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Fibrinolytic enzymes of *Bacillus* spp.: an overview

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Abstract

Failed hemostasis leads to the formation of undesirable blood clots in the blood vessels leading to the condition called thrombosis, which is a primary cause of deaths among cardiovascular diseases world-wide. Conventional thrombolytic agents such as streptokinase, tissue plasminogen activator, urokinase, has several limitation such as higher cost of production as well as their side effects have forced the researchers to find alternative and safer fibrinolytic enzymes. Various fibrinolytic enzymes have been purified and characterized from different sources including fermented foods. Many such fermented foods are rich sources of fibrinolytic enzyme producing *Bacillus* spp. which have the potential to become candidates for preventing thrombosis related disorders. In this review, we have made an attempt to present the importance of *Bacillus* spp. as a source of fibrinolytic enzymes. Details about production, specificity, and diversity in N-terminal sequences and biotechnological approaches for its application has also been described.

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Introduction

Heart diseases accounts for the highest number of deaths all over the world (World life expectancy data, 2014). Thrombosis is one of the leading causes of mortality among cardiovascular diseases worldwide. According to National Commission on Macroeconomics and Health report, it was predicted that in India, there was about 64 million cardiovascular disease (CVD) cases in 2015 (NCMH, 2005). This indicates the high risk factor of CVDs in Indian population. An undesirable blood clot in the blood vessel is referred as "thrombus" (Lopez-Sendon *et al*., 1995). Although under normal physiological condition these fibrin blood clots in blood vessels will be automatically degraded by plasmin or fibrinolysin but under certain abnormal homeostasis condition blood clots are not lysed that leads to "thrombosis". Accumulation of this thrombus will hinder the smooth flow of blood and may lead to conditions such as myocardial infarction and related disorders. Thrombosis is defined by various pathological terms based on the site of formation of thrombus such as deep vein thrombosis and coronary thrombosis.

Mechanism of action of fibrinolytic enzymes

The inherent fibrinolytic enzyme in the body includes t-PA (tissue plasminogen activators), urokinase, these are plasminogen activators, which converts inactive plasminogen to active plasmin that degrades fibrin. Streptokinase and staphylokinase from bacterial sources also belongs to this category of plasminogen activators. Currently many of the above plasminogen activators are under clinical use to treat thrombosis condition. Besides its limitations of higher price, antigenicity of streptokinase and bleeding complications limits their usage (Bode *et al.*, 1996). The increasing percentage of cardiovascular cases and death due to thrombosis all over the world has attracted the researchers to identify alternative thrombolytic agents. Thus, it is essential to explore novel sources of fibrinolytic enzymes along with biotechnological approach to improve the same for the prevention and management of heart diseases globally. A large number of fibrinolytic enzymes have been identified from various sources such as Bacteria, fungi, snake venom and algae. Among bacteria, the members of the genus *Bacillus* have been studied largely for fibrinolytic enzymes and for their properties. Figure 1, indicates the formation of fibrin from fibrinogen and action of plasminogen activators, plasmin and *Bacillus* enzymes. Orally administrable thrombolytic agents have drawn the attention of researchers which includes functional foods or drugs along with the discovery of "Nattokinase" and its beneficial effects. The presence of fibrinolytic enzymes in food is a functional attribute. This has led to exploration for the similar and better kind of fibrinolytic enzyme extensively from various food sources. Development of novel and a better fibrinolytic enzyme is also a

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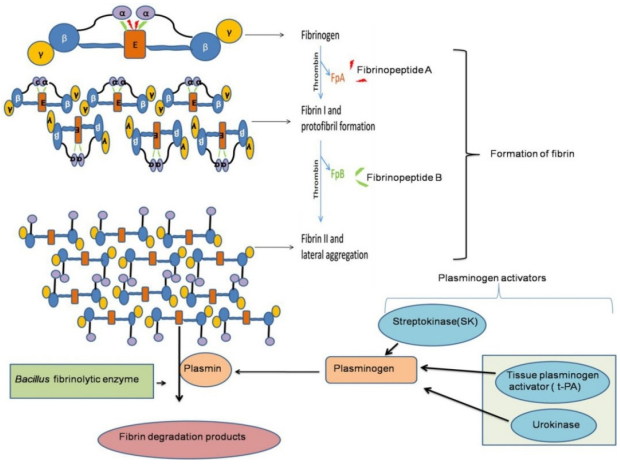

