CHAPTER II

Review of literature

2.1 Overview of fibrinolytic enzyme

Fibrin, the principal protein in blood coagulation, is degraded by fibrinolytic enzymes, peptidase-acting proteases belonging to the hydrolase enzyme class (EC 3.4) [1]. Consequently, under normal homeostasis, they are essential for breaking up blood clots, also called as thrombi, that allow blood to flow freely across the veins. There are two ways that the fibrinolytic enzyme works. Through a process known as direct fibrinolysis, they can directly break down insoluble fibrin that already exists into smaller pieces known as products of fibrin degradation. By turning circulating plasminogen into effective plasmin which acts on the fibrin and restores normal vascular function, they can indirectly aid fibrin lysis.

There have been studies on the production, purification, and characterisation of fibrinolytic enzymes from a variety of fermented foods, bacteria, algae, insects, plants, snakes, and earthworms, among other possible sources [2-4]. Microbial sources derived from fermented foods have garnered significant interest in the commercial manufacturing of fibrinolytic enzymes because of their beneficial characteristics, including high substrate precision, inexpensive production costs, and extensive availability. [5-7]. Furthermore, it has been established that proteases derived from microorganisms cause little adverse effects in individuals, particularly those with inherited disabilities [6]. Therefore, the pharmaceutical industry and drug screening program research has concentrated on new microbial sources for synthesizing fibrinolytic enzymes [8,9].

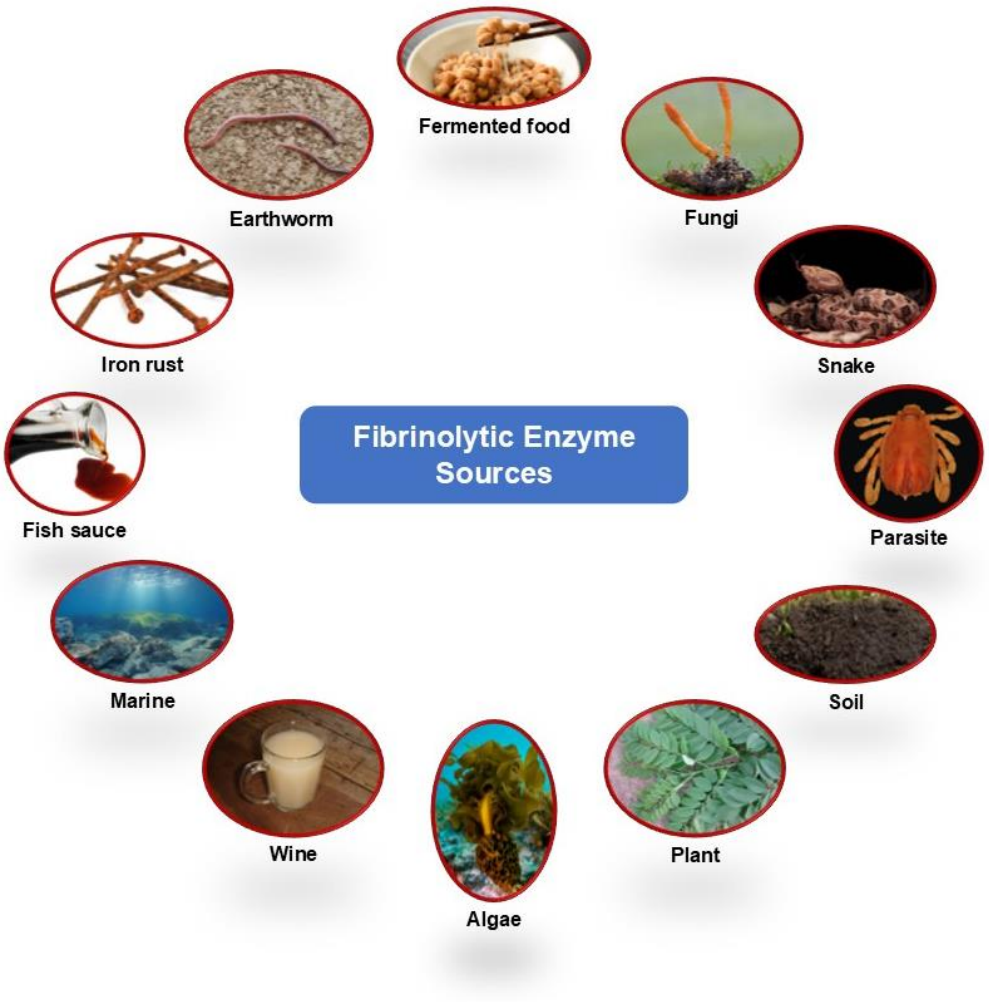

Figure 2.1: Various sources of fibrinolytic enzyme

2.2 Non-food sources of fibrinolytic enzymes

A wide variety of natural sources include large amounts of fibrinolytic enzymes. For instance, they have been found in non-food sources like bacteria [10], fungi, algae [4], earthworms [11], marine microbes and animals [12], soil microorganisms [13], plants [14], and snake venom [15]. Of these, snake venom is the primary source of non-microbial fibrinolytic enzymes. Potent fibrinolytic enzymes are also produced by insects, such as dung beetles [16], caterpillars [17], and praying mantises [18]. The vampire bat's salivary plasminogen activator is the most researched of the higher organisms [19]. Furthermore, numerous microbes and marine algae also produce potent fibrinolytic enzymes [20]. More research is required to ascertain their safety and effectiveness, even if several displayed potential as cardiovascular therapy.

