

Systematic analysis on the obtaining of fibrinolytic fungi enzymes

Análise sistemática sobre a obtenção de enzimas fibrinolíticas a partir de fungos

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Abstract

Fibrinolytic proteases operate directly on fibrin clot and are able to maintain blood flow. Fungi show up as viable sources for obtaining this enzyme. The purpose of this systematic review is to is to unveil all the information concerning production, purification and characterization of fibrinolytic proteases by fungi. The search was conducted in ScienceDirect, PubMed and Scopus databases, using as keywords “(Fibrinolytic enzyme) OR (Fibrinolytic protease) AND (Fungal or Fungus or Fungi)”. Delimiting period of 10 years (2011-2021). The results obtained were filtered by selection criteria, and review articles or articles outside the scope of this work were excluded. The articles were evaluated and scored (0-10) according to pre-established criteria. None of the studies obtained the score 10, however the study with the highest score (9) presented relevant data in all criteria analyzed, obtaining fibrinolytic enzyme from *Xylaria curta*. Among the 21 selected articles, 12 different genera appear and Submerged Fermentation and purification of Serino-proteases were more described. This work also observed a greater representation of purification and characterization steps, indicating the need for attention to cultivation process and enzymatic application. It is clear that the production of these enzymes by fungi is pertinent towards the high recovery observed even after purification and its tendency for pharmaceutical application.

Keywords: Protease; Thrombolytic; Mushroom; Filamentous fungi; Fermentation; Purification.

Resumo

Proteases fibrinolíticas destacam-se por atuarem diretamente no coágulo de fibrina e conseguir manter o fluxo sanguíneo. Fungos despontam na literatura como fontes viáveis na obtenção dessa enzima. Desta forma, o propósito

desta revisão sistemática é analisar as informações disponíveis a respeito da produção, purificação e caracterização de proteases fibrinolíticas por fungos. A pesquisa foi conduzida nas bases ScienceDirect, PubMed e Scopus, utilizando como palavras-chave “ (Fibrinolytic enzyme) OR (Fibrinolytic protease) AND (Fungal or Fungus or Fungi) ”. Delimitando período de 10 anos (2011-2021). Os resultados obtidos foram filtrados por critérios de seleção, sendo excluídos artigos de revisão e artigos fora do escopo deste trabalho. Os artigos foram avaliados e pontuados (0-10) segundo critérios pré-estabelecidos. Nenhum dos trabalhos obteve pontuação máxima, entretanto o trabalho com maior pontuação (9) apresentou dados relevantes em todos os critérios analisados, obtendo enzima fibrinolítica de *Xylaria curta*. Dentre os 21 artigos selecionados foram descritos 12 gêneros diferentes e o uso de Fermentação Submersa e a purificação de Serino proteases foram mais descritos. Este trabalho também observou maior representação de etapas de purificação e caracterização, indicando a necessidade de atenção ao cultivo e aplicação enzimática. Está claro que a produção destas enzimas por fungos é pertinente tendo em vista a alta recuperação observada mesmo após purificação e sua tendência a aplicação farmacêutica.

Palavras-chave: Protease; Trombolítico; Cogumelo; Fungos filamentosos; Fermentação; Purificação.

Resumen

Las proteasas fibrinolíticas destacan por actuar directamente sobre el coágulo de fibrina y mantener el flujo sanguíneo. Los hongos aparecen en la literatura como fuentes viables para la obtención de esta enzima. Así, el propósito de esta revisión sistemática es analizar la información disponible sobre la producción, purificación y caracterización de proteasas fibrinolíticas por hongos. La búsqueda se realizó en las bases de datos ScienceDirect, PubMed y Scopus, utilizando como palabras clave “ (Enzima fibrinolítica) OR (Proteasa fibrinolítica) AND (Fungal or Fungus or Fungi) ”. Plazo delimitador de 10 años (2011-2021). Los resultados obtenidos fueron filtrados por criterios de selección, excluyendo artículos de revisión y artículos fuera del campo de aplicación de este trabajo. Los artículos fueron evaluados y puntuados (0-10) según criterios preestablecidos. Ninguno de los estudios obtuvo la mayor puntuación, sin embargo el estudio con mayor puntuación (9) presentó datos relevantes en todos los criterios analizados, obteniendo la enzima fibrinolítica de *Xylaria curta*. Entre los 21 artículos seleccionados, se describieron 12 géneros diferentes y se describió más el uso de Fermentación Sumergida y la purificación de Serino proteasas. Este trabajo también observó una mayor representación de los pasos de purificación y caracterización, lo que indica la necesidad de atención al cultivo y la aplicación enzimática. Es evidente que la producción de estas enzimas por hongos es pertinente en vista de la alta recuperación observada incluso después de la purificación y su tendencia a la aplicación farmacéutica.

