Review

Marine Microbial Fibrinolytic Enzymes: An Overview of Source, Production, Biochemical Properties and Thrombolytic Activity

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Abstract: Cardiovascular diseases (CVDs) have emerged as a major threat to global health resulting in a decrease in life expectancy with respect to humans. Thrombosis is one of the foremost causes of CVDs, and it is characterized by the unwanted formation of fibrin clots. Recently, microbial fibrinolytic enzymes due to their specific features have gained much more attention than conventional thrombolytic agents for the treatment of thrombosis. Marine microorganisms including bacteria and microalgae have the significant ability to produce fibrinolytic enzymes with improved pharmacological properties and lesser side effects and, hence, are considered as prospective candidates for large scale production of these enzymes. There are no studies that have evaluated the fibrinolytic potential of marine fungal-derived enzymes. The current review presents an outline regarding isolation sources, production, features, and thrombolytic potential of fibrinolytic biocatalysts from marine microorganisms identified so far.

Keywords: cardiovascular diseases; fibrinolytic enzymes; marine microorganisms; thrombolytic activity

1. Introduction

Thrombosis is a major cause of cardiovascular diseases (CVDs) including acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure and stroke, and it is a leading cause of death worldwide [1]. With population growth, aging and changing lifestyles, thrombotic diseases have become a more serious problem [2]. Thrombin catalyzes the conversion of fibrinogen to fibrin, which is a key component of blood clots or thrombi [3]. Public health data of CVDs is well documented by the World Health Organization (WHO). As for WHO, in 2016 alone, CVDs caused 17.9 million deaths globally [4], with a prediction that approximately 23.3 million people will be affected by 2030 [5]; hence, CVDs are emerging as a global health concern as well as economic burden [2]. Thrombosis is known as one of the foremost causes of CVDs and is characterized by the formation of fibrin clots. During normal physiological conditions, there is a homeostatic balance in the formation and degradation of fibrin; however, in some pathological disorders, it is unbalanced, resulting in aggregation of fibrin resulting in thrombosis [6,7]. The rapid dissolution of blood clots and the re-establishment of blood flow are critical for treating thrombotic diseases effectively.

Fibrinolytic enzymes are deemed as the most promising medication for the clinical treatment of thrombosis [6]. They can be grouped according to their function as plasminogen activators (e.g., tissue-type plasminogen activator t-PA, urokinase plasminogen activator u-PA, streptokinase and plasmin-like (e.g., nattokinase) and lumbrokinase fibrinolytic enzymes [8].
Plasminogen activators hydrolyze fibrin by the production of plasmin, and the latter can directly break down fibrin clots [9]. To date, a recombinant tissue-type plasminogen activator (rt-PA) is the only commercial thrombolytic agent with FDA approval. However, clinical data revealed its short time window along with its potential neurotoxicity, and hemorrhages resulted in the possible failure of t-PA treatment [10–12] in addition to its short half-life [13–15] and low effectiveness [16]. For instance, the high cost and undesirable side effects prompted investigators to explore the cost-effective and safer thrombolytic agents [17].

In the last decades, many fibrinolytic enzymes from natural resources, such as snakes [18], earthworms [19,20], insects [21], plants [22], mushrooms [23], microorganisms [24,25] and fermented foods such as Chungkook-jang [26] and Tempeh [27], have been identified and studied. Even though these enzymes have been characterized from a wide range of different sources, microbial fibrinolytic enzymes are considered attractive tools due to their features, such as enhanced specificity [8], low production cost [8], comparatively high yield [28] and the possibility to be genetically modified by recombinant DNA technology and protein engineering approaches [29]. Marine ecosystems serve as a reservoir of microorganisms producing important therapeutic metabolites, especially enzymes [30–39], but they remain largely unexplored to date. Due to the wide biodiversity of the marine environment, marine microorganisms can provide a diverse array of enzymes for biotechnological development, with possible improved pharmacological properties and lesser side effects [40]. Although many other research studies must be carried out to assess the toxicity of these enzymes, evidence showed minimal side effects upon their application to humans [40,41]. In this view, particular attention should be paid to possible allergic properties of microbial fibrinolytic enzymes [42]. Hence, fibrinolytic enzymes from marine sources have gathered clinical interest during these decades.

The current review presents an overview regarding the resources, production, properties and thrombolytic activity of fibrinolytic enzymes from marine microbes identified so far.

2. Marine Microorganisms as Sources of Fibrinolytic Enzyme

Marine microorganisms are important resources of fibrinolytic enzymes. These enzymes possess potential efficacy for health augmentation and nutraceutical use, and their application could prevent cardiovascular diseases effectively [28]. Marine microorganisms producing fibrinolytic enzymes, including bacteria (Streptomyces lusitanus [43], Streptomyces radiopugnans VITSD8 [44], Streptomyces violaceus VITYGM [45], Pseudomonas aeruginosa KU1 [46,47], Alteromonas piscicida [48], Pseudoalteromonas sp. IND11 [49], bacterial strain GPJ3 [50], Marinobacter aquaeolei MS2-1 [51], Bacillus flexus [52], Bacillus subtilis [53], Bacillus subtilis HQS-3 [54], Bacillus vallismortis [55], Bacillus subtilis D21-8 [56], Bacillus pumilus BS15 [29], Bacillus subtilis WR350 [57], Bacillus subtilis JS2 [58], Bacillus velezensis BS2 [59], Bacillus subtilis ICTF-1 [41], Shewanella sp. IND20 [60], Serratia rubidaca KUAS001 [61] and Serratia marcescens subsp. sakuensis [62–64], Arthospira platensis [65]) and microalgae (Chlorella vulgaris [66,67], Dunaliella tertiolecta [68] and Tetraselmis subcordiformis [69]), are summarized in Table 1. As shown in Table 1, marine microorganisms that are classified to the genus Bacillus are considered as the most valuable resources for the production of fibrinolytic enzymes, while there are no studies that have evaluated the fibrinolytic potential of marine fungal-derived enzymes. It should be noted that the catalytic activity of fibrinolytic enzymes can be improved by chemical modifications and mutant selection [70,71].
### Table 1. Marine sources of fibrinolytic enzymes.

