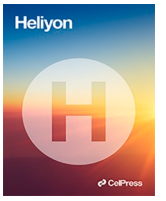
Heliyon 10 (2024) e26668

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Review article

# Diverse origins of fibrinolytic enzymes: A comprehensive review

Chinmay Hazare, Prashant Bhagwat, Suren Singh, Santhosh Pillai *

*Department of Biotechnology and Food Science, Faculty of Applied Sciences, University of Technology, P.O. Box 1334, Durban, 4000, South AfricaDurban*

ARTICLE INFO

*Keywords:*
Cardiovascular diseases
Nattokinase
Therapeutic enzymes
Thrombolytic drugs
Staphylokinase
Streptokinase
Fermented foods

ABSTRACT

Fibrinolytic enzymes cleave fibrin which plays a crucial role in thrombus formation which otherwise leads to cardiovascular diseases. While different fibrinolytic enzymes have been purified, only a few have been utilized as clinical and therapeutic agents; hence, the search continues for a fibrinolytic enzyme with high specificity, fewer side effects, and one that can be mass-produced at a lower cost with a higher yield. In this context, this review discusses the physiological mechanism of thrombus formation and fibrinolysis, and current thrombolytic drugs in use. Additionally, an overview of the optimization, production, and purification of fibrinolytic enzymes and the role of Artificial Intelligence (AI) in optimization and the patents granted is provided. This review classifies microbial as well as non-microbial fibrinolytic enzymes isolated from food sources, including fermented foods and non-food sources, highlighting their advantages and disadvantages. Despite holding immense potential for the discovery of novel fibrinolytic enzymes, only a few fermented food sources limited to Asian countries have been studied, necessitating the research on fibrinolytic enzymes from fermented foods of other regions. This review will aid researchers in selecting optimal sources for screening fibrinolytic enzymes and is the first one to provide insights and draw a link between the implication of source selection and *in vivo* application.

## 1. Introduction

Global mortality is predominantly attributed to cardiovascular diseases (CVDs), standing as the foremost factor behind fatalities [1]. The number of people dying of heart diseases has gone from more than 2 million cases in 2000 to nearly 17.9 million in 2019 [2]. Furthermore, the incidence of cardiovascular damage has been positively associated with higher COVID-19 mortality, thus further increasing the global burden [3]. The most common reason behind cardiovascular disorders like ischemic heart disease (acute coronary syndrome), peripheral vascular disease (PVD), pulmonary embolism (PE), venous thromboembolism (VTE) and stroke is thrombosis, resulting in the blocking of artery or vein by a blood clot (thrombi) [4]. Principally, the formation of a blood clot is the body's natural mechanism to seal the vessel wall injury (Fig. 1); given this, it is to wonder how the formation of blood clots contributes to the development of thrombosis. It is a well-established fact that under normal physiological conditions, an optimal balance between coagulation and anticoagulation of blood is maintained by a tightly regulated process called hemostasis. This is achieved by two oppositely acting systems, namely 'coagulation', to prevent blood loss during injury and 'anticoagulation', to maintain blood fluidity [5]. However, an imbalance of any kind in this state of equilibrium can lead to prolonged bleeding at the site of the wound, internal

* Corresponding author.
*E-mail addresses:* hazarechinmay@gmail.com (C. Hazare), pkbhagwat9988@gmail.com, prashantb@dut.ac.za (P. Bhagwat), singhs@dut.ac.za (S. Singh), santhoshk@dut.ac.za (S. Pillai).

https://doi.org/10.1016/j.heliyon.2024.e26668
Received 8 February 2024; Accepted 16 February 2024
Available online 22 February 2024

bleeding, or thrombosis.

The aggregation of platelets, fibrin, and thrombin in the arteries causes arterial thrombosis, contributing to the emergence and spreading of atherosclerotic plaque, resulting in myocardial infarction [6,7]. The thrombus formed can be treated either by removing or preventing them from forming in the first place. The process of dissolution of thrombi is called thrombolysis, whereas degradation of only the fibrin mesh around the blood clot is called fibrinolysis [8]. The treatment of thrombosis includes using various anticoagulants, antiplatelet medications, fibrinolytic medicines, and surgical techniques [9]. Currently, patients with CVDs are administered with plasmin activators, such as streptokinase, alteplase (t-PA), anistreplase, reteplase (r-PA), and tenecteplase (TNK-*t*-PA), which are commercially available and FDA-approved thrombolytic drugs. However, due to their limited affinity for fibrin and short half-life, they must be constantly injected to be effective, but they also cause other negative effects like internal haemorrhages and allergic responses [10].

Fibrinolytic enzymes, on the other hand, have specificity for fibrin, enabling them to directly lyse the pre-existing thrombi (Minh et al., 2022). Fibrinolytic enzymes are prevalently found in a wide variety of sources including food as well as non-food sources, however, are predominantly found in microbes isolated from these sources. Fibrinolytic enzymes have also been discovered from non-food sources such as snake venom, earthworms, plants, and algae. However, the search for safe, effective, and economical fibrinolytic enzymes continues due to the inherent side effects of current fibrinolytic medicines. While those from food sources, especially fermented foods have various advantages over those currently in use. Microbial fibrinolytic enzymes of food origin are gaining momentum due to their ubiquitous nature, low cost of production, high substrate specificity, and reduced toxicity. The current impetus is on exploring traditional fermented foods as sources of fibrinolytic enzymes due to their rich microbial diversity and long-standing history of safe consumption. This review is an attempt to provide valuable insights into fibrinolytic enzymes and their sources, current thrombolytics, and innate mechanisms of both clot formation as well as dissolution. By categorising fibrinolytic enzymes purified from microbes isolated from food and non-food sources, this review facilitates researchers' search for better fibrinolytic enzymes. In particular, it will help researchers compare microbial fibrinolytic enzymes isolated from food and non-food sources and determine if there are any significant differences in efficacy or other properties. It will also assist the food industry in identifying specific food sources that could be used in the creation of fermented food products with potential health benefits. By marketing such products as functional foods, the industry could tap into growing consumer demand for foods offering health benefits beyond basic nutrition. In summary, the readers of the review will be facilitated to focus their research efforts, compare different types of fibrinolytic enzymes and their sources, and identify potential applications in the pharmaceutical and food industries.

## 2. Fibrinolytic enzymes

Fibrinolytic enzymes are peptidase-acting proteases which belong to the hydrolase class of enzymes (EC 3.4) [11], that degrades fibrin, the major protein involved in blood clotting. Thus, they play a huge role in dissolving blood clots, also known as thrombi, which

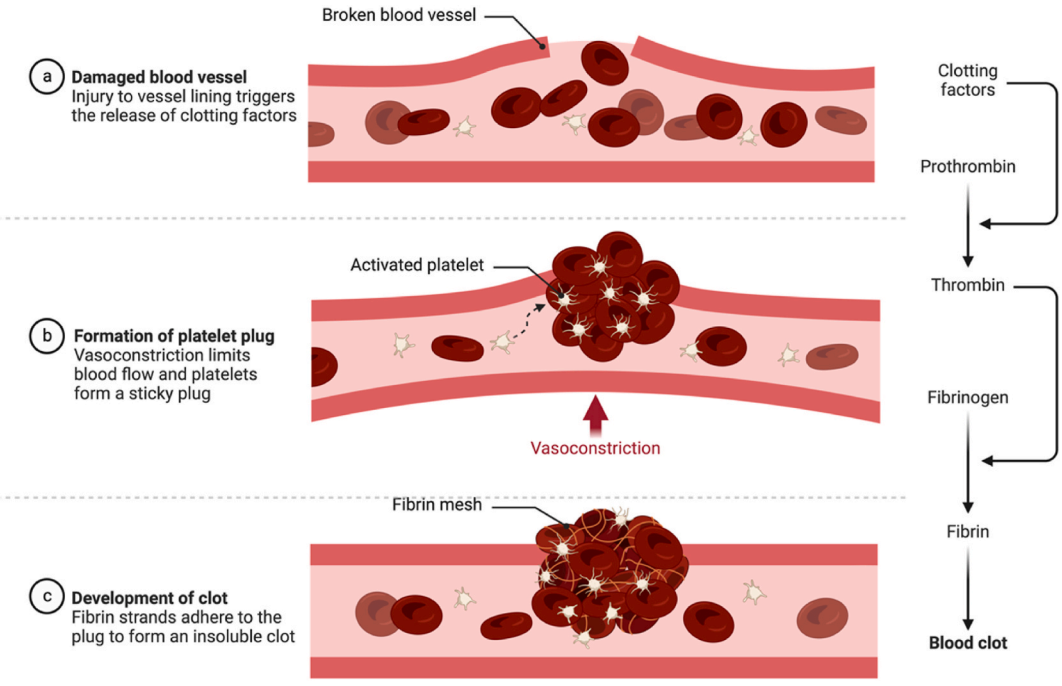



**Fig. 1.** A simplified representation of the coagulation system illustrating how clot formation safeguards against blood loss resulting from the vascular injury.

ensure smooth blood flow through the vessels during normal homeostasis. The fibrinolytic enzymes work in two ways. They can either indirectly promote the lysis of fibrin by activating circulating plasminogen, converting it into active plasmin that acts on the fibrin, the process being called indirect fibrinolysis, or it can directly degrade pre-existing insoluble fibrin into smaller fragments called fibrin degradation products by direct fibrinolysis, thereby restoring typical vascular function.

In 1958, the initial human trials of Streptokinase, a fibrinolytic enzyme sourced from haemolytic Streptococci, were conducted [12]. This was before the discovery and advancement of tPA and uPA, but it was not until the 1980s, when the advent of recombinant DNA technology facilitated large-scale production, that these agents gained prominence. Initially, thrombolysis as a treatment was used only for patients with myocardial infarction. However, during the mid-1990s, tPA was given approval for a specific purpose: treating patients diagnosed with acute ischemic stroke, with certain restrictions [13]. The most used enzyme-based fibrinolytic medicines are urokinase, streptokinase, genetically modified tissue plasminogen activator (*r*-tPA), and nattokinase. First-generation fibrinolytic drugs include streptokinase and tPA, and second and third-generation fibrinolytic agents include alteplase and staphylokinase, respectively [10]. These drugs exert their effects via diverse mechanisms and exhibit varying levels of selectivity towards fibrin clots. Furthermore, fibrinolytic enzymes lyse pre-existing thrombus, unlike warfarin and heparin, which only prevent thrombosis extension and recurrence. Administration of streptokinase and urokinase to patients with pulmonary embolism increases the likelihood of clot clearance, three times compared to patients treated with heparin alone [14].

Streptokinase is a thrombolytic drug derived from *Streptococci*, mainly used to treat acute myocardial infarction and pulmonary thromboembolism. Its mechanism of action involves converting plasminogen to plasmin, thus improving the body's natural fibrinolytic processes. However, as it originates from a haemolytic bacterial source, it triggers an antigenic response, and upon repeated administration results in the formation of neutralizing antibodies and causes allergic reactions [15]. Urokinase is another such thrombolytic drug which acts by activating plasminogen and was first identified in 1947 in human urine [16]. On the other hand, *r*-tPA is made using recombinant DNA technology and acts as a substitute for endogenous tPA that activates the fibrinolytic system. It doesn't elicit any allergic reaction and possesses high specificity towards the clot. The drawback, however, is its short half-life and the requirement for continuous drug infusion to obtain maximum efficacy. Other adverse effects may include gastrointestinal bleeding due to the lack of site-specificity, but severe anaphylaxis is uncommon [15]. Fibrinolytic enzyme therapy is costly, for example the intravenous administration of urokinase, and further may lead to undesirable side effects such as the occurrence of acute resistance to reperfusion, bleeding complications and coronary reocclusion [17]. As a result, researchers have tried isolating fibrinolytic enzymes from various sources, including non-microbial and microbial sources of food and non-food origin, in order to improve the specificity and efficacy of fibrinolytic therapy. Fibrinolytic enzymes have been purified from microbes isolated from non-food sources such as soil, marine environments and directly from plants, snake venom, and algae. Food sources are rich in microbial fibrinolytic enzymes mostly comprise fermented foods such as Japanese Natto [18], Korean Chungkook-Jang soy sauce [19] as well as non-fermented food sources such as edible honey mushrooms [20], to name a few. Fibrinolytic enzymes isolated from food sources are gaining traction because they show the ability to dissolve blood clots efficiently and restore normal vascular function [21]. Enzymes from fermented foods, especially those of microbial origin, can be mass-produced cost-effectively and can even be administered as supplements. Nattokinase, for instance, was the first fibrinolytic enzyme to be purified from a food source. In 1980, Prof. Hiroyuki Sumi, a Japanese researcher at the Chicago University Medical School, discovered that natto can dissolve artificial fibrin. Sumi and his team extracted an enzyme from natto that not only degraded fibrin but also a plasmin substrate. He named this novel, fibrinolytic enzyme "nattokinase". It is a serine protease purified from natto; a Japanese traditional food produced from the fermentation of soybeans with the bacterium, *Bacillus subtilis* (natto) [18].

Numerous microorganisms have been discovered as source of fibrinolytic enzymes. For instance, *Streptococcus hemolyticus* produces streptokinase (Banerjee et al., 2004), *Bacillus subtilis* produces nattokinase (Weng et al., 2017), *Bacillus* sp. Produces bafibrinase (Mukherjee et al., 2012), *Serratia* sp. also contributes to this group (Taneja et al., 2017). Additionally, the fibrinolytic enzymes derived from Bacillus sp. isolated from traditional fermented foods have already been developed as effective food additives and medications for treatment or to prevent the onset of thrombosis and related disorders. According to Peng, Huang, Zhang and Zhang [22], the microbial fibrinolytic enzyme subtilisin DFE from *Bacillus subtilis* demonstrated high specificity and low production cost. A relatively high yield in mass culture was also reported with the potential for further improvement through genetic engineering [23]. In addition, by focusing on specific enzymes like subtilisin DFE, a better understanding of the unique characteristics and potential applications of food-derived microbial fibrinolytic enzymes can be achieved. On the other hand, the microbial and non-microbial fibrinolytic enzymes isolated from non-food sources had low specificity towards fibrin. Moreover, the majority of enzymes investigated from non-food sources have been shown to induce fibrinogenesis, in addition to causing fibrinolysis, thus adversely affecting normal hemostasis [24]. Hence, the unconventional sources have failed to exhibit the expected results and come with significant constraints.

## 3. Fibrinolytic enzyme categories

Fibrinolytic enzymes fall under the family of protease enzymes. Most proteases range in size from ~20 kDa simple catalytic units to 0.7–6 MDa complex proteolytic machinery that cleaves α-peptide bonds and isopeptide bonds of target proteins leading to proteolysis or sometimes even self-hydrolyze their polypeptide chains [25]. They are the largest single family of enzymes, accounting for around 2% of the human genome [26]. Proteases are categorized into six distinct groups based on the active site amino acid or metal ion responsible for catalysing the cleavage of peptide-bond. Among these groups, aspartate, glutamate, and metallopeptidases utilize acid-base catalysis, while serine, cysteine, and threonine peptidases employ covalent catalysis [27]. Similarly, fibrinolytic enzymes, on their mechanism of action can be widely classified into three different types, viz., serine proteases, metalloproteases and serine metalloproteases [28].

### 3.1. Serine protease

Serine proteases, which make up more than one-third of all known proteolytic enzymes, are endopeptidases or types of enzymes that cleave peptide bonds in proteins. These enzymes feature serine at their active site as the nucleophilic amino acid [29]. Fibrinolytic enzymes belonging to the serine protease group display indirect and direct fibrinolytic activity and get inactivated in the presence of the PMSF inhibitor [30]. Serine proteases have been extensively researched for their fibrinolytic characteristics, and it is known that serine proteases such as brinase, plasmin, and trypsin, can directly hydrolyze thrombin, leading to their dissolution [31]. However, fibrinolytic therapy involving the use of serine proteases with direct fibrinolytic action poses the risk of non-specific lysis of clots, that is those necessary for homeostasis, such as those essential for the maintenance of the blood-brain barrier [32]. Until now, tPA and uPA are the only two indirect serine proteases licensed by the Food and Drug Administration [33]. Recently, fibrinolytic enzymes from the serine protease group have been isolated from various sources such as mushroom *Agrocybe aegerita* [34], bacteria *Bacillus flexus BF12* [35], *Streptomyces parvulus* [36], Antarctic krill *Euphausia superba* [37], tree *Pseuderanthemum latifolium* [38] and so on.

### 3.2. Metalloprotease

Metalloproteases comprises of a diverse group of proteolytic enzymes, including both exopeptidases and endopeptidases [39]. Metalloproteases contain one or two divalent metal ions at their active site, coordinated by three amino acid sidechain ligands [40]. In the hydrolysis of the peptide bond, a water molecule plays a crucial role and also serves as a fourth ligand to coordinate with the metal ion in the active form of metallopeptidases. The metal ligands known in metalloproteases include His, Glu, Asp, or Lys [40]. These ligands occur at the catalytic site as follows: His $\gg$ Glu $>$ Asp $>$ Lys. Although most metalloproteases only require one metal ion for catalysis, some have two metal ions that act co-catalytically. Furthermore, fibrinolytic enzymes belonging to the class metalloprotease require metal ions (divalent) e.g., $Hg^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Co^{2+}$, or $Ca^{2+}$ to exert their catalytic activity [41]. Hence, the diagnostic feature of metalloproteases is a chelating agent such as EDTA, which inhibits the enzyme activity. Several studies suggest that metal ions, including $Na^{+}$, $K^{+}$ and $Ca^{2+}$, activate metalloproteases [42,43]. Though many researchers have reported the effect of metal ions on crude fibrinolytic metalloprotease extracts from various sources, only a few have succeeded in purifying fibrinolytic metalloproteases. Some examples include CMase [44], PoFE [45], FVP-I [46], AMMP [46], BKII (Bacillokinase II) [47] and TSMEP I [48].

### 3.3. Serine metalloprotease

Serine metalloproteases are proteases which possess characteristics of both serine as well as metalloproteases and hence are classified as the third category of fibrinolytic enzymes. These enzymes are involved in physiological and pathological processes, such as blood coagulation, fibrinolysis, extracellular matrix remodelling, and immune response. Serine metalloproteases contain a catalytic domain with the characteristic serine residue in the active site, which is responsible for the proteolytic activity of serine proteases [49]. In addition, they have a metal ion site, which is a hallmark of metalloproteases. The presence of both catalytic domains makes these enzymes unique in their ability to perform proteolysis using the mechanism of serine as well as metalloproteases. Examples of serine metalloproteases include M179, CFR15, and AprE176. Velefibrinase from marine *Bacillus velezensis* Z01, is a serine metalloprotease as it was inhibited by PMSF as well as EDTA [50]. Similarly, a fibrinolytic enzyme purified from marine *Serratia marcescens* subsp. *sakuensis* showed dual activity of serine and metalloproteases [51]. Serine metalloproteases have also been purified from a microbial fermented food source, for example, *Bacillus licheniformis* HJ4 isolated from the traditional Korean fermented seafood, Hwangseokae jeotgal [52].

## 4. Thrombolytic agents

### 4.1. First generation thrombolytic agents

The first-generation thrombolytic agents include streptokinase and urokinase. These drugs are non-selective thrombolytic agents and function in more than one way. Firstly, they may function by activating free circulating plasminogen to plasmin, breaking down the blood clots. However, they may also directly lyse fibrin. In addition, they sometimes degrade clotting factors like fibrinogen, thus interfering with the fibrin clot formation process. They have a broad range of applications such as dissolving blood clots, restoring blood flow and preventing or reducing tissue damage and are used to treat conditions such as deep vein thrombosis (DVT) and myocardial infarction [53].

#### 4.1.1. Streptokinase

Streptokinase (SK, EC 3.4.99.22) was serendipitously discovered by Dr William Smith Tillet in the year 1933, which led to development of thrombolytic therapy as a treatment for cardiovascular disorders [54]. Later in the 1950's streptokinase got approval from the FDA to become the first plasminogen activator thrombolytic drug. It is a plasminogen activator of microbial origin secreted by various strains of β-hemolytic *Streptococci* including Lancefield groups A, C, and G. Among these, the *Streptococcus equisimilis* H46A (ATCC 12449 (1945) and ATCC 9542 (1992)) belonging to the Lancefield group C strain of is preferred for streptokinase production. This particular strain stands out for streptokinase production because it lacks erythrogenic toxins and has less demanding growth requirements in comparison to other strains [55].