Palabras clave: Proteasa; Trombolítico; Champiñón; Hongos filamentosos; Fermentación; Purificación.

1. Introduction

The formation of fibrin clots is an important process in the regulation of the hemostatic system as well as its dissolution through fibrinolysis. However, the imbalance caused by some disorders can result in failure in the process of dissolving these clots. The consequent interruption of blood flow can cause serious problems for the affected individual, such as thrombosis, myocardial infarction, stroke, among other diseases linked to the cardiovascular system. Thus, it is extremely important to develop fibrinolytic agents that can be used in this sense, that are functional and have a low production cost (Andrade *et al.*, 2018; Lins *et al.*, 2019). Among the recommended treatments for coping with thrombosis, anticoagulants are the best known. However, in addition to the high production cost, these drugs have dangerous side effects, which can cause adverse bleeding, gastrointestinal discomfort, liver damage, and others. A second alternative is the use of fibrinolytic (or thrombolytic) agents, such as urokinase, streptokinase and tissue plasminogen activator (t-PA). However, these also present problems related to adverse bleeding, in addition to having a short half-life and restricted sources, making it difficult to obtain and apply (Ali *et al.*, 2020).

Proteases with fibrinolytic action stand out for their successful dissolution of clots and maintenance of blood flow. Fibrinolytic enzymes can be classified into serine protease, metalloprotease and serine metalloprotease (Ali *et al.*, 2020; Bin *et al.*, 2009). Those belonging to the first group are able to act both directly and indirectly in fibrin degradation, that is, by cleavage or by activation of plasmin. Metalloprotease with fibrinolytic action are rare, but act in the presence of bivalent metallic ions such as Zn^{2+} , Mg^{2+} , and Ca^{2+} . The serine metalloproteases in turn combine properties of both previously described (Ali *et al.*, 2020; Bin *et al.*, 2009).

These enzymes can be obtained from different sources, being widely produced by different microorganisms (Raju *et al.*, 2013). Furthermore, when obtained from the fermentation of fungi, the production cost is relatively lower and benefits itself from advantages such as its cosmopolitan distribution and ease of large-scale production (Chen *et al.*, 2016).

Given its indispensability, the need to search for new fibrinolytic enzymes is highlighted. The development of new production methods, sources, fermentation conditions, extraction, and purification methodologies has been a strong trend in research in the area, since these enzymes have a wide application spectrum with high biotechnological potential. In this context, this systematic review will discuss the criteria for obtaining, characterizing and purifying fibrinolytic enzymes produced by fungi.

2. Methodology

This research was carried out using the systematic review model, based on the following process: Searches of research articles were performed in popular databases, these being ScienceDirect (<https://www.sciencedirect.com>), PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) and Scopus (<https://www.scopus.com>) using as keywords “(Fibrinolytic enzyme) OR (Fibrinolytic protease) AND (Fungal or Fungus or Fungi)”. For selection of articles, inclusion and exclusion criteria were established based on those proposed by Wanderley (2017), adapted. The selection was made on a two-step analysis, the first step being performed superficially (or initially) taking into account only the title and abstract and the second step (or final step) in more detail analyzing the entire body of the text. Authorial research articles published in the last 10 years (2011-2021) that dealt with the production, purification and/or characterization of fibrinolytic enzymes produced by fungi in general were included in the analysis. Review articles and articles that did not use fungus in the methodology, or did not discriminate against the fungus used, were excluded. Items of the methodology such as how to obtain the enzyme, culture conditions, enzymatic activity, types of analysis and quantification of results were not taken into account for inclusion/exclusion criteria.

From the selected studies, evaluations were made regarding the methodologies for obtaining the enzyme, enzymatic activity, characterization, purification and application. A score was assigned to each of these criteria using the system proposed by Greenhalgh (1997), where each criterion can score 2 when the methodology is adequately present, or complete; can score 1 when the methodology is present but inadequately or incompletely; or it can score 0 when the methodology is not present. A summary of the evaluation and scoring criteria can be seen in Table 1.

Table 1. Evaluation criteria and referent score used to analyze the selected articles.