| Isolated From                                      | Microorganism          | Enzyme         | Reference |
|----------------------------------------------------|------------------------|----------------|-----------|
| Marine sediment from Kovalam beach, Chennai, Tamil Nadu | Streptomyces lusitanus | -              | [43]      |
| Marine brown tube sponges *Agelas conifera*        | Streptomyces radiopugnans | VITSD8       |           |
| Soil samples from South East Coast of India, Chennai | Streptomyces rubiginosus | VITPSS1      |           |
| Marine water sample                                | Streptomyces venezuelae | Thrombinase   | [73]      |
| Mangrove Sediments Pitchavaram, South East Coast of India | Bacillus circulans     | -              | [74]      |
| Marine sediments of Ezhara beach, Kannur District, Kerala, India | *Pseudomonas aeruginosa* | KU1          |           |
| Mangrove sediments of Pulicat Lake, India          | Bacterial strain GPJ3  | -              |           |
| South West Coast of India                          | *Bacillus flexus*      | -              |           |
| Mutagenesis of *B. subtilis* HQS-3                 | *Bacillus subtilis*    | -              |           |
| Surface seawater                                   | *Bacillus vallismortis*| Bvsp          |           |
| Fish scales, Kanyakumari, India                    | *Pseudoalteromonas* sp. IND11 | -        |           |
| Coast of Beihai prefecture of China                | *Bacillus subtilis* HQS-3 | -       |           |
| Deep-sea sediment of Bay of Bengal                 | *Marinobacter aquaeolei* | MS2-1       |           |
| Jeotgal from gul (Oyster, *Crassostrea gigas*), korean fermented food | *Bacillus pumilus* BS15 | AprEBS15     | [29]      |
| Marine niches covering 300 km of the western seacoast of Maharashstra, India | *Bacillus subtilis* ICTF-1 | -       |           |
| Oriyara beach in Kasargod district, Kerala, India | *Serratia rubidaea* KUAS001 | -       |           |
| Jeotgal from munggae (sea squirt), Korean fermented seafood | *Bacillus velezensis* BS2 | AprEBS2     | [59]      |
| Sea mud                                            | *Bacillus subtilis* WR350 | -         |           |
| Sea water collected from a depth of 10 m, 5 km away from Surathkal Coast in the Arabian Sea | *Serratia marcescens* subsp. sakuensis (KU296189.1) | -       | [62-64,70]|
| Jeotgals from salted saeu (small shrimp), Korean fermented seafoods | *Bacillus subtilis* JS2 | AprEJS2     | [58]      |
| Marine isolate                                     | *Shewanella* sp. IND20  | -              |           |
| Jeotgal, Korean fermented seafood                  | *Bacillus licheniformis* KJ-31 | BpKJ-31     | [75]      |
| Culture Collection of Algae, University of Texas, Austin | *Arthrospira (Spirulina) platensis* | -       | [65]      |
| University of Texas, Austin                        | *Chlorella vulgaris*    | -              | [66,67]  |
| Dalian Institute of Chemical Physics, Chinese Academy of Sciences | *Tetraselmis subcordiformis* | -       |           |

### 3. Purification of Fibrinolytic Enzymes

The main purpose of purifying enzymes is to remove other contaminating proteins and other interfering biomolecules. Furthermore, enzyme purification allows the acquisition of insights about structural and functional features of the purified enzyme, as well as foretells its applications [76]. The required level of purity depends on the purpose for which the protein is to be used. If deemed for therapeutic use, the enzyme must have higher level purity and be processed through several subsequent purification steps.

Currently, several approaches have been used for separating and purifying fibrinolytic enzymes from marine microorganisms (Table 2). These approaches involved the
extraction of bacteria with aqueous buffer solution as first step, followed by a concentration/precipitation step using acetone or ammonium sulfate and dialysis [41,43,44,52,62,65,66]. As shown in Table 2, the use of ammonium sulfate for protein precipitation is preferred, as it is a low-cost reagent, highly soluble in water and it is able to stabilize proteins and enzymes [41,44,47,52,62,65]. Further purification is carried out by employing different chromatographic steps (Table 2).

Several purification strategies are listed in Table 2, along with their efficiencies (enzyme specific activities obtained). For example, Barros and colleagues (2020) used ammonium sulfate precipitation (40–70%), acetone precipitation, DEAE-Sephadex (anion exchange) and Superdex 75 (size exclusion) chromatography to purify a fibrinolytic enzyme from *Arthrospira platensis*. The eluted enzyme showed a specific activity of 7,988 U/mg with 32.42-fold purification [65]. A fibrinolytic enzyme from *Bacillus subtilis* ICTF-1 was purified by a three-step procedure. As a first step, ammonium sulfate precipitation was adopted for providing suitable protein concentration, followed by UnoQ Sepharose Strong Anion Exchanger and Butyl Sepharose FF chromatography. The enzyme had a molecular mass of 72 kDa [41].
Table 2. Purification strategies for isolating fibrinolytic enzymes from marine microorganisms.

| Source                  | Enzyme          | Purification Methods                                                                 | Total Protein (mg) | Specific Activity (U mg\(^{-1}\)) | Purification (Fold) | Yield (%) | References |
|-------------------------|-----------------|-------------------------------------------------------------------------------------|--------------------|-----------------------------------|---------------------|-----------|------------|
| *Bacillus flexus*       |                 | Ammonium sulphate precipitation (20%, 40% and 60%), Sephadex G-75 chromatography    | 4.4                | 315.2                             | 5.2                 | 10.8      | [52]       |
| *Bacillus pumilus BS15* | AprEBS15        | Affinity chromatography by HiTrap IMAC FF column                                     | -                  | -                                 | -                   | -         | [29]       |
| *Bacillus velezensis BS2* | AprEBS2        | Affinity chromatography by HiTrap IMAC FF column                                     | -                  | 131.15 m                         | -                   | -         | [59]       |
| *Bacillus subtilis HQS-3* |                | Ammonium sulphate precipitation, alkaline solution treatment, membrane concentration, dialysis, ion exchange and gel filtration chromatography | 12                 | 62,745                            | 30                  | 13        | [54]       |
| *Bacillus subtilis JS2* | AprEJS2         | Affinity chromatography by HiTrap IMAC FF column                                     | -                  | -                                 | -                   | -         | [58]       |
| *Serratia marcescens subsp. sakuensis* |             | Ammonium sulfate precipitation (40%), dialysis, Fast protein liquid chromatography | 0.03               | 1033                              | 21.08               | 19.38     | [62]       |
| *Pseudomonas aeruginosa* |                 | Ammonium sulphate precipitation (50–80%), DEAE Sepharose, Sepharose 6B chromatography | 0.8 mg·mL\(^{-1}\) | 1491.50                           | 13.52               | 17.79     | [47]       |
| *Bacillus licheniformis* | KJ-31           | DEAE-Sepharose FF column and gel filtration chromatography (HiPrep 16/60 Sephacryl S-200 HR column) | 3.2                | 242.8                             | 19                  | 0.2       | [75]       |
| *Bacillus subtilis ICTF-1* |                | Ammonium sulfate precipitation (0–60%), UnoQ Sepharose Strong Anion Exchanger, Butyl Sepharose FF chromatography | 0.669              | 280                               | 32.42               | 7.5       | [41]       |
| *Streptomyces lusitanus* |                 | Ammonium sulfate precipitation (60%), dialysis, size exclusion gel filtration chromatography | -                  | -                                 | -                   | -         | [43]       |
| *Streptomyces radiopugnans* | VITSD8          | Ammonium sulfate precipitation (0–85%), dialysis, ion-exchange chromatography, Size exclusion chromatography | 1.1                | 3891                              | 22.36               | 35        | [44]       |
| *Arthospira platensis*  |                 | Ammonium sulfate precipitation (40–70%), anion exchange (DEAE-Sephadex), size exclusion (Superdex 75) chromatography | 0.02 mg·mL\(^{-1}\) | 7988                             | 32.72               | 28.85     | [65]       |
| *Chlorella vulgaris*    |                 | Acetone precipitation, anion exchange chromatography HiTrapTM DEAE FF column          | 2.0                | 1834.6                            | 2                   | 4.0       | [66]       |
4. Biochemical Characterization of Marine Microbial Fibrinolytic Enzymes