Figure 1. Formation and degradation of fibrin- Fibrinogen is composed of three polypeptides chains (Aα, Bβ and γ chains), action of thrombin on fibrinogen to release fibrinopeptide A and fibrinopeptide B in sequence leads to cross linking among fibrinogen molecules to form fibrin. Streptokinase, Tissue plasminogen activator and Urokinase acts on plasminogen and converts it into plasmin, which in turn degrades fibrin to fibrin degradation products. Bacillus enzymes generally are direct acting fibrinolytic enzymes on fibrin and forms fibrin degradation products.

prerequisite in the current era due to an alarming rate of cardiovascular disease cases all over the world (WHO, 2013). The diverse group of genus *Bacillus* have become the treasure for several fibrinolytic enzymes. The available literature gives the general account on the microbial fibrinolytic enzyme and in this mini-review, we have exclusively focused on *Bacillus* spp. for their fibrinolytic enzymes, application potential and future prospective.

Fibrinolytic enzymes

The enzymes that help in dissolving the undesirable blood clots are referred as fibrinolytic enzymes. Various fibrinolytic enzymes have been identified from fermented foods such as Natto, Chungkook-Jang, Tofuyo, fermented fish such as Skipjack Shikara, Kimchi, fermented shrimp paste, Douchi, Meju and many Asian fermented foods (Mine *et al.*, 2005). Major producers of fibrinolytic enzymes from these fermented foods were identified as *Bacillus* spp. Considering this, here we present an overview of food-grade *Bacillus* spp. as sources of fibrinolytic enzymes. In addition, various methods employed for identification and quantification of fibrinolytic activity have been described along with biotechnological and genomic approach for their enhanced production. Some of the important *Bacillus* species and their fibrinolytic enzymes with their molecular weights are summarized in Table 1.

The nattokinase was the first reported fibrinolytic enzyme from "natto" a traditional Japanese food, with proven reports on their health benefits, safe nature and with its low cost than conventional thrombolytics (Sumi *et al.*, 1987; 1989). In India, fermented foods associated with *Bacillus* are popular such as Bekang, Hawijar, Kinema, Tungrymbai, Tungtoh, Aakhone, Peruyaan, Bemerthu, Maseura, Idli, Dosa batters (Soni *et al.*, 1986; Soni, 2007; Tamang *et al.*, 2012). There are very few reports on these foods as a sources of novel fibrinolytic enzyme producing *Bacillus* spp. and hence needs further exploration.

Bacillus *as a source of fibrinolytic enzymes*

Fibrinolytic enzymes have been reported from various bacterial species including *Streptococcus, Staphylococcus, Vibrio, Paenibacillus, Chryseobacterium, Pseudomonas* (Kotb, 2013). However, *Bacillus* spp. are the major group of microorganisms that are well-known producer of fibrinolytic enzymes. Discovery of nattokinase from "Natto" a traditional food from Japan (Sumi *et al.*, 1987) and its beneficial effects, led to exploration of both fibrinolytic enzymes and such enzyme producing Bacillus species from many traditional foods. A large number of fibrinolytic enzymes have been reported from different *Bacillus* spp. from variety of sources (including certain marine source by Mahajan *et al.*, 2012), such as *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. cereus, B. firmus, B. sphaericus* (Fujita *et al.*, 1993; Kim *et al.*, 1996; Peng *et al.*, 2003; Seo and Lee, 2004). Most of these fibrinolytic enzymes belong to subtilisin family of proteases (Peng *et al.*, 2005). The N-terminal sequence of few important *Bacillus* fibrinolytic enzymes are presented in Figure 2. Among these fibrinolytic enzymes, conserved regions are presented in the box. There is also variation observed among each other at certain positions, which may result in diversity in the properties of fibrinolytic enzymes such as molecular weight, optimal pH and temperature (Peng *et al.*, 2005). Subtilisin DFE and Subtilisin QK-2 have same molecular weight of 28 kDa and indicates most amino acid sequence are conserved. However, slight variation in their sequence at certain position has changed their optimum pH and temperature for their activity. The intervention of research into this area may help in developing the safer thrombolytic agent for the therapeutic usage and help society in prevention and treatment of the disease. Analysis of the whole genome of different species in genus *Bacillus*, gives insight into the number of genes involved in the production of various proteases. These may help in identifying as well as understanding the potential of *Bacillus* which may help in exploring for new fibrinolytic enzymes.