Streptokinase, a single-chain polypeptide, consisting of 414 amino acid residues, possesses a molar mass of 47 kDa and exerts its

fibrinolytic action via indirect fibrinolysis, by activating the circulatory plasminogen [56]. Jackson and Tang (1982) were the first to establish the complete amino acid sequence of streptokinase. The enzyme's structure comprises three domains linked together with flexible loops [57]. The enzyme's action is indirect, as it converts plasminogen into active plasmin through a sequence of protein-protein interactions that form the streptokinase-plasmin complex in a 1:1 ratio. As a result of this interaction, the complex breaks down the arginine-valine bond within plasminogen, leading to the formation of this proteolytic enzyme 'plasmin', which effectively degrades the thrombus matrix [54]. The thrombolytic activity thus aids in the removal of blood clots and artery blockages, which are the primary causes of myocardial infarction and heart attacks [58].

The World Health Organization lists streptokinase as one of the essential thrombolytic medications. Commercially available streptokinase is a sterile, purified preparation of the protein formulated as a lyophilized powder. It is a first-generation plasminogen activator frequently utilized, partly in thrombolytic therapy in developing countries because of its economic viability compared to its counterparts [59]. Although streptokinase has successfully saved many lives since its first clinical usage, it is accompanied by some negative side effects. As a protein derived from a hemolytic bacteria, it can stimulate the production of antibodies in humans, making the drug inactive and preventing its re-administration for at least six months after its first application [60]. Additionally, in the case of previous Streptococcal infections, administration of streptokinase may increase the risk of severe, potentially fatal, allergic reactions in humans. Furthermore, it being a non-fibrin-specific plasminogen activator, could result in bleeding. Notwithstanding the limitations, the efficacy and cost-effectiveness of SK have made it an indispensable medicine in medical facilities, emergency rooms, and intensive care units. Furthermore, efforts are underway to modify streptokinase to increase its half-life, reduce immunogenicity, reduce production costs, and boost biological efficacy.

#### *4.1.2. Urokinase/urokinase-type plasminogen activator*

MacFarlane and Pilling (1947) [61] discovered an unnamed novel fibrinolytic enzyme in human urine, which was named as urokinase half a decade later by Sobel, Mohler, Jones, Dowdy and Guest [62]. Urokinase is derived from various sources, including macrophages, endothelial cells, certain tumor cells, and renal epithelial cells. In its inactive state, urokinase exists as a single polypeptide glycosylated zymogen comprising 411 amino acids, referred to as pro-urokinase, pro-uPA, or scu-PA [63]. This pro-urokinase is composed of three distinct domains: a growth factor domain (GFD) spanning amino acids 1 to 49, a kringle domain (KD) covering amino acids 50 to 131, both situated at the N-terminus, and a serine protease domain (P) spanning amino acids 159 to 411, situated at the C-terminus. The N-terminal and C-terminal domains are connected by a linker segment comprising amino acids 132 to 158 [64]. After being secreted, the glycosylated zymogen (pro-uPA) undergoes a two-round proteolytic process before being converted to its active form and this is accomplished by proteolytic enzymes like plasmin and others like nerve growth factor-g, cathepsin B and L, trypsin, thermolysin, kallikrein, and mast cell tryptase while plasmin being the most effective [65–68]. In addition, pro-uPA can also be cleaved at various sites by proteases such as elastase and thrombin [65]. Upon the first round of proteolysis, the linker region between Lys 158 and Ile 159 is cleaved, resulting in two chains that are still linked by a disulphide bond and have a molecular weight of 54 kDa. Subsequently, a second round of proteolysis occurs at the peptide bond between Lys 135 and Lys 136, leading to the complete separation of the two chains of uPA. Consequently, an inactive amino-terminal fragment (ATF) forms, comprising the kringle domain and the growth factor domain (GFD), while resulting in the formation of an active low molecular weight form of uPA (33 kDa), containing the serine protease domain [15]. Finally, both pro-uPA and the two-chain variants of uPA, or tcu-PA, comprising of an active serine protease domain and an inactive ATF with growth factor domain and kringle domain, bind to their receptor, uPAR, with similar affinity. This similarity in binding affinity is likely attributed to the presence of GFD and the amino-terminal fragment (ATF) [69]. In the past, human urine used to be the prime source of urokinase production, but currently, it is produced using tissue culture and recombinant DNA techniques (expressed in *E. coli*). Its clinical use in treating cardiovascular disorders has been approved by the FDA due to its notable plasminogen activation activity [70].

### *4.2. Second generation thrombolytic agents*

The need for targeted thrombolysis drove the development of second-generation thrombolytic agents, as their predecessors caused non-specific fibrin degradation and degradation of other hemostatic proteins, ultimately leading to haemorrhage. Specifically, the major motivation for developing these agents was to improve their selectivity and specificity towards fibrin-rich thrombi, while minimizing the risk of bleeding complications associated with systemic fibrinolysis. Second-generation thrombolytic agents offer a more promising approach to treating thrombotic disorders by addressing these limitations. Second-generation thrombolytics include alteplase, reteplase, and tenecteplase. These drugs are more selective than first-generation thrombolytics and only activate plasminogen bound to fibrin in blood clots. This makes them more effective and reduces the risk of bleeding. Second-generation thrombolytics are used to treat conditions such as acute ischemic stroke and pulmonary embolism.

#### *4.2.1. Anistreplase or Anisoylated plasminogen-streptokinase activator complex (APSAC) or Eminase)*

Anistreplase is a derivative of streptokinase, chemically modified to form an equimolar complex with human lys-plasminogen of 131 kDa. This complex consists of the catalytic site of plasminogen acylated with *p*-amidinophenyl p'anisate hydrochloride (APAN), which acts as a reversible acylating compound [71]. Through the acylation process, the compound is rendered catalytically inactive, allowing for its administration as a single-bolus injection and safeguarding it from neutralization by plasmin inhibitors. After injection, the deacylation process commences with the hydrolysis of the *p*-anisate group, leading to the formation of the active plasminogen-streptokinase complex and initiating the thrombolysis process [59]. This strategy allows for the drug to be sustained in circulation for a considerably longer period. Compared to streptokinase, Anistreplase demonstrates improved plasma stability and

possesses a longer half-life, approximately 90–105 min, enhanced fibrin binding, as well as a shorter administration time (60 min to 2–5 min). Furthermore, APSAC exhibits a greater degree of selectivity towards plasminogen bound to a blood clot [16]. The single-bolus dose regimen of APSAC is particularly suitable for pre-hospital Acute Myocardial Infarction (AMI) treatment. According to the 'Third International Study of Infarct Survival' (ISIS-3) which underwent a 3 × 2 factorial trial to compare the three thrombolytic drugs to each other, APSAC and streptokinase were found to be similarly effective in improving patient outcomes and reducing mortality rates. Furthermore, APSAC is similar to streptokinase in terms of its ability to trigger an immune response (antigenicity) and has a similar safety profile [59].

#### 4.2.2. Prourokinase (r-scuPA, saruplase, pro-uPA)

Pro-urokinase is an inactive precursor of urokinase and is also referred to as 'single chain urokinase-type plasminogen activator' or 'scu-PA'. This naturally occurring glycoprotein consists of 411 amino acid residues and has been studied as a potential fibrinolytic agent. A notable benefit of scu-PA lies in its ability to promote clot-specific lysis in the presence of fibrin, which is not observed with tcu-PA due to its lack of fibrin specificity [72]. Furthermore, *in vivo*, pro-uPA has the capability to undergo cleavage, resulting in the formation of the two-chain urokinase, subsequently leading to the generation of plasmin [73]. Compared to urokinase, pro-uPA has a longer half-life of more than 24 h and can achieve complete lysis of fibrin in 90 min, while urokinase requires 180 min for fibrinolysis [74].

Prourokinase (*r*-proUK) can be produced using recombinant technology using bacterial systems (*E. coli*) as well as mammalian cell lines and has undergone many clinical studies. Prolyse®, is a recombinant prourokinase (*r*-proUK) by Abbott Laboratories effective for myocardial infarction and stroke (Sun et al., 1998; Ouriel 2002). The efficacy of direct local intraarterial thrombolysis using intra-arterial administration of prourokinase (Prolyse; Abbott Laboratories, Abbott Park, IL) is effective in treating large-vessel middle cerebral artery occlusions within 6 h of a stroke. The success of this treatment approach has been shown through anecdotal case series and a clinical trial called the 'Prolyse in Acute Cerebral Thromboembolism' or (PROACT) II study conducted by Furlan, Higashida, Wechsler, Gent, Rowley, Kase, Pessin, Ahuja, Callahan and Clark [75]. Unfortunately, the study did not lead to approval of Prolyse or the intraarterial method by the US Food and Drug Administration due to inadequate statistical power in the randomized trial.

#### 4.2.3. Tissue plasminogen activator (t-PA)

Tissue plasminogen activator (tPA or PLAT, EC 3.4.21.68) is an endogenous serine protease, secreted into the bloodstream by endothelial cells [76]. In their pioneering work, Rijken, Wijngaards, Zaal-de Jong and Welbergen [77] initially isolated tPA from the human uterus and subsequently confirmed that vascular endothelial cells synthesize and secrete tPA into the bloodstream. This 70 kDa single-chain polypeptide human tPA, which is found on chromosome 8, is composed of 527 amino acid molecules. It contains 35 cysteine residues involved in forming 17 disulphide bonds, demonstrating the disulfide-rich protein model in the human body [78]. It is a glycoprotein with five domains: a) 47-amino-acid fibronectin type I domain (F, 4–50 residues), b) an epidermal growth factor domain (EGF, 50–87 residues), c) kringle 1 (K1, 87–176 residues), d) kringle 2 (K2, 176–262 residues), all four located N-terminally and the fifth, e) a serine protease domain (P, residues 276–527), located C-terminally [79]. The main role of the tissue plasminogen activator is to catalyze the conversion of plasminogen to plasmin, a pivotal enzyme involved in dissolving blood clots. Specifically, tPA binds to fibrin on the surface of the clot and cleaves plasminogen at the Arg561-Val562 peptide bond, resulting in the active form of plasmin, a serine protease. Subsequently, plasmin disintegrates the cross-links between fibrin molecules, leading to the clot's dissolution. Fortunately, plasmin is only active for a short period, preventing the fibrinolytic system from being activated indefinitely [76]. Considering its ability to catalyze the degradation of thrombus, it is categorized as the second-generation thrombolytic drug and is used in treating acute myocardial infarction, pulmonary embolism, and cerebrovascular thrombotic stroke [59].

Tissue plasminogen activator, in contrast to uPA, exhibits a strong affinity for fibrin and significantly enhances (~500-fold) plasminogen activation. This allows fibrin to self-destruct, which is necessary as it only serves as a temporary matrix [80]. Despite the advancements in endovascular clot removal (ECR), thrombolysis using tPA (alteplase) continues to be the principal mode of treatment for acute ischemic stroke. However, tPA has a limited plasma half-life of 4–6 min due to rapid liver clearance, which is one of its disadvantages [81]. In emergency situations, using tPA intravenously poses challenges for drug administration. Administering a 100% bolus of tPA is not sufficient for sustaining its lytic function over an extended period. To achieve an effective removal of blood clots, a 10% bolus followed by a 1-h infusion of tPA is necessary. Nevertheless, this approach has its drawbacks, and a more practical solution, especially in time-sensitive conditions like acute ischemic stroke, would be to use a thrombolytic agent with an extended plasma half-life [13]. Additionally, the presence of fibrin greatly enhances the activity of the plasminogen activator, while its activity is weak in the absence of fibrin. Moreover, the inherent plasminogen activator inhibitor 1 (PAI-1) inhibits its activity *in vivo* by binding to tPA and forming an inactive complex, and the amino acids 296–299 are critical for this inhibition. The liver then removes this inactive complex from the circulation via the scavenger receptor named LDL receptor-related protein 1 (LRRP1), which explains why it has a short half-life [82]. These practical considerations were the driving force behind developing third-generation thrombolytic agents.

#### 4.2.4. Alteplase

Alteplase is a recombinant tissue plasminogen activator (t-PA) that has been cloned to be fibrin-specific. It is produced in Chinese hamster ovary (CHO) cells using recombinant DNA technology, which results in a t-PA that is similar but not identical to native plasminogen due to potential differences in post-translational modifications. It contains a triple mutation consisting of T103 N and N117Q within the K1 domain, as well as the substitution of residues 296–299 with tetra-alanine in the protease domain. These mutations are crucial for providing a prolonged half-life and resistance to the inhibitory action of plasminogen activator inhibitor-1 (PAI-1), unlike t-PA [83]. In 1986, human t-PA became the first therapeutic protein from recombinant mammalian cells to obtain market

approval [84]. Similar to the native t-PA, it consists of a single 70-kDa polypeptide chain containing 527 amino acids. When administered intravenously, a 100 mg dose of alteplase is estimated to decrease plasma fibrinogen levels by 16–33%. It is marketed by the brand name Activase®, Actilyse and Cathflo and has a half-life of about 4–6 min. It is produced from a human melanoma cell line and native human t-PA. Alteplase, being a recombinant form of human t-PA and not bacterial-derived, is non-immunogenic, display excellent selectivity and affinity for fibrin [85]. It may activate clot-bound plasminogen more effectively than circulating plasminogen, leading to plasminogen conversion at the clot site rather than in systemic circulation. However, its use is associated with an elevated risk of bleeding, intracerebral haemorrhage, and a shorter plasma half-life [86].

### *4.3. Third generation thrombolytic agents*

These are modified versions of the second-generation thrombolytics, designed to have an even higher affinity for fibrin, a longer half-life, lower bleeding risks and convenience compared to earlier generations. Examples include tenecteplase and desmoteplase. They are associated with even lower bleeding complication rates and are more convenient to administer, as they can be given as a single bolus injection.

#### *4.3.1. Tenecteplase (TNK)*

Tenecteplase (TNK) is a tissue plasminogen activator (tPA) variant developed in 1994. TNK is very similar to tPA, differing from it by only six amino acids and sharing 97% amino acid identity with tPA. TNK has a molecular weight of 65 kDa with 527 amino acid residues and has three-point mutations wherein Thr103 is replaced with Asn, Asn 117 with Gln, and Lys296-His297-Arg298-Arg299 each with alanine [87]. This has led to an increase in its plasma half-life to 30 min while retaining its advantageous properties. These modifications have improved its resistance to against the action of plasminogen activator inhibitor-1 (PAI-1) and resulted in a longer-lasting tPA-like molecule that has a greater affinity towards fibrin. TNK is more fibrin selective and has a 14-fold increased affinity towards fibrin compared to its counterpart tPA. Its extended half-life allows for rapid administration in a single bolus, enabling its use outside a hospital setting. The drug is manufactured using Chinese hamster ovary (CHO) cells and is marketed in the United States as TNKase™ (Genentech, Inc.) and in Europe as Metalyse® (Boehringer Ingelheim GmbH). *E. coli* has been commonly used to produce recombinant tPA, but *Pichia pastoris*, a methylotrophic yeast, has also been identified as a viable alternative for both eukaryotic and prokaryotic expression systems for *r*-tPA production [59].

#### *4.3.2. Staphylokinase (specific)*

Staphylokinase (SAK, EC 3.4.99.22), although an extracellular protein produced by *Staphylococcus aureus* is considered a third-generation fibrinolytic enzyme as it selectively activates plasminogen at the site of clot formation [53]. It is also referred to as staphylococcal fibrinolysin or Müller's factor consisting of 136 amino acid residues and is a single-chain polypeptide with a 15.5 kDa molecular weight [88]. Staphylokinase shares a similar thrombolytic activity with streptokinase wherein it acts by converting plasminogen to plasmin. It has the ability to form 1:1 stoichiometric complex with plasmin or plasminogen, thereby triggering the activation of additional plasminogen molecules and eventually dissolving the blood clot [59]. However, staphylokinase naturally produced by *S. aureus* causes a high immune response when injected intravenously as a drug, while obtaining high yields of staphylokinase during production also poses challenges. To address this challenge, researchers have utilized recombinant DNA technology and protein engineering to develop various expression systems capable of producing engineered recombinant staphylokinase molecules. These modifications aim to enhance the fibrinolytic activity and overcome the limitations of the existing drug. The goal is to provide recombinant staphylokinase with higher yields, improved fibrinolytic activity, and reduced immunogenicity. Although third-generation recombinant thrombolytic agents have shown promise in improving fibrin specificity and efficacy while reducing bleeding risk, more clinical trials are needed to confirm these findings.

In the last few decades, extensive clinical trials have been conducted on various medications that act as fibrinolytic agents and anticoagulants via various mechanisms and treatment methods. Since the 1980s, the FDA has approved several fibrinolytic enzymes, listed in Table 1 while patents related to fibrinolytic enzymes are listed in Table 2.

**Table 1**
FDA approved thrombolytic agents (Food and Drug Administration 2021).

| Fibrinolytic drugs | Commercial name | Manufacturer | Year approved |
|---|---|---|---|
| u-PA (Urokinase) | Abbokinase® | Abbott Labs | 1978 |
| t-PA (Alteplase) | Activase® | Genentech, Inc. | 1987 |
| t-PA (Alteplase) | Cathflo | Genentech, Inc. | 1996 |
| Reteplase | Retavase® and Rapilysin | Boehringer Mannheim and GmbH | 1996 |
| FIX (Factor IX) | BeneFIX® | Pfizer | 1997 |
| FVIIa (Factor VIIa) | NovoSeven® and NovoSeven® RT | Novo Nordisk Pharmaceuticals Inc. | 1999 |
| TNK-tPA (Tenecteplase) | TNKase™ and Metalyse® | Genentech, Inc. | 2000 |
| t-PA (Alteplase) | Activase® | Genentech, Inc. | 2002 |
| Thrombin (Thrombin in bandages for topical use) | THROMBIN-JMI | GenTrac | 2006 |

## 5. Scope for identification of newer and novel fibrinolytic enzymes from untapped sources

Fibrinolytic enzymes can be obtained from a variety of sources, including directly from food and non-food sources as well as microbes isolated from these sources. Food sources of fibrinolytic enzymes include fermented as well as non-fermented foods. Non-food sources of fibrinolytic enzymes include microbes isolated from soil and marine environment, fungi, animals, etc. While non-food sources of fibrinolytic enzymes are generally known to be potent, food sources have the added benefit of being easily accessible, cost-effective, safer for oral consumption and may provide additional health benefits beyond their fibrinolytic activity. For example, fibrinolytic enzymes such as bromelain [96] and papain [97] from pineapple and papaya, respectively, have been shown to possess anti-inflammatory and digestive benefits, while nattokinase is associated with cardiovascular health benefits [98]. Additionally, consuming fibrinolytic enzymes in the form of whole foods may offer a more balanced and varied approach to obtaining these enzymes instead of relying solely on supplements or medication. Detailed information on the sources of fibrinolytic enzymes is discussed in the following sections, including specific enzymes, their properties, and potential health benefits.

### 5.1. Major non-food sources of fibrinolytic enzymes

Fibrinolytic enzymes are abundantly present in a diverse range of natural sources. For example, they have been reported from non-food sources (Table 3) such as microbes from soil [99], plants [100], marine microbes and fauna [101], earthworms [102], bacteria [103], algae [104], fungi [34], snake venom [105] and so on, out of which snake venom constitutes the largest source of non-microbial fibrinolytic enzyme. The invertebrates including caterpillars [106], praying mantis [107] and dung beetles [108] also produce strong fibrinolytic enzymes. Among the higher organisms, the salivary plasminogen activator from vampire bats is the most studied [109]. In addition, strong fibrinolytic enzymes are also produced by marine algae and many microorganisms [110]. Although some of them offered hope as cardiovascular treatments, more research is needed to determine their safety and efficacy.

There are certain disadvantages associated with using non-food sources for fibrinolytic enzymes. Firstly, the extraction process for these enzymes from most non-food sources such as snake venom, earthworms, plants, etc. can be more complex and time-consuming. Furthermore, the yield may be relatively lower than that of microorganisms isolated from food sources due to their high production capability and cost-effective media requirements. Additionally, non-food sources often yield less specific and less potent enzymes that may not be as effective in treating thrombotic disorders [11]. Furthermore, many non-food sources of fibrinolytic enzymes, such as snake venom and earthworms, are associated with toxicity and immunogenicity, which limit their therapeutic use. The production of these enzymes often requires significant ethical considerations, as well as specialized handling and disposal procedures. These factors can increase the cost and complexity of developing fibrinolytic enzymes from non-food sources, making them less attractive as compared to those purified from microbes isolated from food sources, which offer greater ease of production and purification and a lower risk of toxicity and immunogenicity. Thus, while non-food sources of fibrinolytic enzymes may provide promising leads for novel enzymes, their drawbacks make them unsuitable for practical applications in the medical field. However, these enzymes may provide a basis for developing novel drugs, and their study may yield important insights into the biological mechanisms underlying fibrinolysis. Some examples of fibrinolytic enzymes from non-food sources and their advantages and disadvantages are outlined below.