4.1. Physicochemical Properties of Fibrinolytic Enzymes

4.1.1. Molecular Weight and Effect of pH, Temperature, Inhibitors and Ions

Table 3 provides a detailed overview of the significant physicochemical characteristics of marine microbial fibrinolytic enzymes, including molecular mass, optimal pH and temperature. The molecular mass of the purified marine microbial fibrinolytic enzymes varied significantly, ranging from as low as 21 kDa in an actinomycete (Streptomyces lusitanus) \[43\] to as high as 72 kDa in a cyanobacterium (Arthrospira platensis) \[65\]. Most marine microbial fibrinolytic enzymes have optimum pH fluctuating from neutral to alkaline values, ranging from 6 \[65\] to 7 \[43,44,62\] and from 8 \[29,52,58–60\] to 9 \[41,75\]. The optimal temperature of marine microbial fibrinolytic enzymes ranges between 33 \(^\circ\)C \[44\] to 60 \(^\circ\)C (Bacillus flexus) \[52\].

| Source | Enzyme | Molecular Weight (kDa) | pH Opt. | Temp. Opt. (°C) | Activator/Co-Factor (Metal Ions) | Inhibitor Class | References |
|--------|--------|------------------------|---------|-----------------|----------------------------------|-----------------|------------|
| Bacillus flexus | - | 32 | 8 | 60 | Mg\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\) and Hg\(^{2+}\) | - | [52] |
| Bacillus pumilus BS15 | AprEBS15 | 27 | 8 | 40 | K\(^{+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) | Na\(^{+}\), Fe\(^{3+}\), Mn\(^{2+}\), Co\(^{2+}\), PMSF, EDTA and EGTA | Serine protease | [29] |
| Bacillus velezensis BS2 | AprEBS2 | 27 | 8 | 37 | Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\) | Fe\(^{2+}\), Zn\(^{2+}\), K\(^{+}\), Co\(^{2+}\), PMSF, EDTA, SDS | Serine protease | [59] |
| Bacillus licheniformis KJ-31 | BpKJ-31 | 37 | 9 | 40 | - | - | Alkaline serine protease | [75] |
| Bacillus subtilis JS2 | AprEJS2 | 24 | 8 | 40 | K\(^{+}\), Mn\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\) | PMSF, EDTA, EGTA | Serine protease | [56] |
| Bacillus subtilis HQS-3 | AprEJS2 | 26 | 8 | 45-50 | Mn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\) | PMSF, EDTA, Cu\(^{2+}\), Zn\(^{2+}\) and Co\(^{2+}\) | Serine metallo-protease | [54] |
| Marinobacter aquaeolei MS2-1 | - | 39 | 8 | 50 | DTT | - | Thiol-dependent serine protease | [51] |
| Bacillus subtilis ICTF-1 | - | 28 | 9 | 50 | Ca\(^{2+}\) | Zn\(^{2+}\), Fe\(^{3+}\), Hg\(^{2+}\) and PMSF | Serine protease | [41] |
| Bacillus subtilis var. subtilis Bvsp | Bvsp | 34.4 | 6.5 | 54 | Ca\(^{2+}\), Zn\(^{2+}\) and Ba\(^{2+}\) | Na\(^{+}\), K\(^{+}\), NH\(_4^+\) and Mg\(^{2+}\), PMSF, AEDSF, SDS, Guanidine-HCl, Urea and Isopropyl alcohol | Alkaline serine protease | [55] |
| Serratia marcescens subsp. sauketensis | - | 43 | 7 | 55 | Mn\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\) | PMSF, EDTA | Serine metallo-protease | [62] |
| Pseudomonas aeruginosa KU1 | - | -50 | - | - | Na\(^{+}\), K\(^{+}\) and Co\(^{2+}\) | Fe\(^{3+}\), Mn\(^{2+}\) and Zn\(^{2+}\) | Metalloprotease | [47] |
| Shewanella sp. IND20 | - | 55.5 | 8 | 50 | Ca\(^{2+}\) and Mg\(^{2+}\) | - | - | [60] |
| Streptomyces lusitanus | - | 21 | 7 | 37 | - | - | - | Serine endopeptidase | [44] |
| Streptomyces radiopugnans VITSD8 | - | 38 | 7 | 33 | - | - | - | Serine metallo-protease | [44] |
| Streptomyces rubiginosus VITPS1 | - | 45 | - | - | - | - | - | - | [72] |
| Arthrospira platensis | - | 72 | 6 | 40 | Fe\(^{2+}\) | PMSF | Serine metallo-protease | [65] |
| Chlorella vulgaris | - | 45 | - | - | Fe\(^{2+}\) | PMSF, EDTA | Serine metallo-protease | [66] |

Moreover, some studies have focused on the effect of chemical reagents and metal ions to delineate and characterize catalysis by these novel fibrinolytic enzymes. Table 3
summarizes the effect of metal ions as well as inhibitors on the fibrinolytic enzyme activities. Indeed, according to the specific chemical functionality in their active site, fibrinolytic enzymes can be classified as metalloproteases, serine proteases and serine metalloproteases. As shown in Table 3, the majority of fibrinolytic enzymes from *Bacillus* spp. belongs to serine proteases, and their activity is inhibited by PMSF (Phenyl Methyl Sulphonyl Fluoride). Proteolytic enzymes possessing an active group (OH) from serine amino acid in the catalytic site are recognized as serine proteases. During inhibition of catalytic activity, sulfonyl group of PMSF binds irreversibly to the serine OH group in the active site [77]. In addition, a metalloprotease from *Pseudomonas aeruginosa* KU1 is repressed by some metal ions, e.g., Mn$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$ [47]. Similarly, the activities of fibrinolytic enzymes that belong to serine metalloprotease are dependent on divalent metal ions, such as Mn$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$, for enzymes from *Serratia marcescens* subsp. *sakaiens* [62], Fe$^{2+}$ for enzymes from *Arthospira platensis* [65] and *Chlorella vulgaris* [66]; thus, their catalyses were inhibited by chelating agents such as EDTA (ethylenediaminetetraacetic acid) and EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid).

4.1.2. Fibrinogen Lytic Activity

The efficacy of fibrinolytic enzymes is determined by two different mechanisms: indirectly activating the plasminogen and directly acting on fibrins [78]. In Table 4, the activity of different fibrinolytic enzymes isolated from marine microbial sources reported in terms of direct or indirect fibrinogen lytic activity is listed. As shown in Table 4, marine *Bacillus* enzymes are generally directly acting on fibrin forming or fibrin degradation products. AprEBS2 isolated from *Bacillus velezensis* B52 revealed high A$\alpha$ fibrinolytic activity, followed by moderate B$\beta$ and mild $\gamma$ chains fibrinolysis [59]. Nevertheless, fibrinolytic enzymes from *Bacillus pumilus* BS15 [29] and *Bacillus subtilis* JS2 [58] displayed no $\gamma$-chain lysis. *Bacillus licheniformis* KJ-31 was one of the microorganisms that only produced fibrinolytic enzymes with high A$\alpha$ fibrinogen lytic activity [75].