Table 1. Reported *Bacillus* species with their fibrinolytic enzyme and their molecular weights

Bacillus sp.	Fibrinolytic enzyme	Molecular weight in kDa	Reference
B. subtilis			
B. subtilis	Nattokinase	27.7	Sumi *et al.*, 1987.
B. subtilis 168	Vpr and Wpr	63 and 52	Park *et al.*, 2002.
B. subtilis BK-17	BK-17	31	Jeong *et al.*, 2001.
B. subtilis	BK11	-	Jeong *et al.*, 2004.
B. subtilis QK-02	QK1 and QK2	42 and 28	Ko et al., 2004.
B. subtilis 168	Vpr and Wpr	-	Kho *et al.*, 2005.
B. subtilis A26	Subtilisin BSF1	-	Agrebi *et al.*, 2009.
B. subtilis KCK-7	KCK-7	44	Paik *et al.*, 2004.
B. subtilis TP-6	TPase	-	Kim *et al.*, 2006.
B. subtilis	FS33	-	Wang *et al.*, 2006.
B. subtilis LD-8547	LD8547	-	Wang *et al.*, 2008.
B. subtilis	Subtilisin E	-	Wong *et al.*, 1984.
B. subtilis K42	Co^{2+}-Metallo-protease K42	-	Hassanein *et al.*, 2011.
B. subtilis A1	Bacillokinase II	31.4	Jeong *et al.*, 2004.
B.subtilis ICTF-1	Fibrinolytic enzyme	28	Mahajan *et al.*, 2012.
B. subtilis BR21	Multiple proteases	14, 21, 35 and 46	Yogesh and Halami, 2015a.
B. subtilis YJ1	Nattokinase	27.5	Yin *et al.*, 2010.
Bacillus subtilis LD-8547	DFE	-	Yuan *et al.*, 2012.
B. subtilis KCTC 3014	Vpr	-	Choi *et al.*, 2010
B. amyloliquefaciens CH51	AprE51	27	Kim *et al.*, 2009.
B. amyloliquefaciens MJ5-41	AprE5-41	27	Jo *et al*., 2011.
B.amyloliquefaciens LSSE-62	Subtilisin DJ-4	-	Wei *et al.*, 2011.
B. amyloliquefaciens DC-4	Subtilisin DFE	28	Peng *et al.*, 2003.
B. amyloliquefaciens FCF-11	FCF-11	18.1	Kotb *et al.*, 2014.
B. licheniformis			
B. licheniformis KJ-31	bpKJ 31	37	Hwang *et al.*, 2007.
B. licheniformis CH3-17	AprE3-17	-	Jo *et al*., 2011.
B. licheniformis B4	-	50	Al-Juamily and Al-Zaidy, 2013.
Other *Bacillus* spp.			
Bacillus sp. DJ-4	Subtilisin DJ-4	29	Kim and Choi, 2000.
Bacillus sp.	SMCE	-	Fujita *et al.*, 1993.
Bacillus sp. KA 38	Jeot gal enzyme	41	Kim *et al.*, 1997.
B. vallismortis Ace02	Ace02	28	Kim *et al.*, 2007.
B. cereus NK1	URAK	-	Deepak *et al.*, 2010.
Bacillus sp. A5-S20-I	Bafibrinase	-	Mukerjee *et al.*, 2012.
B. spharicus	Thrombinase	18.6	Balaraman and Prabhakaran, 2007.
Bacilluis sp. CK-11	CK	28.2	Kim *et al.*, 1996.
Bacillus sp.DJ-2	bpDJ-2	-	Choi *et al.*, 2005.
Bacillus sp. KDO-13	-	45	Lee *et al.*, 2001.
B. natto NRRL 3666	-	-	Mahajan *et al*., 2010.
Bacillus polymaxa NRC-A	-	18.0	Mahmoud *et al*., 2011.
B. firmus NA-1	-	-	Seo and Lee, 2004.

Assay methods for the identification and quantification of fibrinolytic activity

There are several methods which have been employed for quantification of fibrinolytic activity. Some of the routinely used methods include, basic fibrin plate assay (Astrup and Mullertz, 1952), measuring liberated tyrosine (Anson, 1938), quantification through chromogenic synthetic substrates for plasmin such as D-Val-Leu-Lys-pNA (V7127) or using azocasein (Hummel *et al.,* 1965).

