Int. J. Biosci. Nanosci. Volume 4, Issue 07, 2017: 120-129

ISSN: 2349-5251

International Journal of Biosciences and Nanosciences

Journal homepage: www.ijbsans.com

Bacterial Proteases for Thrombolytic Activity-A Review

Ramasamy Amsaveni[1]*, Veluswamy Bhuvaneshwari[1], Manokaran Kalaiselvi[2]

[1]Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore - 641029, Tamil Nadu, India.

[2]Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India.

Received: May 2017; **Accepted;** June 2017

*Author to whom corresponding should be addressed

Email: amsabiotech@gmail.com

Abstract Bacteria are well known for their ability to excrete enzymes into the environment. *Bacillus* sp. being industrially important organisms produces a wide variety of extra-cellular enzymes including proteases. Enzymes are a specialized protein produced in an organism which is capable in catalyzing a specific chemical reaction. Enzymes are delicate protein molecules necessary for life. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers. Thrombotic diseases are responsible for alarming rates of mortality and morbidity worldwide. The clinical intervention to cure these disorders is carried out by the administration of thrombolytic agents. The commercial drugs available so far exhibit some side effects. So there should be immediate search for effective natural thrombolytic drug from the natural sources to overcome the side effects.

Keywords *Bacteria, Enzymes, Proteolytic, Thrombolytic drug*

1. Protease

Protease enzyme performs proteolysis, which is catabolism of protein by hydrolysis of the peptide bonds. Alkaline proteases have various applications in industrial products and processes such as detergents, food, pharmaceuticals and leather. For the alkaline protease production, a number of microbial strains were screened using skimmed milk agar media and gelatin hydrolysis method from different soil samples. Different fermentation parameters such as media, optimum media pH, optimum incubation and temperature were tried so far to optimize the maximum production of enzyme from the source organism (Priyanka *et al.*, 2015).

Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are (physiologically) necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale (Chouyyok *et al.*, 2005). They are generally used in detergents (Barindra *et al.*, 2006), food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Paranthaman *et al.*, 2009). Proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by Fungi. Proteases having pH optima in the range of 7.0 or around are called neutral protesaes. Neutral proteases are mainly of plant origin while proteases having optimum activity at pH range 8 and above are classified as alkaline proteases (Alnahdi, 2012).

Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins (Raju *et al.*, 1994; Haq *et al.*, 2006). The molecular weight of proteases ranges from 18-90 kDa. These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi. Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium (Sidney and Lester, 1972). Several species of strains including fungi (*Aspergillus flavus*, *A. melleu*, *A. niger*, *Chrysosporium keratinophilum*, *Fusarium graminarum*, *Penicillium griseofulvin*, *Scedosporium apiosermum*) and bacteria (*Bacillus licheniformis*, *B. firmus*, *B. alcalophilus*, *B. amyloliquefaciens*, *B. proteolyticus*, *B. subtilis*, *B. thuringiensis*) are reported to produce proteases (Ellaiah *et al.*, 2002).

The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of a microbial population. The most important among these are the medium, incubation temperature and pH. The pH of the fermentation medium is reported to have substantial effect on the production of proteases (Al-Shehri, 2004). However, some microorganisms produce heat stable proteases which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature (Al-Shehri, 2004; Haq *et al.*, 2006).

The extracellular proteases are commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (Fujita *et al.*, 1993). Of these, strains of Bacillus sp. dominate the industrial sector (Gupta *et al.*, 2002). Fibrinolytic protease is well known as a sub class of protease, which has an ability to degrade fibrin (Wong *et al.*, 2004).

Fibrinolytic drugs are widely used for the management of atherothrombotic diseases such as acute or prior myocardial or cerebral infarction, ischemic stroke and venous thromboembolism (Mahboubi *et al.*, 2012). Thrombolytic therapy has been a major advance in the management of acute myocardial infarction (White, 2010). Accumulation of fibrin in the blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular diseases (Kim and Choi, 2000). These diseases are the leading causes of death throughout the world (Mine *et al.*, 2005). Thrombolytic agents convert plasminogen to plasmin, lyse the clot by breaking down the fibrin contained in a clot. Currently fibrinolytic enzymes that dissolve blood clots and show promise for thrombosis therapy have been successfully identified from various sources. A wide range of microorganisms has been screened for their fibrinolytic properties (Takeno *et al.*, 1999).