#### 5.1.1. Soil

The soil is rich in minerals, nutrients and salts, and variations in temperature, humidity and pH provide an ideal environment for a wide range of microorganisms. Fibrinolytic activity has been reported from various microbes isolated from soil including bacteria [99], fungi [123], protozoa [124] etc. For example, Xin et al. (2018) discovered a novel extracellular fibrinolytic enzyme producing *Bacillus tequilensis* from a soil sample in Zhuai city, China. The enzyme was thermostable at 45 °C and could be stored in normal conditions. Another novel fibrinolytic enzyme-producing microorganism namely *Stenotrophomonas maltophilia* designated as Gd2 strain, was isolated from soil samples of the Godavari river basin in India by Khursade, Galande, Krishna and Prakasham [125]. It exhibited 74.88% clot lysis *in vitro* on fibrin with 36 $Umg^{-1}$ specific activity which was higher than many other fibrinolytic enzymes reported from *B. subtilis* strain A1 (Jeong et al., 2004), *B. cereus* NS-2 (Bajaj et al., 2013), *B. Subtilis* natto B-12 (Wang et al., 2009) and *Bacillus* sp. KDO-13 (Lee et al., 2001). However, the above-mentioned studies did not check for non-specific proteolysis of other blood proteins

**Table 2**
Patents on fibrinolytic enzymes and sources.

| Fibrinolytic enzyme/Substance/ Source | Patent number | Year | Patent description | Reference |
|---|---|---|---|---|
| *Bacillus amyloliquefaciens* | CN104694424B | 2017 | Fibrinolytic strain isolated from fermented soybean | [89] |
| Nanorod | US9545383B2 | 2017 | An agent that prevents blood coagulation and reverses the effect of the thrombin inhibitor | [90] |
| Fibrinolytic nanoparticles | US9675560B2 | 2017 | Use of nanoparticles for the controlled lysis of blood clots | [91] |
| Nattokinase | CN103238829B | 2015 | A novel nattokinase enteric coated capsule. | [92] |
| Streptokinase | US9115210B2 | 2015 | Methods to produce novel mutants of streptokinase with improved therapeutic properties | [93] |
| Nattokinase | CN103977397B | 2014 | Nattokinase oral emulsion. | [94] |
| Sea earthworm fibrinolytic enzyme | CN102757973B | 2013 | The cDNA sequence and amino acid sequence of a sea earthworm fibrinolytic enzyme | [95] |

**Table 3**
Fibrinolytic enzyme producers and their non-food sources.

| Non-food sources | Bacteria/fungus isolated | Reference |
|---|---|---|
| Earthworm (*Lumbricus rubellus*) | – | [111] |
| Marine worm (*Sipunculus nudus*) | *Bacillus amyloliquefaciens GXU-1* | [112] |
| Desert soil (Pakistan) | *Bacillus tequilensis* ZMS-2 | [113] |
| Earthworm (*Pheretima vulgaris*) | – | [114] |
| Garbage dump soil (India) | *Serratia* sp. KG-2-1 | [42] |
| Marine source (India) | *Serratia marcescens* sp. sakuensis | [51] |
| Marine source (India) | *Serratia rubidaea* KUAS001 | [115] |
| Plant roots | *Paenibacillus graminis* MC 2213 | [116] |
| Sea-mud (China) | *Bacillus subtilis* D21–8 | [117] |
| Soil | *Bacillus atrophaeus* V4 | [118] |
| Soil | *Streptomyces fumanus* | [119] |
| Soil (India) | *Bacillus cereus* RSA1 | [120] |
| Soil (India) | *Bacillus* sp. IND12 | [121] |
| Stem of *Catharanthus roseus* (India) | *Xylaria curta*[a] | [122] |

[a] Enzyme xylarinase has been reported from *Xylaria curta*.

which could further lead to low platelet count and other complications when used as a therapeutic.

### 5.1.2. Marine environment

Marine environments offer a vast and diverse range of flora, fauna, and microorganisms, many of which have the potential to produce novel fibrinolytic enzymes. Recent studies have revealed the presence of various fibrinolytic enzymes from marine sources, including bacteria, fungi, and algae. Marine-derived enzymes have been found to exhibit distinct properties and unique modes of action, which could lead to the development of new and more effective thrombolytic agents. For instance, enzymes from marine bacteria and fungi have been shown to have high fibrin specificity [126], while enzymes from marine algae possess high thermal stability and resistance to proteolysis [127]. Fibrinolytic enzymes have been purified from various marine microorganisms; some examples include *Alteromonas piscicida* [128], *Marinobacter aquaeolei* MS2-1 [129], *Streptomyces lusitanus* [130], *Pseudomonas aeruginosa* KU1 [131], and microalgae like *Tetraselmis subcordiformis* [132], *Chlorella vulgaris* [104], *Dunaliella tertiolecta* [17]. The first reported fibrinolytic protease purified from *Streptomyces radiopugnans* VITSD8 was isolated from marine sponges [101]. The marine microbe *Serratia marcescens* subsp. *sakuensis* (KU296189.1) isolated from Arabian Sea near Surathkal, India has been identified as another source of fibrinolytic enzymes. This enzyme exhibited a higher clot lysis rate of 38% for 3 h, which is more than what was observed with streptokinase and heparin. Notably, when tested, the fibrinolytic enzyme displayed no proteolysis activity towards blood plasma proteins, including haemoglobin, transferrin, and ɣ-globulins (Krishnamurthy and Belur 2018). Krishnamurthy et al. (2018) modified carboxylate residues of the same enzyme to achieve a 19-fold increase in specific activity. However, these studies were not performed *in vivo* at physiological conditions, which are needed to confirm their toxicity, immune response, and stability. The marine environment is, however, vast and a largely unexplored source of fibrinolytic enzymes, and further research is necessary to fully harness the potential of these marine resources.

### 5.1.3. Earthworms

Earthworm, for centuries, has been used as a traditional medicine to treat thrombosis in some Asian countries. For example, in China, Japan, Korea, and some other Asian countries, dried, powdered earthworm preparations called “Earth-dragon” is utilized as a traditional folk treatment for heart diseases [133]. It has been noted that proteases with the capability to degrade thrombus are abundant in earthworms. Such enzymes, referred to as earthworm fibrinolytic enzymes (EFE), are secreted by the alimentary tract of earthworms and have shown significant potential in treating blood clotting disorders. For example, EfP-II and EfP–III–1 are present in the epithelial cells of the alimentary canal surrounding the clitellum of *Eisenia fetida.* However, these enzymes have broad substrate specificity, so that the earthworms can utilize a wide range of microbes and proteins during the digestive process. Hence EFE’s show an inherent non-specificity which in turn is detrimental to the human system [134]. The first earthworm fibrinolytic enzyme was characterised from *Lumbricus rubellus*, hence earning the term lumbrokinase (LK), which is currently categorized as a category of fibrinolytic enzymes [135].

Fibrizyme is another fibrinolytic enzyme from a different type of earthworm, *Eisenia andrei*. Apart from these, other species such as *Eisenia andrei, E. fetida*, *Lumbricus bimastus* etc., are also identified as fibrinolytic enzyme producers. However, the uniqueness of these earthworms is their ability to produce more than one thrombolytic protease. For example, *Lumbricus rubellus* and *Eisenia fetida* produce six and seven different lumbrokinase types [136,137]. Notably, they display a greater degree of specificity towards fibrin when compared to plasminogen activators like t-PA and urokinase. Lumbrokinases are fibrinolytic enzymes known for their ability to activate plasminogen, reducing platelet aggregation, and thinning blood, among other functions. Their molecular weights range from 20 to 35 kDa and are active across a wide pH range of (pH 1–11) [138]. Animal models were used to demonstrate that lumbrokinase obtained from *Eisenia andrei* exhibit the potential to be developed as oral drug [139,140]. Lumbrokinase can also be produced recombinantly using *Pichia pastoris* making it easier for purification and production at a large scale compared to its natural source [141]. In comparison to other fibrinolytic enzymes, lumbrokinase is considered safer and carries a reduced risk of bleeding complications [142], However, there is a commercially available lumbrokinase product named Boluoke®, which has gained approval from

the China Food and Drug Administration [143].

#### 5.1.4. Snake venom

Fibrinolytic enzymes play a major role in assisting the spread of toxic components of snake venom throughout the circulatory system after the snake envenomates its prey. By acting on fibrinogen, fibrin (ogen)olytic enzymes break down fibrin-rich clots and aid in preventing further clot formation. A number of serine and metalloproteases are found in snake venom, including fibrinolytic proteases. Since thrombolytic drugs should remain active in the presence of serine protease inhibitors in mammalian blood, the fibrinolytic proteases found in snake venom could potentially serve as a model for creating new thrombolytic drugs that are suitable for therapeutic applications [144]. Two types of fibrinolytic enzymes are found in snake venom: α-fibrin (ogen)ase and β-fibrin (ogen)ase. As a result of this distinguishing trait, fibrinolytic snake venom enzymes have been developed as a prospective therapeutic medicine for treating occlusive thrombi. Fibrolase, a zinc fibrinolytic metalloproteinase (23 kDa), is derived from the venom of the southern copperhead snake (*Agkistrodon contortrix*). Its mechanism of action involves cleaving the α- and β-chains of fibrinogen, while leaving the γ-chain unaffected [145]. *In vitro* and *in vivo* studies have demonstrated the ability of fibrolase to dissolve fibrin clots directly without the activation of plasminogen. This enzyme effectively breaks down human blood clots and fibrin in a dose-dependent manner. In an animal model of arterial thrombosis, a single intravenous bolus treatment with fibrolase could dissolve femoral artery clots. To prevent rethrombosis after thrombus breakdown by plasminogen activators, arginyl-glycyl-aspartic acid (RGD) containing drugs are effective. As a result, fibrolase was modified by covalently attaching an RGD-like peptide, resulting in a more potent fibrinolytic enzyme. By binding to the fibrinogen receptor integrin IIb3 on platelets, this chimera was demonstrated to retain fibrinolytic activity and prevent platelet aggregation, resulting in thrombus lysis and rethrombosis inhibition [146]. Alfimeprase is another recombinant fibrinolytic enzyme, developed as a clinical agent, modified from fibrolase which directly acts on fibrin [147,148]. Given that alfimeprase advanced to phase II and III studies, none of the studies was completed due to the lack of a significant difference compared to the placebo [149]. However, alfimeprase is currently listed as an orphan medication in Europe for treating acute peripheral arterial obstruction [150].

#### 5.1.5. Plants

Plants, particularly plant lattices, are an abundant source of proteolytic enzymes, which play a crucial role in host-pathogen interactions. Most of the plant derived proteolytic enzymes are classified as serine or cysteine proteases [151]. *In vitro* studies have reported that crude extracts of plant proteases can dissolve clots and display remarkable stability across a broad temperature and pH ranges. For example, *Bacopa monnieri* [152], *Fagonia arabica* [153], *Leea indica* [154], *Urena sinuate* [155], *Trema orientalis* [156], *Leucas aspera* and *Clausena suffruticosa*, are few examples of the plants that produce thrombolytic enzymes. Latex enzymes showing thrombolytic potential have been purified from various plants, such as *Wrightia tinctoria, Calotropis gigantea*, *Cynanchum puciflorum, Synadenium grantii, Pergularia extensa, and Asclepias curassavica* are either serine proteases or cysteine proteases.

Cysteine proteases from fruits of *Pseudananas macrodontes, Bromelia hieronym* and *B. balansae* were examined for their potential thrombolytic properties. Surprisingly, they exhibited procoagulant activity at low concentration and fibrinolytic activity at higher concentration [157]. Additionally, extracts from *Litchi chinensis* were found to possess antiplatelet properties along with thrombolytic and anticoagulant properties, with their fibrinolytic activity increasing with the increase in dose [158]. Fibrinolytic enzymes are also reported in the latex of *Plumeria rubra* and *Cryptostegia grandiflora*. Rajesh, Shivaprasad, Gowda, Nataraju, Dhananjaya and Vishwanath [159] found that enzymes from *C. gigantea* exhibited potent thrombolytic activity however it showed a high risk of haemorrhage, whereas there was not risk of haemorrhage in enzymes from *S. grantii* and *W. tinctoria*. On the other hand, a fibrinogenolytic enzyme Eumiliin was purified and characterized from the latex of *Euphorbia milii* var. hislopii. This enzyme, a 30 kDa cysteine protease, demonstrated remarkable stability across a broad range of temperatures and pH, losing its activity only at 80 °C and primarily degrading the fibrinogen α chain [160]. A serine protease which exists as a homodimer named 'EuP-82', with fibrinogenolytic potential was isolated from *Euphorbia* cf. *lactea*. It was stable over a wide range of pH and temperatures [161]. Hirtin, a serine protease with a molecular weight of 34 kDa was extracted from *Euphorbia hirta*'s latex, acted on both fibrin as well as fibrinogen. It was stable between a pH range of 4–8 and temperatures ranging from 30 to 70 °C. During fibrinogenolysis, Hirtin displayed a higher affinity towards the Aα chain of fibrinogen [162]. Likewise, AMP-48, another serine fibrinolytic enzyme, was isolated from the latex of *Artocarpus heterophyllus*. This protease, with a molecular weight of 48 kDa, hydrolyses fibrinogen, completely dissolving the α subunit followed by partial degradation of β and γ subunits, similar to other fibrinolytic enzymes from plat sources [161].

### 5.2. Food sources of fibrinolytic enzymes

The global research on functional foods and nutraceuticals is rapidly advancing. Scientists are actively engaged in exploring the health benefits of foods, delving into their functional constituents, understanding biochemical structures, and deciphering the underlying mechanisms of their physiological roles. These discoveries are reshaping the nutritional paradigm, where food constituents now extend from their role as just a dietary necessity for sustaining life and growth to promoting prevention, management, or delay of the onset of chronic diseases in later stages of life. Similarly, since ancient times, herbal remedies have been used to heal diseases with minimum adverse effects. Their ease of administration is the most appealing aspect, as they are a common part of many diet regimes. In fact, the first fibrinolytic enzyme purified from fermented food and is now widely researched, nattokinase, was traditionally used as a folk medicine for the treatment of heart and vascular diseases [18].

Fibrinolytic enzymes are also known to be found in a variety of foods, for example Japanese natto [18], edible honey mushroom [20] and Korean chungkook-Jang soy sauce [19]. Researchers have successfully purified enzymes from these foods and isolated

microbes associated with them, along with characterization of their physiochemical properties. Although fibrinolytic enzymes have been isolated from fermented and non-fermented foods, most studies have focused on those derived from fermented foods. The fact that so many fibrinolytic enzymes have been found in fermented foods strongly implies that these foods include many fibrinolytic producers. Furthermore, fibrinolytic enzymes purified from food sources are safe for oral administration. Particularly, it was found that fibrinolysis was enhanced when fibrinolytic enzyme extracted from Japanese natto was orally administered in dogs with experimentally induced thrombosis [163]. More importantly, when human subjects were given nattokinase orally, the fibrinolytic activity, levels of t-PA, and fibrin degradation by-products in plasma were found to double. This effect is associated with increased concentrations of D-dimer, FDP, and antithrombin in the blood, while a significant decrease in factor VIII was observed [164].

In contrast to fibrinolytic enzymes purified from non-food sources, those obtained from food sources have demonstrated minimal side effects, making them a more favourable option for use as nutraceuticals. The safety of these enzymes for oral consumption has been widely documented, with ease of purification and mass production [165]. Unlike enzymes derived from animal sources such as earthworms and snake venom, studying food-derived enzymes does not require ethical clearance. Furthermore, these enzymes are typically fibrin-specific and do not exhibit haemolytic properties [166] or cytotoxic effects [167], thus increasing their potential medical application. Investigating traditional foods from diverse regions as sources of fibrinolytic enzymes could promote healthy dietary habits with the aim of identifying a nattokinase-like enzyme or potentially discovering a more effective and safer fibrinolytic enzyme.

### 5.2.1. *Non-fermented food sources of fibrinolytic enzymes*

Non-fermented food sources too are potential candidates as sources of fibrinolytic enzymes as well as microbes producing fibrinolytic enzymes. While fibrinolytic enzymes currently used are primarily derived from non-food sources, only a limited number of non-fermented food sources have been explored for their fibrinolytic activity. Some studies have identified fruits, rice, bovine milk, and mushrooms as potential food sources of fibrinolytic enzymes (Table 4). Similarly, mushrooms which are generally known to possess various biological activities, also have a significant amount of fibrinolytic activity. Edible mushrooms such as Oysters (*Pleurotus ostreatus)* [168], Quélet (*Pleurotus eryngii*) [169], Shiitake (*Lentinula edodes*) [170] and Pioppino (*Agrocybe aegerita*) [34] are known to possess fibrinolytic activity. In a recent investigation, Petraglia, Latronico, Liuzzi, Fanigliulo, Crescenzi and Rossano [20] showed that oyster mushrooms, Shiitake, Quélet and Pioppino possessed both fibrinolytic and fibrinogenolytic properties. However, the experiments were carried out using crude extracts of fruiting bodies and further experiments need to be carried out using purified enzymes. Though studies have shown the presence of fibrinolytic enzymes in edible mushrooms, highlighting their functional food properties but have not shed any light on the enzymes' large-scale production and purification for pharmaceutical applications. In this regard, a handful of attempts have been made to clone fibrinolytic genes from mushrooms in a single-celled organism for cost-effective production [45,171].

### 5.2.2. *Fermented food sources of fibrinolytic enzymes*

Fermented foods have long been recognized as a rich source of bioactive compounds with various health benefits. In particular, fermented foods have shown promise as potential sources of fibrinolytic enzymes and their producers, which are crucial for the dissolution of blood clots. Numerous studies have investigated the fibrinolytic activity of fermented foods (Table 5). These enzymes have demonstrated fibrinolytic properties *in vitro* and animal models, suggesting their potential therapeutic applications.