Fibrin Plate assay- Plate assay with several modifications have been developed and is still

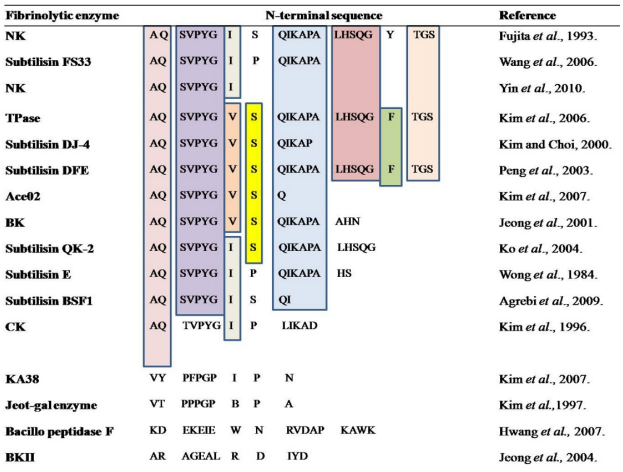

Figure 2. N-terminal sequences of fibrinolytic enzymes from *Bacillus* spp. (Sequence homology are presented inside the box)

a popular choice for the detection as well as quantification of fibrinolytic activity. Here, the formation of clear zone due to degradation of fibrin by the enzyme is considered as the base of fibrinolytic activity. Astrup and Mullertz (1952) have developed an improved fibrin plate method that involved the use of fibrinogen at the concentration of 0.1-0.2% and clotted using thrombin. The enzyme solution is being placed on the clotted surface and incubated for 18-24 hr at 37°C, and the area of fibrin digestion is quantitatively measured. The dimension of the area converted to concentration and measured using a reference curve. Many researchers have followed the above method with some modifications. Lassen (1953) and Batra (1966) described a modified fibrin plate method where congo red was used to stain plate surface containing fibrin film. Jespersen and Astrup (1983) have described optimal conditions and reproducible fibrin plate method.

In another method, fibrinogen solution (5 mg/7 ml of 0.1 M Barbitone buffer of pH 7.8) with thrombin (10 U) and mixed in 7 ml of agarose (1%) and plated. The plate is being heated at 80°C for 30 min for plasminogen free plate. Plasminogen (5 U) can be added in case of plasminogen rich plate method. The activity of the enzyme indicated by clear zone on the plate and is being quantified by measuring the diameter of the clear zone and by comparing with the standard graph generated by plasmin or urokinase at various concentrations (Wang *et al.*, 2006). In our laboratory, we have screened several Bacillus spp. for fibrinolytic enzyme production using fibrin plate assay. The representative plate-assay of results of our laboratory study is shown in Figure 3 (a).

In our study, we have employed a common protein staining dye for SDS-PAGE gels i.e. Coomassie Brilliant Blue (CBB R-250) to stain fibrin plates (Yogesh and Halami, 2015a). This method enables the visualization of clear zones on the fibrin plate and also provides the contrast between clear zone and un-degraded fibrin (with the blue background). Human plasma can be used in the place of fibrin to determine the fibrinolytic activity of the enzyme as followed by Wang *et al.* (2008) where, plasma (0.75%) was used as a base substrate and was added into agar prepared in Tris-HCl buffer (20 mM, pH 8) and thrombin (4 NIH Units)for the plate assay. In all the above methods the quantity of fibrin used in the assays differs based on the methods and modifications employed and also the enzyme solution is either placed on the surface or incorporated into the hole made in the plate. The quantification of fibrinolytic activity using fibrin plate is usually done using a standard curve generated by standard plasmin or urokinase (Wang *et al.*, 2008; Kotb, 2012).

Spectrophotometric methods to assess the fibrinolytic activity

Insolubility of fibrin poses an important limitation on assay of fibrinolytic enzymes as well to study their kinetics. The solubilised fibrin may not reflect the factual fibrinolytic activity of the enzymes, because under the natural conditions, fibrin is an insoluble protein. Measuring liberated Tyrosine- Many researchers have attempted explaining fibrinolytic activity using modified method of Anson (1938). In this method tyrosine liberated from degradation of fibrin is measured to quantify the enzyme activity. Here, enzyme preparation (100 µl) is being pre-incubated in 300 µl of reaction mixture containing 10 mM $CaCl_2$, 0.1 m Tris-HCl (pH 7.8) at 30°C for 5 min, followed by addition of 300 µl of fibrin solution (1.2% w/v). After incubation for 10 min, the reaction is stopped by addition of 600 µl of stop solution containing 0.22 M Sodium acetate, 0.33 M acetic acid and 0.11 M trichloroacetic acid. The reaction mixture is then centrifuged and the supernatant is measured at 275 nm. The fibrinolytic activity is measured with respect to tyrosine standard curve. One unit of activity is defined as the increase in absorbance of 0.01 per minute at 275 nm.