Various thrombolytic agents have been used in the therapeutic treatment of thrombosis, but due to their high cost and hemorrhagic side effects, new sources of these agents have been sought after. Fibrinolytic enzymes produced by microorganisms, have the potential to inhibit blood coagulation and are able to degrade the fibrin. Some potential microorganisms like bacteria of the genus *Bacillus*, *Cyanobacteria*, fungi, and *Streptomyces* have been described as sources of fibrinolytic agents (Banerjee *et al.*, 2013). Thrombolytic drugs are widely used for the management of cerebral venous sinus thrombosis patients. Several *in vitro* models have been developed to study clot lytic activity of thrombolytic drugs, but all of these have certain limitations. There is need of an appropriate model to check the clot lytic efficacy of thrombolytic drugs (Prasad *et al.,* 2006).

Various types of thrombosis are responsible for an increasing number of deaths each year. The formation of a blood clot in a blood vessel (intravascular thrombosis) is one of the main causes of CVDs. The major protein component of blood clots, fibrin, is formed from fibrinogen via proteolysis by thrombin. Meanwhile, fibrin clots can be hydrolyzed by plasmin to avoid thrombosis in blood vessels. In an unbalanced situation due to some disorders, the clots are not hydrolyzed, and thus thrombosis occurs

(Lopez-Sendon *et al.*, 1995). So, several investigations are being pursued to enhance the efficacy and specificity of fibrinolytic therapy, and microbial fibrinolytic enzymes have attracted much more medical interest in recent decades (Tough, 2005).

To produce environmental eco-friendly products and product out puts chemical process are being replaced by enzymes like proteases (Abebe *et al.*, 2014). The production of enzymes is central to the modern biotechnology industry. The technology for producing and using commercially important enzyme products combines the discipline of microbiology, genetics, biochemistry and engineering .Enzymes are biocatalysts produced by living cells to bring about specific biochemical reaction generally forming parts of the metabolic processes of cells (Mohammad *et al.*, 2013).

Bacterium secretes proteases to hydrolyse the peptide bonds. Proteases are involved in digesting long protein chains into short fragments, splitting the peptide bonds that link amino acid residues. Some of them can detach the terminal amino acids from the protein chain (exopeptidases, such as amino peptidases, carboxypeptidase A); the other attack internal peptide bonds of a protein (endopeptidases, such as trypsin, chymotry-psin, pepsin, papain, and elastase). Proteases are also a type of exotoxin, which is virulence factor in bacteria pathogenesis. Bacteria exotoxic protease destroys extracellular structures. Protease enzymes used extensively in the bread industry as a bread improver. Most of the work is focused on alkaline protease producing thermophiles (Purva *et al.*, 1998).

The usage of protease for thrombolytic therapy by oral administration has assessed. Intravenous administration of urokinase and streptokinase has been widely used for thrombosis therapy but these enzymes have low specificity to fibrin and are expensive (Kim *et al.*, 1996). Although plasminogen activators and urokinase are still widely used in thrombolytic therapy today, their expensive prices and undesirable side-effects, such as the risk for internal hemorrhage within the intestinal tract when orally administrated, have prompted researchers to search for cheaper and safer resources. Therefore, microbial fibrinolytic enzymes have also attracted much more medical interest during recent decades. Based on the above ideas the presented study was carried out with the following objectives.

2. Alkaline proteases

Alkaline proteases are produced by mammalian tissues, higher animals, plants, fungi, actinomycetes, bacteria etc. However microbial alkaline proteases have gained significance in commercial production due to inherent advantages of microbial systems like short doubling times, less space requirement, easy genetic manipulations etc. Alkaline proteases of neutraphilic as well alkaliphilic bacterial, fungal and insect origins are utilized for commercial exploitation (Anwar and Saleemuddin, 1998).

3. Nitrogen source

Both organic and inorganic nitrogen sources are used for production of alkaline proteases. Inorganic nitrogen sources like ammonium sulfate, potassium nitrate and sodium nitrate were found to enhance the production (Sinha and Satyanarayana, 1991; Banerjee and Bhattacharyya, 1992). However organic nitrogen sources are found to be more scoring than the inorganic nitrogen sources in production of alkaline proteases (Chandrasekaran and Dhar, 1983; Chaphalkar and Dey, 1994). Soya bean meal increased production in several cases (Tsai *et al.*, 1988; Cheng *et al.*, 1995). Corn steep liquor (CSL) was also tried successfully by many researchers (Fujiwara and Yamamoto, 1987; Malathi and Chakraborty, 1991). Although addition of amino acids enhanced the production (Ikura and Horikoshi, 1987), addition of glycine and casamino acids has resulted in decreased enzyme production (Ong and Gaucher, 1976).

