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AN OVERVIEW ON MICROBIAL FIBRINOLYTIC PROTEASES

E. Venkata Naga Raju*[1] and G. Divakar [2]

Department of Biotechnology, Acharya Nagarjuna University [1], Nagarjuna Nagar, Guntur - 522 510, Andhra Pradesh, India
Department of Biotechnology & Microbiology, Acharya & B.M. Reddy College of Pharmacy [2], Soldevanahalli, Hesaraghatta, Banglore- 560107, Karnataka, India

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Correspondence to Author:

E. Venkata Naga Raju

Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur - 522 510, Andhra Pradesh, India

E-mail:venkatanagarajue@gmail.com

ABSTRACT: Microbial fibrinolytic proteases are of considerable interest in view of their specific activity and stability at biological pH. This review describes different isolation methods which enable the screening and selection of promising organisms for industrial production. Further, strain improvement using mutagenesis and/or recombinant DNA technology can be applied to augment the efficiency of the producer strain to a commercial status. The various nutritional and environmental parameters affecting the production of fibrinolytic proteases are delineated. The purification and properties of these fibrinolytic proteases is discussed, and the use of fibrinolytic proteases is highlighted.

INTRODUCTION: Enzymes are delicate protein molecules necessary for life. Enzymes are well known biocatalysts that perform a multitude of chemical reactions in the metabolism of almost all organisms viz., plants, animals, fungi, bacteria and viruses [1] and are commercially exploited in the detergent, food, leather processing, pharmaceutical, diagnostics, and fine chemical industries. Of the > 3000 different enzymes described to date the majority have been isolated from mesophilic organisms [2]. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Proteases are proteolytic enzymes which catalyze the hydrolysis of proteins based upon their structures or properties of the active site.

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There are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral and alkaline proteases [3, 4].

Proteases are obtained from plants, animal organs and microorganisms, with the majority obtained from microbial sources. Currently, a large proportion of commercially available proteases are derived from bacteria and fungi. Selection of the right organism plays a key role in obtaining high yield of desirable enzymes. On the other hand, it is a well-known fact that extra cellular proteases production in a microorganism is greatly influenced by media components, especially carbon and nitrogen sources and metal ions along with other growth parameters [4].

Cardio vascular such as high blood pressure, acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, stroke etc. are the primary causes of death. CVDs are the number one cause of death globally: more people die annually from CVDs

than from any other cause [5]. Throughout the world, according to data of World Health Organization (WHO) an estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths[5]. Of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke [6]. Low and middle income countries are disproportionally affected: over 80% of CVD deaths take place in low and middle-income countries and occur almost equally in men and women [5]. The number of people, who die from CVDs, mainly from heart disease and stroke, will increase to reach 23.3 million by 2030 [5, 6, 7]. CVDs are projected to remain the single leading cause of death [7].

Fibrinolytic protease is well known as a sub class of protease, which has an ability to degrade fibrin [8, 9, 10, 11, 12]. Fibrin is normally formed from Fibrinogen by the action of Thrombin (E C 3.4.21.5). It is sliced by plasmin (EC 3.4.21.7) which is activated from plasminogen by tissue plasminogen activator [13]. In balanced condition, fibrin clots are hydrolysed by plasmin to avoid thrombosis in blood vessels.

However, in unbalanced condition, as a result of pathophysiological disorders, the clots are not hydrolysed. Deposition of fibrin in blood vessels normally increases thrombosis and other cardiovascular diseases such as high blood pressure, acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, stroke etc. [14, 15].

Urokinase type plasminogen activator, tissue plasminogen activator, bacterial plasminogen activator and streptokinase are typical thrombolytic agents used for therapeutic purposes [16]. Based on working mechanism, thrombolytic agents are of two types, one is plasminogen activator which activates plasminogen into active plasmin to degrade fibrin and the other is plasmin like proteins which directly degrade fibrin [17]. Moreover, on the basis of catalytic mechanism, microbial fibrinolytic enzymes are classified into three types, serine protease (eg. Nattokinase), metallo protease (eg. Armillaria mellea metallo protease), mixture of both serine and metallo protease (eg.Protease from Streptomyces) [18]. Despite widespread uses, thrombolytic agents such as t-PA and urokinase are expensive. They exhibit low fibrin specificity and have undesired side effects such as gastrointestinal bleeding, resistance to repercussion and allergic reactions [19]. Therefore, continuous efforts have been focused in the search of safer and less expensive thrombolytic agents from diverse sources but one must respect biodiversity. Investigation of extracellular fibrinolytic enzymes is a central issue in enzymology due to their wide applications in clinical, pharmaceutical, food and bioremediation process.