Table 4. Fibrino(ogen) lytic activity of various marine microbial enzymes.

| Source                   | Enzyme  | Reference                  | Mode of Action | References |
|--------------------------|---------|----------------------------|----------------|------------|
| *Bacillus velezensis* BS2| AprEBS2 | Strong A$\alpha$-fibrinogenase and moderate $\beta$-fibrinogenase activities | Direct         | [59]       |
| *Bacillus pumilus* BS15 | AprEBS15| Strong A$\alpha$-fibrinogenase and moderate $\beta$-fibrinogenase activities | Direct         | [29]       |
| *Bacillus subtilis* JS2  | AprEJS2 | Strong A$\alpha$-fibrinogenase and moderate $\beta$-fibrinogenase activities | Direct         | [58]       |
| *Bacillus licheniformis* KJ-31 | BpKJ-31 | Strong A$\alpha$ and fibrino (geno) lytic activity | Direct | [75] |
| *Bacillus subtilis* HQS-3 | -      | Hydrolyzed A$\alpha$ chain of fibrin, followed by the $\beta$ chain and finally the $\gamma$–$\gamma$ chain | Direct | [54] |
| *Bacillus vallismortis*  | Bvsp   | Digest A$\alpha$- and B$\beta$-chains readily, but the $\gamma$-chain of fibrinogen slowly | Direct | [55] |

4.2. Amidolytic and Kinetic Properties of Marine Microbial Fibrinolytic Enzymes

Microbial fibrinolytic enzymes display amidolytic (or pro-coagulant) activity, which is assessed using different synthetic chromogenic substrates (Table 5). Most of the studied enzymes show high specificity towards N-Succ-Ala-Ala-Pro-Phe-pNA, classifying them as serine proteases [29,41,58,59,75]. In addition, kinetic parameters including the Michaelis constant [79], rate of reaction ($V_{\text{max}}$) and the turnover number ($k_{\text{cat}}$) help understand the specificity and affinity of an enzyme for a particular substrate [6]. Table 6 summarizes the kinetic properties of selected fibrinolytic enzymes isolated from marine microorganisms in different reaction conditions by using both natural and synthetic substrates.
### Table 5. Kinetic properties of fibrinolytic enzymes.

| Source               | Enzyme      | Substrate Specificity     | $V_{\text{max}}$ | $K_m$  | $k_{\text{cat}}$ | $k_{\text{cat}}/K_m$ | Reference |
|----------------------|-------------|---------------------------|------------------|--------|-----------------|----------------------|-----------|
| *Bacillus velezensis* BS2 | AprEBS2     | N-Succ-Ala-Ala-Pro-Phe-pNA | 39.68 µM min⁻¹   | 0.15 mM | 18.14 s⁻¹       | $1.25 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ | [59]     |
| *Bacillus pumilus* BS15 | AprEBS15    | N-succinyl-al-a-la-pro-phe-pNA | 21.88 µM min⁻¹   | 0.26 mM | 10.02 s⁻¹       | $3.83 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ | [29]     |
| *Bacillus subtilis* JS2 | AprEJS2     | N-Succ-Ala-Ala-Pro-Phe-pNA | 16.71 µM min⁻¹   | 0.09 mM | 7.66 s⁻¹        | $8.51 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ | [58]     |
| *Bacillus subtilis* ICF-1 | -           | N-Succ-Ala-Ala-Pro-Phe-pNA | -                | -      | -               | -                     | [41]     |
| *Bacillus licheniformis* KJ-31 | BpKJ-31   | N-Succ-Ala-Ala-Pro-Phe-pNA | -                | -      | -               | -                     | [75]     |
| *Serratia marcescens* subsp. sakuenis | -    | Fibrin                    | 15.873 µmol min⁻¹ | 0.66 mg mL⁻¹ | 12.21 min⁻¹ | 18.32 mL mg⁻¹ min⁻¹ | [62]     |
| *Bacillus subtilis* | Fibase      | -                         | 0.03 mM min⁻¹    | 2.7 mmol L⁻¹ | -              | -                     | [71]     |
| *Bacillus vallismortis* | Bvsp      | Fibrin                    | 49.8 g mL⁻¹ min⁻¹ | 0.319 g mL⁻¹ | 4.35 min⁻¹   | 13.63 mL mg⁻¹ min⁻¹ | [55]     |

### Table 6. Cloning and expression parameters used for fibrinolytic enzymes production.

| Bacterial Strain | Gene    | Primer | Cloning Host   | Cloning Vector | Expression Host | Expression Vector | References |
|------------------|---------|--------|----------------|----------------|-----------------|-------------------|------------|
| *Bacillus velezensis* BS2 | aprEBS2 | CH51-F (5′-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3′, BamHI site underlined) CH51-R (5′-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3′, EcoRI site underlined) | *B. subtilis* WB600 | pHY300PLK | *E. coli* BL21 (DE3) | pETBS2 | [59] |
| *Bacillus subtilis* JS2 | aprEJS2 | CH51-F (5′-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3′, BamHI site underlined) and CH51-R (5′-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3′, EcoRI site underlined) | *B. subtilis* WB600 | pHY300PLK | *E. coli* BL21 (DE3) | pHYS2 | [58] |
| *Bacillus pumilus* BS15 | aprEBS15 | CH51-F (5′-AGGATCCCAAGAGAGCGATTGCGGCTGTGTAC-3′, BamHI site underlined) and CH51-R (5′-AGAATTCTTCAGAGGGAGCCACCCGTCGATCA-3′, EcoRI site underlined) | *B. subtilis* WB600 | pHY300PLK | *E. coli* BL21 (DE3) | pHYBS15 | [29] |
| *Bacillus vallismortis* | Bvsp | BVSPF (5′-CGCGGATCC-ATGCAAGGTGAAATTAGGTTAATTCCATATTT-3′) containing BamHI I and BVSPR (5′-CGCGGATCCATGCAAGGTGAAATTAGGTTAATTCCATATTT-3′, Xho I sites (underlined)) | - | - | *E. coli* BL21 (DE3) | pGEX-6P-bvsp | [55] |
| *Marinobacter aquaeolei* MS2-1 | - | SPro F (5′-GGG TCG AGT TAG CGG CAA GGA-3′) and SPro R (5′-GGG TCG AGT TAG CGG CAA GGA-3′, Xho I sites (underlined)) | *E. coli* | pGEM-T | *E. coli* BL21 (DE3) | pET-28a(+) | [51] |
| *Tetraselmis subcordiformis* | rt-PA | bar1F (5′-TCTGCACCATTCTCAACACCCTAC-3′), bar1R (5′-TCTGACCATTCTCAACACCCTAC-3′), rpa3F (5′-TCTGACCATTCTCAACACCCTAC-3′) and rpa3R (5′-TCTGACCATTCTCAACACCCTAC-3′, Xho I sites (underlined)) | - | - | *E. coli* Top10 | pSVrPA/CaMVbar | [69] |
5. Production of Marine Microbial Fibrinolytic Enzymes