4. pH and Temperature

The pH of the medium has a huge influence over the enzyme production. The initial pH of the medium was kept at neutral for *Bacillus pumulis* c 172 (pBX 96) (Yaoyu *et al.*, 1997) and in few others an alkaline pH of 9.5 was maintained for alkaline protease production by *Bacillus* sp. GX 6644 (Durham *et al.*, 1987). In certain fermentation processes the pH of the medium maintained by addition of certain buffering agents

(Giesecke *et al.*, 1991). Temperature of the process has to be maintained throughout at an optimal level with control measures. Optimum temperature for alkaline protease production was found to vary in different bacteria. A temperature optimum of 30 °C, 35 °C and 37 °C were reported for *Bacillus pumulis, Bacillus licheniformis and Bacillus subtilis* respectively (Qiu *et al.*, 1990; Sen and Satyanarayana, 1993; Hameed *et al.*, 1996). However a temperature optimum of 55oC was recorded for *Bacillus coagulans* and *Bacillus stearothermophilus* (Gajju *et al.*, 1996; Dhandapani and Vijayaragavan, 1994).

5. Purification of the Enzyme

Purification is a crucial step in the study and application of a biocatalyst. Characterization, applicability and commercial potential of an enzyme depend on its ease of purification. But in general each purification step results in some amount of product or activity loss. In order to develop an economically feasible biocatalytic process it very important to research for a purification strategy that incurs minimal loss of product/activity. Till date several alkaline proteases from varying sources have been purified to homogeneity.

As such there are no specific rules in this regard but in general purification strategy of an alkaline protease usually comprises of few steps like separation of culture from fermentation broth by centrifugation or filtration followed by concentration of the culture supernatant since most of the alkaline proteases are extracellular in nature. The fermentation broth is concentrated by any one of the methods like ultrafiltration (Kang *et al.*, 1999; Smacchi *et al.*, 1999), salting out by solid ammonium sulphate (Kumar 2002; Hutadilok-Towatana *et al.*, 1999) or solvent extraction by using either acetone (Kumar et al. 1999; Thangam and Rajkumar 2002) or ethanol (El- Shanshoury *et al.*, 1995).

Other methods used for concentration of alkaline proteases include lyophilization (Manonmani and Joseph 1993) heat treatment of enzyme (Rahman *et al.*, 1994) or use of activated charcoal (Aikat *et al.*, 2001), or PEG-35,000 (Larcher *et al.*, 1996) or temperature-sensitive hydrogel (Han *et al.*, 1995). Affinity chromatography has been used for purification of alkaline proteases by several researchers. The most widely used affinity matrices include hydroxyapatite (Kobayashi *et al.*, 1996), Sephadex-4-phenylbutylamine (Ong and Gaucher, 1976), immobilized N-benzoyloxycarbonyl phenylalanine agarose (Larcher *et al.*, 1996), immobilized casein glutamic acid (Manonmani and Joseph 1993), aprotinin-agarose (Petinate *et al.*, 1999), and casein-agarose (Hutadilok-Towatana *et al.*, 1999). HIC basically uses the property of hydrophobicity to separate proteins from one another. HIC has been extensively used in FPLC in various columns, such as Mono-Q HR 5/5 (Rattray *et al.*, 1995; Smacchi *et al.*, 1999), Econo-pac Q (Yeoman and Edwards 1997), and Mono S 5/10 (Yum *et al.*, 1994).

Various natural protein substrates such as soya protein, whey, casein, gelatin *etc* are of used in preparation of highly functional protein hydrolysates (Fujimaki *et al.*, 1970; Perea *et al.*, 1993; Kumar and Takagi, 1999). These protein hydrolysates are used in infant food formulations and fortification of fruit juices and soft drinks (Neklyudov *et al.*, 2000; Ward 1985). Perea *et al* in 1993 used cheese whey which is an abundant liquid by-product of dairy industry to produce whey protein hydrolysate in an industrial bioconversion process by treating it with alkaline protease. Protein hydrolysates also play an important role in the regulation of blood pressure and are also used in therapeutic dietary products. Protein hydrolysate produced by treating sardine muscle with alkaline protease of *Bacillus licheniformis* has been reported with inhibitory activity over the angiotensin-I converting enzyme (Matsui *et al.*, 1993).

A protein hydrolysate rich in methionine with application in hypoallergenic infant food formulations has been produced from alkaline protease of *Bacillus amyloliquefaciens* by Kumar and Takagi in 1999. Proteolytic modification of soya proteins has been found to have an enhancing affect over its functional properties. Products like soya sauce were produced by treatment with proteases from olden days. Further, lean meat waste was converted into edible products by treatment with commercial alkaline proteases (O'Meara and Munro, 1984). Alkaline elastases and thermophilic alkaline proteases which efficiently catalyze the connective tissue proteins and muscle fiber proteins hold a great promise as meat tenderizing enzymes (Takagi *et al*., 1992; Wilson *et al*., 1992). Alkaline proteases with keratinolytic activity (B72 from *B. subtilis* and *B. licheniformis* PWD-1) have been used for converting feather waste and other keratin containing waste materials into proteinacious fodder for animals (Dalev, 1994; Cheng *et al*., 1995).