Sources of Microbial Fibrinolytic Enzyme: Microorganisms are important resources for thrombolytic agents. Streptokinase from *Streptococcus hemolyticus* and Staphylokinase from *Staphylococcus aureus* were earlier proved to be effective in thrombolytic therapy [20]. Over the years, more fibrinolytic enzymes from various microbes have been discovered in succession, such as nattokinase (NK) from *Bacillus natto* and Subtilisin DFE and substilisin DJ-4 from *Bacillus amyloliquefacien* [21, 22, 23]. The microorganisms producing fibrinolytic enzymes including bacteria, actinomyces, fungi and algae. *Streptomyces megaspores* SD5, isolated from the water of a hot spring, can produce a strong thermo stable fibrinolytic enzyme [24].

Some kinds of fungi have also been found to produce the protease with high fibrinolytic activity for example *Asperigillusochraceus* 513 [25], *Fusariumoxysporum* [26], *Penicillumchrysogenum* [27], *Rhizopuschinesis* 12 [28]. In addition, Matsubara *et al* [29] found the fibrinolytic enzymes from marine algae *Codiumlatum*, *Codiumdivaricatum*, and *Codiumintricatum*. Lee *et al* [30] recently purified the fibrinolytic enzyme, designated as AMMP, from cultural mycelia of the mushroom *Armillariamella*.

The genus *Bacillus* from traditional fermented foods is an important one among the microorganisms that have been found to produce the fibrinolytic enzymes. In 1987, *B.natto* producing NK was first screened from a traditional Japanese soyabean fermented food named natto [23]. Subsequently some other *Bacilli* from different fermented foods were discovered to produce fibrinolytic enzymes. They are *B.amyloliquefaciens* DC-4 from the Chinese soyabean fermented food named douche [22], *Bacillus sp* CK from the Korean fermented soyabean sauce named Chungkook-Jang [31], *Bacillus sp*. strains DJ-4 from the Koreandoen-

jang [21, 32] and *Bacillus sp*.KA38 from the Korean salty fermented fish called Jeot-gal [33]. Yoon *et al* [34] systematically screened the fibrinolytic enzyme producing strains from many commercial and homemade fermented foods including natto, chungkook jang, doen jang, jeotgal, and the Indonesian fermented food and successfully isolated the strain *Enterococcus faecalis* producing higher fibrinolytic activity. These exciting findings imply the possibility of consuming fermented foods to prevent cardiovascular diseases. Suzuki *et al* [35] reported that dietary supplementation of natto could shorten euglobulin clot lysis time (ECLT), which is used to evaluate the total intrinsic fibrinolytic activity in plasma. At the time, dietary natto extract did not prolong bleeding time, indicating the safety of natto to be developed as a functional food.

Isolation and selection of Fibrinolytic Protease Microorganisms: The primary stage in the development of an industrial fermentation process is to isolate strain(s) capable of producing the target product in commercial yields. This approach results in intensive screening programs to test a large number of strains to identify high producers having novel properties. The conventional practice with many extracellular microbial products is to grow a large number of organisms on agar plate media and to relate each organism's production capability to the radius of the product's zone of diffusion around the colony. In the course of designing a medium for screening fibrinolytic proteases, it is essential that the medium should contain likely inducers of the product and be devoid of constituents that may repress enzyme synthesis.

Normally, fibrinolytic protease organisms are isolated by surface plating on a fibrin agar medium and subsequent screening for the desired characteristics. The organisms are further grown on specific media for estimating fibrinolytic, proteolytic, amylolytic, lipolytic, or cellulolytic activities using appropriate substrates such as fibrin, skim milk or casein, starch, tributyrin, butter fat, or carboxymethylcellulose [36, 37, 38, 39, 40, 41]. The isolates exhibiting desired level of activity are chosen and maintained on slants for further use. The most commonly used general medium for the isolation of fibrinolytic protease organisms was described by Astrup [42].

Production of Microbial Fibrinolytic Proteases: Most of the microorganisms produce fibrinolytic proteases, though interest is limited only to those that yield substantial amounts. It is essential that these organisms be provided with optimal growth conditions to increase enzyme production. The culture conditions that promote fibrinolytic protease production were found to be significantly different from the culture conditions promoting cell growth [43]. The cost of enzyme production and downstream process is the major obstacle against the successful application of protease in the industry [44]. For fibrinolytic enzymes, many attempts in laboratories have been done to improve expression of the fibrinolytic enzyme, including selection of an ideal culture medium, optimization of environmental conditions and over expression by genetically engineered strains.