Using modified natural substrates

Many assay methods based on the use of modified natural substrates or the use of synthetic substrates are being routinely used for quantification and kinetics studies on fibrinolytic enzymes. Azocasein assay- The chromogenic protein- azocasein, was employed as a substrate for plasmin and the activity was measured by quantification of liberated p-nitroaniline (pNA) from degradation of azocasein

as described by Hummel *et al.* (1965).In this method, the enzyme preparation (100 µl) is mixed with 100 µl of azocasein (0.5%) and incubated at 37°C for 30 min. The reaction is being terminated with the addition of 10% ice-cold trichloroacetic acid (TCA). The reaction mixture was centrifuged at 10,000 rpm for 15 min and 400 µl of supernatant was gently mixed with an equal volume of 0.5 N NaOH. The colour developed as a result of liberated p-nitroaniline was read at 440 nm. The increase in 0.01 OD compared to control is defined as 1 unit of protease activity.

Using synthetic Plasmin substrates

V7127D (Val-Leu-Lys-pNA) is a well-known synthetic substrate used to assess the fibrinolytic activity. This method involves, 100 µl of reaction mixture containing 0.1 mM synthetic substrate, 5 µl of enzyme solution and 20 mM of Tris –HCl (pH 8.0) is incubated for 2 min at 37°C. Absorption is measured at 405 nm to quantify the p-nitroaniline (pNA) liberated from the degradation of substrate. One unit of activity is defined as nMol of substrate hydrolysed per minute per ml by the enzyme used.

T-4626 (Nα-p-Tosyl-L-arginine methyl ester or TAME) and Nα-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) are synthetic substrate for plasmin, used to measure esterase activity. The activity using these synthetic substrates are measured at 247 and 254 nm, respectively. One unit of esterase activity is defined as, an increase in absorbance of 0.01 OD during the first 5 min of the reaction at 37°C.

Zymogram assay

Zymogram was employed by many researchers to detect the presence of proteases using different protein substrates such as fibrinogen, gelatine and casein. This is one of the sensitive techniques for the detection of fibrinolytic enzymes (Kim *et al*., 1998). Briefly, the enzyme preparation is mixed with non-reducing 2X zymogram loading buffer (1:5 v/v) and loaded onto 10% acrylamide gels containing substrate fibrinogen (0.12%) along with 10 U of thrombin, and were run at 12 mA current at 4°C. The gel after electrophoresis, treated with 10 mM Tris-HCl buffer (pH 7.4) containing 2.5% triton X-100 for 20-40 min under constant shaking. The gel is then washed thrice with distilled water and incubated in zymogram reaction buffer containing 30 mM Tris-HCl (pH7.4) supplemented with 200 mM NaCl, 10 mM $CaCl_2$ for 12-18 h at 37°C. The staining is done using CBB (Coomassie Brilliant Blue-R250), followed destaining and observed for zone of clearance. The clear zones indicates the activity of the enzymes.

In our laboratory, we have isolated several native

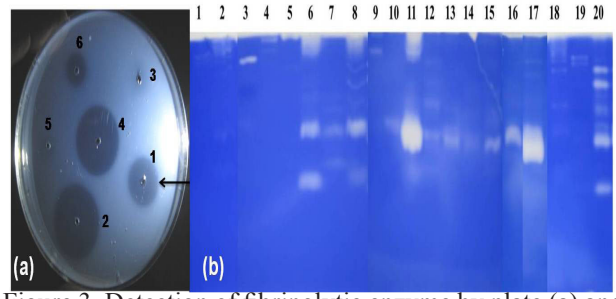

Figure 3. Detection of fibrinolytic enzyme by plate (a) and gel (b) assay.

(a) Culture filtrate of 1. *Bacillus subtilis* 168, 2, 4, 5, Culture filtrate of different native *Bacillus* isolates. 3. Negative control (Tris-HCl 20 mM (pH 7.4) and 6.Positive control (Human Plasmin). Arrow indicates clear zone of enzyme activity.
(b) Fibrin zymogram pattern of various *Bacillus* isolates form food sources. The cell free supernatants from different native *Bacillus* isolates (Lane 1-19) representing zymogram pattern of their fibrinolytic enzymes. 20- *B. subtilis* 168. Clear zone indicates the presence of fibrinolytic enzymes.