*5.2.2.1. Japanese fermented foods.* Natto is an age-old traditional Japanese food with a cheese-like consistency from fermented soybeans. It is believed that natto consumption contributes to the longevity of the Japanese population and was traditionally used to treat CVDs. The mechanism behind the overall cardiovascular health of natto consumption was established by Professor Sumi and his team in 1987 [18]. They discovered that natto contained a protease called nattokinase, a potent fibrinolytic enzyme produced by *Bacillus natto*. Since then, researchers from China, Korea, Japan, and the USA have done a significant number of studies and have independently confirmed that nattokinase (NK) is responsible for positive effects on cardiovascular health. It is a 28 kDa alkaline serine protease with 275 amino acid residues which exhibits potent fibrinolytic activity and high substrate specificity [179]. Nattokinase (NK) exhibits multifaceted actions, going beyond fibrin degradation and thrombus dissolution. Notably, it converts endogenous

**Table 4**
Some fibrinolytic enzymes from non-fermented food sources.

| Source | Scientific nomenclature | Enzyme | Reference |
|---|---|---|---|
| Pineapple | *Ananas comosus* | Bromelain | [172] |
| Edible mushroom | *Cordyceps militaris* | – | [173] |
| Shiitake mushroom | *Lentinus edodes* GNA01 | LEFE | [170] |
| Quélet | *Pleurotus eryngii* | – | [169] |
| Pioppino | *Agrocybe aegerita* | ACase | [34] |
| White fungus | *Tremella fuciformis* | Tfase | [174] |
| Wild edible mushroom | *Sparassis crispa Wulf*. Ex. Fr. | Wulfase | [175] |
| White elf mushroom | *Pleurotus ferulae* | – | [176] |
| Oyster mushroom | *Pleurotus ostreatus* | – | [168] |
| Monkey head mushroom | *Hericium erinaceum* | Herinase | [177] |
| Hon-shimeji | *Lyophyllum shimeji* | – | [178] |

**Table 5**
Fibrinolytic enzymes purified from fermented foods or microbes isolated from fermented foods.

| Source | Key ingredient | Bacterial/fungal strain | Enzyme | Country | Reference |
|---|---|---|---|---|---|
| Ba-bao Douchi | Soybean | *Bacillus subtilis* DC33 | Subtilisin FS33 | China | [191] |
| Cheonggukjang | Soybean | *Bacillus subtilis* HK176 | AprE176 | Korea | [182, |
| | | *Bacillus subtilis* HK176 | M179 | | 192] |
| | | *Bacillus* sp. *strain* | CK 11–4CK | | |
| | | *Bacillus amyloliquefaciens* | | | |
| Cooked Indian rice | Rice | *Paenibacillus* sp. IND8 | – | India | [193] |
| Doenjang | Soybean | *Bacillus amyloliquefaciens* RSB34 | AprE34 | Korea | [194] |
| | | *Bacillus* sp. DJ-4 | Subtilin DJ-4 | | |
| Dosa batter | Rice, Black gram | *Bacillus amyloliquefaciens* MCC2606 | CFR15 | India | [195] |
| Douche | Black soya bean | *Bacillus amyloliquefaciens* Jxnuwx-1 | – | China | [195, |
| | | *Bacillus subtilis* DC27 | DFE27 | | 196] |
| | | *Strain* XY-1 | DFE | | |
| Fermented rice | Rice | *Bacillus cereus* IND5 | – | India | [197] |
| Fermented shrimp paste | Shrimp | *Bacillus* sp. Nov. SK006 | - | Japan | [198] |
| Gembus | Soybean | *Bacillus pumilus 2. G* | – | Indonesia | [199] |
| Hwangseokae jeotgal | Small yellow croaker | *Bacillus licheniformis* HJ4 | AprEHJ4 | Korea | [52] |
| Jeotgal | Sea squirt (munggae) | *Bacillus velezensis* BS2 | AprEBS2 | Korea | [200] |
| Jotgal | Pickled opossum | – | JP-I and JP-II | Korea | [184] |
| | shrimp | *Bacillus subtilis* JS2 | AprEJS2 | | |
| Kimchi | Fermented vegetables | *Bacillus subtilis* ZA400 | BsfA | Korea | [201] |
| Kinema | Soybean | *Bacillus halotolerans* | – | Korea | [202] |
| Meju | Soybean | *Bacillus amyloliquefaciens* MJ5–41 | AprE5–41 | Korea | [203] |
| Natto | Soybean | *Bacillus subtilis* RJAS19 | Nattokinase | Japan | [193] |
| Saeu Jeotgal | Shrimp | *Bacilllus subtilis SJ4* | – | Korea | [204] |
| Skipjack Shiokara | Marine fish | - | Katsuwokinase (KK) | Japan | [181] |
| Tempeh | Soybean | *Fusarium* sp. BLB | FP | Indonesia | [205] |
| Traditional fermented soybean paste products | Soybean | *Bacillus amyloliquefaciens* | – | Vietnam | [206] |

pro-urokinase to urokinase and active plasminogen to plasmin through elevating t-PA levels while inhibiting PAI-1 levels [180]. Additionally, it is non-toxic, lacks side effects when administered orally in prescribed doses, and has a low production cost in comparison to fibrinolytic enzymes purified from non-food sources such as streptokinase, urokinase, lumbrokinase, etc. [59]. Surprisingly, after several *in vitro*, *in vivo,* and clinical trials, nattokinase has still not obtained FDA approval or GRAS status, hence not regulated as a food ingredient. However, it is currently marketed as a dietary supplement rather than a drug and is regulated by FDA under the Dietary Supplement Health and Education Act (DSHEA) of 1994.

Similarly, another microbial fibrinolytic enzyme has been purified from a Japanese fermented food shiokara [181]. However, it is important to note that the fibrinolytic enzyme katsuwokinase purified from shiokara is made from fermented marine fish and no further studies were conducted to confirm the toxicological safety, *in vivo* immune response, and bioavailability of the enzyme. Recently, another such study was conducted on a similar Japanese traditional fermented fishery product called funazushi which is the predecessor to modern sushi. The study showed that the extract from the fermented food increased the activity of plasmin and t-PA and decreased the activity of PAI-1 thus suggesting the antithrombotic role of funazushi. Nevertheless, the study was conducted using the extract of the fermented food, on the contrary it is important to identify the molecules responsible for the antithrombotic role.

*5.2.2.2. Korean fermented foods.* Since the discovery of nattokinase in 1987, other Asian traditional fermented foods have been screened for microbial fibrinolytic enzymes. For example, the enzyme CK from *Bacillus* sp. CK 11–4 was the first fibrinolytic enzyme to be purified from Korean fermented food, chungkook-jung or cheonggukjang [19]. Chungkook-jung is made by fermenting soybeans until a paste with a coarser texture is obtained. Over the years, other *Bacillus* strains producing fibrinolytic enzymes have been isolated from chungkook-jung; for example, *B. amyloliquefaciens* strains CH86-1 (Lee et al. 2010) and CH51 [182,183].

Jeotgal or jotgal or jeot-gal is a fermented food made from whole fish, internal organs, and/or meat, and has been found to contain fibrinolytic enzymes to the likes of Japanese shiokara. Jeotgal is also used as one of the ingredients for the production of Kimchi, another popular fermented food. Several fibrinolytic producers have been isolated from jeotgal and their enzymes have been purified and characterised. To mention a few, salt-resistant fibrinolytic enzyme was characterised from *Bacillus licheniformis* HJ4. Considering its strong fibrinolytic activity, the organism's GRAS status, and significant salt tolerance, *B licheniformis* HJ4 could be used as a promising starter culture in salted fermented foods [52]. In addition, fibrinolytic organisms have also been purified from microorganisms isolated from fermented foods. For example, *Bacillus subtilis* SJ4 from saeu (shrimp) jeotgal, *Bacillus velezensis* BS2 from the sea squirt jeotgal etc [52]. In another study, Kim, Ri and Choe [184] successfully purified two metalloproteases, JP-I and JP-II, directly from the supernatant of jeotgal. While the researchers successfully isolated the enzyme without the tedious process of isolating the microorganism, isolating the organism allows for several additional applications and characterization.

Kimchi, made by spontaneously fermenting vegetables such as cabbage, green onion, radish, and seasoning such as red pepper,

garlic, leek, and ginger [185] was also screened for fibrinolytic producers and their enzymes. Similarly, fibrinolytic enzymes and their genes were identified and cloned from two strains namely, *Bacillus subtilis* and *B. velezensis* isolated from Kimchi [186]. Overall, the growth of various lactic acid bacteria is observed in Kimchi, which could be the possible fibrinolytic producers, however, this needs to be further investigated and confirmed.

*5.2.2.3. Indian fermented foods.* Fermented foods have been a dietary staple in the Indian subcontinent for centuries, contributing to the region's rich culinary heritage and potentially offering several health benefits. For example, screening a diverse range of Northeast Indian fermented soy based foods and fish products for fibrinolytic activity revealed that the fibrinolytic activity in the soy based foods came from microbial origin, predominantly from *Bacillus* spp. which could either be nattokinase or nattokinase like enzymes [187]. Meanwhile, the fibrinolytic activity found in fish products was attributed to the endogenous source. Similarly, hawaijar which is another traditional vegan soy based fermented food from Manipur, India and is also known to possess high fibrinolytic activity [188]. Furthermore, detailed studies need to be conducted and further production and purification of these enzymes are essential to characterize and identify the nature of these enzymes. Fibrinolytic enzymes have also been screened from dosa and fermented rice (Table 5) however, it is essential to consider several other Indian fermented foods such as kanji, panta ilish, fermented bamboo shoots etc., for screening potential fibrinolytic enzymes and their producers.

*5.2.2.4. Chinese fermented foods.* Douchi is a Chinese soybean fermented food to the likes of Japanese natto and Korean chungkookjung from which fibrinolytic enzymes have been successfully purified. A highly potent fibrinolytic enzyme producing *Bacillus subtilis* DC27 was isolated and the extracellular enzyme DFE27 was purified from the fermentation broth of Douchi. The DFE27 enzyme acts directly on fibrin and converts plasminogen to plasmin, suggesting its potential use as a thrombolytic agent [189]. However, various other Chinese fermented foods need to be explored for fibrinolytic enzymes. For example, suan cai which translates to sour vegetables and is similar to Korean kimchi, fermented tofu, stinky tofu which is made by soaking tofu in fermented brine and Chinese fermented shrimp paste [190]. Although microbial fibrinolytic producers with promising fibrinolytic potential have been derived from various fermented foods, most studies are limited to Asian fermented foods. This motivates further exploration of fermented foods beyond Asian countries to discover potential novel fibrinolytic producers. However, further research is needed to fully characterize these fibrinolytic enzymes purified from microbes isolated from fermented foods, evaluate their stability, bioavailability, and safety, and assess their clinical efficacy. Understanding the potential of fermented foods as a natural source of fibrinolytic enzymes could pave the way for developing novel therapeutic interventions for cardiovascular and clot-related disorders.

## 6. Strategies to enhance the production and efficacy of microbial fibrinolytic enzymes

The main barrier to the effective use of fibrinolytic enzymes in the medical field is the cost of enzyme manufacturing and downstream processes. Numerous efforts have been made to increase the expression of fibrinolytic enzymes, including selecting the optimum culture medium, optimizing environmental conditions, and overexpressing the enzyme using genetically modified strains. Studies on the optimization of production media are essential to increase the fibrinolytic enzyme yield at optimum substrate concentration. Several strategies have been used to improve the scale-up and recovery of fibrinolytic enzymes through media optimization.

The constituents of the media have a significant effect on influencing the production and secretion of the enzyme since different microbes possess diverse physiological characteristics [207]. For instance, soybean is the major constituent of several fermented foods such as natto, douchi, tempeh, chungkook-jang etc., from which microbial fibrinolytic enzymes have been purified and studied. However, yeast extract, meat extract or casein showed better fibrinolytic enzyme production in comparison with soybean meal, soy peptone etc. when used as a sole nitrogen source [208]. The presence of protein substrates usually influences the bacteria to secrete fibrinolytic enzymes to utilize the substrate. For instance, the optimization of nattokinase was carried out using soy-peptone as sole carbon and nitrogen source which exhibited a remarkable five-fold increase in enzyme production. The optimized enzyme activity reached 58 FU/mg, presenting a substantial enhancement compared to the 11 FU/mg activity of the unoptimized medium. This significant increase underscores the efficacy of the optimization strategy involving soy-peptone, emphasizing its potential for improving nattokinase productivity [207]. For the large-scale production of enzymes, cheaper raw materials are essential to bring down the cost of production. For example, an increase of 4-fold in the production of the enzyme by the bacteria *Xanthomonas oryzae* IND3 was observed when cow dung, a low-cost substrate was used as a component in the production media [209]. Anh, Mi, Huy and Van Hung [210] used shrimp shell powder for increased fibrinolytic enzyme production using *Bacillus* species M2 and the resultant yield was 2.32 times higher than the control medium. Additionally, inorganic salts, particularly $MgSO_4$ and $CaCl_2$, were general and common constituents for most of the optimized production media, indicating their importance and influence on fibrinolytic enzyme production [211].

Generally, fermentation under submerged condition is considered an important criterion for producing fibrinolytic enzymes. However, Peng and his team have employed solid-state fermentation process in which wheat bran was utilized as a major constituent in the medium for fibrinolytic enzyme production from *B. subtilis* IMR-NK. About 9.2-fold increase was observed in the yield of the fibrinolytic enzyme [207]. Optimizing environmental factors like temperature too plays an important role in enhancing enzyme production. For example, the optimal temperature of *Bacillus pumilus* 2. g for enzyme synthesis was 50 °C because the strain was isolated from an Indonesian fermented food Douchi which involves boiling soybeans before starting the fermentation process [199].

Optimizing fermentation processes to maximize the production of fibrinolytic enzymes is essential to make it efficient and cost-

effective. Response Surface Methodology (RSM) and Artificial Neural Network (ANN) are two techniques commonly used to optimize fermentation conditions for fibrinolytic enzymes. RSM is a common yet simpler statistical technique that involves designing a series of experiments to determine the relationship between various input variables and the response variable (in this case, fibrinolytic enzyme production). Using RSM, researchers can identify the optimal conditions for fermentation and determine how changes in each input variable affect the response variable. Jhample, Bhagwat and Dandge [212] used RSM to optimize fibrinolytic enzyme production and found a 20-fold increase (from 38.19 units to 796.8 units) in the enzyme activity. In contrast, ANN is a complex yet more accurate

**Table 6**
Physiochemical properties of fibrinolytic enzymes.

| Source | Enzyme | pH Opt. | Temp. Opt. (°C) | Mass (kDa) | Inhibitor | Activator/co-factor | Class | Reference |
|---|---|---|---|---|---|---|---|---|
| *Bacillus atrophaeus* V4 | – | 7 | 35 | 36 | PMSF, EDTA | – | Serine metalloprotease | [118] |
| *Bacillus amyloliquefaciens* GUTU06 | – | 5 | 45 | 28 | $Zn^{2+}$, $Mg^{2+}$, $Na^{+}$, $Ca^{+2}$, | $Fe^{2+}$, $K^{+}$ | – | [224] |
| *Bacillus amyloliquefaciens* GXU-1 | FEB-1 | 8 | 37 | 30 | $Fe^{2+}$, $K^{+}$, $Mg^{2+}$, $Na^{+}$, $Ca^{+2}$, PMSF, EDTA | – | Serine metalloprotease | [112] |
| *Aspergillus versicolor* ZLH-1 | Versiase | 5 | 40 | 37.3 | EDTA, BGTA | – | Metalloprotease | [225] |
| *Bacillus velezensis* Z01 | Velefibrinase | 7 | 40 | 32.3 | $Cu^{2+}$, $Fe^{2+}$, $Mn^{2+}$, $Zn^{2+}$, PMSF, EDTA, $\beta$-mercaptoethanol | $Ca^{+2}$, $Mg^{+2}$ | Serine metalloprotease | [50] |
| *Pheretima vulgaris* | EPF3 | 7.8 | 50 | 25 | PMSF, SBTI, TPCK, EDTA, $Cu^{2+}$, $Fe^{2+}$ | – | Serine protease | [226] |
| *Bacillus licheniformis* HJ4 | *aprEHJ4* | 8 | 40 | 24 | – | – | – | [52] |
| *Bacillus velezensis* SN -14 | – | 8.5 | 37 | 28 | $Fe^{2+}$, $Zn^{2+}$, $Li^{+}$ | $Ca^{+2}$, $Mg^{+2}$, $Ba^{2+}$, $Fe^{2+}$ | – | [219] |
| Jotgal (Korean traditional fermented food) | JP I | 8.1 | 50 | 36 | $Cu^{2+}$, EDTA | – | Metalloprotease | [184] |
| Jotgal (Korean traditional fermented food) | JP II | 9.9 | 45 | 36 | $Cu^{2+}$, $Fe^{3+}$, EDTA | $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2}$ | Metalloprotease | [184] |
| *Bacillus cereus* RSA1 | – | 8 | 50 | 40 | DFP | $Mn^{2+}$, $Zn^{2+}$, and $Cu^{2+}$, DTT and β-mercaptoethanol | Serine protease | [120] |
| *Bacillus velezensis* BS2 | – | 8 | 37 | 27 | $Fe^{3+}$, $Zn^{2+}$, $K^{+}$, $Co^{2+}$, PMSF, EDTA, SDS | $Mg^{+2}$, $Ca^{+2}$, $Mn^{+2}$ | Serine protease | [41] |
| *Bacillus amyloliquefaciens* Jxnuwx-1 | – | 7.6 | 41 | 29 | $Fe^{3+}$, $Fe^{2+}$, PMSF, EDTA, SBTI | – | Serine metalloprotease | [227, 228] |
| *Bacillus subtilis* DC27 | DFE27 | 7 | 45 | 29 | PMSF | – | Serine protease | [228] |
| *Streptomyces radiopugnans* VITSD8 | – | 7 | 33 | 35 | – | – | – | [101] |
| *Bacillus subtilis JS2* | AprEJS2 | 8 | 40 | 27 | PMSF, EDTA, EGTA | $K^{+}$, $Mn^{2+}$, $Mg^{2+}$, $Zn^{2+}$ | Serine protease | [229] |
| *Bacillus tequilensis* | – | 10.5 | 45 | 27 | $Cu^{2+}$, $Zn^{2+}$, $Fe^{3+}$, $\beta$ -mercaptoethanol | $Na^{+}$, $K^{+}$, $Mn^{2+}$, $Mg^{2+}$ and $Ba^{2+}$, PMSF, EDTA | Serine protease | [99] |
| *Serratia marcescens* subsp. *sakuensis* | – | 7 | 55 | 43 | PMSF, EDTA | $Mn^{2+}$, $Mg^{2+}$, $Zn^{2+}$ | Serine metalloprotease | [51] |
| *Bacillus amyloliquefaciens* MCC2606 | CFR15 | 10.5 | 45 | 32 | PMSF, EDTA | $Mn^{2+}$ | Serine metalloprotease | [195] |
| *Chlorella vulgaris* | – | – | | 45 | PMSF, EDTA | $Fe^{2+}$ | Serine metalloprotease | [104] |
| *Bacillus amyloliquefaciens* RSB34 | AprE34 | 8 | 40 | 27 | $Mn^{2+}$ $Fe^{2+}$, PMSF | $Mg^{2+}$, $Zn^{2+}$, $K^{+}$ | Serine protease | [194] |
| *Serratia* sp. KG-2-1 | – | 8 | 40 | 52 | $Hg^{2+}$, $Ca^{2+}$, $Fe^{3+}$ | $Na^{+}$, $Mn^{2+}$, $K^{+}$, $Cu^{2+}$ and $Ba^{2+}$ | Metalloprotease | [42] |
| *Xylaria curta* | Xylarinase | 8 | 35 | 33 | $Fe^{2+}$, $Zn^{2+}$, EDTA and EGTA | $Ca^{2+}$ | Metalloprotease | [122] |
| *Cordyceps militaris* | – | 7.2 | 37 | 28 | SBTI, $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$ | $Mn^{2+}$, $Ca^{2+}$, $Fe^{3+}$, $Fe^{2+}$ | Serine protease | [173] |
| *Pleurotus ferulae* | – | 4,5,8 | 50 | 20 | EGTA, EDTA, $Cu^{2+}$, $Mg^{2+}$ | – | Metalloprotease | [176] |

computational model that simulates the behaviour of neurons in the human brain based on artificial intelligence (AI). In fermentation optimization, ANN can be used to develop a predictive model that considers multiple input variables (such as pH, temperature, and substrate concentration) and their interactions to predict the optimal conditions for fibrinolytic enzyme production. ANN and genetic algorithm (GA) are used for better prediction capability and to improve the ability to optimize non-linear problems. RSM and ANN have been successfully used to optimize fermentation conditions for fibrinolytic enzymes, leading to increased enzyme production and improved yields. Joji, Santhiagu and Salim [213] successfully used ANN to optimize the media to give an output of 1.8-fold higher enzyme production. These techniques can potentially reduce the time and cost associated with developing fibrinolytic enzymes [214].

Low gene expression of the fibrinolytic enzymes and complexities involved in downstream processing also contribute to the high cost of production of these enzymes. To address this, implementing cloning techniques and heterologous expression systems offers a potential solution. By cloning and expressing the active enzymes in a host organism, it is possible to enhance the yield of these enzymes and simplify their downstream processing. For instance, the gene encoding a fibrinolytic enzyme was successfully cloned from the mushroom *Cordyceps militaris* and expressed in the yeast *Pichia pastoris* by Katrolia, Liu, Zhao, Kopparapu and Zheng [171]. Similarly, a 1.34-fold increase in enzyme activity was observed by cloning the fibrinolytic gene in *Bacillus subtilis,* originally found in *B. pumilus* [215]. Apart from cloning the gene of interest, Yao, Meng, Le, Lee, Jeon, Yoo and Kim [216] replaced and engineered a more efficient promoter which also increased the fibrinolytic enzyme production. Remarkably, the team led by Buniya et al. engineered a hybrid enzyme by combining staphylokinase and streptokinase in *E. coli* BL21 (DE3). Notably, their findings revealed that the recombinant enzyme exhibited complete clot lysis when compared to the native streptokinase [217]. This underscores the significance of exploring recombinant enzymes further, as demonstrated by their enhanced thrombolytic efficacy. Similarly, enzyme engineering using chemical modification was carried out for a fibrinolytic enzyme isolated from *Serratia marcescens* subsp. *sakuensis* achieving a remarkable 219-fold enhancement in enzyme activity [218]. In another independent study by Lu et al., chemical modifications in *Bacillus velezensis* SN-14 fibrinolytic enzyme improved the thermal and acid-base stability of the enzyme [219]. Nonetheless, comprehensive investigations into the *in vivo* effects and stability of these modified enzymes are imperative for a thorough understanding of their potential applications.