Selection of medium components is usually critical for the fermentative production of fibrinolytic enzymes. Since different microbes possess diverse physiological characteristics, it is necessary to optimize nutrient components and environmental conditions for cell growth and fibrinolytic enzyme production [45, 46]. For instance, soluble starch or dextrin is the best carbon source for *B. amyloliquefaciens DC-4* due to the strong amylase activity [47, 48]. The optimal temperature of *S.megasporus SD5* for enzyme synthesis is 55^{o}C because the strain was isolated from a hot spring [49]. In some cases, fibrin was found to enhance the enzyme production, suggesting that fibrin, as a substrate of fibrinolytic enzyme, could activate or induce enzyme production during cultivation [48, 49].

Although the traditional one at a time optimization statergy is simple and easy, it frequently fails to locate the region of optimal response because the comprehensive effect of factors is not taken into consideration. Liu *et al* [50]employed the statistical methods fractional factorial design (FFD) and central composit design (CCD) to optimize the fermentation media for production of NK and finally increased the fibrinolytic activity to 1,300±60 U/ml, about 6.5 times higher than the original one [51].

Liquid fermentation is usually considered as the first choice for bacteria, whereas solid-state fermentation (SSF) is favoured by fungi. SSF has numerous advantages for enzyme production, such

as low waste water output, low operating costs, and large productivity. Tao *et al* [52, 53] systematically studied the production of the fibrinolytic enzyme from *F.oxysporum* with different SSF methods, largely increasing the production and reducing the cost. With a view to develop an economically feasible technology, research efforts are mainly focused on:

(i) Improvement in the yields of fibrinolytic proteases; and

(ii) Optimization of the fermentation medium and production conditions.

Improvement of yield by recombinant and non-recombinant methods: Strain improvement plays a key role in the commercial development of microbial fermentation processes. As a rule, the wild strains usually produce limited quantities of the desired enzyme to be useful for commercial application [54]. However, in most cases, by adopting simple selection methods, such as spreading of the culture on specific media, it is possible to pick colonies that show a substantial increase in yield [55]. The yield can be further improved by the use of mutagens or antibiotics and the adoption of special techniques or procedures for detecting useful mutants.

The advent of protein engineering and sophisticated molecular technologies has opened possibilities for screening variants of enzymes and tailor-made proteins from microorganisms with enhanced production yields which may be of interest for specific commercial applications [56]. Currently, two conceptionally different strategies are available for generation of protein-engineered variants: random and site-directed mutagenesis. With random mutagenesis, a large number of variants are produced, but the success of this approach largely depends on the availability of efficient screening procedures to identify variants with improved properties.

Site-directed mutagenesis depends on the access to structural or biochemical data to reduce the number of variants to be constructed, as every protein variant is purified and individually tested for improvements. For producing mutated enzymes, the two approaches are optimally used in combination with each other.

Promising variants generated and identified by random mutagenesis often can be improved by further site-directed introduction of known advantageous mutations. Recently, Lai *et al* [57] successfully doubled the specific activity of fibrinolytic enzyme through random mutagenesis *in vitro* by the chemical ethyl methane sulfonate (EMS).

In molecular biology applications, *Bacillus subtilis* has been recognized as a good host for the expression of foreign proteins with pharmacological activities because of its non pathogenecity and capability of screening functional extracellular proteins to the culture medium [58]. Subtilisin DFE was actively expressed in the protease deficient strain *B. substilis* WB 600 [59]. Furthermore, the native promoter of subtilisin DFE gene was replaced by that of α-amylase gene from *B. amyloliquefaciens DC-4*, resulting in a sharp increase in fibrinolytic activity from 80 to 200 urokinase units per millilitre [60]. Liu and song [61] succeeded in the functional expression of NK in *B.subtilis* as well. When expressed in *E.coli*, NK and subtilisin DFE formed insoluble aggregates without enzymatic activity [48].

However, two published papers reported the successful expression of active NK and subtilisin DFE in *E. coli* [62, 63]. Both papers took advantage of the principle that the extracellular protease subtilisin from the genus *Bacillus* is synthesized as Pre-pro enzymes, and its pro peptide may function as an intra-molecular chaperon to facilitate correct folding of protease domine [64, 65]. Zhang *et al* [63] showed that substilisin DFE was highly expressed in *E. coli* BL21(DE3) as fusion protein of Trx-prosubtilisin DFE via the expression vector pE32a and that strong fibrinolytic activity was detected in both soluble fraction and inclusion bodies fraction after in vitro renaturation. Moreover, the fusion proteins are easily purified and refolded in a column to activate enzyme. Most importantly, Trx-peptides can be automatically cleaved during in vitro refolding to form the mature subtilisin DFE.