Bacillus spp. from various fermented food sources such as idli batter, dosa batters, Bekang, Hawaijar, cereals and legumes with the ability to produce fibrinolytic enzymes (Yogesh and Halami, 2015a; 2015b). The cultures were grown in Luria Bertani (LB) broth supplemented with 1% soybean powder. The cell-free supernatants of these were analysed using fibrin zymogram as indicated in Figure 3 (b).

Our results indicated the ability of the *Bacillus* to produce number of fibrinolytic enzymes ranging from one to many. Several *Bacillus* isolates produced multiple clear zones indicates the potential of *Bacillus* isolates to produce multiple proteases with fibrinolytic activity, indicating huge potential of *Bacillus* spp. as a treasure of many fibrinolytic enzymes. This vast potential of genus *Bacillus* to produce variety of fibrinolytic enzymes need to be exploited and individual enzymes need to be studied for the discovery and development of novel, better and safer fibrinolytic enzymes.

Diversity in fibrinolytic enzymes- N-terminal sequence of fibrinolytic enzymes

Many researchers have reported fibrinolytic enzymes with variation in their molecular weights. Table 1 indicates reported species of *Bacillus* and their fibrinolytic enzymes and variation in molecular weights. Figure 2, indicates the similarity and variation in N-terminal sequence of fibrinolytic enzymes and most of the reported fibrinolytic enzymes have shown their similarity.

Variation in amino acid sequences of fibrinolytic enzymes and their relation to the activity

Most of the fibrinolytic enzymes from *Bacillus*

spp. belongs to subtilisin family of serine proteases and inhibited by PMSF (Phenyl Methyl Sulphonyl Fluoride) and acts better on synthetic substrates of Plasmin. The N-terminal sequences of several fibrinolytic enzymes indicated that there were several conserved amino acids, however, the changes at certain key position in the sequence indicates the diversity in the sequence, which also attributes to diverse properties of the enzymes. Figure 2 represents the N-terminal sequence of important fibrinolytic enzymes and conserved amino acids are indicated in the box. The homology of the amino acid sequences of most of these fibrinolytic enzymes are similar to that of subtilisin. However, the molecular weight of most of these individual enzymes ranged from 18-63 kDa (Table 1).

The changes in the amino acid sequence has profound effect on the activity of the enzymes. For instance the enzyme CK from *Bacillus s*p.CK and Subtilisin DJ-4 from *Bacillus* sp. DJ-4 have amino acid sequence similarity to that of subtilisin, that are found to exhibit eight and four times higher activity respectively than their related enzyme Subtilisin Carlsberg (Kotb, 2013). The variations were also observed in their optimum pH, temperature range and stability. This property can be attributed to the replacement of amino acids at the key positions of the enzymes due to evolutionary changes. The detailed study on sequences and their effect on property of the enzyme may help designing an improved fibrinolytic enzymes with the replacement of amino acids and which are more potent and fibrin-specific as well as active at physiological condition.

Enhanced production of fibrinolytic enzymes

Mutation studies and genetic engineering has been employed for strain improvement and/or media optimization leads to enhanced production of fibrinolytic enzymes. Mahajan *et al.* (2012) have employed L18 – Orthogonal array method successfully for media optimization and which enhanced nattokinase production to 2.6 folds higher with the yield of 8814 U/ml compared to un-optimized media (3420 U/ml). Liu *et al.* (2005) have optimized media using statistical experimental method employing Central Composite Design (CCD) which showed nattokinase activity of 1300 U/ml. Agrebi *et al.* (2009) used hulled grain of wheat as inexpensive substrate for media optimization using Plackett-Burman statistical design and response surface methodology. The observed increase in fibrinolytic enzyme production was 4.2 fold compared with initial medium. The statistical methods were extensively employed to obtain optimal media composition and physiological condition for higher yield of enzyme. Liquid state fermentation is considered as the most preferred method for production of enzymes (Peng *et al.*, 2005; Mahajan *et al*., 2010). However, Chang *et al.* (2000) have used solid-state fermentation with wheat bran as medium for production of fibrinolytic enzyme from a mutant of *B. subtilis* IMR-NK and purified the enzyme up to 9.2 fold with the specific activity of 4400 U/mg protein. Deepak *et al.* (2008) used four variables (Glucose, Peptone, $CaCl_2$, and $MgSO_4$) at pH 7.5 and have employed response surface methodology (RSM) and central composite rotary design (CCRD) for fermentation media optimization for Nattokinase production by *B. subtilis.* The optimized media had the composition of Glucose -1%,Peptone- 5.5%, $MgSO_4$ - 0.2%, $CaCl_2$- 0.5% and optimized parameters were pH-7.5, 37^{o}C, 10 h. The study indicated the significant effect of peptone on Nattokinase production. Agrebi *et al.* (2009) have used hulled wheat grains as one of the media component and observed 4.2 fold increase in enzyme production from *B. subtilis* A26. In another study, Anh *et al.* (2015) showed that the shrimp shell powder also can be used as raw material for enzyme production and obtained 2.32 fold increase in enzyme production with *Bacillus* species M2. An interesting observation that $MgSO_4$ and $CaCl_2$ are found commonly in most of the optimized media and known to influence the production of fibrinolytic enzymes.