There are specific drawbacks to employing fibrinolytic enzymes derived from non-food sources. First, extracting these enzymes from the majority of non-food sources, including

plants, earthworms, snake venom, etc., can be time-consuming and more difficult. Furthermore, because of their high production capacity and economical media needs, the amount produced can be less compared to that of microbes obtained from food sources. Moreover, enzymes from non-food sources are frequently less strong and selective, which may limit their ability to cure thrombotic diseases [1].

The toxicity and immunogenicity of several non-food sources, like earthworms and snake venom, constrain the therapeutic use of fibrinolytic enzymes. Creating these enzymes entails significant ethical concerns, including specialized handling and disposal standards. Compared to fibrinolytic enzymes obtained from food-source microorganisms, which are more straightforward to generate and purify and provide a reduced risk of toxicity and immunogenicity, the development of fibrinolytic enzymes derived from non-food sources can be costlier and more intricate. Non-food sources of fibrinolytic enzymes might present hopeful avenues for new enzymes; nonetheless, their disadvantages render them inappropriate for actual therapeutic use. These enzymes provide a basis for evolving novel pharmaceuticals, and investigating them might uncover essential insights into the molecular mechanisms governing fibrinolysis.

Table 2.1: Fibrinolytic enzymes from different non-food sources

Source	**Species**	**Reference**
Fungi	*Pycnoporus coccineus*	[21]
	Cochliobolus hawaiiensis Alcorn AUMC 8606	[22]
	Cordyceps militaris	[23]
	Mucor subtilissimus UCP 1262	[9]
	Pleurotus sajor-caju CTM10057	[24]
	Aspergillus terreus SH72	[25]
	Pleurotus ferulae	[26]
	Xylaria curta	[27]
Algae	*Arthrospira platensis*	[28]
	Chlorella vulgaris	[4]
	Undaria pinnatifida	[29]
	Ulva pertusa	[30]
Parasite	*Rhipicephalus microplus*	[31]

Earthworm	*Pheretima vulgaris*	[32]
	Pheretima posthumous	[33]
	Lumbricus rubellus	[34]
	Eisenia fetida	[35]
Sandworm	*Perinereis aibuhitensis*	[36]
Marine	*Bacillus velezensis* Z01	[37]
	Holothuria scabra	[38]
	Bacillus flexus	[39]
	Pseudomonas aeruginosa KU1	[40]
	Serratia rubidaea KUAS001	*[41]*
	Streptomyces radiopugnans VITSD8	[12]
	Fictibacillus sp. SKA27	[42]
Soil	*Alcaligenes aquatilis* PJS_1	[43]
	Bacillus pseudomycoides MA02	[10]
	Bacillus subtilis Egy	[44]
	Bacillus cereus RSA1	[45]
	Stenotrophomonas sp. KG-16-3	[46]
	Bacillus tequilensis	[13]
Plant	*Gliricidia sepium* seeds	[47]
	Ficus carica latex	[48]
	Petasites japonicus	[49]
	Cnidoscolus urens (L.) Arthur leaves	[50]
	Aster yomena (Kitam.) Honda	[51]
Snake	*Bothrops colombiensis*	[52]
	Trimeresurus mucrosquamatus	[53]
Bovine milk	*Streptococcus agalactiae* EBL-31	[54]
	Pseudomonas aeruginosa CMSS	[55]
Iron rust	*Bacillus cereus*	[56]

2.3 Fibrinolytic enzymes from food sources

The investigation of nutraceuticals and functional foods is rapidly proliferating worldwide. Scientists are continually investigating the medical advantages of foods, their functional

components, their biochemical structures, and the processes underpinning their biological activities. The nutritional paradigm is evolving due to these findings, as food elements are now seen as essential for sustenance and growth and as tools for preventing, managing, or delaying the beginning of chronic illnesses in future life. Likewise, since antiquity, herbal medications have been employed to address ailments with minimal contrary consequences. Their most appealing characteristic is their ease of administration since they are a fundamental component of several dietary regimens. Nattokinase, the inaugural fibrinolytic enzyme found in fermented food and presently under intensive investigation, was historically used as a traditional cure for vascular and heart ailments. [57].

A wide range of foods, including Korean chungkook-Jang soy sauce [58], edible honey mushrooms [59], and Japanese natto [57], are known to contain fibrinolytic enzymes. Enzymes from these foods have been effectively purified, and bacteria associated with them have been discovered. Their physiochemical characteristics have also been characterized. Although fibrinolytic enzymes have been found in both fermented and non-fermented foods, most research have focused on those derived from fermented foods. Given the abundance of fibrinolytic enzymes discovered in fermented foods, it is quite likely that these foods also contain fibrinolytic producers in a large number.

Moreover, oral administration of pure fibrinolytic enzymes from dietary sources is safe. In dogs with clinically induced thrombosis, it was explicitly discovered that oral treatment of fibrinolytic enzyme derived from Japanese natto increased fibrinolysis [60]. More significantly, it was discovered that the fibrin breakdown by-products, t-PA levels, and fibrinolytic activity in plasma doubled when nattokinase was administered orally to human participants. In the blood, the higher concentration of FDP, antithrombin, and D-dimer, are linked to this effect, although factor VIII was shown to have significantly decreased [61].