Rating criteria	Score		
	0	1	2
(A) Production	Absent	Without variables	With variables
(B) Characterization	Absent	Partial	Total
(C) Fibrinolytic Activity	Absent	Fibrin plate	Spectrophotometry
(D) Purification	Absent	Partical	Total
(E) Aplications	Absent	-	Present

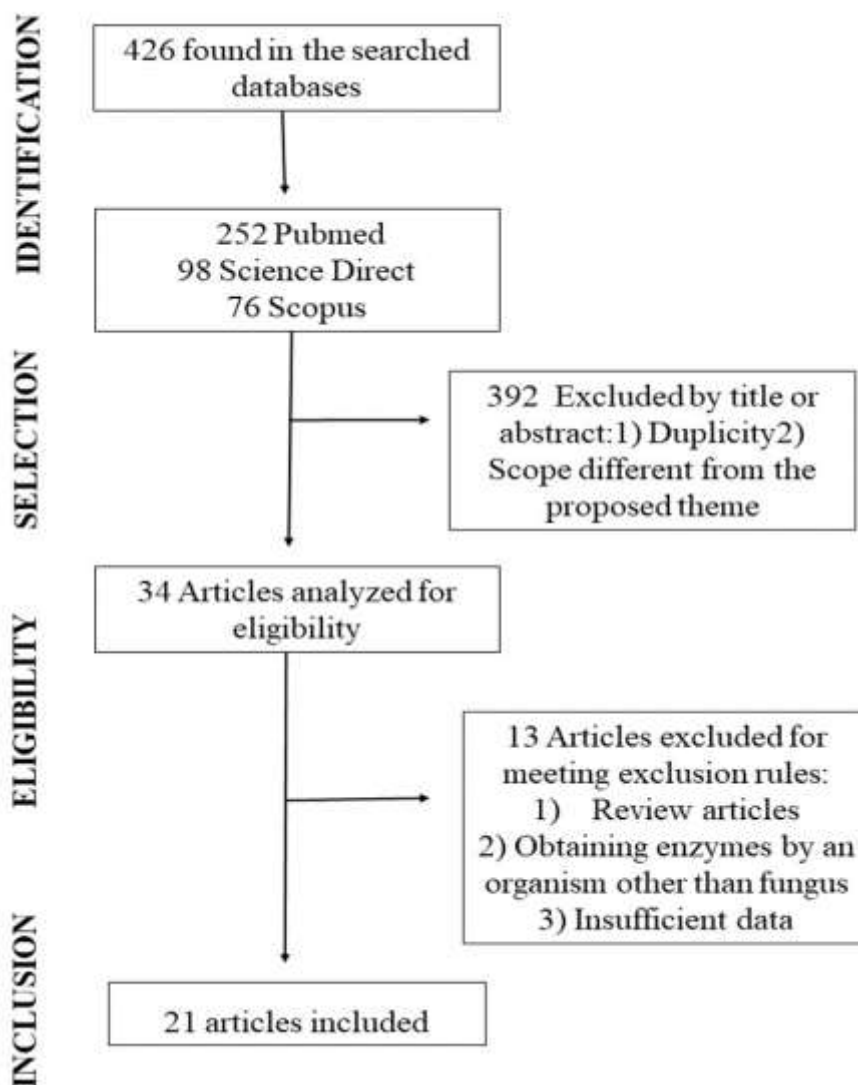
Source: Authors (2022).

3. Results and discussion

3.1 Selection of articles

In phase 1 (article selection), 426 citations were identified in the electronic databases. After the initial evaluation, duplicate articles were removed and with objectives different from those addressed in this work, leaving 34 publications. The comprehensive evaluation of the full articles was completed and 13 articles were excluded, with 21 citations remaining. A flowchart detailing the process of identifying, including, and excluding studies is shown in Figure 1.

Figure 1. Flowchart of the article selection process using inclusion and exclusion criteria.



Source: Authors (2022).

3.2 Characteristics of articles and punctuation

A summary of the main characteristics of the included studies is provided in Table 2. As described in the methodology, the articles were selected in the period 2011-2021, among those selected 11 were published in the last five years, only one article was published in the year 2021 and one in 2020. All studies evaluated the acquisition of fibrinolytic enzymes by fungi, as well as some level of purification and characterization of the enzyme. In contrast, not all articles reported in detail the fermentation conditions, especially articles that used mushrooms.

Table 2. Main characteristics of selected articles.

