution of the artificially produced clot/thrombus (Figure 5B). These methodologies are considered complementary for the quality of the results in studies involving the determination of the fibrinolytic enzyme (Ferreira et al., 2010; Galo and Colombo, 2009).

Figure 5 - Illustration of the methods for determining fibrinolytic activity via the fibrin plate method (A) and artificial thrombi formation (B).



Source: Authors (2022).

The fibrin plate method is a qualitative method for direct measurement of halo appearance (Flute, 1964), observed by cup plate test for enzyme detection via degradation of the halo formation (Sousa et al., 2008). The artificial thrombi method is an in vitro quantitative method (Figure 7) performed by the formation of fibrin clots formed through the reaction of thrombin and fibrinogen (Astrup & Mullertz, 1952), and the clot dissolving capacity by fibrinolytic enzymes present in a sample for determination of fibrinolytic activity is quantified using spectrophotometry (Wang et al., 2014).

Table 2 - List of mushroom species and biochemical characterization of enzymes synthesized under different growing conditions.

Species	Acquisition of the Extract		Biochemical Characterization						Reference
	Cultivation Conditions	Fermentation	pH	Temperature	Metal ions	Inibidores	Molecular Mass	Enzyme	
Pleurotus sajor-caju	BDA- lentil flour, YE, glucose, KH_2PO_4	SF-28 °C 3 days	9.5	70 °C	Ca^{2+}, Mg^{2+}, Fe^{2+}	PMSF and DFP	~65 kDa	Protease SPPS	Benmrad et al. (2019)
Pleurotus ferulae	Basidiomes in sterile saline	-	4.0-8.0	50 °C	Cu^{2+}, Mg^{2+}	EGTA and EDTA	~20 kDa	Metaloprotease	Choi et al. (2016)
Cordyceps militaris	Sucrose and soybean meal	SF 23 °C	7.4	37 °C	Fe^{2+}	PMSF, Aprotinin and Pepstatin	~ 32 kDa	Serina Aspártica	Liu et al. (2015)
Pleurotus ostreatus	BDA-Glucose-KH_2PO_4-$MgSO_4$-soymilk	SF - 25°C 6 days	7.4-7.8	37 °C-45 °C	Ca^{2+}, K^{+}	EDTA, SBTI and Pepstatin	~18 kDa	Metaloprotease	Liu et al. (2014)
Lyophyllum shimeji	Basidiome obtained from the Yedang Mushroom Company	-	8.0	37 °C	Cu^{2+}, Co^{2+}	PMSF and TPCK	~21 kDa	Serina Metaloprotease	Moon et al. (2014)
Auricularia polytricha	Basidiome obtained from a mushroom farm (lyophilized)	-	7.0	37 °C	-	-	~66 kDa	-	Ali et al. (2014)
Hericium erinaceum	Basidiome obtained from the Korean Mushroom Company	-	5.5-7.0	30 °C	Ca^{2+}, Mg^{2+},Mn^{2+}	PMSF-TPCK and Aprotinin	~51 kDa	Metaloprotease	Choi et al. (2013)
Coprinus comatus	Sucrose and soybean meal	SF-25 °C 6 days	5.0	25 °C	-	-	-	-	Liu et al. (2012)

Submerged fermentation (SF). Source: Authors (2022).

4. Conclusion

In general, this review paper summarized the main characteristics of the mushroom species that produce proteolytic enzymes with fibrinolytic action. As such, it can be seen that mushroom biocompounds meet the criteria of the pharmaceutical industries for obtaining and producing in the short term, and also have low cost, mild synthesis conditions, ease of use and wide specificity for substrates, when compared with other microorganisms. The most studied species were of the genus Pleurotus, and it is believed that this was due to the easy acquisition of the basidiomes.

Few species of mushrooms have been studied in recent years and not all studies that have already been published can be considered complete; either because they have not purified or because they have not biochemically characterized the fibrinolytic enzymes. Thus, more studies are needed to recognize the biotechnological properties of mushrooms and define their applications in the most diverse sectors of industries, not only in commercial mushroom species, but also in native species.