Food-derived fibrinolytic enzymes have shown fewer adverse effects than those isolated from non-food sources, which makes them a better choice for usage as nutraceuticals. These enzymes are easily purified and produced in large quantities, and their safety for oral intake has been well-proven [62]. Studying enzymes derived from food does not need ethical approval, unlike enzymes from animal sources like snake venom and earthworms. These enzymes' potential medical use is increased because they are usually fibrin-specific and lack cytotoxic [63] or haemolytic effects [64]. In order to find a nattokinase-like enzyme or a more potent and secure fibrinolytic enzyme, traditional foods from various geographical areas could

be investigated as possible sources of fibrinolytic enzymes, encouraging healthy eating practices. The fibrinolytic enzymes formed by the *Bacillus* sp. isolated from fermented food will be discussed further in the review.

Table 2.2: Fibrinolytic enzymes from different food sources.

Source	**Species**	**References**
Hawaijar, Indian traditional food	*Bacillus subtilis*	[65]
Asian traditional fermented shrimp paste	*Bacillus* sp. SK006	[66]
fermented milk of *Vigna unguiculata*	*Bacillus subtilis* VITMS2	[67]
Myeolchi Jeotgal, a popular Korean fermented seafood	*Bacillus velezensis* CJ1	[68]
traditionally fermented natto	*Bacillus subtilis*	[69]
Japanese fermented soybean natto	*Bacillus subtilis* G8	[70]
Ntété (squash commonly known in Congo Brazzaville NTETE wrapped in leaves and cooked in a smothered state)	*Bacillus*	[71]
Moromi (an Indonesian soybean-based fermented food)	*Bacillus subtilis* K2	[72]
Fish sauce	*Bacillus velezensis* SW5	[73]
Chinese traditional fermented black soya bean (douchi)	*Bacillus amyloliquefaciens* Jxnuwx-1	[74]
Kimchi, traditional Korean fermented vegetable	*Bacillus subtilis* K3, *Bacillus velezensis* K208	[75]
Douchi, traditional fermented soy bean food	*Bacillus subtilis* DC27	[76]
Sea squirt (muggae) jeotgal, traditional fermented seafood	*Bacillus sp.* BS2	[77]
Fermented rice	*Xanthomonas oryzae* IND3	[5]
Dosa batter	*Bacillus amyloliquefaciens* MCC2606	[78]
Fermented rice	*Bacillus cereus* IND1	[79]

Gul (Oyster) jeotgal, Korean fermented sea food	*Bacillus pumilus* BS15	[80]
Jeotgal, salted fermented Korean sea food	*Bacillus subtilis* JS2	[81]
Fermented natto granulat	*Bacillus subtilis* C142	[82]
Doenjang, traditional Korean fermented soy food	*Bacillus amyloliquefaciens*	[83]
Tuak, an indonesian palm wine	NA	[84]

2.4 Production of fibrinolytic enzyme by recombinant cloning and expression method

The extensive manufacturing of fibrinolytic enzymes by recombinant technology has attracted increased interest due to advancements in biotechnology, owing to its high efficiency and low cost. Currently, recombinant technology employing modified strains, cells, or plants is the predominant method for the industrial manufacture of bacterial and fungal enzymes. *Escherichia coli*, *Lactococcus lactis*, and *Bacillus subtilis* are among the bacteria used to generate fibrinolytic enzyme. However, because of its cost and convenience, *E. coli* is one of the most widely utilized bacteria for the overexpression of several fibrinolytic enzyme [62].

However, recovering significant amounts of these enzymes is often difficult because recombinant proteins aggregate to form insoluble and inactive inclusion bodies [85,86]. Most protein is lost when the inclusion body's protein is refolded and solubilized. Furthermore, some fibrinolytic enzymes are serine proteases, which hydrolyze the bacterial proteins and produce an inadequate yield of enzymes. Due to their greater sensitivity, Western blots can detect the expressed fibrinolytic enzyme, but SDS-PAGE gels cannot clearly show it without further purification [87-90]. To get beyond these obstacles, researchers have tried a variety of strategies. Several refolding solutions are being studied to improve the renaturation of fibrinolytic enzyme inclusion bodies [91,92]. In order to prevent inclusion body formation in *E. coli*, secretory expression has also been used. While active nattokinase can be effectively expressed in *E. coli* using signal peptides, the activity is not as high as that of native nattokinase [89]. There is still a need to find additional ways to boost the activity and output of fibrinolytic enzymes produced in *E. coli*.

Bacillus, particularly *Bacillus subtilis*, is believed to a suitable host strain for the industrial synthesis of fibrinolytic enzyme. *B. subtilis's* high-level secretory system and culture simplicity make it a well-researched host for heterologous proteins. Furthermore, *B. subtilis* is regarded by the U.S. Food and Drug Administration as a food-grade species that poses no safety risks [93]. However, it can simultaneously create several proteases, which complicates fibrinolytic enzyme purification and hydrolyses the produced fibrinolytic enzyme. To address this issue, strains of B. subtilis lacking extracellular protease have been developed. Fibrinolytic enzyme is currently produced using *B. subtilis* WB600 (six-protease-gene-deficient) [94,95], WB700 (seven-protease-gene-deficient) [96], and WB800 (eight-protease-gene-deficient) [97,98]. Table 3 provides a summary of the wide variety of heterologous expression systems that are employed to produce fibrinolytic enzymes.

Table 2.3: Parameters for cloning and expression utilized for the production of fibrinolytic enzymes.