6. Thrombolytic activity

Thrombolytic therapy, with its ability to produce rapid clot lysis, has long been considered an attractive alternative. Thrombolytic drugs like tissue plasminogen activator (t-PA), urokinase, streptokinase etc. play a crucial role in the management of patients with CVST. In India though streptokinase and urokinase are widely used due to its lower cost, as compared to otherVarious methods were developed to measure the clot lysis activity of thrombolytic drugs. The best way to study thrombolytic drugs is through in vitro clot lysis model (Mosnier *et al*., 2010).

Fibrinolytic activity and serum antifibrinolysin were estimated in normal pregnant women, during and after labour. The decreased fibrinolytic activity found during labour returned to non-pregnant levels within24 hours of delivery. During the same period, the serum antifibrinolysin was rapidly diminished. It was suggested that the post-partum increase in fibrinolytic activity to non-pregnant levels due to alterations in the fibrinolytic system itself, as well as to changes in circulating antifibrinolysin (Tung *et al*., 2008).

Fibrinolytic drugs are widely used for the management of atherothrombotic diseases such as acute or prior myocardial or cerebral infarction, ischemic stroke and venous thromboembolism. Quite a lot of in vitro models have been developed to study clot lytic activity of fibrinolytic drugs, but all of these have certain limitations. There is need of a rapid method to check and quantify the clot lytic efficacy of fibrinolytic drugs precisely. In the present study, an attempt has been made to curtail two novel methods to study fibrinolysis in a simplified and easy way using standard fibrinolytic dugs, plasmin and streptokinase. Fibrin clots were allowed to form in microcentrifuge tubes using plasma separated from the whole blood from healthy mice or directly using fibrinogen and thrombin. After lysis by various doses of plasmin and streptokinase, fluid was removed and its volume was measured. Difference obtained in volume taken before and after plasma clot lysis was expressed as percentage of fibrinolysis. Recently blood clot formation has been a severe problem of blood circulation. Thrombus or embolus hinders the blood flow by blocking the blood vessel therefore depriving tissues of normal blood flow and oxygen. These consequence yield necrosis of the tissue in that area. Thrombin formed blood clot from fibrinogen and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). The purpose of a fibrinolytic drug is to dissolve thrombin in acutely occluded coronary arteries thereby to restore blood supply to ischemic myocardium, to limit necrosis and to improve prognosis (Laurence *et al*., 1992).

For the treatment of myocardial infarction, many thrombolytic agents are used. Among them, streptokinase is remarkable and widely used. Moreover, Tissue-type Plasminogen activator is more effective and safer than either urokinase or streptokinase type activators. It is noted that all available thrombolytic agents still have significant deficiencies, including the necessity of large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Therefore, steps are taken to develop improved recombinant variants of these drugs in order to minimize deficiencies of the available thrombolytic drugs (Adams *et al*., 2006)

7. Streptokinase

Streptokinase is an extracellular metallo-enzyme produced by beta-haemolytic *Streptococcus* and is used as an effective and cheap clot-dissolving medication in some cases of myocardial infarction (heart attack) and pulmonary embolism. It belongs to a group of medications known as fibrinolytics. Fibrinolytic enzymes producing *Aspergillus japonicum* KSS 05 strain were screened for the production by fibrin plate assay method. The maximum zone of fibrin hydrolysis were found 6 mm diameter. Further the *Aspergillus japonicum* KSS 05 were employed for the production by submerged fermentation and it showed 235 IU by pH 6, temperature 30 °C and 1 mL inoculums size (Yadav and Siddalingeshwara, 2015).

Streptokinase is one of the major blood-clot-dissolving agents used in many medical treatments. With the cloned streptokinase gene (skc), production of the secreted streptokinase from various *Bacillus subtilis* strains. The original skc promoter and signal sequence replaced with the *B. subtilis* levan sucrase promoter and signal sequence. *B. subtilis* is a modified skc produces streptokinase at a comparable level with WB600 as the expression host, a C-terminally-processed streptokinase was observed. Streptokinase derivatives resistant to C-terminal degradation were engineered. This showed a 2.5- would potentially be a thrombolytic agent (Elnager *et al.*, 2014).