Based on the accomplished analyzes,, the characterization of the fibrinolytic enzymes present in mushrooms, it is considered necessary realize more studies to discover the biotechnological properties and to be able to better define their applications in the pharmaceutical products industry to assist in the treatment of thrombosis.

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References

Acosta, G. A., Fonseca, M. I., Fariña, J. I., & Zapata, P. D. (2022). Exploring Agaricomycetes from the Paranaense rainforest (Misiones, Argentina) as an unconventional source of fibrinolytic enzymes. *Mycologia*. 114(2):242-253.10.1080/00275514.2022.2035148

Aguilar, J. G., & Sato, H. H. (2018). Microbial proteases: Production and application in obtaining protein hydrolysates. *Food Research International*, 103, 253-262.

Ali, A. M. M., et al. (2022). Production of fibrinolytic enzymes during food production. In: *Current Developments in Biotechnology and Bioengineering*, p. 157-187. https://doi.org/10.1016/B978-0-12-823506-5.00018-7

Ali, A. M., & Bavisetty, S. C. B. (2020). Purification, physicochemical properties, and statistical optimization of fibrinolytic enzymes especially from fermented foods: a comprehensive review. *International Journal of Biological Macromolecules*, v. 163, p. 1498-1517.

Ali, S. M., Ling, T. C., Sekaran, M., Y. S. T., Jegadeesh, R., & Vikineswary, S. (2014). Recovery and partial purification of fibrinolytic enzymes of Auricularia polytricha (Mont.) Sacc by an aqueous two-phase system. *Separation and Purification Technology*, 122 359–366. http://dx.doi.org/10.1016/j.seppur.2013.11.016

Altaf, F., Wu, S., & Kasim, V. (2021). Role of Fibrinolytic Enzymes in Anti-Thrombosis Therapy. *Front Mol Biosci*, 8:680397. 10.3389/fmolb.2021.680397.

Ângelo, R. S. (2010). Enzimas hidrolíticas. In: Esposito, E., Azevedo, J. L. (Org.). *Fungos: uma introdução à biologia, bioquímica e biotecnologia*. Caxias do Sul: Educs, (2ª edição).

Anna, D., Protopopova, R. I., Litvinov, D. K., Galanakis, C., Nagaswami, N. A., Barinov, A. R., Mukhitov, Dmitry, V. K., & John. W. W. (2017). Morphometric characterization of fibrinogen's αC regions and their role in fibrin self-assembly and molecular organization. *Nanoscale*. 10.1039/c7nr04413e

Astrup, T., & Mullertz, S. (1952). The fibrin platemethod for estimating of fibrinolyticactivity. *Arch Biochem Biophys*; 40: 346–51p. http://dx.doi.org/10.1016/0003-9861(52)90121-5

Bajaj, B. K., Singh, S., Khullar, M., Singh, K., & Bhardwaj, S. (2014). Optimization of fibrinolytic protease production from Bacillus subtilis I-2 using agro-residues. *Braz Arch Biol Technol* 57:653–662. https://doi.org/10.1590/S1516-8913201402132

Banerjee, G., & Ray, A. K. (2017). Impact of microbial proteases on biotechnological industries. *Biotechnology and Genetic Engineering Reviews*, 33(2), 119-143.