Species	**Gene**	**Primer**	**Cloning vector/ Cloning host**	**Expression vector/ Expression host**	**Reference**
B. mojavensis LY-06	rAprY	forward (5′-TGTGGATCCGTGAGAAGCAAAAAATTGTGGATCA-3′, BamHI site underlined) reverse primers (5′-CCGCTCGAGTTGTGCAGCTGCTTGTACGT-3′, XhoI site underlined)	peT28a(+) *E. coli* BL21 (DE3)	-NA-	[99]
B. subtilis	*aprEFS M4*	aprE-F (5'-GCGAATTCGCCGCATCTGTGTCTTTG-3', EcoRI site underlined)	pHY300PLK *B. subtilis* WB600	pET26b(+) *E. coli* BL21 (DE3)	[100]

		aprE-R (5'-GCGAATTCGAGAACAGAGAAGCCGCT-3', EcoRI site underlined) CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3', BamHI site underlined) CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3', EcoRI site underlined) pET-F (5'-AGAGGATCCGATGGCAGGGAAATCA-3', BamHI site underlined) pET-R (5'-AGACTCGAGCTGAGCTGCCGCCTG-3', XhoI site underlined)			
B. licheniformis HJ4	*aprEHJ4*	HJ4-F (5′-CCGACGATCATGGAACGGAT-3′) HJ4-R (5′-GCGGTCTATTCATACTTTCGAACC-3′) pETHJ4-F (5′-AGAGGATCCGATGGCTCAGCCGGCG-3′, BamHI site underlined) pETHJ4-R (5′-AGACTCGAGTTGAGCGGCAGCTTC-3′, XhoI site underlined)	pGEM-T Easy *E. coli* DH5α	pET26(b +) *E. coli* BL21(DE3)	[91]
B. velezensis CJ1	aprECJ1	CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC- 3', BamHI site underlined)	pHY300PLK *E. coli* DH5α	*B. subtilis* WB600	[68]

		CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA3', EcoRI site underlined)			
B. subtilis natto	*aprN*	-NA-	pUC57	pHBM905BDM *E. coli* XL10-Gold	[101]
B. subtilis CH3-5	*aprE3-5*	aprE3-5-F (5'-CGCGGATCCGGG**TCTACT**AAAATATTATTCCATCTAT**TAC AAT**AAATTC -3') Pcry3A-F (5'-CGCGGATCCGGG**TTGCAA**AAAATATTATTCCATCTAT**TAA GCT**AAATTC -3') P10-F (5'-CGCGGATCCGGG**TTGACA**AAAATATTATTCCATCTAT**TAA ACT**AAATTC -3') PSG1-F (5'-CGCGGATCCGGG**TTGACA**AAAATATTATTCCATCTAT**TAC AAT**AAATTC -3')	pHY300PLK *B. subtilis* WB600	-NA-	[102]

		PsrfA-F (5'-CGCGGATCCGGG**GTGATA**AAAATATTATTCCATCTAT**TAA ACT**AAATTC -3') *aprE3-5*-R (5'- GCGAATTCGAGAACAGAGAAGCCGCT -3')			
B. subtilis (K3) *B. velezensis* (K208)	*aprE*	CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC- 3', BamHI site underlined) CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA- 3', EcoRI site underlined)	pGEM-T-Easy *E. coli* DH5α	-NA-	[75]
B. velezensis BS2	*aprEBS2*	CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3', BamHI site underlined) CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3', EcoRI site underlined) pETBS2-F (5'-AGAGGATCCGATGGCAGGGAAATC-3', BamHI site underlined) pETBS2-R (5'- AGACTCGAGCTGAGCTGCCGCCTG-3', XhoI site underlined)	pHY300PLK *B. subtilils* WB600	pET26b (+) *E. coli* BL21 (DE3)	[77]

Bacillus strain, SJ4	*aprESJ4*	CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3', BamHI site underlined) CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3', EcoRI site underlined)	pGEM-T Easy vector *E. coli* DH5α	-NA-	[103]
Bacillus cereus strain AB01	Bacifrinase	FP2 (5'-CCGCTCGAGTTGATTCTTTGTAGCTGAGTTATCTGC-3', XhoI site underlined) RP2 (5'-CGCGGATCCATGGGATATTACGACGGACCA-3', BamHI site underlined)	pET19b *E. coli* DH5α cells	*E. coli* BL21 DE3	[104]
-NA-	NAT, DFE	Fnatto (5′-TGTTTGCKTTARCGTTAATCTTTACGATGG-3′, K = G or T, R = A or G) Rnatto (5′-TTATTGWGCWGCYGCYTGTACGTTGAT-3′, W = A or T, Y = C or T) Fnatto_XhoI (5′-TCTGCGCTCGAGGCCGGAAAAAGCAGT-3′) Rnatto_BamHI (5′-TTCACTGGATCCTTATTGGGCAGCTGCTTGTACG-3′) for proNAT ForDFE_NdeI (5′-CCGGCT CATATGGCAGGGAAATCAAAC-3′)	pGEM-T Easy vector	pET16b(+) *E. coli* BL21 (DE3)	[105]

		RevDFE_BamHI (5′-TTCACTGGATCCTTATTGTGCAGCTGCTTGTA-3′) for proDFE			
B. pumilus BS15	*aprEBS15*	CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3', BamHI site underlined) CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3', EcoRI site underlined) pET-F (5'-AGAGGATCCGATGGCAGGGAAATC-3', BamHI site underlined) pET-R (5'-AGACTCGAG CTGAGCTGCCGCCTG-3', XhoI site underlined)	pHY300PLK *B. subtilis* WB600	pET26b(+) *E. coli* BL21 (DE3)	[80]
B. subtilis JS2	*aprEJS2*	CH51-F (5′-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3', BamHI site underlined) CH51-R (5′-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3′, EcoRI site underlined) pETJS2-F (5′-AGAGGATCCGATGGCAGGGAAATC-3′, BamHI site underlined) pETJS2-R (5′-AGACTCGAGCTGAGCTGCCGCCTG-3′, XhoI site underlined)	pHY300PLK *B. subtilils* WB600	pET26b(+) *E. coli* BL21 (DE3)	[81]