5.1. Construction of Genetically Engineered Strains

Gene cloning, mutagenesis and recombinant DNA technology have also been employed for the overexpression of fibrinolytic enzymes in bacterial hosts and to engineer their catalytic properties. For example, Yao and colleagues (2018) achieved significantly higher fibrinolytic activity of the recombinant fibrinolytic enzyme from *Bacillus pumilus* BS15 [29]. In another example, Che and colleagues (2020), using gene dosage, codon optimization and process optimization, achieved high expression and secretion of a fibrinolytic enzyme (fibase) isolated from marine *Bacillus subtilis* [71]. Hence, a combination of culture media optimization and recombinant DNA technology has been effectively employed for augmenting the enzyme titer. Table 6 indicates some of the heterologously expressed fibrinolytic enzymes from marine microorganisms.

5.2. Fermentation Approach

The production cost of an enzyme is one of the challenging factors for the industrial sector. The commercial obtainability of microbial fibrinolytic enzymes needs high yield at the lowest possible costs [80]. Hence, fermentation approaches are highly remarkable in cutting down the cost of production for an enzyme. For instance, selected submerged fermentations can improve production yield and efficiency. Similarly, Anusree and colleagues (2020), by using submerged fermentation, were able to improve the expression of fibrinolytic enzyme from a bacterium *Serratia rubidaea* KUAS001 obtained from marine milieus [61]. In addition, Pan and colleagues (2019) showed the utilization of non-sterile submerged fermentation to minimize the production cost of enzymes from *Bacillus subtilis* D21-8 [56]. Moreover, several researchers showed that the use and application of diverse statistical tools, such as Box–Behnken design [46], two-level full factorial design (2^5) [49,52,60], response surface methodology [52,60,72,81], Plackett–Burman design [64,81], one-factor experiment [64], L_18-orthogonal array method [41] and central composite experimental design [49,67], are useful approaches for optimizing physico-chemical parameters for the production of fibrinolytic enzymes. For example, Farraj and colleagues (2020), applying a two-level full factorial design and response surface methodology, were able to increase the expression of the fibrinolytic enzyme isolated from *Bacillus flexus* using a solid state fermentation process. They demonstrated an enhanced production of fibrinolytic enzymes up to 3.5-fold [52].

6. Thrombolytic Activity of Marine Microbial Fibrinolytic Enzymes

The effective treatment of CVDs relies on thrombolysis agents, such as microbial fibrinolytic enzymes [82,83]. Microorganisms have been utilized to produce fibrinolytic enzymes since ancient times. In the last decades, researchers have intensively reported on the production of fibrinolytic enzymes from marine microorganisms. For example, a study carried out by Hwang et al. (2007) showed that BpKJ-31 is a promising candidate as a health-promoting biomaterial that does not induce bleeding [75]. Studies on in vitro lysis of clots by a purified fibrinolytic enzyme from the marine *Serratia marcescens* resulted in 38% clot lysis, which was significantly higher than that reported by streptokinase and heparin [62]. In addition, in the study carried by Gowthami and colleagues (2021), the fibrinolytic enzyme isolated from bacterial strain GPJ3 displayed digestion of blood clot completely under in vitro condition and exhibited potent activity on wound healing of macrophages [50]. The characteristics of the recombinant fibase from a marine *Bacillus subtilis* suggest its potential use for the treatment and/or prevention of thrombosis [71]. Moreover, purified PEKU1, a novel fibrinolytic protease from *Pseudomonas aeruginosa* KU1, has exceptional potential for being developed as a therapeutic agent to treat CVDs [47].

7. Conclusions

The scientific community already effectively utilizes all available information on fibrinolytic enzymes. As a future prospective, the community should focus on the exploration
of novel sources of fibrinolytic enzymes, especially from the marine environment. Marine microbial fibrinolytic enzymes have immense therapeutic potential as target drugs to prevent or cure CVDs. Extensive studies on these enzymes promises to develop cost effective, safe and preventive solutions for the management of cardiac diseases. The new trend for developing and improving thrombolytic agents is to enhance its fibrin specificity and binding efficacy. Further optimization of production parameters is also required to design economical, effective and safe drugs. Thus, the use of marine microbial fibrinolytic enzymes as thrombolytic agents might be auspicious and a safe option in future.