FUNGUS	OBTAINING THE ENZYME				FIBRINOLYTIC ACTIVITY		CHARACTERIZATION			REFERENCE
	Fermentation	Temp.	Substrate	Time (days)	Method	Specific Activity (U/mg)	pH and temp.	Inhibitors	Mass	
<i>Paecilomyces tenuipes</i>	-	-	-	-	Fibrin plate	2,6	pH 5, 35°C	PMSF	14 kDa	(Kim <i>et al.</i> , 2011)
<i>Cordyceps militaris</i>	-	-	-	-	Fibrin plate	5,8	pH 7, 40°C	PMSF	34 kDa	(Choi <i>et al.</i> , 2011)
<i>Ganoderma lucidum</i>	SmF	30°C	MCDBF	15	Fibrin zymography	350	-	-	-	(Kumaran <i>et al.</i> , 2011)
<i>Aspergillus oryzae</i>	SmF	28°C	Liquified rice	7	Fibrin plate	5,5	PH 6, 50°C	PMSF	30 kDa	(Shirasaka <i>et al.</i> , 2012)
<i>Hericium erinaceum</i>	-	-	-	-	Spectrophotometry	0,41	pH 7, 30°C	EDTA, EGTA	51 kDa	(Choi <i>et al.</i> , 2013)
<i>Auricularia polytricha</i>	-	-	-	-	Fibrin plate	10,83	PH 7, -	-	66 kDa	(Mohamed-ali <i>et al.</i> , 2014)
<i>Pleurotus ostreatus</i>	SmF	25°C	Glucose, KH ₂ PO ₄ , MgSO ₄ , Soy milk.	6	Fibrin plate	5,2	pH 7,4, 45°C	EDTA	18,2 kDa	(Liu <i>et al.</i> , 2014)
<i>Lyophyllum shimeji</i>	-	-	-	-	Fibrin plate	5,2	pH 8, 37°C	PMSF, EDTA	21 kDa	(Moon <i>et al.</i> , 2014)
<i>Rhizopus microsporus</i>	SmF	28°C	Solution with soy (1%), peptone (1%) and dextrin (2%)	2,5	Fibrin plate	43,2	pH 7, 37°C	-	24,5 kDa	(Zhang <i>et al.</i> , 2015)
<i>Cordyceps militaris</i>	SmF	23°C	Soy (5%) and Sucrose (2%)	5	Fibrin plate	40,7	pH 7,4, 37°C	PMSF	32 kDa	(Liu <i>et al.</i> , 2015)
<i>Neurospora sitophila</i>	SSF	28°C	Soybeans and Wheat Bran	2	Fibrin plate	9,2	pH 7,6, 41°C	PMSF, Aprotinin, SBTI	-	(Liu <i>et al.</i> , 2016)
<i>Xylaria curta</i>	SSF	28°C	Rice bran	15	Fibrin plate	1,1	pH 8, 35°C	EDTA, EGTA	33 kDa	(Meshram <i>et al.</i> , 2016)
<i>Mucor subtilissimus</i>	SSF	25°C	Wheat bran	3	Spectrophotometry	57,1	-, 37°C	PMSF	-	(Nascimento <i>et al.</i> , 2016)
<i>Xylaria curta</i>	SmF	28°C	Czapek Dox	7	Fibrin plate	3,99	pH 8, 35°C	EDTA, EGTA	33 kDa	(Meshram <i>et al.</i> , 2016)
<i>Cordyceps militaris</i>	SmF	23°C	Soy (5%) and Sucrose (2%)	5	Fibrin plate	40,7	pH 7,2, 37°C	SBTI	28 kDa	(Liu <i>et al.</i> , 2017)

<i>Mucor subtilissimus</i>	SSF	25°C	Wheat bran	3	Spectrophotometry	2,54	pH 8, 40°C	PMSF	20 kDa	(Nascimento <i>et al.</i> , 2017)
<i>Pleurotus ferulae</i>	-	-	-	-	Fibrin zymography	21,01	pH 4, 5 e 8, 50°C	EDTA, EGTA	20 kDa	(Choi <i>et al.</i> , 2017)
<i>Neurospora sitophila</i>	SSF	28°C	Soybean residue and Wheat bran	2	And fibrin plaque	9,2	pH 7,4, 50°C	PMSF, SBTI, TPCK	49 kDa	(Deng <i>et al.</i> , 2018)
<i>Mucor subtilissimus</i>	SmF	30°C	Wheat or soy bran (MS-2)	4	Fibrin plate	4,83	PH 7, 50°C	PMSF, β-mercaptoethanol, EDTA	52 kDa	(Clementino <i>et al.</i> , 2019)
<i>Mucor subtilissimus</i>	SSF	25°C	Wheat bran	3	Spectrophotometry	26,1	pH 9, 40°C	-	70 kDa	(Nascimento <i>et al.</i> , 2020)
<i>Agrocybe aegerita</i>	-	-	-	-	Spectrophotometry	7,24	PH 7,6, 47°C	PMSF, SBTI	31,4 kDa	(Li <i>et al.</i> , 2021)

*SmF: Submerged Fermentation. SSF: Solid State Fermentation. Source: Authors (2022).