Barbosa, E. E. P., Pimenta, L., Brito, A. K. P., Martim, S. R., & Teixeira, M. F. S. (2020). Cultivo de cogumelo comestível em resíduos lignocelulósicos de floresta tropical para produção de proteases / Mushroom cultivation edible in lignocellulosic residues from rainforest for protease production. *Brazilian Journal of Development*, 6(11), 92475–92485. https://doi.org/10.34117/bjdv6n11-598

Benmrad, O., M., Mechri, S., Zaraî, J. N., et al. (2019). Purification and biochemical characterization of a novel thermostable protease from the oyster mushroom Pleurotus sajor-caju strain CTM10057 with industrial interest. *BMC Biotechnol* 19, 43. https://doi.org/10.1186/s12896-019-0536-4

Bezerra, V. H. S., Cardoso, S. L., Fonseca-Bazzo, Y., Silveira, D., Magalhães, P. O., & Souza, P. M. (2021). Protease Produced by Endophytic Fungi: A Systematic Review. *Molecules*, 26 (22):7062. Published 2021 Nov 22.10.3390/molecules26227062

Brito, A. K. P., Pimenta, L., Barbosa, E. E. P., Batista, S. C. P., Coelho, M. P. S. L. V., Castillo, T. A., Martim, S. R., & Teixeira, M. F. S. (2021). Evaluation of tropical forest substrates for cultivation and production of proteases by Pleurotus djamor. *Research, Society and Development*, 10(3), e31810313385. https://doi.org/10.33448/rsd-v10i3.13385

Buba, J. C. (2018). Produção de protease com atividade fibrinolítica por cultivo submerso de *Mucor subtilissimus* em biorreator. *Universidade de São Paulo*. http://www.teses.usp.br/teses/disponiveis/3/3137/tde28022018-133041/pt-br.php

Cardoso, K. B. B., Nascimento, M. C., Batista, A. C., Oliveira, V. M., Nascimento, T. P., Batista, J. M. S., Costa, R. M. P. B., Pastrana, L., & Porto, A. L. F. (2022). Systematic analysis on the obtaining of fibrinolytic fungi enzymes. *Research, Society and Development*, 11(2), e13611225449, 2022. 10.33448/rsd-v11i2.25449.

Cho, I. H., Choi, E. S., Lim, H. G., & Lee, H. H. (2004). Purification and characterization of six fibrinolytic serine-proteases from earthworm Lumbricus rubellus. *J Biochem Mol Biol*, 37 (2):199-205.10.5483/bmbrep.2004.37.2.199.

Choi, B. S., Sapkota, K., Choi, J. H., Shin, C. H., Kim, S., & Kim, S. J. (2013). Herinase: a novel bi-functional fibrinolytic protease from the monkey head mushroom, Hericium erinaceum. *Appl Biochem Biotechnol*, Jun; 170 (3):609-22. 10.1007/s12010-013-0206-2. Epub 2013 Apr 7. PMID: 23564433.

Choi, J. H., Kim, D. W., Kim, S., & Kim, S. J. (2016). Purification and partial characterization of a fibrinolytic enzyme from the fruiting body of the medicinal and edible mushroom Pleurotus ferulae. *Prep Biochem Biotechnol*, Jul 3;47(6):539-546. 10.1080/10826068.2016.1181083.

Coelho, M., Figueiredo, A. S. F., Martim, S. R. M., & Teixeira, M. F. S. T. (2022). Ciclo de produção de cogumelos comestíveis cultivados em resíduos lignocelulósicos da fruticultura Amazônica: um estudo de caso. *Concilium*, 284–294. https://doi.org/10.53660/CLM-116-138

Das, G., & Prasad, M. P. (2010). Isolation, purification & mass production of protease enzyme from Bacillus subtilis. *International Research Journals of Microbiology*, 1(2), 026-031, April.

Erez, E., Fass, D., & Bibi, E. (2009). How intramembrane proteases bury hydrolytic reactions in the membrane. *Nature*, 459 (7245):371-378.10.1038/nature08146

Fasim, A., More, V. S., & More, Sunil, S. (2021). Large-scale production of enzymes for biotechnology uses. *Current Opinion in Biotechnology*, v. 69, p. 68-76. https://doi.org/10.1016/j.copbio.2020.12.002.