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References

1. Raskob, G.; Angchaisuksiri, P.; Blanco, A.; Buller, H.; Gallus, A.; Hunt, B.; Hylek, E.; Kakkar, A.; Konstantinides, S.; McCumber, M. Ist he steering committee for world thrombosis day. Thrombosis: A major contributor to global disease burden. Arter. Thromb Vasc Biol. 2014, 34, 2363–2371. [CrossRef]
2. Roth, G.A.; Mensah, G.A.; Johnson, C.O.; Addolorato, G.; Ammirati, E.; Baddour, L.M.; Barengo, N.C.; Beaton, A.Z.; Benjamin, E.J.; Benziger, C.P. Global burden of cardiovascular diseases and risk factors, 1990–2019: Update from the gbd 2019 study. J. Am. Coll. Cardiol. 2020, 76, 2982–3021. [CrossRef]
3. Winter, W.E.; Flax, S.D.; Harris, N.S. Coagulation testing in the core laboratory. Lab. Med. 2017, 48, 295–313. [CrossRef]
4. Roth, G.A.; Johnson, C.; Abajobir, A.; Abd-Allah, F.; Abera, S.F.; Abyu, G.; Ahmed, M.; Aksut, B.; Alam, T.; Alam, K. Global, regional, and national burden of cardiovascular diseases for 10 causes, 1990 to 2015. J. Am. Coll. Cardiol. 2017, 70, 1–25. [CrossRef] [PubMed]
5. World Health Organization (WHO). NCDs Mortality and Morbidity. 2016 [Updated 2016; Cited]. Available online: http://www.who.int/gho/ncd/mortality_morbidity/en/ (accessed on 20 November 2021).
6. Kotb, E. Activity assessment of microbial fibrinolytic enzymes. Appl. Microbiol. Biotechnol. 2013, 97, 6647–6665. [CrossRef] [PubMed]
7. Chapin, J.C.; Hajjar, K.A. Fibrinolysis and the control of blood coagulation. Blood Rev. 2015, 29, 17–24. [CrossRef]
8. Peng, Y.; Yang, X.; Zhang, Y. Microbial fibrinolytic enzymes: An overview of source, production, properties, and thrombolytic activity in vivo. Appl. Microbiol. Biotechnol. 2005, 69, 126–132. [CrossRef] [PubMed]
9. Chitte, R.R.; Deshmukh, S.V.; Kanekar, P.P. Production, purification, and biochemical characterization of a fibrinolytic enzyme from thermophilic Streptomyces sp. MCMB-379. Appl. Biochem. Biotechnol. 2011, 165, 1406–1413. [CrossRef]
10. Simoons, M.L.; de Jaegere, P.; van Domburg, R.; Boersma, E.; Maggioni, A.; Franzosi, M.; Leimberger, J.; Califf, R.; Schröder, R.; Knatterud, G. Individual risk assessment for intracranial haemorrhage during thrombolytic therapy. Lancet 1993, 342, 1523–1528. [CrossRef]
11. Fromm, R.E., Jr.; Hoskins, E.; Cronin, L.; Pratt, C.M.; Spencer, W.H., III; Roberts, R. Bleeding complications following initiation of thrombolytic therapy for acute myocardial infarction: A comparison of helicopter-transported and nontransported patients. Ann. Emerg. Med. 1991, 20, 892–895. [CrossRef]
12. Niego, B.E.; Freeman, R.; Puschmann, T.B.; Turnley, A.M.; Medcalf, R.L. t-PA-specific modulation of a human blood-brain barrier model involves plasmin-mediated activation of the Rho kinase pathway in astrocytes. Blood J. Am. Soc. Hematol. 2012, 119, 4752–4761. [CrossRef]
13. Eisenberg, P.R.; Sherman, L.A.; Tiefenbrunn, A.J.; Ludbrook, P.A.; Sobel, B.E.; Jaffe, A.S. Sustained fibrinolysis after administration of t-PA despite its short half-life in the circulation. Thromb. Haemost. 1987, 57, 035–040. [CrossRef]
14. Nilsson, T.; Wallen, P.; Mellbring, G. In vivo metabolism of human tissue-type plasminogen activator. Scand. J. Haematol. 1984, 33, 49–53. [CrossRef]
15. Tsikouris, J.P.; Tsikouris, A.P. A review of available fibrin-specific thrombolytic agents used in acute myocardial infarction. Pharmacother. J. Hum. Pharmacol. Drug Ther. 2001, 21, 207–217. [CrossRef]
16. Matsuo, O.; Rijken, D.; Collen, D. Comparison of the relative fibrinogenolytic, fibrinolytic and thrombolytic properties of tissue plasminogen activator and urokinase in vitro. Thromb. Haemost. 1981, 45, 225–229. [CrossRef]
17. Lu, F.; Lu, Z.; Bie, X.; Yao, Z.; Wang, Y.; Lu, Y.; Guo, Y. Purification and characterization of a novel anticoagulant and fibrinolytic enzyme produced by endophytic bacterium Paenibacillus polymyxa EJS-3. *Thromb. Res.*, 2010, 126, e349–e355. [CrossRef] [PubMed]

18. Zhang, Y.; Wisner, A.; Xiong, Y.; Bon, C. A novel plasminogen activator from snake venom: Purification, characterization, and molecular cloning. *J. Biol. Chem.* 1995, 270, 10246–10255. [CrossRef] [PubMed]

19. Wu, J.X.; Zhao, X.Y.; Pan, R.; He, R.Q. Glycosylated trypsin-like proteases from earthworm *Eisenia fetida*. *Int. J. Biol. Macromol.* 2007, 40, 399–406. [CrossRef] [PubMed]

20. Iannucci, N.; Camperi, S.; Cascone, O. Purification of lumbrokinase from *Eisenia fetida* using aqueous two-phase systems and anion-exchange chromatography. *Sep. Purif. Technol.* 2008, 64, 131–134. [CrossRef]

21. Ahn, M.Y.; Hahn, B-S.; Ryu, K.S.; Kim, J.W.; Kim, I.; Kim, Y.S. Purification and characterization of a serine protease with fibrinolytic activity from the dung beetles, *Carabus mauomensis*. *Thromb. Res.* 2003, 112, 339–347. [CrossRef]

22. Chung, D.M.; Choi, N.-S.; Maeng, P.J.; Chun, H.K.; Kim, S.-H. Purification and characterization of a novel fibrinolytic enzyme from chive (*Allium tuberosum*). *Food Sci. Biotechnol.* 2010, 19, 697–702. [CrossRef]

23. Katrolia, P.; Liu, X.; Zhao, Y.; Kopparapu, N.K.; Zheng, X. Gene cloning, expression and homology modeling of first fibrinolytic enzyme from mushroom (*Cordyceps militaris*). *Int. J. Biol. Macromol.* 2020, 146, 897–906. [CrossRef] [PubMed]

24. Lv, F.; Zhang, C.; Guo, F.; Lu, Y.; Bie, X.; Qian, H.; Lu, Z. Expression, purification, and characterization of a recombined fibrinolytic enzyme from endophytic *Paenibacillus polymyxa* EJS-3 in *Escherichia coli*. *Food Sci. Biotechnol.* 2015, 24, 125–131. [CrossRef]

25. Taneja, K.; Badaj, B.K.; Kumar, S.; Dilbaghi, N. Production, purification and characterization of fibrinolytic enzyme from *Serratia* sp. KG-2-1 using optimized media. *3 Biotech* 2017, 7, 184. [CrossRef] [PubMed]

26. Kim, W.; Choi, K.; Kim, Y.; Park, H.; Choi, J.; Lee, Y.; Oh, H.; Kwon, I.; Lee, S. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain ck 11-4 screened from Chungkook-Jang. *Appl. Environ. Microbiol.* 1996, 62, 2482–2488. [CrossRef] [PubMed]

27. Sugimoto, S.; Fuji, T.; Morimaya, T.; Johdo, O.; Nakamura, T. The fibrinolytic activity of a novel protease derived from a tempeh producing fungus, *Fusarium* sp. BLB. *Biosci. Biotechnol. Biochem.* 2007, 71, 2184–2189. [CrossRef]

28. Mine, Y.; Wong, A.H.K.; Jiang, B. Fibrinolytic enzymes in Asian traditional fermented foods. *J. Biosci. Bioeng.* 2018, 126, 687–6892. [CrossRef]

29. Yao, Z.; Kim, J.A.; Kim, J.H. Gene cloning, expression, and properties of a fibrinolytic enzyme secreted by *Bacillus pumilus* bs15 isolated from gut (oyster) junction. *Biotechnol. Bioprocess Eng.* 2018, 23, 293–301. [CrossRef]

30. Barzkar, N.; Sohail, M.; Jahromi, S.T.; Nahavandi, R.; Khodadadi, M. Marine microbial L-glutaminase: From pharmaceutical to food industry. *Appl. Microbiol. Biotechnol.* 2015, 105, 443–446. [CrossRef]

31. Barzkar, N.; Sohail, M.; Jahromi, S.T.; Gozari, M.; Poormozaffar, S.; Nahavandi, R.; Hafezieh, M. Marine bacterial esterases: Emerging biocatalysts for industrial applications. *Appl. Biochem. Biotechnol.* 2021, 193, 1187–1214. [CrossRef]