Regarding the score, none of the selected articles reached the maximum score, as can be seen in Table 3. Most articles (85% of the selected articles) scored between 6 and 8 (38%, 28% and 19%, respectively), however only 52.4% scored above 7, only one article reached a score of 9, Meshram *et al.* (2016) evaluated the production of fibrinolytic enzyme by *Xylaria curta* under solid state fermentation, obtaining specific activity of up to 9.22 U/mg, the authors were able to perform and publish in a single work the production conditions, with evaluation of different agro-waste as substrate, enzymatic characterization, purification by chromatographic techniques and application. Among the criteria evaluated, the presence of some application activity of the studied enzyme was the least scored (only 38.1% of the articles), revealing the deficiency in more in-depth studies on the enzymes produced, providing guidance for the enzymes produced.

Table 3. Score of selected articles.

REFERENCE	A	B	C	D	E	TOTAL
(KIM <i>et al.</i> , 2011)	0	2	1	2	0	5
(CHOI <i>et al.</i> , 2011)	0	2	1	2	0	5
(KUMARAN <i>et al.</i> , 2011)	1	1	1	2	1	7
(SHIRASAKA <i>et al.</i> , 2012)	1	2	1	2	0	6
(CHOI <i>et al.</i> , 2013)	1	2	2	2	0	7
(MOHAMED ALI <i>et al.</i> , 2014)	1	0	2	1	0	4
(LIU <i>et al.</i> , 2014)	1	2	1	2	0	6
(MOON <i>et al.</i> , 2014)	1	2	1	2	0	6
(ZHANG <i>et al.</i> , 2015)	1	2	1	2	0	6
(LIU <i>et al.</i> , 2015)	1	2	1	2	1	8
(LIU <i>et al.</i> , 2016)	1	2	1	2	1	8
(MESHARAM <i>et al.</i> , 2016)	2	2	1	2	1	9
(NASCIMENTO <i>et al.</i> , 2016)	1	2	2	1	0	5
(MESHARAM <i>et al.</i> , 2016)	1	2	1	2	1	8
(LIU <i>et al.</i> , 2017)	1	2	1	2	0	6
(NASCIMENTO <i>et al.</i> , 2017)	1	2	2	2	0	7
(CHOI <i>et al.</i> , 2017)	1	1	1	2	1	7
(DENG <i>et al.</i> , 2018)	1	2	1	2	0	6
(CLEMENTINO <i>et al.</i> , 2019)	2	2	2	1	0	7
(NASCIMENTO <i>et al.</i> , 2020)	1	1	2	2	1	8
(LI <i>et al.</i> , 2021)	0	2	1	2	1	7

*(A): Production: with variables (2), without variables (1), absent (0);

(B): Characterization: total (2), partial (1) and absent (0);

(C): Fibrinolytic Activity: Fibrin plate (1) or spectrometer (2);

(D): Purification: total (2), partial (1) or absent (0);

(E): Applications: present (2) or absent (0).

Source: Authors (2022)

3.3 Fungus

Twelve different genera were observed in the fungi used in the selected articles, (*Paecilomyces*, *Agrocybe*, *Aspergillus*, *Rhizopus*, *Cordyceps*, *Pleurotus*, *Mucor*, *Xylaria*, *Ganoderma*, *Neurospora*, *Hericium*, *Auricularia*, *Lyophyllum*), among these, most articles used fungi of the Ascomycota or Basidiomycota phylum, they make up more than 98% of terrestrial fungi, with the Ascomycota phylum being considerably richer in number of species (Wang *et al.*, 2010). The phylum Zygomycota, considered in flux, was present in 5 articles, of which 4 were represented by the genus *Mucor*. These fungi are characterized by rapid growth and abundant spore production (Zabell & Morell, 2020).

Filamentous fungi are known as excellent enzyme producers, being known mainly for the high recovery of proteases (Souza *et al.*, 2015). 8 articles among those analyzed (38.1%) used some kind of filamentous fungus, indicating that although

there is a tendency to use these microorganisms, they can still be further explored, especially taking into account biological diversity and the possibility of genetic alteration. Other reported advantages about its application take into account logistical and financial issues, since the production of protease by filamentous fungi benefits from its extracellular secretion, facilitating extraction and purification since there is no need for cell lysis, reducing steps in the process of obtaining these biomolecules (Batista *et al.*, 2020; Martinez-Medina *et al.*, 2019). The use of cell disruption, in addition to adding more steps to the process, also makes it more complete, since the lysis fragments characterize a greater number and diversity of contaminating molecules and an increase in the viscosity of the medium. In addition to requiring a greater number of operations during purification, consequently making the final cost higher than those of extracellular products (Pessoa-JR, 2020).