Moreover, AI technologies can be used to predict enzyme kinetics and substrate specificity, providing insights into enzyme behaviour and aid the optimization of reaction conditions [220]. By unravelling complex protein-protein interactions involved in fibrinolysis, AI will facilitate the identification of critical molecular products and potential intervention targets. Additionally, AI tools can be used to integrate diverse datasets and enable bioinformatics analysis for understanding enzyme regulation, biomarker identification, patient stratification, and personalized treatment approaches [221]. Finally, AI algorithms can optimize production and purification processes, improving the efficiency and cost-effectiveness of fibrinolytic enzyme manufacturing [220]. For example [213], used AI based ANN in combination with a genetic algorithm (GA) to optimize fibrinolytic enzyme production and found that these AI-based tools were better at giving predictions than the conventional RSM. Integrating AI technologies with experimental approaches holds immense promise in advancing our understanding and application of fibrinolytic enzymes in therapeutic and diagnostic contexts. Thus, these advancements offer opportunities to address cost-related concerns and pave the way for more efficient and economically viable production of fibrinolytic enzymes in the future.

## 7. Biochemical characterisation

Biochemical characterization of fibrinolytic enzymes from various sources is essential to gain valuable insights into their properties. They should be subjected to detailed analysis, which includes determining their molecular weight, optimal temperature, and pH, inactivators, activators and classification into serine proteases, metalloproteases, and serine metalloproteases. A comprehensive summary of these biochemical properties of fibrinolytic enzymes can be found in Table 6.

The molecular weight of the reported pure fibrinolytic enzymes has been observed to vary, spanning from as low as 14 kDa to as high as 97 kDa. Most of these enzymes are identified as serine proteases [120,200] or metalloprotease [42,184] along with some enzymes that possessed characteristics of both metalloprotease and serine protease, namely serine metalloproteases [196]. It is noteworthy to stress that fibrinolytic enzymes' pH compatibility is particularly important, especially for applications involving intravenous administration, considering the blood's pH of 7.4. It has been observed that most fibrinolytic enzymes exhibit high activity within a pH range close to neutral or slightly alkaline (Table 6). Ensuring a pH range compatible with the physiological conditions of the bloodstream becomes crucial for maximizing the efficacy of these enzymes in therapeutic applications. Therefore, when selecting and designing fibrinolytic enzymes for intravenous use, their pH activity profile must be carefully considered to ensure optimal function within the physiological environment. Serine fibrinolytic enzymes belonging to serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 8.0 and 10 except for *Staphylococcus* sp. strain AJ which is active at pH 2.5–3.0. The molecular weights of most of the fibrinolytic enzyme's range between 20 and 45 kDa, and the isoelectric points are around 8.0 [222,223]. The highest and lowest optimal temperatures recorded were 70 °C (CK protease from *Bacillus* sp. strain CK 11–4) and 20 °C (*Streptomyces venezuelae*), respectively; however, it is important to select those active at physiological temperature. To characterize serine fibrinolytic enzymes, phenylmethylsulfonyl fluoride (PMSF), diisopropylfluorophosphate (DFP), or E−64 are used, which irreversibly inhibit all serine proteases. Similarly for the metalloprotease class, divalent metal ions are necessary for their activities, for example, $Ca^{+2}$, $Mg^{+2}$, $Mn^{+2+}$ for JP II [184], and $Mg^{2+}$, $Fe^{3+}$ for cMase [44]. Hence chelating agents such as EDTA are generally used while characterizing metalloproteases. The optimal pH for these enzymes lies within the range of 6.0–7.0. Notably, they exhibit a remarkable specificity towards fibrin, surpassing other proteases known for their broad substrate specificity. Moreover, some of these fibrinolytic enzymes possess the ability to activate plasminogen, further augmenting the process of fibrinolysis. Although many of these enzymes exhibit comparative advantages over other currently used fibrinolytic enzymes, a comprehensive investigation is

necessary to evaluate their potential for *in vivo* applications. While several microbial fibrinolytic enzymes have been studied to some extent, there is a scarcity of complete characterization studies that could determine the efficacy of these enzymes for *in vivo* use.

Apart from the crucial characterizations mentioned above, other essential activities need to be examined to ascertain their efficiency. These include substrate specificity, stability under physiological conditions, resistance to proteolytic degradation, immunogenicity, and potential interactions with other drugs or compounds. Ideally, a fibrinolytic enzyme should exhibit high specificity towards fibrin, the main component of blood clots, while minimizing interactions with other blood components such as haemoglobin, collagen, myoglobin, and other blood components. However, achieving absolute specificity can be challenging due to the structural similarities or shared binding sites between these components. Additionally, studies on enzyme pharmacokinetics, biodistribution, and toxicity assessments are crucial for evaluating their suitability for *in vivo* applications. This comprehensive characterization is vital for providing valuable insights into the potential clinical translation of these fibrinolytic enzymes. By focusing on these aspects, future research can establish clearer directions for developing and utilising these enzymes in clinical practice, thereby advancing the field of fibrinolysis.

## 8. Future prospect and further research

The research on microbial fibrinolytic enzymes from fermented foods is limited to a few Asian countries as illustrated in Fig. 2. However, the rich global culinary traditions extend far beyond Asian countries and a slight expansion is observed in recent studies conducted since 2020, particularly in countries such as Italy and Indonesia. Therefore, a plethora of foods including traditional fermented foods from Latin America, the Middle East, Africa, Europe, and other regions, need to be screened for fibrinolytic enzymes and their microbial producers. Furthermore, these traditional fermented foods from diverse countries may harbour fibrinolytic enzymes distinct from those found in Asian counterparts. To harness this untapped potential, a multifaceted approach is warranted. In addition to exploring new food sources, an in-depth enzymatic characterization, paired with techniques such as bioinformatic analysis of the purified enzyme on sequencing, is necessary. Furthermore, implementing omics techniques such as metagenomics and metatranscriptomics to screen fibrinolytic enzymes directly from food sources can unveil unique enzymes especially those from non-culturable microbes or even from non-microbial sources with tremendous potential applications in both the food and pharmaceutical industries. By amalgamating traditional wisdom with cutting-edge technology, we can unlock a treasure trove of novel enzymes, enriching our understanding and broadening the scope of applications for these valuable biocatalysts.

## 9. The failure of fibrinolytic enzymes in clinical trials and possible rectifying mechanisms

Fibrinolytic enzymes, despite their noteworthy efficacy in preclinical trials, have faced hurdles in clinical trials, primarily attributed to issues such as variable pharmacokinetics, immunogenic responses, and challenges in achieving optimal therapeutic concentrations at target sites. Moreover, the complex interplay of these enzymes within the intricate *in vivo* milieu adds another layer of complexity. In examining the challenges encountered by fibrinolytic enzymes in clinical trials, it is essential to consider the journey of specific candidates. Despite initial promise demonstrated in *vitro* and animal model studies, the translation of these enzymes to clinical applications has faced substantial hurdles [148]. A notable case is alfimeprase, where the progression from discovery to Phase III trials spanned over two decades. The Phase I trial, focusing on chronic peripheral arterial occlusion (PAO), showcased the safety profile and thrombolytic activity of alfimeprase, establishing its potential for treating PAO with minimal risk of haemorrhage. However, as alfimeprase advanced to Phase II and III trials (NAPA-1, NAPA-2, and NAPA-3), challenges emerged, including the observed correlation between the length of the thrombus and the success of lysis. Notably, the efficacy of alfimeprase seemed compromised in shorter thrombi, prompting considerations of potential flaws in the side hole catheter delivery mechanism [149]. Similar inconsistencies were seen in the case of nattokinase trials. In the NAPS trial, nattokinase exhibited null effects on atherosclerosis progression, blood pressure, and various cardiovascular risk factors [230]. Additionally, other nattokinase studies reported positive outcomes, including reductions in fibrinogen [231] and blood pressure [232], yet inconsistencies across trials raise questions

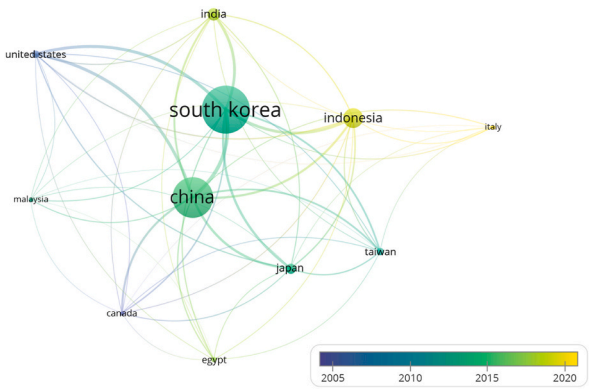



**Fig. 2.** Geographic distribution of research on fibrinolytic enzymes from fermented foods from the year 2000–2023 (Generated using VOSviewer).

about generalizability. Despite the potential fibrinolytic and thrombolytic attributes observed in *vitro* and animal models, limited data from human studies hinder the conclusive validation of these enzyme's clinical efficacy. The multifactorial nature of cardiovascular diseases and variations in study designs underscore the complexity of fibrinolytic enzyme application, necessitating further research to elucidate optimal dosage, delivery mechanisms, and patient selection criteria for successful clinical outcomes. These findings underscore the complexity of translating fibrinolytic enzymes from preclinical success to clinical triumph, necessitating ongoing exploration of alternative delivery methodologies to address limitations encountered during the later stages of clinical development. To rectify these shortcomings, ongoing research endeavours focus on innovative delivery systems, dosage optimization, and the exploration of modified enzyme variants with enhanced stability and reduced immunogenicity. Addressing these challenges is crucial for realizing the full therapeutic potential of fibrinolytic enzymes, thereby paving the way for their successful integration into clinical applications.

## 10. Conclusion

This review has evaluated the drawbacks and benefits of current thrombolytic agents and emphasized the need to explore alternative sources of fibrinolytic enzymes. Although in use for thrombolytic therapy, current fibrinolytic enzymes have negative side effects. The review has shed light on the significance of selecting appropriate sources for screening microbes capable of producing fibrinolytic enzymes, considering factors such as immunogenicity, scalability for mass production, and cost-effectiveness. The extensive discussion throughout the review emphasizes how the selection of the source of fibrinolytic enzyme and fibrinolytic enzyme producers can significantly influence the properties of the fibrinolytic enzyme. Considering these factors is crucial for optimizing the development and utilization of fibrinolytic enzymes with desirable characteristics for clinical applications. For instance, salt-resistant fibrinolytic enzymes were successfully purified from *Bacillus licheniformis* HJ4, which was isolated from jeotgal, a Korean fermented food made from salted seafood or meat. Furthermore, fibrinolytic enzymes purified from marine algae have been shown to exhibit high thermal stability, while those from earthworm gut display non-specific fibrinolytic activity. In addition, fibrinolytic enzymes from snake venom exhibit resistance to inherent fibrinolytic enzyme inhibitors from human blood, as naturally they function to facilitate the spreading of toxins in their prey. These findings provide compelling evidence that the characteristics of fibrinolytic enzymes are critically dependent on their producers and the environments in which they are produced. Thus, it is crucial to consider the source of the enzyme when studying its properties and potential applications.

The review highlights the advantages of fibrinolytic enzymes isolated from microbes derived from food sources over non-food sources. Food-derived fibrinolytic enzymes, like nattokinase, can be used as oral supplements. However, extensive research needs to be carried out to fully understand the immunogenicity of fibrinolytic enzymes purified from food sources. It should be emphasized that conventional fermented foods lack a universal nomenclature and may exhibit inconsistencies in spelling or even have multiple designations, leading to difficulties in data synthesis and compilation. It also highlights the significant gaps in the vast array of fibrinolytic enzyme sources yet to be explored. For example, since 1980 till today, fibrinolytic enzymes from fermented foods of a few countries have been screened but are primarily confined to the Asian continent. This emphasizes the potential and need for exploring the vast resources of fermented foods from Africa, Latin America, and the Middle East. A literature survey of the prevalence of heart diseases along with their food habits, including consumption of traditional foods across different communities globally, should be conducted, which could act as a strong platform to further research the link between food habits, fibrinolytic enzymes, and CVDs. Overall, further research in this field is required to advance our understanding of fibrinolysis and to develop novel and effective thrombolytic agents for managing thrombotic diseases.

*Funding*

This work was supported by the National Research Foundation, Republic of South Africa under grant numbers [UID 138097 and MND 200630538954].

## Code availability

No codes were utilized during this study.

## CRediT authorship contribution statement

**Chinmay Hazare:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Prashant Bhagwat:** Supervision, Writing – review & editing. **Suren Singh:** Supervision, Writing – review & editing, Resources. **Santhosh Pillai:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## References


[1] M. Amini, F. Zayeri, M. Salehi, Trend analysis of cardiovascular disease mortality, incidence, and mortality-to-incidence ratio: results from global burden of disease study 2017, BMC Publ. Health 21 (1) (2021) 401, https://doi.org/10.1186/s12889-021-10429-0.

[2] I. WHO, Cardiovascular Diseases (CVDs), Cardiovascular Diseases (CVDs), WHO, 2019 (Errata).

[3] A. Hessami, A. Shamshirian, K. Heydari, F. Pourali, R. Alizadeh-Navaei, M. Moosazadeh, S. Abrotan, L. Shojaie, S. Sedighi, D. Shamshirian, N. Rezaei, Cardiovascular diseases burden in COVID-19: systematic review and meta-analysis, Am. J. Emerg. Med. 46 (2021) 382–391, https://doi.org/10.1016/j.ajem.2020.10.022.

[4] F. Gul, S.F. Janzer, Peripheral Vascular Disease, StatPearls, 2021 [Internet].

[5] M. Tomaiuolo, L.F. Brass, T.J. Stalker, Regulation of platelet activation and coagulation and its role in vascular injury and arterial thrombosis, Interventional cardiology clinics 6 (1) (2017) 1.

[6] N. Mackman, Triggers, targets and treatments for thrombosis, Nature 451 (7181) (2008) 914–918, https://doi.org/10.1038/nature06797.

[7] B. Furie, B.C. Furie, Mechanisms of thrombus formation, N. Engl. J. Med. 359 (9) (2008) 938–949, https://doi.org/10.1056/NEJMra0801082.

[8] G. Cesarman-Maus, K.A. Hajjar, Molecular mechanisms of fibrinolysis, Br. J. Haematol. 129 (3) (2005) 307–321, https://doi.org/10.1111/j.1365-2141.2005.05444.x.

[9] N. Mackman, W. Bergmeier, G.A. Stouffer, J.I. Weitz, Therapeutic strategies for thrombosis: new targets and approaches, Nat. Rev. Drug Discov. 19 (5) (2020) 333–352, https://doi.org/10.1038/s41573-020-0061-0.

[10] A. Krishnamurthy, P.D. Belur, S.B. Subramanya, Methods available to assess therapeutic potential of fibrinolytic enzymes of microbial origin: a review, Journal of Analytical Science and Technology 9 (1) (2018) 1–11, https://doi.org/10.1186/s40543-018-0143-3.

[11] A.M. Moula Ali, S.C.B. Bavisetty, Purification, physicochemical properties, and statistical optimization of fibrinolytic enzymes especially from fermented foods: a comprehensive review, Int. J. Biol. Macromol. 163 (2020) 1498–1517, https://doi.org/10.1016/j.ijbiomac.2020.07.303.

[12] A.J. Johnson, C.W. Mc, The lysis of artificially induced intravascular clots in man by intravenous infusions of streptokinase, J. Clin. Invest. 38 (9) (1959) 1627–1643, https://doi.org/10.1172/JCI103941.

[13] R.L. Medcalf, C.B. Keragala, The fibrinolytic system: mysteries and opportunities, Hemasphere 5 (6) (2021) e570.

[14] E. Kotb, The biotechnological potential of fibrinolytic enzymes in the dissolution of endogenous blood thrombi, Biotechnol. Prog. 30 (3) (2014) 656–672, https://doi.org/10.1002/btpr.1918.

[15] F. Altaf, S. Wu, V. Kasim, Role of fibrinolytic enzymes in anti-thrombosis therapy, Front. Mol. Biosci. 8 (2021) 476, https://doi.org/10.3389/fmolb.2021.680397.

[16] S.S. Kumar, A. Sabu, Fibrinolytic Enzymes for Thrombolytic Therapy, Therapeutic Enzymes: Function and Clinical Implications, 2019, pp. 345–381.

[17] P.E.d.C. e Silva, R.P. Bezerra, A.L.F. Porto, An overview about fibrinolytic enzymes from microorganisms and algae: production and characterization, Journal of Pharmaceutical and Biological Sciences 4 (1) (2016) 1–11.

[18] H. Sumi, H. Hamada, H. Tsushima, H. Mihara, H. Muraki, A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet, Experientia 43 (10) (1987) 1110–1111, https://doi.org/10.1007/BF01956052.

[19] W. Kim, K. Choi, Y. Kim, H. Park, J. Choi, Y. Lee, H. Oh, I. Kwon, S. Lee, Purification and characterization of a fibrinolytic enzyme produced from Bacillus sp. strain CK 11-4 screened from Chungkook-Jang, Appl. Environ. Microbiol. 62 (7) (1996) 2482–2488, https://doi.org/10.1128/aem.62.7.2482-2488.1996.

[20] T. Petraglia, T. Latronico, G.M. Liuzzi, A. Faniglulo, A. Crescenzi, R. Rossano, Edible mushrooms as source of fibrin (ogen) olytic enzymes: comparison between four cultivated species, Molecules 27 (23) (2022) 8145, https://doi.org/10.3390/molecules27238145.

[21] J. Xu, M. Du, X. Yang, Q. Chen, H. Chen, D.-H. Lin, Thrombolytic effects in vivo of nattokinase in a carrageenan-induced rat model of thrombosis, Acta Haematol. 132 (2) (2014) 247–253, https://doi.org/10.1159/000360360.

[22] Y. Peng, Q. Huang, R.-h. Zhang, Y.-z. Zhang, Purification and characterization of a fibrinolytic enzyme produced by Bacillus amyloliquefaciens DC-4 screened from douchi, a traditional Chinese soybean food, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 134 (1) (2003) 45–52, https://doi.org/10.1016/S1096-4959(02)00183-5.

[23] Y. Ghasemi, F. Dabbagh, A. Ghasemian, Cloning of a fibrinolytic enzyme (subtilisin) gene from Bacillus subtilis in Escherichia coli, Mol. Biotechnol. 52 (1) (2012) 1–7, https://doi.org/10.1007/s12033-011-9467-6.

[24] R. Saini, S. Saini, S. Sharma, Potential of probiotics in controlling cardiovascular diseases, J. Cardiovasc. Dis. Res. 1 (4) (2010) 213, https://doi.org/10.4103/0975-3583.74267.

[25] M. Rai, M. Curley, Z. Coleman, F. Demontis, Contribution of proteases to the hallmarks of aging and to age-related neurodegeneration, Aging Cell 21 (5) (2022) e13603, https://doi.org/10.1111/acel.13603.

[26] Q. Li, L. Yi, P. Marek, B.L. Iverson, Commercial proteases: present and future, FEBS Lett. 587 (8) (2013) 1155–1163, https://doi.org/10.1016/j.febslet.2012.12.019.

[27] J.S. Ramírez-Larrota, U. Eckhard, An introduction to bacterial biofilms and their proteases, and their roles in host infection and immune evasion, Biomolecules 12 (2) (2022) 306, https://doi.org/10.3390/biom12020306.

[28] J.R. Simkhada, P. Mander, S.S. Cho, J.C. Yoo, A novel fibrinolytic protease from Streptomyces sp. CS684, Process Biochem. 45 (1) (2010) 88–93, https://doi.org/10.1016/j.procbio.2009.08.010.

[29] M.J. Page, E. Di Cera, Serine peptidases: classification, structure and function, Cell. Mol. Life Sci. 65 (2008) 1220–1236, https://doi.org/10.1007/s00018-008-7565-9.

[30] N. Barzkar, Z. Khan, S.T. Jahromi, S. Pourmozaffar, M. Gozari, R. Nahavandi, A critical review on marine serine protease and its inhibitors: a new wave of drugs? Int. J. Biol. Macromol. 170 (2021) 674–687, https://doi.org/10.1016/j.ijbiomac.2020.12.134.

[31] L. Jeffries, D. Buckley, The detection and differentiation of fibrinolytic enzymes in bacteria, J. Appl. Bacteriol. 49 (3) (1980) 479–492, https://doi.org/10.1111/j.1365-2672.1980.tb04723.x.

[32] B.P. Ayanti, S.N. Ethica, A.R. Sulisytaningtyas, S.S. Dewi, D.S. Zilda, Prospective Purification and Assay of Thrombolytic Protease from Bacillus Sp. HSFI-10 Isolated from Sand Sea Cucumber for Antithrombotic Agent Development, 7th International Conference on Biological Science (ICBS 2021), Atlantis Press, 2022, pp. 404–415, https://doi.org/10.2991/absr.k.220406.057 (Errata).