Mutation based enhancement

Mutation is one of the popular methods for the increased production of metabolites. Using this technique, several mutants of *Bacillus* were developed for the enhanced production of fibrinolytic enzymes. The mutant of *B. subtilis* IMR-NK1 was used for the production of natto (Chang *et al.*, 2000), by solid state fermentation using wheat bran as substrate. Zhang *et al.* (2006) have constructed four mutants of subtilisin to demonstrate the importance of hydrogen bonds in the catalytic triad for catalysis using molecular dynamics (MD) simulations. The results indicated that the H-bonds are important for catalysis and also for binding of substrates.

A strong fibrin-specific enzyme was purified and studied from a γ-radiated stable mutant *B. subtilis* LD 8547 over wild type enzyme producing strain (Wang *et al*., 2008).In another study, the method of site-directed mutagenesis was used to enhance the oxidative stability of subtilisin nattokinase that expressed in *E. coli* (Weng *et al*., 2009). A mutant library was generated by Yongjun *et al.* (2011) using three homologous genes from

Table 2. Heterologously expressed fibrinolytic enzymes of *Bacillus* spp., host and vector system employed

Fibrinolytic enzyme	Host	Vector system used	Reference
B. subtilis neutral proteases	*Lactococcus lactis* MG1363	pMG36e	Van de Guchte *et al.*, 1990.
Subtilisin DFE	*B. subtilis* WB600	pSUGV4	Peng *et al.*, 2004.
Subtilisin DFE	*B. subtilis* WB600	pSUGV4	Xiao *et al.*, 2004.
Subtilisin DFE	*E. coli* BL21(DE3)	pET32a	Zhang *et al.*, 2005.
Vpr	*E. coli* BL21(DE3)	pET-21a(+)	Kho *et al.*, 2005.
Nattokinase	*Lactococcus lactis* NZ9000	pXB622	Liang *et al.*, 2007.
AprE2	*B. subtilis* WB600	pHY3-5	Ju *et al.*, 2007.
Nattokinase	*Spodoptera frugiperda* (SF9) Insect cells	recombinant baculo virus, rv-egfp-NK	Li *et al.*, 2007.
Nattokinase	*B. subtilis*	T7 expression system	Chen *et al.*, 2010.
Vpr	*E. coli*	pGEX-2T	
Bacillopeptidase F	*B. subtilis* WB600	pHY300PLK	Kwon *et al.*, 2011.
AprE3-17	*B. subtilis* WB600	pHY300PLK	Jo *et al.*, 2011.
Subtilisin	*E. coli* BL21(DE3)	pET-15b	Ghasemi *et al.*, 2012.
AprE176	*E. coli* BL21(DE3)	pET26b(+)	Jeong *et al.*, 2015.

B. natto, *B. amyloliquefaciens* and *B. licheniformis* and demonstrated that the DNA family shuffling to improve the fibrinolytic activity of Nattokinase. They also analysed the surface conformational changes in the substrate binding pocket analysis through molecular modelling. Venkataraju and Divakar (2013) used physical mutagen (UV irradiation), chemical mutagens EMS (Ethyle Methane Sulfonate) and EtBr (Ethidium Bromide) for mutagenesis in strain improvement in *B. cereus* and obtained potent strain GD55 for optimum fibrinolytic protease production. An error prone PCR was employed for a major fibrinolytic enzyme coding gene apr176 from *B. subtilis* HK176. The cloned gene was over expressed in *E. coli* BL21(DE3). The mutant with one amino acid substitution showed highest fibrinolytic activity and also increased thermo-stability (Jeong *et al.*, 2015).