Although filamentous fungi are gaining ground in the production of enzymes, obtaining fibrinolytic proteases from mushrooms is significantly higher (61.9%), with several authors highlighting their efficiency and safety, especially in the use of edible mushrooms, studied by presence of natural bioactives and used in folk medicine as herbal medicine. Currently, several mushroom extracts with antioxidant, antitumor, immunomodulatory, antifungal, hypotensive and anticoagulant activities are available in the literature (Kim *et al.*, 2011; Li *et al.*, 2021). It is noteworthy that the use of fungi in general has advantages related to fermentation under various environmental conditions, such as pH, temperature, light, and the variety of substrates that can be used as nutrients (Haq *et al.*, 2004).

3.4 Cultivation Conditions

The production of fibrinolytic protease is extremely influenced by the composition of the fermentation medium, especially carbon and nitrogen sources and physical factors such as temperature, pH, agitation, inoculum concentration and fermentation time. Each organism is able to reach the maximum enzyme production in a specific condition, therefore, the optimization of the medium components and the culture parameters is essential in the biological process. This review analyzed 21 studies, of which only 2 evaluated different conditions during fermentation. Meshran *et al.* (2016) evaluate the effect of several sources of nitrogen and carbon as substrate, such as: rice bran, wheat bran, eggshell, orange peel and banana peel. No analysis regarding physical factors was performed. In contrast, Clementino *et al.* (2019) used the extractive fermentation technique to obtain pre-purified enzyme in a single step, varying not only the culture medium (wheat bran and soybean bran) but also the composition and concentration of the aqueous two-phase system applied to 2³ factorial planning. The optimization of the composition of the medium and the physical conditions are applied to increase the production of enzymes and it is an important parameter in the development of an economically viable process.

One of the main factors that must be taken into account in the production of proteases is the type of fermentation adopted. Currently, the most used technique is Submerged Fermentation (FS), with lower viscosity and possibility of agitation and aeration (Castro *et al.*, 2011). In contrast, Solid State Fermentation (FES) reduces the likelihood of bacterial contamination and results in a more concentrated extract. The main difference between the two fermentation models is the amount of free water present (Santos *et al.*, 2018). Among the articles analyzed that used fungi of the phylum Basidiomycota as producers, five did not report fermentation parameters, two used FS and none FES. As for the fungi of the phylum Ascomycota, six used FES, six used FS and two did not propose fermentation parameters. FES has been increasingly used in the production of fungi as it simulates its natural environment, favoring growth (Soccol *et al.*, 2017), in addition to meeting the industrial demand for the use of ecological and economic resources, as it enables the application of agro-industrial residues. Still on the use of FES, the use of substrates such as wheat, soybean and rice bran was observed among the selected works, the former being present in 83% of fermentations alone or in some type of composition.

The physical parameters described in the analyzed studies can be seen in Table 1. The temperature conditions varied between 23°C and 30°C. These conditions are advantageous for use in industry, considering that the temperatures used are easily

reached, facilitating maintenance during this important step (Mamo *et al.*, 2020). Another important factor during fermentation is the pH of the medium, however only one paper presents this information, Xiao-Ian *et al.* (2014) used a synthetic medium (soy milk, glucose, KH_2PO_4 and MgSO_4) with pH 7 in FS at 25° C, more information can be seen in table 2. The lack of information regarding such important parameters affects the quality of the research, hindering the reproduction and introduction of fungi with great biotechnological potential in the industry.

3.5 Fibrinolytic Activity

The presence of proteases with fibrinolytic action can be verified in the laboratory using different methodologies. The Fibrin Plate Lysis Area (ALPF) test is considered a direct measure of fibrinolytic activity (Flute and MCPPath, 1964), this methodology allows the confirmation of the presence of fibrinolytic enzymes from the formation of a degradation halo. More modern methodologies use spectrophotometry to quantify fibrinolytic proteases. In this case, the enzyme is applied in a tube containing an artificially produced clot, the reaction occurs for a predetermined time, at the end, the clot dissolution can be noticed (Wang *et al.*, 2011), the reaction must be stopped and the reading performed in a spectrophotometer.