Chiang *et al* [62] indicated that either nattokinase or pronattokinase could be over expressed in *E.coli* as a recombinant protein fused to the C terminus of olesin, a unique structural protein of seed oil bodies, by a linker polypeptide intein.

After reconstitution of artificial oil bodies, active NK was released through self-splicing of intein induced by temperature alteration and spontaneous cleavage of the propeptide [62].

Optimization of Fermentation medium: Fibrinolytic proteases are generally produced by submerged fermentation. In addition, solid state fermentation processes have been exploited to a lesser extent for production of these enzymes [66, 67, 68]. In commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly toward:

(i) Evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes;

(ii) Requirement of divalent metal ions in the fermentation medium; and

(iii) Optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation.

In addition, no defined medium has been established for the best production of fibrinolytic proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production.

Nitrogen source: In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components [69]. The fibrinolytic protease production is dependent on the availability of both carbon and nitrogen sources in the medium [68]. Although complex nitrogen sources are usually used for fibrinolytic protease production, the requirement for a specific nitrogen supplement differs from organism to organism. Low levels of fibrinolytic protease production were reported with the use of inorganic nitrogen sources in the production medium [43, 68, 70].

Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium [22, 24]. However, one report indicated no repression in the fibrinolytic protease activity with the use of ammonium salts [43]. An increase in fibrinolytic protease production by the addition of ammonium sulphate was also observed by Amrita Raja and Nancy Khess [43]. Similarly, sodium nitrate was found to be stimulatory for fibrinolytic protease production [43]. Substitution of silver nitrate in the basal medium with sodium nitrate increased enzyme production even more [71].

On the contrary, several reports have demonstrated the use of organic nitrogen sources leading to higher fibrinolytic protease production than the inorganic nitrogen sources [43, 44, 72, 73]. Tingwei and Rueilan[74] recorded maximum enzyme yields using a combination of 3% soybean meal. Rice chaff, Sessamum oil cake and Sunflower Oil Cake were found to be a cheap and suitable source of nitrogen by some workers [66, 68, 75, 76.]

Apart from serving as a nitrogen source, rice chaff, sessamum oil cake and sunflower oil cake also provide several micronutrients, vitamins, and growth-promoting factors. Suitable nitrogen sources as substitutes for rice chaff, sessamum oil cake and sunflower oil are still being evaluated. Peptone (1%) and yeast extract (1–2%) also serve as excellent nitrogen sources [44, 68]. Addition of certain amino compounds was shown to be effective in the production of extracellular fibrinolytic proteases by *Bacillus lichniformis* B4 local isolate [44]. However, aspartate appeared to have inhibitory effects on both protease and fibrinolytic protease production [77].

Carbon source: Studies have also indicated a reduction in fibrinolytic protease production due to catabolic repression by glucose [43, 44, 74, 78]. On the other hand the low yields of fibrinolytic protease production with the lowering of pH brought about by the rapid growth of the organism[66, 70, 79, 80, 81]. In commercial practice, high carbohydrate concentrations repressed fibrinolytic protease production. Therefore, carbohydrate was added either continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited and thereby reduce the power requirements [66].

Increased yields of fibrinolytic proteases were reported by several workers who used different sugars such as maltose [43, 44], mannitol [44], glucose [74, 78] and fructose [68, 77].

However, a repression in enzyme synthesis was observed with these ingredients at high concentrations.

Metal ion requirement: Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese, and molybdenum are required in the fermentation medium for optimum production of fibrinolytic proteases. However, the requirement for specific metal ions depends on the source of enzyme. The use of $MgSo_4$, $AgNO_3$, $CaCl_2$, $MnCl_2$ at a concentration of 0.1-0.5mM or NaN_3 at a concentration of 0.1- 0.5mM resulted in an increase in fibrinolytic protease activity in *Bacillus subtilis*, β-hemolytic [71, 73, 79], *Oidiodendron flavum* [82], *Schizophyllum commune* [83, 84], *Pseudomonas aeruginosa* [43], *Bacillus lichniformis* B4 [44], *Rhizomucor miehei* [78], *Ganoderma lucidum* VK 12 [85], *Escherichia coli* [86], *Candida guilliermondii* [66], *Bacillus cereus GD55* [77].