[33] U. Food, D. Administration, Drugs@ FDA: FDA Approved Drug Products, 2022 (Errata).

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

[35] T.S. Raj, S. Athimoolam, P. Vijayaraghavan, Biosynthesis and characterization of a novel fibrinolytic alkaline serine protease from newly isolated Bacillus flexus BF12 for biomedical applications, Curr. Pharmaceut. Biotechnol. 22 (5) (2021) 706–717, https://doi.org/10.2174/1389201021666201117094714.

[36] V.N. Alencar, M.C. Nascimento, J.V. Ferreira, J.M. Batista, M.N.D. Cunha, J.M. Nascimento, R.V. Sobral, M.T. Couto, T.P. Nascimento, R.M. Costa, Purification and characterization of fibrinolytic protease from *Streptomyces parvulus* by polyethylene glycol-phosphate aqueous two-phase system, An Acad. Bras Ciências 93 (2021) e20210335.

[37] G.-Y. Qian, G. Lim, S.-J. Yin, J.-M. Yang, J. Lee, Y.-D. Park, Biochemical study of fibrinolytic protease from Euphausia superba possessing multifunctional serine protease activity, Protein Pept. Lett. 28 (6) (2021) 651–664, https://doi.org/10.2174/0929866527666201112123714.

[38] L.V. Truong, B.S. Paulsen, V.H. Bac, A novel serine protease from pseuderanthemum latifolium B. Hansen: characterization and fibrino (geno) lytic activities, Nat. Prod. Res. 35 (16) (2021) 2640–2646, https://doi.org/10.1080/14786419.2019.1656626.

[39] H. Nagase, Metalloproteases, Current Protocols in Protein Science 24 (1) (2001) 21.4.1–21.4.13.

[40] A. Vaidya, V.S. Nair, J.J. Georrge, S. Sp, Comparative analysis of thermophilic proteases, J. Life Sci. Bioinform. Pharm. Chem. Sci. 4 (2018) 65–91.

[41] Z. Yao, J.A. Kim, J.H. Kim, Characterization of a Fibrinolytic Enzyme Secreted by Bacillus Velezensis BS2 Isolated from Sea Squirt Jeotgal, 2019, https://doi.org/10.4014/jmb.1810.10053.

[42] K. Taneja, B.K. Bajaj, S. Kumar, N. Dilbaghi, Production, purification and characterization of fibrinolytic enzyme from Serratia sp. KG-2-1 using optimized media, 3 Biotech 7 (3) (2017) 1–15, https://doi.org/10.1007/s13205-017-0808-4.

[43] A.E. Sales, F.A.S.D. de Souza, J.A. Teixeira, T.S. Porto, A.L.F. Porto, Integrated process production and extraction of the fibrinolytic protease from Bacillus sp. UFPEDA 485, Appl. Biochem. Biotechnol. 170 (7) (2013) 1676–1688, https://doi.org/10.1007/s12010-013-0306-z.

[44] L. Cui, M.S. Dong, X.H. Chen, M. Jiang, X. Lv, G. Yan, A novel fibrinolytic enzyme from Cordyceps militaris, a Chinese traditional medicinal mushroom, World J. Microbiol. Biotechnol. 24 (4) (2008) 483–489, https://doi.org/10.1007/s11274-007-9497-1.

[45] M.-H. Shen, J.-S. Kim, K. Sapkota, S.-E. Park, B.-S. Choi, S. Kim, H.-H. Lee, C.-S. Kim, H.-S. Chun, C.-I. Ryoo, Purification, characterization, and cloning of fibrinolytic metalloprotease from Pleurotus ostreatus mycelia, J. Microbiol. Biotechnol. 17 (8) (2007) 1271–1283.

[46] S.-E. Park, M.-H. Li, J.-S. Kim, K. Sapkota, J.-E. Kim, B.-S. Choi, Y.-H. Yoon, J.-C. Lee, H.-H. Lee, C.-S. Kim, Purification and characterization of a fibrinolytic protease from a culture supernatant of Flammulina velutipes mycelia, Biosci., Biotechnol., Biochem. (2007), https://doi.org/10.1271/bbb.70193, 0708030527-0708030527.

[47] Y.-k. Jeong, J.H. Kim, S.-w. Gal, J.-e. Kim, S.-s. Park, K.-t. Chung, Y.-H. Kim, B.-W. Kim, W.-H. Joo, Molecular cloning and characterization of the gene encoding a fibrinolytic enzyme from Bacillus subtilis strain A1, World J. Microbiol. Biotechnol. 20 (7) (2004) 711–717.

[48] J.-H. Kim, Y.S. Kim, Characterization of a metalloenzyme from a wild mushroom, Tricholoma saponaceum, Bioscience, Biotechnology, and Biochemistry 65 (2) (2001) 356–362, https://doi.org/10.1271/bbb.65.356.

[49] E. Kotb, Purification and partial characterization of serine fibrinolytic enzyme from Bacillus megaterium KSK-07 isolated from kishk, a traditional Egyptian fermented food, Appl. Biochem. Microbiol. 51 (2015) 34–43.

[50] Y. Zhou, H. Chen, B. Yu, G. Chen, Z. Liang, Purification and characterization of a fibrinolytic enzyme from marine Bacillus velezensis Z01 and assessment of its therapeutic efficacy in vivo, Microorganisms 10 (5) (2022) 843, https://doi.org/10.3390/microorganisms10050843.

[51] A. Krishnamurthy, P.D. Belur, A novel fibrinolytic serine metalloprotease from the marine Serratia marcescens subsp. sakuensis: purification and characterization, Int. J. Biol. Macromol. 112 (2018) 110–118, https://doi.org/10.1016/j.ijbiomac.2018.01.129.

[52] Y. Meng, Z. Yao, H.G. Le, S.J. Lee, H.S. Jeon, J.Y. Yoo, J.H. Kim, Characterization of a salt-resistant fibrinolytic protease of Bacillus licheniformis HJ4 isolated from Hwangseokae jeotgal, a traditional Korean fermented seafood, Folia Microbiol. (2021) 1–9, https://doi.org/10.1007/s12223-021-00878-w.

[53] R. Nedaeinia, H. Faraji, S.H. Javanmard, G.A. Ferns, M. Ghayour-Mobarhan, M. Goli, B. Mashkani, M. Nedaeinia, M.H.H. Haghighi, M. Ranjbar, Bacterial staphylokinase as a promising third-generation drug in the treatment for vascular occlusion, Mol. Biol. Rep. 47 (2020) 819–841, https://doi.org/10.1007/s11033-019-05167-x.

[54] M. Vachher, A. Sen, R. Kapila, A. Nigam, Microbial therapeutic enzymes: a promising area of biopharmaceuticals, Current Research in Biotechnology 3 (2021) 195–208, https://doi.org/10.1016/j.crbiot.2021.05.006.

[55] P.K. Vadla, M. Tummuru, D. Kumar, Purification and characterization of recombinant Streptokinase expressed in E. coli from Streptococcus equisimilis with N-terminal methionine, Jurnal Teknologi Laboratorium 8 (1) (2019) 8–17, https://doi.org/10.29238/teknolabjournal.v8i1.153.

[56] H. Malke, J.J. Ferretti, Streptokinase: cloning, expression, and excretion by Escherichia coli, Proc. Natl. Acad. Sci. USA 81 (11) (1984) 3557–3561, https://doi.org/10.1073/pnas.81.11.3557.

[57] R. Aneja, M. Datt, S. Yadav, G. Sahni, Multiple exosites distributed across the three domains of streptokinase co-operate to generate high catalytic rates in the streptokinase–plasmin activator complex, Biochemistry 52 (49) (2013) 8957–8968, https://doi.org/10.1021/bi400142s.

[58] A. Banerjee, Y. Chisti, U. Banerjee, Streptokinase—a clinically useful thrombolytic agent, Biotechnol. Adv. 22 (4) (2004) 287–307, https://doi.org/10.1016/j.biotechadv.2003.09.004.

[59] Adivitiya, Y.P. Khasa, The evolution of recombinant thrombolytics: current status and future directions, Bioengineered 8 (4) (2017) 331–358, https://doi.org/10.1080/21655979.2016.1229718.

[60] P. Juhlin, P.Å. Boström, A. Torp, A. Bredberg, Streptokinase antibodies inhibit reperfusion during thrombolytic therapy with streptokinase in acute myocardial infarction, J. Intern. Med. 245 (5) (1999) 483–488, https://doi.org/10.1046/j.1365-2796.1999.00485.x.

[61] R. MacFarlane, J. Pilling, Fibrinolytic activity of normal urine, Nature 159 (4049) (1947), 779-779.

[62] G. Sobel, S. Mohler, N. Jones, A. Dowdy, M. Guest, Urokinase-an Activator of Plasma Profibrinolysin Extracted from Urine, 20814, American Journal of Physiology, AMER PHYSIOLOGICAL SOC 9650 ROCKVILLE PIKE, BETHESDA, MD, 1952, pp. 768–769 (Errata).

[63] D. Rijken, H. Lijnen, New insights into the molecular mechanisms of the fibrinolytic system, J. Thromb. Haemostasis 7 (1) (2009) 4–13, https://doi.org/10.1111/j.1538-7836.2008.03220.x.

[64] R. Egelund, T.E. Petersen, P.A. Andreasen, A serpin-induced extensive proteolytic susceptibility of urokinase-type plasminogen activator implicates distortion of the proteinase substrate-binding pocket and oxyanion hole in the serpin inhibitory mechanism, Eur. J. Biochem. 268 (3) (2001) 673–685.

[65] M. Schmitt, L. Goretzki, F. Jänicke, J. Calvete, M. Eulitz, H. Kobayashi, N. Chucholowski, H. Graeff, Biological and clinical relevance of the urokinase-type plasminogen activator (uPA) in breast cancer, Biomed. Biochim. Acta 50 (4–6) (1991) 731–741.

[66] M.S. Stack, D.A. Johnson, Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase), J. Biol. Chem. 269 (13) (1994) 9416–9419, https://doi.org/10.1016/S0021-9258(17)36896-5.

[67] R. Sadasivan, R. Cherian, A. Wyatt, Urokinase-type plasminogen activator expression in human prostate carcinomas, Am. J. Med. Sci. 312 (1) (1996) 8–11, https://doi.org/10.1016/S0002-9629(15)41739-2.

[68] L. Goretzki, M. Schmitt, K. Mann, J. Calvete, N. Chucholowski, M. Kramer, W.A. Günzler, F. Jänicke, H. Graeff, Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-uPA) by the cysteine protease cathepsin L, FEBS Lett. 297 (1–2) (1992) 112–118, https://doi.org/10.1016/0014-5793(92)80339-I.

[69] N. Mahmood, C. Mihalcioiu, S.A. Rabbani, Multifaceted role of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR): diagnostic, prognostic, and therapeutic applications, Front. Oncol. 8 (2018) 24, https://doi.org/10.3389/fonc.2018.00024.

[70] R.R.A. Kadir, U. Bayraktutan, Urokinase plasminogen activator: a potential thrombolytic agent for ischaemic stroke, Cell. Mol. Neurobiol. 40 (2020) 347–355, https://doi.org/10.1007/s10571-019-00737-w.

[71] S. Sherry, Pharmacology of anistreplase, Clin. Cardiol. 13 (S5) (1990) 3–10, https://doi.org/10.1002/clc.4960131303.

[72] M. Moser, C. Bode, Pharmacology and clinical trial results of saruplase (scuPA) in acute myocardial infarction, Expet Opin. Invest. Drugs 8 (3) (1999) 329–335, https://doi.org/10.1517/13543784.8.3.329.

[73] A.A. Ismail, B.T. Shaker, K. Bajou, The plasminogen–activator plasmin system in physiological and pathophysiological angiogenesis, Int. J. Mol. Sci. 23 (1) (2021) 337, https://doi.org/10.3390/ijms23010337.

[74] P. Proctor, M.A. Leesar, A. Chatterjee, Thrombolytic therapy in the current ERA: myocardial infarction and beyond, Curr. Pharmaceut. Des. 24 (4) (2018) 414–426.

[75] A. Furlan, R. Higashida, L. Wechsler, M. Gent, H. Rowley, C. Kase, M. Pessin, A. Ahuja, F. Callahan, W.M. Clark, Intra-arterial prourokinase for acute ischemic stroke: the PROACT II study: a randomized controlled trial, JAMA 282 (21) (1999) 2003–2011, https://doi.org/10.1001/jama.282.21.2003.

[76] T.N. Jilani, A.H. Siddiqui, Tissue Plasminogen Activator, StatPearls Publishing, 2021. StatPearls [Internet].

[77] D. Rijken, G. Wijngaards, M. Zaal-de Jong, J. Welbergen, Purification and partial characterization of plasminogen activator from human uterine tissue, Biochim. Biophys. Acta Protein Struct. 580 (1) (1979) 140–153, https://doi.org/10.1016/0005-2795(79)90205-8.

[78] S.-O. Yang, G.H. Nielsen, K.M. Wilding, M.A. Cooper, D.W. Wood, B.C. Bundy, Towards on-demand E. coli-based cell-free protein synthesis of tissue plasminogen activator, Methods and Protocols 2 (2) (2019) 52, https://doi.org/10.3390/mps2020052.

[79] R. Nedaeinia, H. Faraji, S.H. Javanmard, G.A. Ferns, M. Ghayour-Mobarhan, M. Goli, B. Mashkani, M. Nedaeinia, M.H.H. Haghighi, M. Ranjbar, Bacterial staphylokinase as a promising third-generation drug in the treatment for vascular occlusion, Mol. Biol. Rep. 47 (1) (2020) 819–841, https://doi.org/10.1007/s11033-019-05167-x.

[80] V.W.C. Van Hinsebergh, Annemie, P. Koolwijk, Role of fibrin matrix in angiogenesis, Ann. N. Y. Acad. Sci. 936 (1) (2001) 426–437, https://doi.org/10.1111/j.1749-6632.2001.tb03526.x.

[81] B. Gu, Y. Huang, E.L. Manchester, A.D. Hughes, S.A.M. Thom, R. Chen, X.Y. Xu, Multiphysics modelling and simulation of thrombolysis via activated platelet-targeted nanomedicine, Pharmaceut. Res. 39 (2022) 41–56.

[82] C. Seillier, P. Hélie, G. Petit, D. Vivien, D. Clemente, B. Le Mauff, F. Docagne, O. Toutirais, Roles of the tissue-type plasminogen activator in immune response, Cell. Immunol. 371 (2022) 104451, https://doi.org/10.1016/j.cellimm.2021.104451.

[83] E. Mohammadi, K. Mahnam, A. Jahanian-Najafabadi, H.M.M. Sadeghi, Design and production of new chimeric reteplase with enhanced fibrin affinity: a theoretical and experimental study, J. Biomol. Struct. Dyn. 39 (4) (2021) 1321–1333, https://doi.org/10.1080/07391102.2020.1729865.

[84] F.M. Wurm, Production of recombinant protein therapeutics in cultivated mammalian cells, Nat. Biotechnol. 22 (11) (2004) 1393–1398.

[85] M. Gupta, S. Saxena, V. Meshram, Microbial clot busters: an overview of source, production, properties and fibrinolytic activity, in: J. Singh, V. Meshram, M. Gupta (Eds.), Bioactive Natural Products in Drug Discovery, 2020, pp. 485–516.

[86] G. Broocks, R. McDonough, M. Bechstein, U. Hanning, C. Brekenfeld, F. Flottmann, H. Kniep, M.T. Nawka, M. Deb-Chatterji, G. Thomalla, Benefit and risk of intravenous alteplase in patients with acute large vessel occlusion stroke and low ASPECTS, J. Neurointerventional Surg. 15 (1) (2023) 8–13, https://doi.org/10.1136/neurintsurg-2021-017986.

[87] B.A. Keyt, N.F. Paoni, C.J. Refino, L. Berleau, H. Nguyen, A. Chow, J. Lai, L. Pena, C. Pater, J. Ogez, A faster-acting and more potent form of tissue plasminogen activator, Proc. Natl. Acad. Sci. USA 91 (9) (1994) 3670–3674, https://doi.org/10.1073/pnas.91.9.3670.

[88] S.T. Mannully, C. Shanthi, K.K. Pulicherla, Lipid modification of staphylokinase and its implications on stability and activity, Int. J. Biol. Macromol. 121 (2019) 1037–1045, https://doi.org/10.1016/j.ijbiomac.2018.10.134.

[89] B.T.Z. Wang, Xiaolan Li, Yang Lin, Yang Huilin, One Plant of Bacillus Amyloliquefaciens Strain for Being Isolated from Producing Protease in Fermented Soya Bean, Google Patents, Jiangxi Normal University, China, 2017 (Errata).

[90] S.H.B. Kimberly Hamad-Schifferli, Helena de Puig Guixe Blood clotting control, Google Patents, Massachusetts Institute of Technology, US, 2017 (Errata).

[91] B.S. Anand Ramamurthi, Andrew Sylvester Nanoparticles for Controlled Lysis of Blood Clots, Google Patents, Cleveland Clinic Foundation, US, 2017 (Errata).

[92] X.L. Liu Huizhou Wei, Mingfang Xie Yuchun, Li Haojian Yang, Liangrong, A Kind of Nattokinase Enteric Capsule and Preparation Method Thereof, Google Patents, Institute of Process Engineering of CAS, China, 2015 (Errata).

[93] N.M. Shekhar Kumar, Girish Sahni Mutants of Streptokinase and Their Covalently Modified Forms, Google Patents, Council of Scientific and Industrial Research (CSIR), India, 2015 (Errata).

[94] Y.C.H. Chen Jiepeng Chen, Qiu Xuelian Duan Lili, Hu Liusong Ji Yeyu, Cai Guizhu Xu Zhikai, Ye Honglin Honglin, Xiaoying Huang, Nattokinase Oral Emulsion and Preparation Method Thereof, Google Patents, Shuan Jun Bio Tech Ltd Guangdong, China, 2014 (Errata).

[95] J.Y.W. Zhao Chunling Ju, Jin Xin Chen, Limei Lianbo, cDNA Sequence and Amino Acid Sequence of Lugworm Fibrinolytic Enzyme with Thrombolysis Activity, Google Scholar, Weifang Medical University, China, 2013 (Errata).

[96] S. Badar, M. Azarkan, A.H. Mekkawy, J. Akhter, K. Pillai, R. El Mahyaoui, K. Ke, L. Cavanaugh, D.L. Morris, Comparison of proteolytic, cytotoxic and anticoagulant properties of chromatographically fractionated bromelain to un-fractionated bromelain, Am. J. Tourism Res. 13 (5) (2021) 4309.

[97] H.R. Yang, M.N. Zahan, Y. Yoon, K. Kim, D.H. Hwang, W.H. Kim, I.R. Rho, E. Kim, C. Kang, Unveiling the potent fibrino (geno) lytic, anticoagulant, and antithrombotic effects of papain, a cysteine protease from carica papaya latex using κ-carrageenan rat tail thrombosis model, Int. J. Mol. Sci. 24 (23) (2023) 16770.

[98] A. Bilawal, Z. Jiang, Y. Li, B. Qi, Isolation, Bioactivity, Identification, and Commercial Application of Soybean Bioactive Peptides, CRC Press, 2022, pp. 217–234. Phytochemicals in Soybeans.

[99] X. Xin, R.R. Ambati, Z. Cai, B. Lei, Purification and characterization of fibrinolytic enzyme from a bacterium isolated from soil, 3 Biotech 8 (2) (2018) 1–8, https://doi.org/10.1007/s13205-018-1115-4.

[100] N.A. Ahamed, I.A. Arif, S. Al-Rashed, A. Panneerselvam, V. Ambikapathy, In vitro thrombolytic potential of fibrinolytic enzyme from Brevibacterium sp. isolated from the root of the plant, Aloe castellorum, J. King Saud Univ. Sci. 34 (3) (2022) 101868, https://doi.org/10.1016/j.jksus.2022.101868.

[101] D. Dhamodharan, Novel fibrinolytic protease producing Streptomyces radiopugnans VITSD8 from marine sponges, Mar. Drugs 17 (3) (2019) 164, https://doi.org/10.3390/md17030164.

[102] W. Yang, W. Wang, Y. Ma, Q. Yang, P. Li, S. Du, Bioevaluation of pheretima vulgaris antithrombotic extract, PvQ, and isolation, identification of six novel PvQ-derived fibrinolytic proteases, Molecules 26 (16) (2021) 4946, https://doi.org/10.3390/molecules26164946.

[103] M. Chandramohan, C.Y. Yee, P.H.K. Beatrice, P. Ponnaiah, G. Narendrakumar, A.V. Samrot, Production, characterization and optimization of fibrinolytic protease from Bacillus pseudomycoides strain MA02 isolated from poultry slaughter house soils, Biocatal. Agric. Biotechnol. 22 (2019) 101371, https://doi.org/10.1016/j.bcab.2019.101371.