B. amyloliquefaciens RSB34	*aprE34*	CH51-F (5'-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3', BamHI site underlined) CH51-R (5'-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA3', EcoRI site underlined) pET-F (5'-AGAGGATCCGATGGCAGGGAAATC-3', BamHI site underlined) pET-R (5'-AGACTCGAGCTGAGCTGCCGCCTG-3', XhoI site underlined)	pGEM-T Easy vector *E. coli* DH5α	pET26b(+) *E. coli* BL21 (DE3)	[83]

2.5 Physiochemical properties of fibrinolytic enzymes produced by *Bacillus* spp.

Numerous studies have been conducted on the physicochemical characteristics of fibrinolytic enzymes, such as their molecular mass (kDa), optimal pH, temperature, the impact of inhibitors and metal ions on the catalytic activity, and amidolytic activity. The key features of *Bacillus* sp. derived fibrinolytic enzymes obtained from fermented food are summarised in Table 4.

Enzymes have molecular masses that range from 23.5 to 68 kDa. Several studies conducted with medications used to treat thrombosis show the benefits of lower molecular weight molecules. These benefits include a more dependable dose-response relationship and fewer adverse effects [76,106]. Most enzymes are more productive in neutral and alkaline conditions, with pH values ranging from 6. to 8. Because of their high catalytic activity and high substrate specificity, studies reveal that the pharmaceutical industry has been very interested in alkaline proteases produced by Bacillus species [107]. The fibrinolytic protease Bacifrinase, which *Bacillus cereus* AB01 produced, had the lowest optimal action temperature, at about 37 °C, according to temperature-related data. The enzyme rAprY, created by *Bacillus mojavensis*, had the greatest optimal temperature at 50 °C. It is important to consider how temperature affects the kinetics of the enzymatic process since high temperatures can cause the enzyme to become denatured, which results in the breakage of its chemical bonds and the loss of its functionality.

Depending on whether an enzyme is serine protease, metalloprotease, or serine metalloprotease, determines how metal ions or inhibitors affect the fibrinolytic activity of the enzyme. The impact of a variety of metal ions, including Na^{+}, K^{+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+}, and Fe^{3+}, on the biological activity of enzymes has been investigated [91,99,100]. Divalent metal ions such as Mg^{2+}, Ba^{2+}, K^{+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} affected the activity of a small number of fibrinolytic proteases, while Co^{2+}, Fe^{3+}, and Zn^{2+} hindered their activity. Certain fibrinolytic metalloproteases need the divalent ions Co^{2+}, Ni^{2+}, and Zn^{2+} for their activity. The most often used irreversible serine protease inhibitors is phenyl methyl sulfonyl fluoride (PMSF). The fibrinolytic activity of metalloproteases is significantly inhibited by inhibitors such as ethyleneglycol bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and ethylene diamine tetraacetic acid (EDTA). On the other hand, both serine and metalloprotease inhibitors prevent the fibrinolytic action of the third "serine metalloprotease" class of enzymes.

The specificity of fibrinolytic enzymes has been evaluated spectrophotometrically using the synthetic substrates N-succinyl-ala-ala-pro-phe-p-nitroanilide, N-succinyl-Ala-Ala-Pro-Phe-pNA, fibrinogen, and H-D-Ile-Pro-Arg-pNA [77,82,100,104].

Table 2.4: Physiochemical characteristics of fibrinolytic enzymes from *Bacillus* spp. isolated from fermented food samples.

Species	**Source**	**Mol. Wt. pH Temperature**	**Activator**	**Inhibitor**	**Substrate specificity**	**References**
B. mojavensis	Douchi	27.7 kDa 8.0, 50 °C	Mg^{2+}, Ba^{2+}, K^{+}, and Ni^{2+}	Mn^{2+}, Ca^{2+}, Fe^{3+}, PMSF, and EDTA	-NA-	[108]
B. subtilis	Traditional Korean fermented food	28 kDa 8.0 40 °C	Ca^{2+}, Mg^{2+} EDTA, and EGTA	Fe^{3+}, Co^{2+}, Mn^{2+}, PMSF, and SDS	K_m and V_{max} values were 0.28 mM and 54.05 μM/min for N-succinyl-ala-ala-pro-phe-p-nitroanilide	[100]
B. licheniformis	Hwangseokae jeotgal, Traditional Korean fermented seafood	27 kDa 8.0 40 °C	Mg^{2+}	K^{+}, Fe^{3+}, Co^{2+}, Zn^{2+}, PMSF, EDTA, and EGTA	K_m and V_{max} values were 0.166 ± 0.013 mM and 8.576 ± 0.235 μM/min for N-succinyl-ala-ala-pro-phe-p-nitroanilide	[91]