8. Proteases from microorganisms

In a study *Trichoderma, Aspergillus, Penicillium, Rhizopus* and *Mucor* were isolated from alkaline soil samples using potato dextrose agar medium. Among the isolates three strains of *Aspergillus* spp., one *Mucor* sp. and one strain of *Curvularia* sp. showed greater proteolytic activity (Palanivel *et al.*, 2013). Fibrinolytic proteases were produced by solid state fermentation using agro industrial substrates. The results for fibrinolytic and protease activities, were obtained with *Mucor subtillissimus* UCP at a temperature of 25 °C for 72 hours. The optimum temperature for the produced enzyme was 45 °C and most of its original activity was retained after being subjected to 80 °C for 120 min. The specificity to chromogenic substrate and the inhibition by PMSF indicates that it is a chymotrypsin-like serine protease (Nascimento *et al.*, 2015).

Six bacterial colonies were isolated from soil and among these, GS-P4 isolate produced highest protease activity and was identified as *Bacillus* sp. by morphological and biochemical test various physiological characters were studied like pH, temperature, fermentation time. The isolated protease results of washing with detergent can be use as biotechnological tool for industrial purpose (Rupali, 2015). *Pseudomonas flourescens* AU17 was isolated from the fish discharged soil sample and it was tested for its ability to protease enzyme. The optimum conditions observed for protease production were temperature 37 °C, and pH 9, 1% wheat bran for carbon source, 0.5 % peptone for protein source.The bacterial isolate has potential that could be commercially exploited to assit in protein degradation in various industial processes (Vinoth *et al.*, 2014).

Ethyl methane sulfonate (EMS) and ethidium bromide treated *Bacillus cereus* GD 55 was proved for optimum production of fibrinolytic protease. The maximum fibrinolytic protease production was observed with fructose 1% inoculum size level 2%, pH 8.0, temperature 35 °C , NH_4NO_3 1% , peptone 1% and incubation time 48 hours in the production medium was studied. EMS&EB-15 mutant strains were found to produce 2-4 fold more enzyme. These findings have more impact on enzyme economy for biotechnological applications of microbial fibrinolytic proteases (Raju, 2013).

For the alkaline protease production, a number of microbial strains were screened using skimmed milk agar media and gelatine hydrolysis method from different soil samples. Among these, strain has been identified as *Bacillus*, on the basis of morphological and biochemical characters using Bergey's Manual of Determinative Bacteriology. Different fermentation parameters such as media, optimum media pH, optimum incubation and temperature were tried to optimize for maximum production of enzyme from the source organism and Luria Bertani media with 3% casein, pH-11 at 37 °C for 48 hours has been showing maximum enzyme production (Patil *et al.,* 2015).

Vijayaraghavan *et al.* (2013) performed the proteolytic activity of the isolates by using bromocresol green reagent on casein/skimmed milk agar plates. Later, a minimum of 0.0015% of bromocresol green dye was incorporated with the substrate agar plates before autoclaving to detect the proteolytic activity of bacteria. The proteolytic activity appeared as a colourless zone, while the rest of the plates were greenish-blue in colour which was pH dependent

S. glomeroaurantiacus VITSDVM6 was shown to be efficient producer of extra cellular protease, which can be beneficial for industries applications (Mohanasrinivasan *et al.*, 2014). Protease producing *Micrococcus luteus* B-07 was screened on milk nutrient agar plates; its identification was done by biochemical tests and genetic analysis. Production of fibrinolytic enzyme from *Micrococcus luteus* B-07 was achieved in soy meal wheat powder broth for which conditions were maintained at 33°C±2°C and pH 7.2. Fibrinolytic activity checked on fibrin agar plate against plasmin as standard and *in-vitro* blood clot lyses with 5% of blood clot. The effect of various environmental factors such as temperature, pH and various metal ions on crude enzyme was read at 660 nm (Aradhye *et al.*, 2015).

Rani and chaudhary (2015) isolated the strains BP1 and BP2 which produced proteolytic enzyme of 1.01μ/ml and 0.73μ/mL after the incubation of 42 hours and 72 hours, respectively at 37±2°C. As the time of incubation increase the proteolytic activity decrease. Both the isolated *Bacillus* strains were producing protease was novel and makes it potential for industrial application

Two strains of clinically isolated *S. aureus* were investigated for the fibrinolytic and thrombolytic properties. Antithrombotic assay results of strains JS7 and JS17 reveals that by prolonged clotting time, the formation of blood clot still occurred even with administration of high amount of the sample. These strains show the fibrinolytic activity and clot lytic activity. A zone of 6.4 mm and 6.5 mm diameter was measured in fibrin plate, which shows its fibrinolytic activity. Artificial blood clot in capillary tube was digested in both the petri plates containing JS7 and JS17 (Ravi *et al.*, 2014).

Protease were isolated and purified from *Bacillus subtilis* and also looked for its potential application in leather making process. The results of this study revealed that the bacterial strain *Bacillus subtilis* is a potent source for protease enzyme. The purification techniques have proceeded successfully without any major difficulties and resulted in an increase in protein concentration. Further, the leather sample processed by using protease is found to have maximum softness. The use of protease in leather processing could eliminate the use of pollution causing chemicals such as sodium, lime and solvents and greatly help to prevent environmental pollution (Sathiya, 2013).