Feijoo-Siota, L., and& Villa, T. G. (2011). Native and Biotechnologically Engineered Plant Proteases with Industrial Applications. *Food Bioprocess Technol*, 4:1066–1088., 4(6), 1066-1088, 2011. 10.1007/s11947-010-0431-4

Ferreira, C. N., Sousa, M. O., Dusse, L. M. S., & Carvalho, M. G. (2010). O novo modelo da cascata de coagulação baseado nas superfícies celulares e suas implicações. *Rev Bras Hematol Hemoter*, 32(5), 416-421. https://doi.org/10.1590/S1516-84842010000500016

Flute, P. T. (1964). *Haemorrhage and fibrinolysis*. Proceedings of the Royal Society of Medicine, 57(7), 603–606.

Fonseca, T. R. B. (2013). *Pleurotus ostreatoroseus* DPUA 1720: Avaliação do Crescimento, Produção de Basidioma e Determinação da Atividade Proteolítica em Resíduos Agroindustriais. *Universidade Federal do Amazonas*.

Galo, L. A., & Colombo, M. F. (2009). Espectrofotometria de longo caminho óptico em espectrofotômetro de duplo-feixe convencional: uma alternativa simples para investigações de amostras com densidade óptica muito baixa. *Química Nova*, 32 (2), 488-492. https://doi.org/10.1590/S0100-40422009000200036

Katrolia, P., Liu, X., Zhao, Y., Kopparapu, N. K., & Zheng, X. (2020). Gene cloning, expression and homology modeling of first fibrinolytic enzyme from mushroom (*Cordyceps militaris*). *Int J Biol Macromol*, 146:897-906. 10.1016/j.ijbiomac.2019.09.212

Kollman, J. M., Pandi, L., Sawaya, M. R., Riley, M., & Doolittle, R. F. (2009). Crystal structure of human fibrinogen. *Biochemistry*, 48(18):3877-3886.10.1021/bi802205g

Li, G., Liu, X., Cong, S., Deng, Y., & Zheng, X. (2021). A novel serine protease with anticoagulant and fibrinolytic activities from the fruiting bodies of mushroom *Agrocybe aegerita*. *Int J Biol Macromol*, 168:631-639. 10.1016/j.ijbiomac.2020.11.118

Liu, X. L., Zheng, X. Q., Qian, P. Z., Kopparapu, N. K., Deng, Y. P., Nonaka, M., & Harada, N. (2014). Purification and characterization of a novel fibrinolytic enzyme from culture supernatant of *Pleurotus ostreatus*. *J Microbiol Biotechnol*, Feb 28;24(2):245-53. 10.4014/jmb.1307.07063.

Liu, X. L., Zheng, X. Q., & Zhang, J. K. (2012). Production of a Fibrinolytic Enzyme from Coprinus comatus YY-20. *Applied Mechanics and Materials*, Vols. 138–139, pp. 1195–1201. https://doi.org/10.4028/www.scientific.net/amm.138-139.1195

Machado, A. R. G., Teixeira, M. F. S., Kirsch, L. S. M., Campelo, C. L., & Oliveira, I. M. A. (2016). Nutritional value and proteases of *Lentinus citrinus* produced by solid state fermentation of lignocellulosic waste from tropical region. *Saudi Journal of Biological Sciences*, 23(5): 621-627. DOI: https://doi.org/10.1016/j.sjbs.2015.07.002

Mander, P., Cho, S. S., Simkhada, J. R., Choi, Y. H., & Yoo, J. C. (2011). A low molecular weight chymotrypsin-like novel fibrinolytic enzyme from *Streptomyces* sp. CS624. *Process Biochemistry*, v. 46, p. 1449–1455. https://doi.org/10.1016/j.procbio.2011.03.016

Mine, Y., Wong, A. H. K., & Jinag, B. (2005). Fibrinolytic enzymes in Asian traditional fermented foods. *Food Research International*, 38:243–250. https://doi.org/10.1016/j.foodres.2004.04.008

Moon, S. M., Kim, J. S., Kim, H. J., Choi, M. S., Park, B. R., Kim, S. G., Ahn, H., Chun, H. S., Shin, Y. K., Kim, J. J., Kim, D. K., Lee, S. Y., Seo, Y. W., Kim, Y. H., & Kim, C. S. (2014). Purification and characterization of a novel fibrinolytic α chymotrypsin like serine metalloprotease from the edible mushroom, Lyophyllum shimeji. *J Biosci Bioeng*, May;117(5):544-50. 10.1016/j.jbiosc.2013.10.019.