B. subtilis SJ4	Saeu jeotgal (small salted shrimp)	68 kDa 7.0, 40 °C	Ca^{2+}, Mg^{2+}, and Mn^{2+}	Co^{2+}, Zn^{2+}, PMSF, EDTA, and EGTA	K_m and V_{max} values were 0.19 mM and 29.76 µM/min for *N*-Succinyl-ala-ala-pro-phe-*p*-nitroanilide	[92]
B. velezensis BS2	Sea squirt (munggae) jeotgal, a traditional Korean fermented seafood	27 kDa 8.0 37 °C	Mg^{2+}, Ca^{2+}, and Mn^{2+}	Fe^{3+}, Zn^{2+}, K, Co^{2+}, and PMSF	K_m and V_{max} values were 0.15 mM and 39.68 µM/l/min for N-succinyl-Ala-Ala-Pro-Phe-pNA	[77]
B. cereus	Alcohol starter culture	42 kDa 7.4 37 °C	-NA-	-NA-	K_m and V_{max} values were 3.08 µM and 66.9 µM/min against fibrinogen	[104]
B. pumilus BS15	Gul (oyster) jeotgal, a Korean fermented sea food	27 kDa 8.0 40 °C	K^+, Mg^{2+}, Zn^{2+}	Na^+, Fe^{3+}, Mn^{2+}, Co^{2+}, PMSF, SDS, EDTA, and EGTA	K_m and V_{max} values were 0.26 mM and 21.88 µM min-1 for N-succinyl-Ala-Ala- Pro-Phe-pNA	[80]

B. subtilis JS2	Jeotgals, salted and fermented Korean sea foods	27 kDa 8.0 40 °C	K^+, and Na^+	Mn^{2+}, Mg^{2+}, Zn^{2+}, PMSF, EGTA, and EDTA	K_m and V_{max} values were 0.09 mM and 16.71 µM/l/min for N-succinyl-ala-ala-pro-phe-p-nitroanilide	[81]
B. amyloliquefaciens RSB34	Doenjang, a traditional Korean fermented soy food	27 kDa 8.0 40 °C	K^+, Mg^{2+}, Zn^{2+}, Fe^{2+}, SDS, EDTA, and EGTA	Mn^{2+}, Ca^{2+}, and PMSF	K_m and V_{max} values of were 0.131 ± 0.026 mM and 16.551 ± 0.316 µM/l/min for N-succinyl-ala-ala-pro-phe-p-nitroanilide	[83]
B. subtilis C142	Fermented Natto granulat	23.5 kDa 6.0 40 °C	-NA-	PMSF	K_m and V_{max} values were 0.34 mM and 0.25 mmoles·mg-1min-1 for H-D-Ile-Pro-Arg-pNA	[82]

- PMSF: Phenylmethylsulfonyl fluoride; EDTA: Ethylenediaminetetraacetic acid; EGTA: Ethylene Glycol Tetraacetic Acid; SDS: Sodium dodecyl sulfate; NA: Not Available

2.6 Fibrinolytic activity assessment

The 340 kDa glycoprotein known as human fibrinogen comprises two symmetrical sections, each comprising three polypeptide chains called Aα, Bβ, and γ. These chains polymerize to produce fibrin when tissue is injured [109,110]. When thrombin interacts with fibrinogen, a polypeptide structure comprising α, β, and γ chains is produced, which is the fibrin molecule [111,112]. Fibrinolytic enzymes have a selectivity of breaking down the Aα and Bβ chains of the fibrinogen molecule, with discrete or even absent destruction of the γ chains, based on the structure of fibrinogen and fibrin molecules. Except for the NatWT enzyme [113], which facilitated the hydrolysis of only α chains, the enzymes are also specific for α and β chains regarding fibrin breakdown.

The fibrin plate method, created by Astrup and Müllertz [114], is typically used to assess fibrinolytic activity. This method involves incubating human fibrin with the fibrinolytic enzyme and then using sodium dodecyl sulphate polyacrylamide gel electrophoresis to evaluate the cleavage pattern.The assessment of the fibrinogenolytic and fibrinolytic activity of the proteases produced by *Bacillus* sp. bacteria isolated from fermented food is shown in Table 5. Analysing the direct hydrolysis of fibrin and/or fibrinogen molecules or the indirect hydrolysis via converting plasminogen into plasmin allowed for the identification of these processes.

Table 2.5: Fibrinolytic properties of fibrinolytic enzymes produced by *Bacillus* spp isolated from fermented food samples.

Enzyme	Fibrinolytic activity	Reference
Nattokinase	Fibrin plate hydrolysis 1038 ± 156 U/ml	[115]
AprEFSM4	Hydrolysis of Aα-chain in 5 min and Bβ-chain in 20 min	[100]
NK-Bs	Clear halo appeared around NK-Bs	[101]
NatWT, NatTK, NatOC, DFE, and DFE_{G169A}	Within 10 minutes, DFE_{G169A} completely broke down all of the fibrin's chains, whereas DFE took more than 70 minutes to do so. While NatWT only selectively acted on the Aα chain, NatTK and NatOC broke down both the Aα and Bβ chains.	[113]
-NA-	Hydrolyzed three chains of fibrinogen/fibrin completely within 4 h	[74]

DFE27	Clear hydrolyzed zones formed in both the plasminogen-free and -rich plates.	[76]
AprEBS2	Hydrolysis of Aα-chain in 10 min and Bβ-chain in 6 h	[77]
CFR15-protease	Hydrolysis of α polymer, γ-γ′ dimer, α-chain, and *β*-chain after 30 h	[78]
AprEBS15	Hydrolysis of Aα-chain in 10 min and Bβ-chain in 3 h	[80]
AprEJS2	Hydrolysis of Aα-chain in 10 min and Bβ-chain in 3 h	[81]
C142	Fibrin plate hydrolysis 19 mm/10 FU Hydrolysis of Aα-chain in 10 min and Bβ-chain in 80 min	[82]
AprE34	Hydrolysis of Aα-chain in 10 min and Bβ-chain in 6 h	[83]

2.7 Pharmacological, thrombolytic, and toxicological assessment of fibrinolytic enzymes derived from *Bacillus* spp

The literature study indicates that whereas several articles detail bacterial fibrinolytic enzyme's biochemical characterization and purification methods, limited research examines their pharmacological properties and toxicological effects.