32. Barzkar, N.; Khan, Z.; Jahromi, S.T.; Poormozaffar, S.; Gozari, M.; Nahavandi, R. A critical review on marine serine protease and its inhibitors: A new wave of drugs? *Int. J. Biol. Macromol.* 2020, 170, 674–687. [CrossRef]

33. Barzkar, N.; Sohail, M. An overview on marine cellulolytic enzymes and their potential applications. *Appl. Microbiol. Biotechnol.* 2020, 104, 6873–6892. [CrossRef]

34. Barzkar, N. Marine microbial alkaline protease: An efficient and essential tool for various industrial applications. *Int. J. Biol. Macromol.* 2020, 161, 1216–1229. [CrossRef] [PubMed]

35. Barzkar, N.; Jahromi, S.T.; Poorsaheli, H.B.; Vianello, F. Metabolites from marine microorganisms, micro, and macroalgae: Immense scope for pharmacology. *Mar. Drugs* 2019, 17, 464. [CrossRef] [PubMed]

36. Jahromi, S.T.; Barzkar, N. Marine bacterial chitinase as sources of energy, eco-friendly agent, and industrial biocatalyst. *Int. J. Biol. Macromol.* 2018, 120, 2147–2154. [CrossRef]

37. Jahromi, S.T.; Barzkar, N. Future direction in marine bacterial agarases for industrial applications. *Appl. Microbiol. Biotechnol.* 2018, 102, 6847–6863. [CrossRef] [PubMed]

38. Barzkar, N.; Homaei, A.; Hemmati, R.; Patel, S. Thermostable marine microbial proteases for industrial applications: Scopes and risks. *Extremophiles* 2018, 22, 335–346. [CrossRef]

39. Iqbal, R.; Barzkar, N.; Selvi, B.; Hamid, M.; Rahman, M. A novel thermotolerant PAE from marine sponges. *Mar. Drugs* 2010, 126, 120–124. [CrossRef] [PubMed]

40. Iqbal, R.; Barzkar, N.; Selvi, B.; Hamid, M.; Rahman, M. A novel thermostable PAE from marine sponges. *Mar. Drugs* 2010, 126, 120–124. [CrossRef] [PubMed]

41. Dhamodharan, D. Novel fibrinolytic protease producing *Streptomyces radiopugnans* VTSD8 from marine sponges. *Mar. Drugs* 2019, 17, 164. [CrossRef] [PubMed]

42. Sharma, C.; Osmolovskiy, A.; Singh, R. Microbial fibrinolytic enzymes as anti-thrombotics: Production, characterisation and prodigious biopharmaceutical applications. *Pharmaceutics* 2021, 13, 1880. [CrossRef]

43. Sodushwar, S.; Devi, C.S. Production of Fibrinolytic Protease from *Streptomyces Lusitanus* Isolated from Marine Sediments; IOP Conference Series: Materials Science and Engineering; IOP Publishing: Bristol, UK, 2017; p. 022048. [CrossRef]

44. Dhamodharan, D. Novel fibrinolytic protease producing *Streptomyces radiopugnans* VTSD8 from marine sponges. *Mar. Drugs* 2019, 17, 164. [CrossRef] [PubMed]
46. Kumar, S.S.; Haridas, M.; Sabu, A. Process optimization for production of a fibrinolytic enzyme from newly isolated marine bacterium *Seudomonas aeruginosa* KU1. *Biocatal. Agric. Biotechnol.* **2018**, *14*, 33–39. [CrossRef]

47. Kumar, S.S.; Haridas, M.; Abdulhameed, S. A novel fibrinolytic enzyme from marine *Pseudomonas aeruginosa* KU1 and its rapid in vivo thrombolysis with little haemolysis. *Int. J. Biol. Macromol.* **2020**, *162*, 470–479. [CrossRef]

48. Demina, N.; Veslopolova, E.; Gaenko, G. The marine bacterium *Alteromonas piscicida*—a producer of enzymes with thrombolytic action. *Izv. Akad. Nauk SSSR. Seria Biol.* **1990**, *3*, 415–419.

49. Vijayaraghavan, P.; Vincent, S.G. Statistical optimization of fibrinolytic enzyme production by *Pseudoalteromonas* sp. IND11 using cow dung substrate by response surface methodology. *SpringerPlus* **2014**, *3*, 60. [CrossRef][PubMed]

50. Gowthami, K.; Madhuri, R.J. Optimization of cultural conditions for maximum production of fibrinolytic enzymes from the local marine bacterial isolates and evaluation of their wound healing and clot dissolving properties. *J. Pharm. Res. Int.* **2021**, *33*, 246–255. [CrossRef]

51. Masilamani, R.; Natarajan, S. 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* **2015**, *70*, 1143–1149. [CrossRef]

52. Al Farraj, D.A.; Kumar, T.S.; Vijayaraghavan, P.; Elshikh, M.S.; Alkufeidy, R.M.; Alkubaisi, N.A.; Alshammari, M.K. Enhanced production, purification and biochemical characterization of therapeutic potential fibrinolytic enzyme from a new *Bacillus flexus* from marine environment. *J. King Saud Univ. Sci*. **2020**, *32*, 3174–3180. [CrossRef]

53. Pan, S.; Chen, G.; Zeng, J.; Cao, X.; Zheng, X.; Zeng, W.; Liang, Z. Fibrinolytic enzyme production from low-cost substrates by marine *Bacillus subtilis*: Process optimization and kinetic modeling. *Biochem. Eng. J.* **2019**, *141*, 268–277. [CrossRef]

54. Huang, S.; Pan, S.; Chen, G.; Huang, S.; Zhang, Z.; Li, Y.; Liang, Z. Biochemical characteristics of a fibrinolytic enzyme purified from a marine bacterium, *Bacillus subtilis* HQS-3. *Int. J. Biol. Macromol.* **2013**, *62*, 124–130. [CrossRef][PubMed]

55. Cheng, Q.; Xu, F.; Hu, N.; Liu, X.; Liu, Z. A novel Ca²⁺-dependent alkaline serine-protease (Bfsp) from *Bacillus* sp. With high fibrinolytic activity. *J. Mol. Catal. B Enzym.* **2015**, *117*, 69–74. [CrossRef]

56. Pan, S.; Chen, G.; Wu, R.; Cao, X.; Liang, Z. Non-sterile submerged fermentation of fibrinolytic enzyme by marine *Bacillus subtilis* harboring antibacterial activity with starvation strategy. *Front. Microbiol.* **2019**, *10*, 1025. [CrossRef]

57. Wu, R.; Chen, G.; Pan, S.; Zeng, J.; Liang, Z. Cost-effective fibrinolytic enzyme production by *Bacillus subtilis* WR350 using medium supplemented with corn steep powder and sucrose. *Sci. Rep.* **2019**, *9*, 6824. [CrossRef][PubMed]

58. Yao, Z.; Kim, J.A.; Kim, J.H. Properties of a fibrinolytic enzyme secreted by *Bacillus subtilis* JS2 isolated from saeu (small shrimp) jeotgal. *Food Sci. Biotechnol.* **2018**, *27*, 765–772. [CrossRef]