Al-Juamily and Al-Zaidy (2013) selected isolates belonging to the genus *Bacillus* for production of fibrinolytic enzyme. The protease enzyme was purified by ammonium sulfate precipitation, ionic exchange with DEAE-cellulose and Sephacryl S-200 filtration. The purification of protease resulted in an enzyme with specific activity of 32.52 unit/mg protein with purification folds 30.11times. An optimum incubation temperature was 37 °C. Purified protease enzyme had a maximum activity at pH 7.0 of phosphate buffer. The molecular weight of the fibrinolytic enzyme was 50118 Dalton, Km and Vmax values of purified fibrinolytic enzyme respectively

Obeid *et al.,* (2015) screened and characterized *Bacillus* spp. that could produce a natural nattokinase with high activity. The study was carried out on 50 samples collected from different regions in Sudan. Primary screening and characterization of the microorganism showed that five samples (10%) were considered as *Bacillus subtilis* according to microscopic and biochemical characteristics. Selective medium was prepared for the extraction and production of nattokinase from these new isolates. The selected isolates *Bacillus subtilis* could produce active nattokinase with inhibition zone diameter ranged from 15-26 mm according to haemolysis and fibrinolytic activity.

9. Metalloproteases

Fibrinolytic enzymes were successively discovered from different microorganisms, the most important among which is the genus *Bacillus* from traditional fermented foods (Mine *et al.*, 2005). The physiochemical properties of these enzymes have been characterized, and their effectiveness in thrombolysis *in vivo* has been further identified. Therefore, microbial fibrinolytic enzymes, especially those from food-grade microorganisms, have potential to be developed as functional food additives and drugs to prevent or cure thrombosis and other related diseases (Hwang *et al.*, 2002). Fibrinolytic enzymes are mainly proteases. These catalyze total hydrolysis of proteins and specifically act on interior peptide bonds (Bayoudh *et al.*, 2000). All living cells produce different types of proteases, but the majority is produced by microorganisms. Many workers have reported that bacteria are high protease producers (Kalisz, 1988).

Proteases are grossly subdivided into two major groups, namely exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four families: serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960).

The fibrinolytic enzymes belonging to metalloprotease require divalent metal ions for their activities, for example Zn2+ (Kim *et al*., 1996), Ca2+ and Mg2+ for AMMP (Lee *et al*., 1999), Co2+ and Hg2+ for enzymes from *Bacillus* sp. KDO-13, 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 (Liu *et al*., 2005).

Acknowledgement: Authors are thankful to the Secretary, Principal and Department of Biotechnology of Kongunadu Arts and Science College for providing facilities.

References

Abebe B, Sago A, Admasu G, Getache H, Kassaand M. 2014. Isolation, Optimization and characterization of protease producing bacteria from soil and water in Gondar town, Northwest Ethiopia. Inte J Bacteriology, Virology and Immunology. 1(3):020-024.

Al-Juamily E, Al-Zaidy B. 2013. Purification and Characterization of Fibrinolytic Enzyme Produced from *Bacillus lichniformis* B4. International Journal of Research in Pure and Applied Microbiology. 2(5):256-266.

Alnandi HS. 2012. Isolation and Screening of Extraceullar Proteases Produced by new isolated *Bacillus* sp. Journal of Applied Pharmaceuticals Sciences. 2(9):071-074.

Al-Shehri MA. 2004. Production and some properties of protease produced by Bacillus licheniformis isolated from Tihamet Aseer, Saudi Arabic. Pakistan Journal of Biological Science. 7:1631-1635.

Anwar A, Saleemuddin H. 1998. Alkaline protease production by alkaliphilic marine bacteria isolated from Marsa-Matrouh (Egypt) with special emphasis on *Bacillus cereus* purified protease. African Journal of Biotechnology. 10(22):4631-4642.

Barindra S, Debashish G, Malay S, Joydeep M. 2006. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma Proteobacterium isolated from the marine environment of the Sundarbans. Process Biochemistry. 41:208- 215.

Chouyyok W, Wongmongkol N, Siwarungson N, Prichnont S. 2005. Extraction of alkaline protease using an aqueous two-phase system from cell free *Bacillus subtilis* TISTR 25 fermentation broth. Process Biochemistry. 40:3514-3518.

Dalev B. 1994. Bergey's manual of systematic bacteriology Vol. 2 Baltimore, MD, Williams and Wolkins 1986. Current Journal of Applied Science. 1(3):211-287.