Thrombolytic activity tests are used primarily to evaluate fibrinolytic enzymes' capacity to break up blood clots that have already formed. In people, these might result in consequences such as ischaemic stroke, acute myocardial infarction, pulmonary embolism, and acute arterial thrombosis [116]. Both in vitro and in vivo techniques are employed to examine the thrombolytic activity of fibrinolytic enzymes. The primary technique used was Prasad et al.'s [117] dissolving of clots in tubes. The thrombolytic effectiveness of the enzyme Bacethrombase in thrombi in rats' tails caused by carrageenan was examined in the study conducted by Majumdar et al. [118], which showed a significant prevention of thrombus formation. It is widespread usage to utilize carrageenan to cause inflammation and thrombosis in animal tails. This material can harm endothelial cells and induce local blood vessel inflammation by releasing inflammatory mediators that aid in the thrombus' development [119].

Animal models (rodents) have been used in investigations to evaluate the pharmacological characteristics of fibrinolytic enzymes. These analyses include determining potential adverse consequences associated with bleeding occurrences. The prothrombin time

(PT), activated partial thromboplastin time (aPTT), thrombin time (TT), and clotting time (CT) are utilized as markers. The time at which fibrinogen transforms into fibrin and causes a blood clot is often examined using these assays [120]. Majumdar et al. [118] found that TT was prolonged, indicating that either fibrin polymerization or thrombin activation was inhibited. Choi et al. [82] also showed that rats given the examined fibrinolytic enzymes had more prolonged PT and aPTT, which may indicate that these proteases have anticoagulant properties. APTT assesses the intrinsic and common route of the coagulation process, whereas PT analyzes the extrinsic and common route. The extrinsic, intrinsic, and common pathways are laid out by the coagulation process. The extrinsic route includes the tissue factor and the initial coagulation processes. Factors VIII, IX, XI, XII, pre-kallikrein, and high molecular weight kininogen are all involved in the intrinsic pathway. The common route, which consists of components V, X, fibrinogen, and prothrombin, is where the intrinsic and extrinsic pathways merge [121].

No haemolytic effects are seen in vitro in the haemolysis assays listed in Table 6, indicating that these proteases can be used safely as thrombolytic agents. Desired clinical features of ideal fibrinolytics include facilitating the rapid breakdown of the thrombus, preventing re-occlusion, avoiding systemic fibrinogenolysis, and posing minimal risk of bleeding [122]. Several tests for cytotoxicity and in vivo toxicity are also listed in Table 6, with no behavioural abnormalities, alterations in mammalian cells, histopathological lesions, or death in animals given fibrinolytic enzyme treatment. According to these findings, *Bacillus*-produced fibrinolytic enzymes are promising candidates for the creation of safe thrombolytic drugs and are recommended for pre-clinical research to evaluate their fibrinolytic capabilities in an animal model [123]. The key details about the pharmacological characteristics, thrombolytic activity, and toxicity assessment of fibrinolytic enzymes produced by *Bacillus* species isolated from fermented foods are shown in Table 6.

Table 2.6: Assessment of pharmacological characteristics, toxicity, and thrombolytic activity of fibrinolytic enzymes produced by *Bacillus* spp.

Enzyme	Pharmacological, thrombolytic and toxicological assessment	Reference
Nattokinase	The estimated clot lysis time on artificial clot was 51.5 ± 2.5 min	[115]
BS IDCC1101	There was no cytotoxic effect on cell viability when treated with HaCaT cell. There were no significant changes in body weight after oral administration of BS IDCC1101 at a dose of 300 and 2000 mg/kg B.W. to SD female rats. In addition, for 14 days in a row, there was no mortality, had any obvious pathological abnormalities, or any unusual necropsy findings.	[124]
rSubtilisin QK	Absorption of subtilisin QK, examined using the ex vivo gut sac model, showed a good linear correlation between the absorption of proteins from the epithelium of the intestine into the sac contents in a time-dependent manner. At a dose of 400 IU/g (100 FU/g) for the acute toxicity trial and 100 IU/g (25 FU/g) for a 28-day subchronic toxicity investigation, exhibited no adverse effects. The detection of D-dimer and FDPs during the in vivo fibrinolysis activity revealed that the high-dosage group had considerably greater D-dimer and FDP concentrations than the low-dose group. Subtilisin QK was administered orally, and its effects on thrombi morphology were examined using the modified κ-carrageenan induced thrombosis rat model. The ear and tail arteries of the thrombosis treated rats showed thrombolysis and re-canalization seven days following treatment, as evidenced by morphological features, fibrinolysis markers, and histological alterations.	[125]