pH and temperature: The important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production. The culture pH also strongly affects many enzymatic processes and transport of various components across the cell membrane [87]. The optimum pH 5-8 reported for maximum fibrinolytic protease production by *Bacillus subtilis*, β-hemolytic [71, 73, 79], *Oidiodendron flavum* [82], *Schizophyllum commune* [83, 84], *Pseudomonas aeruginosa* [43], *Bacillus lichniformis* B4 [44], *Rhizomucor miehei* [78], *Ganoderma lucidum* VK 12 [85], *Escherichia coli* [86], *Candida guilliermondii* [66], *Bacillus cereus GD55* [77]. In view of a close relationship between fibrinolytic protease synthesis and the utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the fibrinolytic protease production, such as the start and end of the fibrinolytic protease production period.

Temperature is another critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood [88]. The optimum temperatures 30°C-40°C reported for maximum fibrinolytic protease production by *Bacillus subtilis* [67, 90], β-hemolytic [71, 73, 79], *Oidiodendron flavum* [82], *Schizophyllum commune* [83, 84], *Pseudomonas aeruginosa* [43], *Bacillus lichniformis* B4 [44], *Rhizomucor miehei* [78], *Ganoderma lucidum* VK 12 [85], *Escherichia coli* [86], *Candida guilliermondii* [66], *Bacillus cereus* [72, 77], *Cordyceps militaris* [80], *Penicillium chrysogenum* SGAD 12 [75], *Bacillus sphaericus* [89].

Aeration and agitation: During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be obtained by:

(i) Variations in the aeration rate;

(ii) Variations in the agitation speed of the bioreactor; or

(iii) Use of oxygen-rich or oxygen-deficient gas phase (appropriate air-oxygen or air-nitrogen mixtures) as the oxygen source [91, 92, 93].

The variation in the agitation speed influences the extent of mixing in the shake flasks or the bioreactor and will also affect the nutrient availability. Optimum yields of fibrinolytic protease are produced at 200 rpm for *Bacillus sphaericus* [89], *Candida guilliermondii* [66], *Streptomyces sp* CS684 at 160 rpm [70] and *Fusarium sp* BLB at 140 rpm [94]. In one study, *Bacillus* cereus NK1 produced increased enzyme titres when agitated at 300 rpm [72]. Similarly, *Bacillus subtilis* A26 exhibited maximum enzyme yield at an agitation rate of 200 rpm [79].

Recovery and Purification Of Fibrinolytic Proteases: Crude preparations of fibrinolytic proteases are generally employed for commercial use. Nevertheless, the purification of fibrinolytic proteases is important from the perspective of developing a better understanding of the functioning of the enzyme [81, 95, 96]. After successful fermentation, when the fermented medium leaves the controlled environment of the fermentor it is exposed to a drastic change in environmental conditions. The rapid lowering of the temperature of the fermented medium (<5°C) becomes indispensable to prevent microbial contamination as well as to maintain enzyme activity and stability.

The removal of the cells, solids, and colloids from the fermentation broth is the primary step in enzyme downstream processing, for which vacuum rotary drum filters and continuous disc centrifuges are commonly used [89, 97, 98, 99].

To prevent the losses in enzyme activity caused by imperfect clarification or to prevent the clogging of filters, it is necessary to perform some chemical pre-treatment of the fermentation broth before commencing separation [100, 101]. Furthermore, the fermentation broth solids are often colloidal in nature and are difficult to remove directly. In this case, addition of coagulating or flocculating agents becomes vital [97].

Flocculating agents are generally employed to effect the formation of larger flocs or agglomerates, which in turn accelerate the solid–liquid separation. Cell flocculation [97] can be improved by neutralization of the charges on the microbial cell surfaces, which includes changes in pH and the addition of a range of compounds that alter the ionic environment. The flocculating agents commonly used are inorganic salts, mineral hydrocolloids, and organic polyelectrolytes. For example, the use of a isooctane proved to be an effective extracting agent at 240 ppm and pH 4.0–7.0, and gave maximum yield of fibrinolytic protease activity [71].

Several schemes have been described for recovery and purification of fibrinolytic enzymes either from the commercially available crude preparations or the fermentation broths of various Microbial sp. viz., *Pseudomonas aeruginosa* [43], *Bacillus lichniformis* B4 [44], *Bacillus subtilis and Streptococci*[71], *Bacillus subtilis strain* GBRC1 [73], *Bacillus subtilis* A26 [79], *Bacillus sphaericus* [89], *Bacillus subtilis* A1 [90], *Bacillus subtilis* DC33 [118], *Bacillus sp* [119], *Candida guilliermondii*[66], *Streptomyces sp* [70 102, 103, 104, 105, 106], *Penicillium chrysogenum* SGAD12[75], *Rhizomucor miehei*[78], *Cordyceps militaris* [80], *Oidiodendron flavum* [82], *Schizophyllum commune* [84], *Ganoderma Lucidum* VK 12 [85] and *Escherichia coli* [86].