Durham H, Demina N, Veslopolova F, Gaenko G. 1987. The marine bacterium *Alteromonas piscicida*-a producer of enzymes with thrombolytic action. Journal of Applied Biochemistry. 3:415-419.

Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B. 2002. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process Biochemistry. 38:615-620.

Fujita M, Nomura K, Hong K, Asada A, Nishimuro S. 1993. Purification and character of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. Biochemistry, Biophysics Research and Communication. 197:1340 -1347.

Giesecke T, *Egorov* N, Landau S, Milovanova F. 1991 Fibrinolytic activity in mono and mixed cultures of *Coryneform* bacteria. 10:86-90.

Gray C, Beg QK, Gupta R. 1985. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enzmye and Microbial Technology. 32:294-304.

Hameed Z, Andreeva A, Ushakova V, Egorov N. 1996. Study of proteolytic enzymes of various strains of *penicillium lilacinum* in relation to their fibrinolytic activity. Journal of Applied Microbiology. 4:417-422.

Haq IU, Mukhtar H, Umber H. 2006. Production of protease by Penicillium chrysogenum through optimization of environmental conditions. Journal of Agriculture Social Science. 2(1):23-25.

Hartley BS. 1960. Proteolytic enzymes. Annual Reviews in Biochemistry. 29:45-72.

Hutadilok-Towatana, Stepanova N, Maksimova A, Lulikova P, Silaev B. 1999. Fractionation of a preparation of fibrinolytic enzymes tricholysin formed from *Trichotecium roseum*

LK. Ex Fr.on carboxymethyl-sephadex C-50. Current Journal of Science. 12:407-410.

Kang L, Klocking HP, Markwardt F. 1999. Thrombolytic and pharmacodynamic properties of *Asperigillus ochraceus* protease. International Journal of Applied Science. 38: 341-349.

Kim SH, Choi NS. 2000. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp strain DJ-4 screened from Doen-Jang. Journal of Bioscience, Biotechnology and Biochemistry. 64:1722-1725.

Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y *et al*., 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp strain CK 11-4 screened from chungkook-Jang. Applied and Environmental Microbiology. 62(7):2482-2488.

Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y *et al*., 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus sp*. strain CK11-4 screened from Chungkook jang. Applied Environmental Microbiology. 62(7): 1488-2482.

Larcher Q, Rahman C, Aikat R. 2001. Production and properties of fibrinolytic enzyme in solid state cultures of *Fusarium pallidoorosem*. Current Journal of Science. 17:943-948.

Lee J, Park S, Choi WA, Lee KH, Jeong YK, Kong IS, Park S. 1999. Production of a fibrinolytic enzyme in bioreactor culture by *Bacillus subtilis* BK-17. Journal of Microbiology and Biotechnology. 9(4):443-449.

Liu JG, Xing JM, Chang TS, Ma ZY, Liu HZ. 2005. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochemistry. 40:2757-2762.

Lopez-Sendon, Kotb E. 1995. Fibrinolytic bacterial enzymes, with thrombolytic activity. world health organization. 3:978 -983.

Mahboubi A, Sadjady SK, Abadi MSS, Azadi S, Solaimanian R. 2012. Biological Activity Analysis of Native and Recombinant, Streptokinase. Iranian Journal of Pharmaceutical Research. 4:1087-1093.

Matsui F, Malke H, Ferretti. 1993. Streptokinase cloning expression and excretion by *Escherichia coli*. Journal of Applied Science. 81:57-61.

Mine Y, Wong AHK, Jiang B. 2005. Fibrinolytic enzymes in Asian traditional fermented foods. Food Research International. 38:243-250.

Mohammad BD, Mastan SA, Nizam R. 2013. Isolation, Characterization and Screening of enzyme producing Bacteria from different Soil Samples. International Journal of Pharma and Biosciences. 4(3):813-824.

Mohanasrinivasan.V, Subathra C, Lalitha M, shruthi P , Naine.S. 2014. Screening, molecular characterization and assessment of profound protease activity from Streptomyces glomeroauranticus VITSDVM6. International Journal of ChemTech Research. 6(8):4027 - 4032.

Nascimento T, Sales E, Porto C, Brandão R, Takaki G, Teixeira J *et al*., 2015. Production and Characterization of New Fibrinolytic Protease from *Mucor subtillissimus* UCP 1262 in Solid-State Fermentation. Advances in Enzyme Research. 3:81-91.

O'Meara, Munro R. 1984. Bergys manual of Determinative Bacteriology *Streptomyces rimoses* extacellular protease Characterization and evaluation of various crude preparations, European Journal of Applied Microbiology and Biotechnology. 8:81-90.

Obeid A, Alawad A.M, Ibrahim HM. 2015. Isolation and Characterization of *Bacillus Subtillus* with Potential Production of Nattokinase. International Journal of Advanced Research. 3:94-101.