Regarding fibrinolytic enzyme quantification methodologies, most of the selected articles (71.42%) used ALPF, more specifically, the methodology described by Astrup and Mullertz (1952). Zhang *et al.* (2015) showed the highest specific activity using ALPF to measure the fibrinolytic activity produced by *Rhizopus microsporus* (43.2 U/mg) in FS. *Cordyceps militaris*, also produced under FS, had a similar activity of 40.7 U/mg (Liu *et al.*, 2015; Liu *et al.*, 2016) Of the 5 studies (23.8%) that used spectrophotometric methodology, 4 were performed with the fungus *Mucor subtilissimus*, which was capable of producing up to 57.1 U/mg of fibrinolytic enzyme in FES (Nascimento *et al.*, 2020). The use of spectrophotometric techniques implies a more assertive quantification of the biomolecule of interest since it is a sensitive and automated technique (Galo and Colombo, 2009).

3.6 Purification

Fibrinolytic enzymes are applied in the pharmaceutical industry, this approach implies the need to achieve a high degree of purity, so it is common to use more than one chromatography in sequence, such as gel filtration, ion exchange, hydrophobic interaction or affinity. Other techniques can still precede the purification process such as salt precipitation or by organic solvents, centrifugation and filtration. These strategies are commonly referred to as pre-purification steps, signaling the process of preparation for purification itself (Guggisberg *et al.*, 2011).

Purification of the enzymes studied in the analyzed articles was classified, according to the proposed methodology, in total (score 2), showing the enzyme purified by more than one chromatographic step; Partial (score 1), showing the enzyme purified in pre-purification, chromatographic or aqueous two-phase system (SDFA) methodologies; or absent (score 0, when no purification methodology is reported. As can be seen in Table 3, of the 21 selected articles, 18 (85.7%) presented fibrinolytic activity of purified enzymes, and none of the articles received a score 0.

Using ALPF to determine the fibrinolytic activity of an enzyme produced by *Paecilomyces tenuipes*, Kim *et al.* (2011). obtained specific activity of 431.81 U/mg and recovery of 3.8% after consecutive steps of CM cellulose, DEAE-sepharose CL6B fast flow, Sephadex G-75 and POROS 20 HQ. The combination of different types of chromatographic columns supports the individualization of the molecule of interest. The same can be seen in the purification of enzyme produced by *Aspergillus oryzae* KSK-3, which used steps followed by precipitation with ammonium sulfate, ultrafiltration and chromatography (DEAE-Toyopearl 650C, Sephacryl S-100HR and Superdex 75 HR 10/30), resulting in specific activity of 1005 U/mg). Already using spectrophotometry to quantify the proteases produced, Choi *et al.* (2013) used the sequence of CM cellulose, DEAE cellulose, Sephadex G-75, HiLoad 16/60 and Superdex 75 to obtain 220.65 U/mg of fibrinolytic enzyme produced by *Hericium erinaceum*.

While Nascimento *et al.* (2017) presents 154.79 U/mg after precipitation in ammonium sulfate, DEAE-Sephadex and Superdex 75 HR10/300 GL.

3.7 Enzymatic characterization

Enzymatic characterization is carried out using processes that involve chemical or physical agents. These agents are capable of affecting the native state of the enzyme and may even lead to denaturation and consequent total loss of activity. Thus, the characterization of produced enzymes, especially when destined for the pharmaceutical industry, is essential.

3.7.1 Optimal pH and Temperature

The optimal pH of the enzymes, that is, the pH range where they showed the greatest fibrinolytic activity, varied between pH 4 and 9, demonstrating a wide range of enzymes capable of maintaining themselves in acidic or alkaline conditions. However, most of the studied enzymes showed optimal pH in the range 7 (38%) and 8 (19%). Choi *et al.* (2017) showed optimal activity of enzyme produced by *Pleurotus ferulae* in three distinct pH ranges (4, 5 and 8), being the only work to report activity at pH 4, however its enzyme was able to maintain stability at 4°C for 12 hours at pH 7, 7.4 and 8, indicating greater interest in these conditions. At the other extreme, only one work showed optimal activity of enzymes produced by *Mucor subtilissimus* at pH 9 (with 23.3 U/mL) (Nascimento *et al.*, 2020). Only two studies did not assess optimal pH or stability, Nascimento *et al.* (2016) presents only data regarding the optimal temperature, however the same authors assess these conditions in another publication (Nascimento *et al.*, 2020). And Kumaram *et al.* (2011) does not report an assessment of these parameters.