Naeem, M., Manzoor, S., Abid, M. U. H., Tareen, M. B. K., Asad, M., Mushtaq, S., Ehsan, N., Amna, D., Xu, B., & Hazafa, A. (2022). Fungal Proteases as Emerging Biocatalysts to Meet the Current Challenges and Recent *Developments in Biomedical Therapies*: An Updated Review. J. Fungi, 8, 109. https://doi.org/10.3390/jof8020109

Pechik, I., Yakovlev, S., Mosesson, M. W., Gilliland, G. L., & Medved, L. (2006). Structural basis for sequential cleavage of fibrinopeptides upon fibrin assembly. *Biochemistry*, Mar 21;45 (11):3588-97. 10.1021/bi0525369. PMID: 16533041; PMCID: PMC2531209.

Pimenta, L., Barbosa, E. E. P., Brito, A. K. P. de, Martim, S. R., & Teixeira, M. F. S. (2021). Processo eco-amigável para selecionar substrato Lignocelulósico para produção de peptidases ácidas / Eco-friendly process to select Lignocellulosic substrate for the production of acid peptidases. *Brazilian Journal of Development*, 7(1), 3469–3479. https://doi.org/10.34117/bjdv7n1-234

Ruiz, M. A., Greco, O. T., Braile, D. M. (2009). Fator de impacto: importância e influência no meio editorial, acadêmico e científico. *Rev Bras Cir Cardiovasc*. 24(3): 273-278. https://doi.org/10.1590/S0102-76382009000400004

Shafee, T. (2014). *Evolvability of a Viral Protease: Experimental Evolution of Catalysis, Robustness and Specificity*. University of Cambridge: Cambridge, UK.

Sharma, C., Osmolovskiy, A., & Singh, R. (2021). Microbial Fibrinolytic Enzymes as Anti-Thrombotics: Production, Characterisation and Prodigious Biopharmaceutical Applications. *Pharmaceutics*. 2021;13 (11):1880. Published Nov 5.10.3390/pharmaceutics13111880

Sharma, M., & Bajaj, B. K. (2017). Optimization of bioprocess variables for production of a thermostable and wide range pH stable carboxymethyl cellulase from *Bacillus subtilis* MS 54 under solid state fermentation. *Environ Prog Sustain Energy*. doi:10.1002/ep.12557

Souza, H. Q., et al. (2008). Seleção de Basidiomycetes da Amazônia para produção de enzimas de interesse biotecnológico. *Ciência e Tecnologia de Alimentos*, v. 28, pg. 116-124, Campinas, SP.

Strehl, L. (2005). O fator de impacto do ISI e a avaliação da produção científica: aspectos conceituais e metodológicos. *Ci. Inf., Brasília*, 34(1), 19-27. https://wp.scielo.org/wp-content/uploads/STREHL-L..pdf

Wang, J., Wu, C., Chen, Y., Chen, C., Hu, S., & Chang, S. (2014). Antihyperglycemic activity of exopolysaccharide produced by mushroom Pleurotus ferulae with submerged liquid culture on streptozotocin-induced diabetic rats. *Journal of Food and Nutrition Research*, 2(7), 419-424.

Weisel, J. W., & Litvinov, R. I. (2017). Fibrin Formation, Structure and Properties. *Subcell Biochem*, 82: 405–456. 10.1007/978-3-319-49674-0_13

Wu, S., et al. (2021). Biocatalysis: enzymatic synthesis for industrial applications. *Angew. Chem. Int*. Ed., 60,88–119. https://doi.org/10.1002/anie.202006648