[104] P.E.d.C. e Silva, R.C. de Barros, W.W.C. Albuquerque, R.M.P. Brandão, R.P. Bezerra, A.L.F. Porto, In vitro thrombolytic activity of a purified fibrinolytic enzyme from Chlorella vulgaris, J. Chromatogr. B 1092 (2018) 524–529, https://doi.org/10.1016/j.jchromb.2018.04.040.

[105] E.F. Sanchez, V.G. Alvarenga, L.S. Oliveira, D.L. Oliveira, M.I. Estevao-Costa, R. Flores-Ortiz, J.A. Eble, A fibrinolytic snake venom metalloproteinase, mutalysin-II, with antiplatelet activity and targeting capability toward glycoprotein GPIbα and glycoprotein GPVI, Biochimie 184 (2021) 1–7, https://doi.org/10.1016/j.biochi.2021.01.016.

[106] A.F.M. Pinto, R. Dobrovolski, A.B.G. Veiga, J.A. Guimarães, Lonofibrase, a novel α-fibrinogenase from Lonomia obliqua caterpillars, Thromb. Res. 113 (2) (2004) 147–154, https://doi.org/10.1016/j.thromres.2004.01.012.

[107] Y.-S. Kim, B.-S. Hahn, S.-Y. Cho, I.-M. Chang, Biochemical characterization of serine proteases with fibrinolytic activity from *Tenodera sinensis* (praying Mantis), Toxicol. Res. 17 (2001) 97–104.

[108] M.Y. Ahn, B.-S. Hahn, K.S. Ryu, J.W. Kim, I. Kim, Y.S. Kim, Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, Catharsius molossus, Thromb. Res. 112 (5–6) (2003) 339–347, https://doi.org/10.1016/j.thromres.2004.01.005.

[109] T. Zhang, M. Zhou, H. Cai, K. Yan, Y. Zha, W. Zhuang, J. Liang, Y. Cheng, Identification, purification, and pharmacological activity analysis of *Desmodus rotundus* salivary plasminogen activator alpha 1 (DSPAα1) expressed in transgenic rabbit mammary glands, Transgenic Res. 31 (2022) 149–163.

[110] C. Sharma, A. Osmolovskiy, R. Singh, Microbial fibrinolytic enzymes as anti-thrombotics: production, characterisation and prodigious biopharmaceutical applications, Pharmaceutics 13 (11) (2021) 1880, https://doi.org/10.3390/pharmaceutics13111880.

[111] L. Stephani, P. Rahayu, D. Retnoningrum, M.T. Suhartono, H. Rachmawati, R.R. Tjandrawinata, Purification and proteomic analysis of potent fibrinolytic enzymes extracted from Lumbricus rubellus, Proteome Sci. 21 (1) (2023) 8, https://doi.org/10.1186/s12953-023-00206-9.

[112] H. Jiang, R. Shi, X. Li, J. Tang, D. Min, Insight into the thrombolytic ability of an extracellular fibrinolytic enzyme from Bacillus amyloliquefaciens GXU-1 isolated from Sipunculus nudus, Protein Expr. Purif. 213 (2024) 106371, https://doi.org/10.1016/j.pep.2023.106371.

[113] Z. Khan, M. Shafique, H.R. Nawaz, N. Jabeen, S.A. Naz, Bacillus tequilensis ZMS-2: a novel source of alkaline protease with antimicrobial, anti-coagulant, fibrinolytic and dehairing potentials, Pak. J. Pharm. Sci. 32 (2019) 1913–1918, https://doi.org/10.1016/j.pep.2023.106371 (4(Supplementary)).

[114] H. Liu, J. Yang, Y. Li, Y. Ma, W. Wang, W. Zhong, P. Li, S. Du, A novel fibrinolytic protein from pheretima vulgaris: purification, identification, antithrombotic evaluation, and mechanisms investigation, Front. Mol. Biosci. 8 (2022) 1388.

[115] M. Anusree, K. Swapna, C.N. Aguilar, A. Sabu, Optimization of process parameters for the enhanced production of fibrinolytic enzyme by a newly isolated marine bacterium, Bioresour. Technol. Rep. 11 (2020) 100436, https://doi.org/10.1016/j.biteb.2020.100436.

[116] M.T.T. Do Couto, A.V. Da Silva, R.V.D.S. Sobral, C.H. Rodrigues, M.N.C. Da Cunha, A.C.L. Leite, M.d.V.B. Figueiredo, J. de Paula Oliveira, R.M.P.B. Costa, A.E. S. Conniff, Production, extraction and characterization of a serine protease with fibrinolytic, fibrinogenolytic and thrombolytic activity obtained by Paenibacillus graminis, Process Biochem. 118 (2022) 335–345, https://doi.org/10.1016/j.procbio.2022.05.005.

[117] S. Pan, G. Chen, R. Wu, X. Cao, Z. Liang, Non-sterile submerged fermentation of fibrinolytic enzyme by marine Bacillus subtilis harboring antibacterial activity with starvation strategy, Front. Microbiol. 10 (2019) 1025, https://doi.org/10.3389/fmicb.2019.01025.

[118] A. Varol, S. Albayrak, H. Ozkan, Y. Demir, M. Taskin, A. Adiguzel, Production, purification and characterization of novel fibrinolytic enzyme from Bacillus atrophaeus V4, Biologia 78 (2) (2023) 591–600.

[119] Z. He, Y. Sun, M. Chu, J. Zhu, Y. Zhang, Q. Tang, G. Osman, L. Jiang, Z. Zhang, Screening of a novel fibrinolytic enzyme-producing Streptomyces from a hyper-arid area and optimization of its fibrinolytic enzyme production, Fermentation 9 (5) (2023) 410, https://doi.org/10.3390/fermentation9050410.

[120] C. Sharma, G.E.M. Salem, N. Sharma, P. Gautam, R. Singh, Thrombolytic potential of novel thiol-dependent fibrinolytic protease from Bacillus cereus RSA1, Biomolecules 10 (1) (2020) 3, https://doi.org/10.3390/biom10010003.

[121] P. Vijayaraghavan, P. Rajendran, S.G. Prakash Vincent, A. Arun, N. Abdullah Al-Dhabi, M. Valan Arasu, O. Young Kwon, Y.O. Kim, Novel sequential screening and enhanced production of fibrinolytic enzyme by *Bacillus* sp. IND12 using response surface methodology in solid-state fermentation, BioMed Res. Int. 2017 (2017) 3909657.

[122] V. Meshram, S. Saxena, K. Paul, M. Gupta, N. Kapoor, Production, purification and characterisation of a potential fibrinolytic protease from endophytic Xylaria curta by solid substrate fermentation, Appl. Biochem. Biotechnol. 181 (4) (2017) 1496–1512, https://doi.org/10.1007/s12010-016-2298-y.

[123] P. Palanivel, L. Ashokkumar, R. Balagurunathan, Production, purification and fibrinolytic characterization of alkaline protease from extremophilic soil fungi, Int. J. Pharma Bio Sci. 4 (2013) 101–110.

[124] F.v. Klink, H. Alizadeh, G.L. Stewart, M.S. Pidherney, R.E. Silvany, Y. He, J.P. McCulley, J.Y. Niederkorn, Characterization and pathogenic potential of a soil isolate and an ocular isolate of Acanthamoeba castellanii in relation to Acanthamoeba keratitis, Curr. Eye Res. 11 (12) (1992) 1207–1220, https://doi.org/10.3109/02713689208999546.

[125] P.S. Khursade, S.H. Galande, P.S. Krishna, R. Prakasham, Stenotrophomonas maltophilia Gd2: a potential and novel isolate for fibrinolytic enzyme production, Saudi J. Biol. Sci. 26 (7) (2019) 1567–1575, https://doi.org/10.1016/j.sjbs.2018.10.014.

[126] N. Barzkar, S.T. Jahromi, F. Vianello, Marine microbial fibrinolytic enzymes: an overview of source, production, biochemical properties and thrombolytic activity, Mar. Drugs 20 (1) (2022) 46, https://doi.org/10.3390/md20010046.

[127] M. Lahaye, Marine algae as sources of fibres: determination of soluble and insoluble dietary fibre contents in some 'sea vegetables', J. Sci. Food Agric. 54 (4) (1991) 587–594, https://doi.org/10.1002/jsfa.2740540410.

[128] N. Demina, E. Veslopolova, G. Gaenko, The marine bacterium *Alteromonas piscicida*–a producer of enzymes with thrombolytic action, Izvestiia Akademii Nauk SSSR, Seriia Biologicheskaia (3) (1990) 415–419.

[129] R. Masilamani, S. Natarajan, Molecular cloning, overexpression and characterization of a new thiol-dependent, alkaline serine protease with destaining function and fibrinolytic potential from *Marinobacter aquaeolei* MS2-1, Biologia 70 (9) (2015) 1143–1149.

[130] S. SudeshWarma, C.S. Devi, Production of fibrinolytic protease from Streptomyces lusitanus isolated from marine sediments, in: IOP Conference Series: Materials Science and Engineering, IOP Publishing, 2017 022048, https://doi.org/10.1088/1757-899X/263/2/022048(Errata.

[131] S.S. Kumar, M. Haridas, S. Abdulhameed, A novel fibrinolytic enzyme from marine Pseudomonas aeruginosa KU1 and its rapid in vivo thrombolysis with little haemolysis, Int. J. Biol. Macromol. 162 (2020) 470–479, https://doi.org/10.1016/j.ijbiomac.2020.06.178.

[132] C. Wu, C. Zheng, J. Wang, P. Jiang, Recombinant expression of thrombolytic agent reteplase in marine microalga Tetraselmis subcordiformis (Chlorodendrales, Chlorophyta), Mar. Drugs 19 (6) (2021) 315, https://doi.org/10.3390/md19060315.

[133] J. Zhao, S.-P. Qi, J. Wu, L. Li, R.-Q. He, Earthworm fibrinolytic enzyme, Stud. Nat. Prod. Chem. 30 (2005) 825–847, https://doi.org/10.1016/S1572-5995(05)80048-1.

[134] J. Zhao, R. Xiao, J. He, R. Pan, R. Fan, C. Wu, X. Liu, Y. Liu, R.-Q. He, In situ localization and substrate specificity of earthworm protease-II and protease-III-1 from Eisenia fetida, Int. J. Biol. Macromol. 40 (2) (2007) 67–75, https://doi.org/10.1016/j.ijbiomac.2006.05.007.

[135] H. Mihara, H. Sumi, T. Yoneta, H. MizuMOTO, R. Ikeda, M. Seiki, M. Maruyama, A novel fibrinolytic enzyme extracted from the earthworm, Lumbricus rubellus, Jpn. J. Physiol. 41 (3) (1991) 461–472, https://doi.org/10.2170/jjphysiol.41.461.

[136] I.-H. Cho, E.-S. Choi, H.-G. Lim, H.-H. Lee, Purification and characterization of six fibrinolytic serine-proteases from earthworm Lumbricus rubellus, BMB Reports 37 (2) (2004) 199–205.

[137] F. Wang, C. Wang, M. Li, L. Gui, J. Zhang, W. Chang, Purification, characterization and crystallization of a group of earthworm fibrinolytic enzymes from Eisenia fetida, Biotechnol. Lett. 25 (13) (2003) 1105–1109.

[138] R. Pan, Z.-J. Zhang, R.-Q. He, Earthworm protease, Applied and Environmental Soil Science (2010) 294258, 2010.

[139] C.K. Lee, J.S. Shin, B.S. Kim, I.H. Cho, Y.S. Kim, E.B. Lee, Antithrombotic effects by oral administration of novel proteinase fraction from earthworm Eisenia andrei on venous thrombosis model in rats, Arch Pharm. Res. (Seoul) 30 (4) (2007) 475–480.

[140] X.M. Yan, C.-H. Kim, C.K. Lee, J.S. Shin, I.H. Cho, U.D. Sohn, Intestinal absorption of fibrinolytic and proteolytic lumbrokinase extracted from earthworm, Eisenia andrei, KOREAN J. PHYSIOL. PHARMACOL. 14 (2) (2010) 71–75, https://doi.org/10.4196/kjpp.2010.14.2.71.

[141] T. Ge, Z.-J. Sun, S.-H. Fu, G.-D. Liang, Cloning of thrombolytic enzyme (lumbrokinase) from earthworm and its expression in the yeast Pichia pastoris, Protein Expr. Purif. 42 (1) (2005) 20–28, https://doi.org/10.1016/j.pep.2005.04.005.

[142] R. Tjandrawinata, D. Yunaidi, L. Susanto, The safety and tolerability of lumbrokinase DLBS1033 in healthy adult subjects, Drug Research 66 (6) (2016) 293–299, https://doi.org/10.1055/s-0035-1569406.

[143] S.S. Kumar, S. Abdulhameed, Therapeutic enzymes, Bioresources and Bioprocess in Biotechnology: Volume 2: Exploring Potential Biomolecules (2017) 45–73.

[144] E.F. Sanchez, R.J. Flores-Ortiz, V.G. Alvarenga, J.A. Eble, Direct fibrinolytic snake venom metalloproteinases affecting hemostasis: structural, biochemical features and therapeutic potential, Toxins 9 (12) (2017) 392.

[145] M. Trikha, S. Schmitmeier, F. Markland, Purification and characterization of fibrolase isoforms from venom of individual southern copperhead (*Agkistrodon contortrix*) snakes, Toxicon 32 (12) (1994) 1521–1531.

[146] S. Swenson, L.R. Bush, F.S. Markland, Chimeric derivative of fibrolase, a fibrinolytic enzyme from southern copperhead venom, possesses inhibitory activity on platelet aggregation, Arch. Biochem. Biophys. 384 (2) (2000) 227–237, https://doi.org/10.1006/abbi.2000.2129.

[147] C.F. Toombs, Alfimeprase: pharmacology of a novel fibrinolytic metalloproteinase for thrombolysis, Pathophysiol. Haemostasis Thrombosis 31 (3–6) (2001) 141–147, https://doi.org/10.1159/000048057.

[148] S.R. Deitcher, C.F. Toombs, Non-clinical and clinical characterization of a novel acting thrombolytic: alfimeprase, Pathophysiol. Haemostasis Thrombosis 34 (4–5) (2006) 215–220, https://doi.org/10.1159/000092427.

[149] S.M. Han, F.A. Weaver, A.J. Comerota, B.A. Perler, M. Joing, Efficacy and safety of alfimeprase in patients with acute peripheral arterial occlusion (PAO), J. Vasc. Surg. 51 (3) (2010) 600–609.

[150] J. Szymańska, K. Kakareko, A. Rydzewska-Rosołowska, I. Głowińska, T. Hryszko, Locked away—prophylaxis and management of catheter related thrombosis in hemodialysis, J. Clin. Med. 10 (11) (2021) 2230, https://doi.org/10.3390/jcm10112230.

[151] A. Domsalla, M.F. Melzig, Occurrence and properties of proteases in plant latices, Planta Med. 74 (7) (2008) 699–711, https://doi.org/10.1055/s-2008-1074530.

[152] T.B. Emran, M.A. Rahman, M.M.N. Uddin, M.M. Rahman, M.Z. Uddin, R. Dash, C. Layzu, Effects of organic extracts and their different fractions of five Bangladeshi plants on in vitro thrombolysis, BMC Compl. Alternative Med. 15 (1) (2015) 1–8, https://doi.org/10.1186/s12906-015-0643-2.

[153] S. Prasad, R.S. Kashyap, J.Y. Deopujari, H.J. Purohit, G.M. Taori, H.F. Daginawala, Effect of Fagonia arabica (Dhamasa) on in vitro thrombolysis, BMC Compl. Alternative Med. 7 (1) (2007) 1–6, https://doi.org/10.1186/1472-6882-7-36.

[154] S.A. Sakib, A.M. Tareq, A. Islam, A. Rakib, M.N. Islam, M.A. Uddin, M.M. Rahman, V. Seidel, T.B. Emran, Anti-inflammatory, thrombolytic and hair-growth promoting activity of the n-hexane fraction of the methanol extract of Leea indica leaves, Plants 10 (6) (2021) 1081, https://doi.org/10.3390/plants10061081.

[155] S. Dibyajyoti, P. Swati, Phytochemical screening and thrombolytic activity of chloroform extract of Urena sinuata (L.), J. Fund. Appl. Sci. 6 (2) (2014) 208–219, https://doi.org/10.4314/jfas.v6i2.7.

[156] A. Azad, S. Lasker, O.I. Irfan-Ur-Rahaman, S. Akter, M. Khairuzzaman, M. Islam, C. Sultana, N. Das, N. Ahmeda, S. Hossain, Comparative studies on antioxidant and thrombolytic activities of methanol and ethylacetate extracts of trema orientalis, Int. J. Innovat. Biol. Chem. Sci. 9 (2016) 47–51.

[157] M.E. Errasti, A. Prospitti, C.A. Viana, M.M. Gonzalez, M.V. Ramos, A.E. Rotelli, N.O. Caffini, Effects on fibrinogen, fibrin, and blood coagulation of proteolytic extracts from fruits of Pseudananas macrodontes, Bromelia balansae, and B. hieronymi (Bromeliaceae) in comparison with bromelain, Blood Coagul. Fibrinolysis 27 (4) (2016) 441–449, https://doi.org/10.1097/MBC.0000000000000531.

[158] H. Mir, N. Perveen, Phyto-chemistry and therapeutic potential of litchi (Litchi chinensis Sonn.): an age-old ingredient in traditional medicine, Indian Journal of Traditional Knowledge (IJTK) 21 (1) (2022) 29–39, https://doi.org/10.56042/ijtk.v21i1.27743.

[159] R. Rajesh, H.V. Shivaprasad, C.D.R. Gowda, A. Nataraju, B.L. Dhananjaya, B.S. Vishwanath, Comparative study on plant latex proteases and their involvement in hemostasis: a special emphasis on clot inducing and dissolving properties, Planta Med. 73 (10) (2007) 1061–1067, https://doi.org/10.1055/s-2007-981575.

[160] K. Fonseca, N. Morais, M. Queiroz, M. Silva, M. Gomes, J. Costa, C. Mamede, F. Torres, N. Penha-Silva, M. Beletti, Purification and biochemical characterization of Eumiliin from Euphorbia milii var. hislopii latex, Phytochemistry 71 (7) (2010) 708–715, https://doi.org/10.1016/j.plaphy.2015.04.012.

[161] J. Siritapetawee, P. Sojikul, S. Klaynongsruang, Biochemical characterization of a new glycosylated protease from Euphorbia cf. lactea latex, Plant Physiol. Biochem. 92 (2015) 30–38, https://doi.org/10.1016/j.plaphy.2015.04.012.

[162] G.K. Patel, A.A. Kawale, A.K. Sharma, Purification and physicochemical characterization of a serine protease with fibrinolytic activity from latex of a medicinal herb Euphorbia hirta, Plant Physiol. Biochem. 52 (2012) 104–111, https://doi.org/10.1016/j.plaphy.2011.12.004.

[163] H. Sumi, H. Hamada, K. Nakanishi, H. Hiratani, Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinases, Acta Haematol. 84 (3) (1990) 139–143, https://doi.org/10.1159/000205051.

[164] Y. Kurosawa, S. Nirengi, T. Homma, K. Esaki, M. Ohta, J.F. Clark, T. Hamaoka, A single-dose of oral nattokinase potentiates thrombolysis and anti-coagulation profiles, Sci. Rep. 5 (1) (2015) 11601, https://doi.org/10.1038/srep11601.

[165] Y. Weng, J. Yao, S. Sparks, K.Y. Wang, Nattokinase: an oral antithrombotic agent for the prevention of cardiovascular disease, Int. J. Mol. Sci. 18 (3) (2017) 523, https://doi.org/10.3390/ijms18030523.

[166] M. Taniguchi, R. Aida, K. Saito, A. Ochiai, S. Takesono, E. Saitoh, T. Tanaka, Identification and characterization of multifunctional cationic peptides from traditional Japanese fermented soybean Natto extracts, J. Biosci. Bioeng. 127 (4) (2019) 472–478, https://doi.org/10.1016/j.jbiosc.2018.09.016.

[167] S.R. Ruiz Sella, T. Bueno, A.A. de Oliveira, S.G. Karp, C.R. Soccol, Bacillus subtilis natto as a potential probiotic in animal nutrition, Crit. Rev. Biotechnol. 41 (3) (2021) 355–369, https://doi.org/10.1080/07388551.2020.1858019.