NK-Bs	Rats given a high or medium dose of FDP and D-dimer had plasma concentrations that were comparable to those of rats treated with vermis kinase, but they were noticeably greater than the negative control. Following NK-B treatment, there was a notable decrease in lumen thrombosis.	[101]
CFR15-protease	Dose and time-dependently increase of APTT and PT values	[78]
Bacifrinase	Dose-dependent increase in human plasma's PT and APTT was observed Collagen-induced PRP aggregation was dose-dependently inhibited, with an IC50 value of 163.0 nM There was no cytotoxicity against HEK and human cells at a dosage of 2 µM, but at 300 nM it showed very slight (2.98%) haemolysis of mammalian erythrocytes. Mice administered with a 2.0 mg/kg (i.v.) showed no signs of toxicity, bleeding problems, mortality, or adverse effects. The Ca clotting time, PT, and APTT were all dose-dependently increased, and at 6 hours after treatment, the fibrinogen content in the PPP of the mice in the treated group was considerably lower than that of the untretaed group.	[104]
C142	At a dose of 20,000 FU/kg, there was an increase in the APTT (1.4 times) and PT (1.34 times) compared to the control group.	[82]
Bacethrombase	Did not exhibit haemolytic activity against the goat blood erythrocytes or cytotoxicity towards the HeLa and HT29 cells at the 15 g/ml dosage. Did not cause any behavioural changes or adverse consequences in the treated rats, nor did it show any signs of toxicity or bleeding problems at a dose of 10 mg/kg Inhibition of tail thrombus development in rats treated with carrageen was exhibited dose-dependently	[118]

	Treated group's fibrinogen level significantly decreased after two hours of treatment, which also dose-dependently delayed the PPP's APTT, PT, and TT.	

2.8 Structural-functional characteristics

An assessment of their structural-functional properties and modes of activity is essential for enzymes to have widespread commercial and medicinal applications. However, the research hasn't focused much on the structural-functional characteristics and intermolecular interactions of fibrinolytic enzymes. The high-affinity (Ca1) and low-affinity (Ca2) calcium binding sites found in subtilisin enzymes are essential for maintaining the enzymes' thermostability and preventing autolysis. Bacillus subtilis HK176-produced fibrinolytic enzymes were found to have improved thermostability because of calcium binding sites (AprE176: 11% and M179: 36%) (Jeong et al., 2015). In contrast to the wild type, molecular modelling study indicated that the 176th residue of M179, threonine, was situated close to the cation-binding site. This likely resulted in M179 binding tightly to Ca2+, increasing M179's thermostability.

The I-TASSER modelled structure of Bacifrinase, a serine fibrinolytic protease that involves the catalytic triad of Asp102, His83, and Ser195, was published in another study. With an ACE of 442.39, an interface area of 2522.80, and a geometric form complementarity score of 19698, the bacifrinase–fibrinogen (B-chain) interaction was consistent [126]. Additionally, when subtilisin K2 was docked against substrate fibrin using the High Ambiguity Driven protein-protein DOCKing (HADDOCK) website, the SWISS-MODEL anticipated a 3D structure of the protein and demonstrated a binding affinity of 19.4 kcal/mol and a Kd value of 6.3X 10^{-15} M. A substantial similarity between the two proteins was shown by the structural superimposition of subtilisin K2 on nattokinase, which provided a root mean square deviation of 0.12 Å. But the locations of the active site residues of nattokinase (Asp32, His64, and Ser221) and subtilisin K2 (Asp19, His51, and Ser208) were different [72]. In another study

conducted by Jain et. al., a significant binding energy of −6.46 kcal/mol between nattokinase and fibrin was found via molecular docking, indicating a strong binding affinity. Ser300, Leu302, and Asp303 are important fibrin binding residues found and verified [115].

Nattokinase is regarded as an antigen, which prevents its use as an injectable therapeutic protein, according to in silico investigation. Vianney et. al., attempted to identify the amino acid residues that had caused the immunogenicity, where the B-cell epitopes of nattokinase were continually and discontinuously predicted using a variety of web servers. S18, Q19, T242, and Q245 were among the four amino acids permitted to undergo mutation after the expected conserved amino acids were eliminated. The purpose of substitution mutation was to reduce native nattokinase's immunogenicity. S18D, Q19I, T242Y, and Q245W were the suggested muteins based on the stability of the altered protein using the Gibbs free energy difference. Several tools were used to develop and validate the mutant nattokinase's 3D model. The protein's stability analysis and physicochemical characteristics showed that the mutation increased stability without altering the nattokinase catalytic site. According to molecular dynamics simulation, the mutation showed comparable conformation, stability, and functionality to the typical nattokinase [127].

In another study from our lab, the ExPASy-ProtParam tool, MEME server, SOPMA, PDBsum tool, CYS-REC tool, SWISS-MODEL, SAVES servers, TMHMM program, GlobPlot, and peptide cutter software were used to analyze the various physiochemical parameters as well as the structural and functional characteristics of the fibrinolytic enzyme sequences produced by the genus *Bacillus*. The catalytic triad of Asp, His, and Ser was conserved in 49 of the sixty fibrinolytic serine protease enzymes and enzymes high thermostability was indicated by the anticipated instability and aliphatic indexes of 1.94–37.77 and 68.9–93.41, respectively. The motif Peptidase S8/S53 domain, which was consistently found in 56 sequences, is reportedly connected with the catalysis of peptide bonds by a catalytic triad process. Comparing the experimental result with the in-silico study results revealed that two enzyme sequences have properties that should be taken into account when developing a probable transformed enzyme because they are thermostable at high pH and show αβ-fibrinogenase activity in both experimental and in-silico investigations [128].

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