Mona *et al* [66] purified fibrinolytic enzyme from *Candida guilliermondii* by using ammonium sulphate saturation, dialysis followed by DEAE-Sepharose column to obtain a 7-8 fold increase in the purity. Jaya Ram Simkhada *et al* [70] purified fibrinolytic enzyme from *Streptomyces sp* by using ammonium sulphate saturation, dialysis, followed by Sepharose CL-6B column and Poros HQ gel filtration to obtain a 10-11 fold increase in the purity.

Jayalakshmi *et al* [73] purified fibrinolytic enzyme from *Bacillus subtilis strain* GBRC1 by using ammonium sulphate saturation, dialysis, followed by DEAE-Sephadex G-75 column to obtain 1-2 fold increase in the purity. Rym Agrebi [79] purified fibrinolytic enzyme from *Bacillus subtilis* A26 by using ammonium sulphate saturation, dialysis, CM-Sepharose, ultrafiltration, sephadex G-100 followed by Mono-Q Sepharose to obtain a 4-5 fold increase in the purity. Seon *et al* [80] purified fibrinolytic enzyme from *Cordyceps militaris* by using ammonium sulphate saturation, dialysis followed by Sephadex G-100 to obtain a 3-4 fold increase in the purity.

Nagwa *et al* [82] purified fibrinolytic enzyme from *Oidiodendron flavum* by using ammonium sulphate saturation, dialysis, DEAE- cellulose column followed by Sephadex G-25 gel filtration to obtain an 8-9 fold increase in the purity. Chung Lun Lu et al[84] purified fibrinolytic enzyme from *Schizophyllum commune* by using ammonium sulphate saturation, dialysis followed by Superdex 75 column to obtain a 9-10 fold increase in the purity. Sekar Kumaran [85] purified fibrinolytic enzyme from *Ganoderma Lucidum* VK 12 by using ammonium sulphate saturation, dialysis followed by DEAE-cellulose column and Sephadex G-100 gel filtration to obtain a 14-15 fold increase in the purity. Balaraman *et al* [89] purified fibrinolytic enzyme (thrombinase) from *Bacillus sphaericus* by using ammonium sulphate saturation, dialysis, Q-Sepharose column followed by Sephacryl S-300 gel filtration to obtain a 90-91 fold increase in the purity.

De Renzo *et al* [102] purified streptokinase from a relatively crude commercial preparation (Varidase; Lederle Laboratories, American Cyanamid, USA). Column chromatography on DEAE-cellulose was followed by column electrophoresis in sucrose density gradients to obtain a five- to six fold increase in purity. Taylor and Botts [104] attained a final specific activity of 100,000 units of streptokinase per mg of protein. This required a combination of ion exchange (DEAE-Sephadex A-50) and gel permeation (Sephadex G-100) chromatography. Tomar [103] purified streptokinase from varidase using a different procedure. Streptokinase was fractionated either by hydroxyl apatite chromatography or ammonium sulphate fractionation.

Precipitation of streptokinase with 40–50% ammonium sulphate resulted in a two to threefold increase in specific activity. The precipitate was recovered by centrifugation and dialyzed against 0.09 M sodium chloride. The dialyzed solution was further purified by gradient elution from a DEAE-cellulose chromatography column. The major peak of eluted activity was concentrated 10-fold by ultrafiltration.

A highly pure streptokinase was recovered from the relatively crude commercial Kabikinase (Kabi Vitram, Sweden) by Einarsson *et al* [105]. Ammonium sulphate fractionation was first used to obtain a crude precipitate of streptokinase. This was re-dissolved and subjected to gel permeation chromatography. The eluted streptokinase fraction was further purified using column chromatography on DEAE-cellulose or DEAE-Sepharose. Several affinity chromatography methods have been discussed for purifying streptokinase [107, 108, 109, 110].

The earliest such procedure used insolubilized di-isopropyl fluorophosphates (DIP) plasmin as the affinity ligand [107]. This ligand was produced by the conversion of plasminogen to plasmin with urokinase and inhibition of the proteolytic activity of plasmin by DIP [107]. Purification through the affinity column caused a 30% decrease in the streptokinase activity, suggesting incomplete inhibition of the plasmin affinity ligand bound to the chromatography matrix. Different affinity purification used a monoclonal antibody ligand [108].