Regarding the optimal temperature, as shown in Table 2, they vary between 35°C and 50°C, comprising 23.9% of the articles with more pronounced fibrinolytic activity at 37°C (Moon *et al.*, 2014; Zhang *et al.*, 2015; Liu *et al.*, 2015; Choi *et al.*, 2017; Liu *et al.*, 2017) and 19% with maximum activity at 50°C (Shirasaka *et al.*, 2012; Deng *et al.*, 2018; Clementino *et al.*, 2019). Two works did not present information regarding this data (Kumaram *et al.*, 2011; Mohamed-Ali *et al.*, 2014). Information regarding the optimum pH and temperature are extremely important for the development of basic and application research, establishing the ground for the development of valid alternatives for the treatment of thrombosis, so it is extremely important that in works where production and characterization of fibrinolytic enzymes, parameters with optimal pH and temperature, as well as stability, are determined.

3.7.2 Inhibitors

Inhibition of fibrinolytic proteases is another relevant segment of the characterization; inhibitors can act on enzymes by modifying structural characteristics that determine their mechanism of action and consequently inhibit enzymatic activity (Vargas, 2020). According to the studies analyzed, several compounds were able to inhibit the activity of the enzymes studied, these were PMSF (serine protease inhibitor) (Kim *et al.*, 2011; Choi *et al.*, 2011; Liu *et al.*, 2017; Moon *et al.*, 2014; Liu *et al.*, 2015; Liu *et al.*, 2016; Nascimento *et al.*, 2016; Nascimento *et al.*, 2017; Shirasaka *et al.*, 2012; Cleentino *et al.*, 2011); EDTA (metalloprotease inhibitor) (Guggisberg *et al.*, 2011; Xiao-Ian *et al.*, 2014; Meshram *et al.*, 2016; Andrade *et al.*, 2018; Meshram *et al.*, 2016; Lins *et al.*, 2019); SBTI (serine protease inhibitor) (Liu *et al.*, 2016; Liu *et al.*, 2017; Deng *et al.*, 2018; Li *et al.*, 2020); Aprotinin (Inhibitor of serine and cysteine protease) (Liu *et al.*, 2016); TPCK (serine protease inhibitor) (Shirasaka *et al.*, 2021); EGTA (metalloprotease inhibitor) (Choi *et al.*, 2013; Meschram *et al.*, 2016; Andrade *et al.*, 2018; Meshram *et al.*, 2016; Lins *et al.*, 2019; Choi *et al.*, 2016) and β -mercaptoethanol (cysteine protease inhibitor) (Clementino *et al.*, 2019).

Of the 21 articles selected, only four did not conduct tests evaluating the inhibition of enzymatic activity, according to these data, 81% of the analyzed articles identified fibrinolytic enzymes as serine proteases and 19% presented data demonstrating the presence of metalloprotease.

3.7.3 Molecular mass

The molecular mass of the enzymes studied in the selected works varies between 14 kDa and 70 kDa, however, more than half of the works (52.4%) present enzymes between 20 and 34 kDa. At the extremes of the spectrum we have Kim *et al.*, (2011) presenting fibrinolytic enzyme (1431.81 U/mg) produced by *Paecilomyces tenuipes* with 14 kDa in size; and Nascimento *et al.*, (2020) presenting fibrinolytic enzyme (43.12 U/mg) produced by *Mucor subtilissimus* with 70 kDa. Three articles did not present data.

4. Final Considerations

Excessive search was performed in databases reporting a total of 426 articles in general, after applying predetermined inclusion and exclusion criteria, 21 articles published between 2011 and 2021 were selected. According to the analysis and scoring criteria, it was observed that 52.4% of the articles had a final score above 7, however only one reached a score 9 presenting information on obtaining the enzyme from the evaluation of the cultivation conditions to the application of the enzyme, and none reached the maximum score (10). Thus, it is important to direct more in-depth research on fibrinolytic enzymes produced by fungi, considering that such molecules show great variation. It is noteworthy that, despite being quite promising, solid state fermentation is still not sufficiently explored, especially in relation to studies with substrate analysis or factorial planning, being present in only two of the analyzed works. Almost all articles presented complete purification and characterization, indicating pH 7-8 and 37°C as optimal conditions for enzyme activity, in addition to highlighting a higher prevalence of serine proteases in the analyzed studies.

The analysis performed was also able to verify the tendency of the prevalence of purification and characterization of enzymes on the production and application in the analyzed works. However, the optimization of cultivation and production parameters can be decisive for the frequency of fibrinolytic activity. Similarly, studies involving the application of the enzyme can also be deterministic in its industrial direction, an essential quality for its future fate as a thrombolytic agent. In this way, this work indicates the need to expand the publications related to the production of fibrinolytic enzymes as well as the evaluation of their applications. As for purification and characterization, despite being more present in the studies studied, as they are such important parts in the development and application of this enzyme, it is necessary to pay attention to the development of a more complex characterization, exploring the physicochemical properties of the enzyme.

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Conflict of interests

The authors declare no conflict of interest.

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