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

60. Vijayaraghavan, P.; Vincent, S.P. A low cost fermentation medium for potential fibrinolytic enzyme production by a newly isolated marine bacterium, *Shewanella* sp. IND20. *Biotechnol. Rep.* **2015**, *7*, 135–142. [CrossRef]

61. Anusree, M.; Swapna, K.; Aguilar, C.; Sabu, A. Optimization of process parameters for the enhanced production of fibrinolytic enzyme by a newly isolated marine bacterium. *Bioresource. Technol. Rep.* **2020**, *11*, 100436. [CrossRef]

62. Krishnamurthy, A.; Belur, P.D. A novel fibrinolytic serine metalloprotease from the marine *Serratia marcescens* subsp. *sakuensis*: Purification and characterization. *Int. J. Biol. Macromol.* **2018**, *112*, 110–118. [CrossRef]

63. Krishnamurthy, A.; Belur, P.D.; Rai, P. Studies on fibrinolytic enzyme from marine *Serratia marcescens* subsp. *sakuensis* (KU296189.1). *Int. J. Appl. Bioeng.* **2017**, *11*, 1–5.

64. Krishnamurthy, A.; Belur, P.D.; Rai, P.; Rekha, P.D. Production of fibrinolytic enzyme by the marine isolate *Serratia marcescens* subsp. *sakuensis* and its in-vitro anticoagulant and thrombolytic potential. *Pure Appl. Microbiol* **2017**, *11*, 1987–1998. [CrossRef]

65. de Barros, P.D.S.; e Silva, P.E.C.; Nascimento, T.P.; Costa, R.M.P.B.; Bezerra, R.P.; Porto, A.L.F. Fibrinolytic enzyme from *Arthrosira platensis* cultivated in medium culture supplemented with corn steep liquor. *Int. J. Biol. Macromol.* **2020**, *164*, 3446–3453. [CrossRef][PubMed]

66. e Silva, P.E.D.C.; de Barros, R.C.; Albuquerque, W.W.C.; Brandão, R.M.P.; Bezerra, R.P.; Porto, A.L.F. In vitro thrombolytic activity of a purified fibrinolytic enzyme from *Chlorella vulgaris*. *J. Chromatogr. B* **2018**, *1092*, 524–529. [CrossRef]

67. Pablos, E.; de Souza, F.; de Barros, R.; Marques, D.; Porto, A.; Bezerra, R. Enhanced production of fibrinolytic protease from microalgae *Chlorella vulgaris* using glycerol and corn steep liquor as nutrient. *Ann. Microbiol. Res.* **2017**, *1*, 9–19.

68. Silva, T.; Barros, P.; Silva, P.; Bezerra, R.; Porto, A. Effects of Metal Ions and Proteases Inhibitors on Fibrinolytic. Available online: [https://www.sbnmicrobiologia.org.br/29cbm-anais/resumos/15/R0528-2.PDF](https://www.sbnmicrobiologia.org.br/29cbm-anais/resumos/15/R0528-2.PDF) (accessed on 20 November 2021).

69. Wu, C.; Zheng, C.; Wang, J.; Jiang, P. Recombinant expression of thrombolytic agent repetase in marine microalga *Tetraselmis subcordiformis* (chlorodendrales, chlorophyta). *Mar. Drugs* **2021**, *19*, 315. [CrossRef][PubMed]

70. Krishnamurthy, A.; Munda, S.; Belur, P.D. Improving the catalytic efficiency of fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* by chemical modification. *Process Biochem.* **2018**, *75*, 79–85. [CrossRef]

71. Che, Z.; Cao, X.; Chen, G.; Liang, Z. An effective combination of codon optimization, gene dosage, and process optimization for high-level production of fibrinolytic enzyme in *Komagataella phaffii* (*Pichia pastoris*). *BMCL* Biotechnol. **2020**, *20*, 63. [CrossRef]

72. Verma, P.; Chatterjee, S.; Keizah, M.S.; Devi, S.C. Fibrinolytic protease from marine *Streptomyces rubiginosus* VTIPS1. *Cardiovasc. Hematol. Agents Med. Chem. (Former. Curr. Med. Chem.-Cardiovasc. Hematol. Agents)* **2018**, *16*, 44–55. [CrossRef]
73. Naveena, B.; Gopinath, K.P.; Sakthiselvan, P.; Partha, N. Enhanced production of thrombinase by Streptomyces venezuelae: Kinetic studies on growth and enzyme production of mutant strain. *Bioreour. Technol.* 2012, 111, 417–424. [CrossRef]

74. Sadeesh Kumar, R.; Rajesh, R.; Gokulakrishnan, S.; Subramanian, J. Screening and characterization of fibrinolytic protease producing Bacillus circulans from mangrove sediments pitchavaram, South east coast of India. *Int. Lett. Nat. Sci.* 2015, 28, 10–16.

75. Hwang, K.-J.; Choi, K.-H.; Kim, M.-J.; Park, C.-S.; Cha, J.-H. Purification and characterization of a new fibrinolytic enzyme of Bacillus licheniformis KJ-31, isolated from korean traditional Jeot-gal. *J. Microbiol. Biotechnol.* 2007, 17, 1469–1476. [PubMed]

76. Bajpai, P. *Xylanolytic Enzymes*; Academic Press: Cambridge, MA, USA, 2014.

77. Koffman, B.; Modarress, K.; Bashirelahi, N. The effects of various serine protease inhibitors on estrogen receptor steroid binding. *J. Steroid Biochem. Mol. Biol.* 1991, 38, 569–574. [CrossRef]

78. Weisel, J.W.; Litvinov, R.I. Fibrin formation, structure and properties. *Fibrous Proteins: Struct. Mech.* 2017, 82, 405–456.

79. Lam, K.; Lloyd, G.; Neuteboom, S.; Palladino, M.; Sethna, K.; Spear, M.; Potts, B. Natural Product Chemistry for Drug Discovery; Buss, A.D., Butler, M.S., Eds.; Royal Society of Chemistry Cambridge: Cambridge, UK, 2010.

80. Silva, R.; Ferreira, H.; Little, C.; Cavaco-Paulo, A. Effect of ultrasound parameters for unilamellar liposome preparation. *Ultrason. Sonochemistry* 2010, 17, 628–632. [CrossRef]

81. Agrebi, R.; Haddar, A.; Hajji, M.; Frikha, F.; Manni, L.; Jellouli, K.; Nasri, M. Fibrinolytic enzymes from a newly isolated marine bacterium Bacillus subtilis A26: Characterization and statistical media optimization. *Can. J. Microbiol.* 2009, 55, 1049–1061. [PubMed]

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

83. Mukherjee, A.K.; Rai, S.K.; Chattopadhyay, P.; Kar, S.K. Bafibrinase: A non-toxic, non-hemorrhagic, direct-acting fibrinolytic serine protease from Bacillus sp. strain AS-S20-I exhibits in vivo anticoagulant activity and thrombolytic potency. *Biochimie* 2012, 94, 1300–1308. [CrossRef]