[168] H.-S. Choi, H.-H. Shin, Purification and partial characterization of a fibrinolytic protease inPleurotus ostreatus, Mycologia 90 (4) (2018) 674–679, https://doi.org/10.1080/00275514.1998.12026956.

[169] W.S. Cha, S.S. Park, S.J. Kim, D. Choi, Biochemical and enzymatic properties of a fibrinolytic enzyme from Pleurotus eryngii cultivated under solid-state conditions using corn cob, Bioresour. Technol. 101 (16) (2010) 6475–6481, https://doi.org/10.1016/j.biortech.2010.02.048.

[170] J.-H. Choi, K.-J. Kim, S. Kim, Purification and antithrombotic potential of a fibrinolytic enzyme from shiitake culinary-medicinal mushroom, *Lentinus edodes* GNA01 (Agaricomycetes), Int. J. Med. Mushrooms 20 (1) (2018) 47–59.

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

[172] H. Ako, A. Cheung, P. Matsuura, Isolation of a fibrinolysis enzyme activator from commercial bromelain, Arch. Int. Pharmacodyn. Ther. 254 (1) (1981) 157–167.

[173] X. Liu, N.-k. Kopparapu, Y. Li, Y. Deng, X. Zheng, Biochemical characterization of a novel fibrinolytic enzyme from Cordyceps militaris, Int. J. Biol. Macromol. 94 (2017) 793–801, https://doi.org/10.1016/j.ijbiomac.2016.09.048.

[174] J.H. Choi, D.W. Kim, S.E. Park, S. Kim, S.J. Kim, Purification and partial characterization of TFase, a fibrinolytic enzyme from the fruiting bodies of the medicinal and edible mushroom, Tremella fuciformis, Appl. Biochem. Microbiol. 51 (6) (2015) 712–719, https://doi.org/10.1134/s0003683815060046.

[175] J.H. Choi, H.J. Lee, S. Kim, Purification and antithrombotic activity of wulfase, a fibrinolytic enzyme from the fruit bodies of the edible and medicinal mushroom Sparassis crispa Wulf, ex. Fr, Applied Biochemistry and Microbiology 52 (6) (2016) 608–614, https://doi.org/10.1134/s000368381606003x.

[176] J.-H. Choi, D.-W. Kim, S. Kim, S.-J. Kim, Purification and partial characterization of a fibrinolytic enzyme from the fruiting body of the medicinal and edible mushroom Pleurotus ferulae, Prep. Biochem. Biotechnol. 47 (6) (2017) 539–546, https://doi.org/10.1080/10826068.2016.1181083.

[177] B.S. Choi, K. Sapkota, J.H. Choi, C.H. Shin, S. Kim, S.J. Kim, Herinase: a novel bi-functional fibrinolytic protease from the monkey head mushroom, Hericium erinaceum, Appl. Biochem. Biotechnol. 170 (3) (2013) 609–622.

[178] S.M. Moon, J.S. Kim, H.J. Kim, M.S. Choi, B.R. Park, S.G. Kim, H. Ahn, H.S. Chun, Y.K. Shin, J.J. Kim, Purification and characterization of a novel fibrinolytic α chymotrypsin like serine metalloprotease from the edible mushroom, *Lyophyllum shimeji*, J. Biosci. Bioeng. 117 (5) (2014) 544–550.

[179] D. Li, L. Hou, M. Hu, Y. Gao, Z. Tian, B. Fan, S. Li, F. Wang, Recent advances in nattokinase-enriched fermented soybean foods: a review, Foods 11 (13) (2022) 1867, https://doi.org/10.3390/foods11131867.

[180] Z.H. Cao, J.M. Green-Johnson, N.D. Buckley, Q.Y. Lin, Bioactivity of soy-based fermented foods: a review, Biotechnol. Adv. 37 (1) (2019) 223–238, https://doi.org/10.1016/j.biotechadv.2018.12.001.

[181] H. Sumi, N. Nakajima, C. Yatagai, A unique strong fibrinolytic enzyme (katsuwokinase) in skipjack “Shiokara” a Japanese traditional fermented food, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 112 (3) (1995) 543–547, https://doi.org/10.1016/0305-0491(95)00100-X.

[182] S.H. Shin, S.W. Hong, K.S. Chung, Purification and characterization of a fibrinolytic enzyme produced by Bacillus amyloliquefaciens HC188, Microbiology and Biotechnology Letters 41 (1) (2013) 33–43, https://doi.org/10.4014/kjmb.1208.08010.

[183] A.R. Lee, G.M. Kim, J.-Y. Park, H.D. Jo, J. Cha, Y.-S. Song, J. Chun, J.H. Kim, Characterization of a 27 kDa fibrinolytic enzyme from Bacillus amyloliquefaciens CH86-1 isolated from cheonggukjang, Journal of the Korean Society for Applied Biological Chemistry 53 (1) (2010) 56–61, https://doi.org/10.3839/jksabc.2010.010.

[184] C. Kim, K. Ri, S. Choe, A novel fibrinolytic enzymes from the Korean traditional fermented food—jotgal: Purification and characterization, J. Food Biochem. 44 (7) (2020) e13255.

[185] J.H. Baek, K.H. Kim, D.M. Han, S.H. Lee, C.O. Jeon, Effects of glutinous rice paste and fish sauce on kimchi fermentation, Lwt 173 (2023) 114253, https://doi.org/10.1016/j.lwt.2022.114253.

[186] Z. Yao, Y. Meng, H.G. Le, S.J. Lee, H.S. Jeon, J.Y. Yoo, D.N. Afifah, J.H. Kim, Isolation of 2 *Bacillus* strains with strong fibrinolytic activities from kimchi, Microbiology and Biotechnology Letters 48 (2020) 439–446.

[187] T.A. Singh, K.R. Devi, G. Ahmed, K. Jeyaram, Microbial and endogenous origin of fibrinolytic activity in traditional fermented foods of Northeast India, Food Res. Int. 55 (2014) 356–362, https://doi.org/10.1016/j.foodres.2013.11.028.

[188] T.A. Singh, G. Nongthombam, G. Goksen, H.B. Singh, G. Rajauria, P.K. Sarangi, Hawaijar–an Ethnic Vegan Fermented Soybean Food of Manipur, India: A Comprehensive Review, Food Research International, 2023 112983, https://doi.org/10.1016/j.foodres.2023.112983.

[189] Y. Hu, D. Yu, Z. Wang, J. Hou, R. Tyagi, Y. Liang, Y. Hu, Purification and characterization of a novel, highly potent fibrinolytic enzyme from *Bacillus subtilis* DC27 screened from Douchi, a traditional Chinese fermented soybean food, Sci. Rep. 9 (1) (2019) 9235.

[190] J. Zhang, C. Zhang, W. Wu, X. Lv, X. Xin, D. Liu, H. Hu, S. Guo, Correlation of the bacterial communities with umami components, and chemical characteristics in Zhejiang xuecai and fermented brine, Food Res. Int. 140 (2021) 109986, https://doi.org/10.1016/j.foodres.2020.109986.

[191] C.T. Wang, B.P. Ji, B. Li, R. Nout, P.L. Li, H. Ji, L.F. Chen, Purification and characterization of a fibrinolytic enzyme of Bacillus subtilis DC33, isolated from Chinese traditional Douchi, J. Ind. Microbiol. Biotechnol. 33 (9) (2006) 750–758, https://doi.org/10.1007/s10295-006-0111-6.

[192] S.J. Jeong, K. Heo, J.Y. Park, K.W. Lee, J.Y. Park, S.H. Joo, J.H. Kim, Characterization of AprE176, a fibrinolytic enzyme from Bacillus subtilis HK176, J. Microbiol. Biotechnol. 25 (1) (2015) 89–97, https://doi.org/10.4014/jmb.1409.09087.

[193] D.J. Kumar, R. Rakshitha, M.A. Vidhya, P.S. Jennifer, S. Prasad, M.R. Kumar, P.T. Kalaichelvan, Production, optimization and characterization of fibrinolytic enzyme by Bacillus subtilis RJAS19, Pakistan J. Biol. Sci. 17 (4) (2014) 529–534, https://doi.org/10.3923/pjbs.2014.529.534.

[194] Z. Yao, X. Liu, J.M. Shim, K.W. Lee, H.-J. Kim, J.H. Kim, Properties of a fibrinolytic enzyme secreted by Bacillus amyloliquefaciens RSB34, isolated from Doenjang, J. Microbiol. Biotechnol. 27 (1) (2017) 9–18, https://doi.org/10.4014/jmb.1608.08034.

[195] Y. Devaraj, S.K. Rajender, P.M. Halami, Purification and characterization of fibrinolytic protease from Bacillus amyloliquefaciens MCC2606 and analysis of fibrin degradation product by MS/MS, Prep. Biochem. Biotechnol. 48 (2) (2018) 172–180, https://doi.org/10.1201/9780429027413.

[196] H. Yang, L. Yang, X. Li, H. Li, Z. Tu, X. Wang, Genome sequencing, purification, and biochemical characterization of a strongly fibrinolytic enzyme from *Bacillus amyloliquefaciens* Jxnuwx-1 isolated from Chinese traditional Douchi, J. Gen. Appl. Microbiol. 66 (2020) 153–162.

[197] G.D. Biji, A. Arun, E. Muthulakshmi, P. Vijayaraghavan, M.V. Arasu, N.A. Al-Dhabi, Bio-prospecting of cuttle fish waste and cow dung for the production of fibrinolytic enzyme from *Bacillus cereus* IND5 in solid state fermentation, 3 Biotech 6 (2) (2016) 231.

[198] Y. Hua, B. Jiang, Y. Mine, W. Mu, Purification and characterization of a novel fibrinolytic enzyme from Bacillus sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste, J. Agric. Food Chem. 56 (4) (2008) 1451–1457, https://doi.org/10.1021/jf0713410.

[199] D.N. Afifah, M. Sulchan, D. Syah, Yanti, M.T. Suhartono, J.H. Kim, Purification and characterization of a fibrinolytic enzyme from Bacillus pumilus 2.g isolated from gembus, an Indonesian fermented food, Prev Nutr Food Sci 19 (3) (2014) 213–219, https://doi.org/10.3746/pnf.2014.19.3.213.

[200] Z. Yao, J.A. Kim, J.H. Kim, Characterization of a fibrinolytic enzyme secreted by *Bacillus velezensis* BS2 isolated from sea squirt jeotgal, J. Microbiol. Biotechnol. 29 (2019) 347–356.

[201] M.-J. Ahn, H.-J. Ku, S.-H. Lee, J.-H. Lee, Characterization of a novel fibrinolytic enzyme, BsfA, from Bacillus subtilis ZA400 in Kimchi reveals its pertinence to thrombosis treatment, J. Microbiol. Biotechnol. 25 (12) (2015) 2090–2099, https://doi.org/10.4014/jmb.1509.09048.

[202] P. Kharnaior, M. Das, J.P. Tamang, Therapeutic and anti-thrombotic properties of some naturally fermented soybean foods of the eastern himalayas, Fermentation 9 (2) (2023) 91, https://doi.org/10.3390/fermentation9020091.

[203] H.D. Jo, H.A. Lee, S.J. Jeong, J.H. Kim, Purification and characterization of a major fibrinolytic enzyme from Bacillus amyloliquefaciens MJ5-41 isolated from Meju, J. Microbiol. Biotechnol. 21 (11) (2011) 1166–1173, https://doi.org/10.4014/jmb.1106.06008.

[204] Z. Yao, Y. Meng, H.G. Le, J.A. Kim, J.H. Kim, Isolation of Bacillus subtilis SJ4 from Saeu (Shrimp) jeotgal, a Korean fermented seafood, and its fibrinolytic activity, Microbiology and Biotechnology Letters 47 (4) (2019) 522–529, https://doi.org/10.4014/mbl.1906.06003.

[205] S. Sugimoto, T. Fujii, T. Morimiya, O. Johdo, T. Nakamura, The fibrinolytic activity of a novel protease derived from a tempeh producing fungus, Fusarium sp. BLB, Biosci., Biotechnol., Biochem. 71 (9) (2007) 2184–2189, https://doi.org/10.1271/bbb.70153.

[206] D. Huy, P. Hao, P. Hung, Screening and identification of *Bacillus* sp. isolated from traditional Vietnamese soybean-fermented products for high fibrinolytic enzyme production, Int. Food Res. J. 23 (1) (2016) 326–331.

[207] Y. Peng, X. Yang, Y. Zhang, Microbial fibrinolytic enzymes: an overview of source, production, properties, and thrombolytic activity in vivo, Appl. Microbiol. Biotechnol. 69 (2005) 126–132, https://doi.org/10.1007/s00253-005-0159-7.

[208] J.-K. Wang, H.-H. Chiu, C.-S. Hsieh, Optimization of the medium components by statistical experimental methods to enhance nattokinase activity, Fooyin Journal of Health Sciences 1 (1) (2009) 21–27, https://doi.org/10.1016/S1877-8607(09)60004-7.

[209] P. Vijayaraghavan, M.V. Arasu, R.A. Rajan, N. Al-Dhabi, Enhanced production of fibrinolytic enzyme by a new Xanthomonas oryzae IND3 using low-cost culture medium by response surface methodology, Saudi J. Biol. Sci. 26 (2) (2019) 217–224, https://doi.org/10.1016/j.sjbs.2018.08.029.

[210] D.B.Q. Anh, N.T.T. Mi, D.N.A. Huy, P. Van Hung, Isolation and optimization of growth condition of *Bacillus* sp. from fermented shrimp paste for high fibrinolytic enzyme production, Arabian J. Sci. Eng. 40 (1) (2015) 23–28.

[211] P. Vijayaraghavan, P. Rajendran, S.G. Prakash Vincent, A. Arun, N. Abdullah Al-Dhabi, M. Valan Arasu, O. Young Kwon, Y.O. Kim, Novel sequential screening and enhanced production of fibrinolytic enzyme by Bacillus sp. IND12 using response surface methodology in solid-state fermentation, BioMed Res. Int. 2017 (2017).

[212] S.B. Jhample, P.K. Bhagwat, P.B. Dandge, Statistical media optimization for enhanced production of fibrinolytic enzyme from newly isolated Proteus penneri SP-20, Biocatal. Agric. Biotechnol. 4 (3) (2015) 370–379, https://doi.org/10.1016/j.bcab.2015.05.006.

[213] K. Joji, A. Santhiagu, N. Salim, Computational modeling of culture media for enhanced production of fibrinolytic enzyme from marine bacterium *Fictibacillus* sp. strain SKA27 and in vitro evaluation of fibrinolytic activity, 3 Biotech 9 (2019) 323.

[214] R. Wu, G. Chen, S. Pan, J. Zeng, Z. Liang, Cost-effective fibrinolytic enzyme production by Bacillus subtilis WR350 using medium supplemented with corn steep powder and sucrose, Sci. Rep. 9 (1) (2019) 6824, https://doi.org/10.1038/s41598-019-43371-8.

[215] Z. Yao, J.A. Kim, J.H. Kim, Gene cloning, expression, and properties of a fibrinolytic enzyme secreted by Bacillus pumilus BS15 isolated from gul (oyster) jeotgal, Biotechnol. Bioproc. Eng. 23 (2018) 293–301, https://doi.org/10.1007/s12257-018-0029-7.

[216] Z. Yao, Y. Meng, H.G. Le, S.J. Lee, H.S. Jeon, J.Y. Yoo, J.H. Kim, Increase of a fibrinolytic enzyme production through promoter replacement of aprE3-5 from Bacillus subtilis CH3-5, J. Microbiol. Biotechnol. 31 (6) (2021) 833.

[217] H.K. Buniya, A.K. Hameed, A.Y. Al-Hayawi, Cloning and expression of staphylokinase-streptokinase recombinant protein in E. coli BL21 (DE3), Biologia 78 (4) (2023) 1113–1117.

[218] A. Krishnamurthy, S. Mundra, P.D. Belur, Improving the catalytic efficiency of fibrinolytic enzyme from Serratia marcescens subsp. sakuensis by chemical modification, Process Biochem. 72 (2018) 79–85.

[219] M. Lu, Z. Gao, S. Xing, J. Long, C. Li, L. He, X. Wang, Purification, characterization, and chemical modification of Bacillus velezensis SN-14 fibrinolytic enzyme, Int. J. Biol. Macromol. 177 (2021) 601–609, https://doi.org/10.1016/j.ijbiomac.2021.02.167.

[220] A. Singh, A. Majumder, A. Goyal, Artificial intelligence based optimization of exocellular glucansucrase production from Leuconostoc dextranicum NRRL B-1146, Bioresour. Technol. 99 (17) (2008) 8201–8206, https://doi.org/10.1016/j.biortech.2008.03.038.

[221] R. Gupta, D. Srivastava, M. Sahu, S. Tiwari, R.K. Ambasta, P. Kumar, Artificial intelligence to deep learning: machine intelligence approach for drug discovery, Mol. Divers. 25 (2021) 1315–1360, https://doi.org/10.1007/s11030-021-10217-3.

[222] S.-H. Kim, N.-S. Choi, Purification and characterization of subtilisin DJ-4 secreted by Bacillus sp. strain DJ-4 screened from Doen-Jang, Biosci. Biotechnol. Biochem. 64 (8) (2000) 1722–1725, https://doi.org/10.1271/bbb.64.1722.

[223] J.H. Ko, J.P. Yan, L. Zhu, Y.P. Qi, Identification of two novel fibrinolytic enzymes from Bacillus subtilis QK02, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 137 (1) (2004) 65–74, https://doi.org/10.1016/j.cca.2003.11.008.

[224] J. Wu, G. Lan, N. He, L. He, C. Li, X. Wang, X. Zeng, Purification of fibrinolytic enzyme from Bacillus amyloliquefaciens GUTU06 and properties of the enzyme, Food Chem. X 20 (2023) 100896, https://doi.org/10.1016/j.fochx.2023.100896.

[225] L. Zhao, X. Lin, J. Fu, J. Zhang, W. Tang, Z. He, A novel Bi-functional fibrinolytic enzyme with anticoagulant and thrombolytic activities from a marine-derived fungus Aspergillus versicolor ZLH-1, Mar. Drugs 20 (6) (2022) 356, https://doi.org/10.3390/md20060356.

[226] H. Liu, J. Yang, Y. Li, Y. Ma, W. Wang, W. Zhong, P. Li, S. Du, A novel fibrinolytic protein from pheretima vulgaris: purification, identification, antithrombotic evaluation, and mechanisms investigation, Front. Mol. Biosci. 8 (2021), https://doi.org/10.3389/fmolb.2021.772419.

[227] H. Yang, L. Yang, X. Li, H. Li, Z. Tu, X. Wang, Genome sequencing, purification, and biochemical characterization of a strongly fibrinolytic enzyme from Bacillus amyloliquefaciens Jxnuwx-1 isolated from Chinese traditional Douchi, J. Gen. Appl. Microbiol. (2019), https://doi.org/10.2323/jgam.2019.04.005.

[228] Y. Hu, D. Yu, Z. Wang, J. Hou, R. Tyagi, Y. Liang, Y. Hu, Purification and characterization of a novel, highly potent fibrinolytic enzyme from Bacillus subtilis DC27 screened from Douchi, a traditional Chinese fermented soybean food, Sci. Rep. 9 (1) (2019) 1–10, https://doi.org/10.1038/s41598-019-45686-y.

[229] Z. Yao, J.A. Kim, J.H. Kim, Properties of a fibrinolytic enzyme secreted by Bacillus subtilis JS2 isolated from saeu (small shrimp) jeotgal, Food Sci. Biotechnol. 27 (3) (2018) 765–772, https://doi.org/10.1007/s10068-017-0299-4.

[230] H.N. Hodis, W.J. Mack, H.J. Meiselman, V. Kalra, H. Liebman, J. Hwang-Levine, L. Dustin, N. Kono, M. Mert, R.B. Wenby, Nattokinase atherothrombotic prevention study: a randomized controlled trial, Clin. Hemorheol. Microcirc. 78 (4) (2021) 339–353.

[231] C.-H. Hsia, M.-C. Shen, J.-S. Lin, Y.-K. Wen, K.-L. Hwang, T.-M. Cham, N.-C. Yang, Nattokinase decreases plasma levels of fibrinogen, factor VII, and factor VIII in human subjects, Nutr. Res. 29 (3) (2009) 190–196.

[232] J.Y. Kim, S.N. Gum, J.K. Paik, H.H. Lim, K.-C. Kim, K. Ogasawara, K. Inoue, S. Park, Y. Jang, J.H. Lee, Effects of nattokinase on blood pressure: a randomized, controlled trial, Hypertens. Res. 31 (8) (2008) 1583–1588.