Another affinity purification scheme used acylated plasminogen or plasmin as the affinity ligand[109]. The acylation of plasminogen or plasmin was carried out with p-nitrophenyl-p-guanidino benzoate (NPGB). Plasminogen acylation with NPGB allowed it to be used as an affinity ligand without requiring activation to plasmin. This probably reduced the plasmin associated proteolysis of streptokinase. Rodriguez et al.[109] used a combination of two affinity matrices for chromatographic purification of recombinant streptokinase. The affinity ligands were human plasminogen and monoclonal antibody against streptokinase. Both ligands were bound to Sepharose as the chromatographic matrix. This purification method produced a preparation with about 50,000 units of activity per mg of protein and a purity of >93%.

Use of immobilized NPGB acylated plasminogen for affinity purification was further reported by Liu *et al* [110]. A solution of urea was the eluent. Approximately ninefold purification was achieved with a yield of >90%. The specific activity of the purified material was 74,000 units/mg. Hernandez-Pinzon *et al* [111] recovered streptokinase by cross-flow ultrafiltration. Approximately 14% of the initial activity was lost as the protein solution was concentrated by eightfold. This loss was associated with denaturation of the streptokinase through unfolding and aggregation. Streptokinase has been purified from the filtrate of a streptococcal fermentation broth using hydrophobic interaction chromatography on phenyl- or octyl-Sepharose column. A gradient elution with 21% ammonium sulfate was used to recover the streptokinase. Further purification involved gel permeation and ion exchange chromatography steps.

Perez *et al* [106] purified a recombinant streptokinase produced by fermentation in E. coli K12. To isolate the streptokinase, the biomass was first recovered by centrifugation and then washed, and the cells were disrupted. The streptokinase inclusion bodies were then dissolved and re-natured. Hydrophobic interaction chromatography was then used to obtain the protein at a purity of >99%. The overall recovery yield was 49%. A similar yield of about 45% and a purity of >97% were attained by Zhang *et al* [112] in recovering recombinant streptokinase from inclusion bodies produced in E. coli. Generalized schemes for the recovery and renaturation of inclusion body recombinant proteins have been published [113].

Reverse-phase HPLC has been used for purifying a bovine plasminogen activator from culture supernatants of the bovine pathogen *S. uberis*[114]. A single protein with a molecular mass of 32 kDa was detected in the eluted active fraction. This plasminogen activator lacked the C-terminal domain that is characteristic of the *S. equisimilis* streptokinase [114].

Other work on *S. uberis* streptokinase has been reported [114, 115] and its mechanism of action has been subjected to closer scrutiny [116]. Commercial production of streptokinase requires special attention to bio safety considerations because the protein is potentially immunogenic to process workers.

In addition, care is necessary if streptokinase is being produced using natural strains of streptococci because all streptokinase producing streptococci are potentially pathogenic. The various safety considerations relevant to production of biopharmaceutical proteins have been discussed by [117]. Cheng Tao Wang *et al* [118] purified fibrinolytic enzyme from *Bacillus subtilis* DC33 by using ammonium sulphate saturation, dialysis, phenyl sepharose 6FF, DEAE-Sepharose FF column followed by Sephadex G-50 gel filtration to obtain a 34-35 fold increase in the purity. Nurulhanis Ahmad Sanusi *et al* [119] purified fibrinolytic enzyme from *Bacillus sp* by using ammonium sulphate saturation, dialysis, DEAE-Sepharose column followed by Sephadex G-75 gel filtration to obtain a 4-5 fold increase in the purity.

Biochemical characterization of Microbial Fibrinolytic Proteases: The enzymatic and physicochemical properties such as molecular weight, substrate specificity, optimal pH, optimal temperature and stability of fibrinolytic proteases from several microorganisms have been studied extensively. Some microbial fibrinolytic enzymes including those from *Streptomyces* [50, 120], *Armillariamellea* [30], and genus *Bacillus* [121, 122] have been purified and characterized. According to their catalytic mechanisms, these enzymes are classified into serine proteases (NK, subtilisin DFE, and CK) and metalloprotease [50, 120].