Ong D, Gaucher H. 1976. Fibrinolytic activity of bacteria from *Pseudomonas* genus. International Journal of Applied Science. 27:845-849.

Paranthaman R, Alagusundaram K, Indhumathi J. 2009. Production of protease from rice mill wastes by Aspergillus niger in solid state fermentation. World Journal of Agriculture Science. 5(3):308-312.

Patil P, Sabale S, Devale A. 2015. Isolation and Characterization of Protease Producing Bacteria from Rhizosphere Soil and Optimization of Protease Production Parameters. International journal of current microbiology and applied science. 2:58 -64.

Prasad S, Agrawal A, Shirin M. 2006. Development of an in vitro model to study clot lysis activity of thrombolytic drugs. Journal of American Science. 3:1546-1784.

Priyanka M, Sheela B, Thangamani C. 2015. Isolation and Characterization of Protease Producing Bacteria from Rhizosphere Soil and Optimization of Protease Production Parameters. Original Research Article. 2:58-64.

Raju E. 2013. *Bacillus Cereus* GD 55 Strain Improvement by Physical and Chemical Mutagenesis for Enhanced Production of Fibrinolytic Protease. International Journal of Pharma Sciences and Research. 4:80-82.

Raju K, Jaya R, Ayyanna C. 1994. Hydrolysis of casein by Bajara protease importance. Biotechnology Coming Decades. 181:55-70.

Ramalakshmi N, Vangalapati M. 2012. Isolation and characterization Of Protease Producing Bacterial From Soil And Estimation Of Protease By Spectrophotometry . International Journal of Science and Technology. 1(1):1-7.

Rani P, Chaudhary N. 2015. Isolation and characterization of protease producing *Bacillus* sp from soil. International Journal of Pharma Sciences and Research. 6:633-640.

Ravi M, Subhashchandra M, Gaddad, Jayaraj Y. 2014. Identification of Antithrombosis, Fibrinolytic and clot lysis activity of *Staphylococcus aureus* in *In vitro*; International Journal of Research in Pure and Applied Microbiology. 4(2):39-42.

Rupali D. 2015. Screening and Isolation of Protease Producing Bacteria from Soil Collected from Different Areas of Burhanpur Region (MP) India. International Journal of Current Microbiology and Applied Science. 4(8):597-606.

Sathiya G. 2013. Prodution of protease from *Bacillus subtilis* and its application in leather making process. International Journal of Research in Biotechnology and Biochemistry. 3(1): 7-10.

Sexton MM, Jones AL, Chaowagul W, Woods ED. 1994. Canadian Journal of Microbiology. 40:903-910.

Sidney F, Lester P. 1972. Methods in Enzymology. Academic Press Incorporation, New York.

Smacchi S, Abdel Fattah F, Ismail AS, Saleh S. 1999. Purification and properties of two fibrinolytic enzymes from *Fusarium oxysporum* N.R.C.I. American Journal of Applied Science. 148:123-128.

Takeno, T., T. Okamura, M. Sera, M. Takana, S. Fukuda and M. Ohsugi, 1999. Screening of fibrinolytic enzymes of microorganisms. The Bulletin of Mukogawa's University Natural of Science, 47:67-72.

Tough 2005. Fibrinolytic bacterial enzymes, with thrombolytic activity. world health organization. 3:978-983.

Tsai R, Voet D, Voet J. 1998. Characterization and optimization of protease enzyme. American Journal of Science. 1:87-95.

Vinoth J, Murugan S, Stalin C. 2014. Optimizaton of alkaline protease production and its fibrinolytic activity from the bacterium *Pseudomonas flourescens* isolated from fish waste discharged soil. African journal of biotechnology. 13(30): 3052-3060.

White H. 2010. Thrombolysis for Acute Myocardial Infarction. Journal of American science. 109(6):2285-2292.

Wu Y, Abeles M . 1995. Partial purification and characterization of protease enzyme from Bacillus subtilis and *Bacillus megatherium*. Application of Biochemistry Biotechnology. 121-124:335-45.

Yadav S, Siddalingeshwara KG. 2015. Screening and biosynthesis of fibrinolytic enzyme from *Aspergillus japonicum*. Journal of Drug Delivery and Therapeutics. 5(6):60-62.

Yaoyu V, Berdzulishvili M, Afanaseva T, Alergant A. 1997. Fibrinolytic activity of pathogenic *Staphylococci* of different origins. Current Medical Chemistry. 6:332-334.

Yeoman JK, Edwards V.1997. Isolation and properties of the fibrinolytic enzyme from *Actinomyces thermovulgaris* culture broth. International Journal of Applied Science. 45: 455-459.