The fibrinolytic enzymes belonging to serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 8.0 and 10. The molecular weights of many microorganisms viz., *Pseudomonas aeruginosa*[43], *Bacillus lichniformis* B4 [44], *Bacillus subtilis and Streptococci* [71], *Bacillus subtilis strain* GBRC1 [73], *Bacillus subtilis* A26 [79], *Bacillus sphaericus* [89], *Bacillus subtilis* A1 [90], *Bacillus subtilis* DC33 [118], *Bacillus sp* [119], *Candida guilliermondii* [66], *Streptomyces sp* [70, 102, 103, 104, 105, 106], *Penicillium chrysogenum* SGAD12 [75], *Rhizomucor miehei* [78], *Cordyceps militaris* [80], *Oidiodendron flavum* [82], *Schizophyllum commune* [84], *Ganoderma Lucidum* VK 12 [85] and *Escherichia coli* [86] range between 27.7 and 44 kDa, and the isoelectric points are about 8.0 [21, 123], with the exception of BPDJ-2 (pI 3.5-3.7) [32].The optimal temperature has a wide range, between 30^{o}C and 70^{o}C [21, 31], mostly about 50^{o}C [59, 122].

Almost all serine fibrinolytic enzymes belong to subtilisin of *Bacillus* origin. They own the same catalytic triad made up of Ser221, His64, and Asp32 and have no intra-molecular disulfide bond. Their fibrinolytic activity can be irreversibly inhibited by phenyl methyl sulfonyl fluoride (PMSF), di isopropyl fluorophosphates (DFP), or E-64 [21, 31, 59, 79]. The fibrinolytic enzymes belonging to metalloprotease require divalent metal ions for their activities, for example Zn^{2+} for Jeot-gal [31], Ca^{2+} and Mg^{2+} for AMMP [45], Co 2+ and Hg^{2+} for enzymes from *Bacillus sp* .KDO-13 [45], so their activities can be inhibited by chelating agents such as EDTA. These enzymes have an optimal pH between 6.0 and 7.0, except one from *R.chinensis 12*, with an optimal pH of 10.5 [50].

All these fibrinolytic enzymes have high substrate specificity to fibrin, different from other proteases with broad substrate specificity. For instance, CK activity degrading fibrin is about eight times higher than that of substilisin Carlsberg, a common alkaline protease with identical N-terminal sequence [31]. Similar examples also include NK and Subtilisin E [125], Subtilisin DFE and subtilisin BPN [59] and subtilisin DJ-4 and subtilisin BPN [21]. The evolutionary changes of the critical amino acid residues in the substrate binding site probably account for this difference. However, more research should be done to completely elucidate this interesting phenomenon and provide some hints of the structure-function relationship. Furthermore, some microbial fibrinolytic enzymes can activate plasminogen and further enhance the fibrinolysis [31].

The fibrinolytic mechanism of NK has been explored more extensively than other microbial fibrinolytic enzymes. NK not only directly cleaves cross-linked fibrin, but also activates the production of t-PA, resulting in the transformation of inactive plasminogen to active plasmin [8, 126]. Sumi *et al* [23] reported the effectiveness of NK capsules in dissolving thrombi in dogs. After blood clots were experimentally induced in a major leg vein of male dogs, each dog was orally administered either four capsules of NK or placebo. The results showed that the blood clots in the dogs that received NK capsules completely dissolved within 5hr of treatment, and normal blood circulation was restored.

However, as a negative control, blood clots in dogs that have received the placebo did not show any sign of thrombolysis even after 18 hrs of treatment. Sumi *et al* [23] and Fujita *et al* [127] investigated the thrombolytic effect of NK on a thrombus in the common carotid of rat where the endothelial cells of the vessel wall have been injured by acetic acid. Animals treated with NK recovered 62% of the arterial blood flow, where as those treated with plasmin regained just 15.8%, and those treated with elastase did not get any recovery. It was concluded that the in vivo thrombolytic activity on NK is stronger than that of plasmin or elastase.

Another human trial involved 12 healthy Japanese volunteers (six men and six women, between 21 and 55 years old). Each participant had 200gm of natto daily before breakfast, and their fibrinolytic activity was tracked through a series of blood plasma tests. The results showed that oral administration of natto (nattokinase) enhanced the ability of participants' activity for 2 to 8 hrs after administration [23].

CONCLUSION: Thrombolytic diseases are today a major cause of morbidity and mortality worldwide. Microbial fibrinolytic enzymes have apparent significance in thrombosis therapy. Therefore great attention has been directed towards a search for microbial thrombolytic agents of various origins with particular reference to agents with more specificity and less toxicity. This review information helps in isolation of promising microbial fibrinolytic enzymes producers for industrial production, strain improvement, optimization, purification, and characterization.

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