Enhancement using potent promoter or alteration in promoter region

Many researchers have employed promoter region of other genes or alteration in the promoter region of the fibrinolytic enzyme to enhance the production of fibrinolytic enzymes. The sequence coding for promoter and the signal peptide of α-amylase gene from *B. amyloliquefaciens* was fused with pro-peptide and mature peptide sequence of fibrinolytic enzyme Subtilisin DFE using an *E.coli/ B. subtilis* shuttle vector (pSUGV4) and Subtilisin DFE gene was successfully expressed in *B. subtilis* WB600 (Xiao *et al.*, 2004).The influence of altered promoter on nattokinase production was demonstrated by Wu *et al.* (2011), where the promoter of NK gene (PaprN) was altered, particularly in -10 region of promoter. This was found to enhance the nattokinase production to 136% and inferring that this can effectively enhance the production of heterologous protein in *B. subtilis.*

Heterologous expression of fibrinolytic enzymes

Several researchers have attempted to produce various fibrinolytic enzymes heterologously in genetically engineered *E. coli, Bacillus subtilis* and even lactic acid bacterial systems. Heterologous expression helps in controlled and enhanced production of fibrinolytic enzymes as well as in ease of purification than the conventional purification methods. Van de Guchte *et al.* (1990) have attempted and expressed a neutral protease gene nprE in *Lactococcus lactis* subsp. *lactis* strain MG1363, using lactococcal expression vector pMG36e. Similarly many fibrinolytic enzymes were expressed. Apart from bacterial cells, eukaryotic systems were also used to express fibrinolytic enzymes, a recombinant NK protein was successfully expressed in *Spodoptera frugiperda* insect cells by Li *et al.* (2007). Table 2 indicates some of the heterologously expressed fibrinolytic enzymes of *Bacillus* spp.

Specificity of fibrinolytic enzymes

The fibrinolytic enzyme is considered more

effective if they possess the characters like specific cleavage of fibrin compared to other proteins or RBCs (Red Blood Corpuscles). Most of the reported *Bacillus* fibrinolytic enzymes are serine proteases or metallo-proteases and acts best on synthetic substrate as plasmin (Peng *et al*., 2005; Kotb, 2013). A fibrinolytic enzyme from *Bacillus* sp. KA38 called as Jeot-gal enzyme was reported to have more preference towards fibrin than α- casein and RBCs (Kim *et al*., 1997). Another fibrinolytic enzyme with specificity towards the α-chain of fibrin was reported in our laboratory, wherein enzyme was found to degrade only the α-chain among the four chains of fibrin (Yogesh and Halami, 2015b). Hence, there is a greater need to identify fibrinolytic enzymes that are specific only to fibrin. However, microbial proteases show broad specificity towards other protein substrates too. In this direction, more number of fibrinolytic enzymes needs to be screened and characterized along with biotechnological approaches such as protein engineering, which may help in improving affinity and specificity of the enzyme towards fibrin. Yangisawa *et al.* (2010) have performed X-ray diffraction and reported the first X-ray diffraction analysis on crystallized Nattokinase (NK). They obtained the diffraction images using synchrotron radiation and the crystallized NK was found to be needle-like crystals and belong to space group C2. They also determined the X-ray structure of non-hydrogen form of undeuterated NK and succeeded in deuteration of NK, which could further facilitate in neutron crystallography that may help in understanding the mechanism of NK. The reported three-dimensional structure of NK and Subtilisin E from *B. subtilis* DB104 are nearly identical and based on the molecular simulation and prediction results it was found that the arrangement of hydrogen around the amino acid serine (Ser-221) probably account for specificity of NK towards the substrates (Yanagisawa *et al.*, 2013).

Safety studies on fibrinolytic enzymes from Bacillus *spp. and their clinical studies*

Sumi *et al.* (1990) have reported dissolution of induced thrombi in a dog model, where blood clots were experimentally induced in leg vein and NK (Nattokinase) capsules were orally administered and complete dissolution of clot was observed within 5 h, leading to restoration of normal blood circulation. Many fibrinolytic enzymes from *Bacillus* spp. were studied for their effect on dissolution of clots in animal models as well as human trials involving dietary supplementation of Natto (Sumi *et al*., 1989) or oral administration of fibrinolytic enzymes.

Omura *et al.* (2004) studied the anti-thrombotic and fibrinolytic effect of the NKCP in humans. The NKCP was extracted as a purified protein layer from *B. subtilis natto* fermentation and the adult subjects were administered with this preparation. A significant increase in fibrinolytic activity was observed in sub-acute and chronic studies where the NKCP was administered to 28 volunteers (for two weeks) and 23 volunteers (for several months). The observation was done for fibrinolytic effect with shortening of euglobulin lysis time. In both trials shortening in euglobulin lysis time was observed and no significant changes with other coagulation and fibrinolytic parameters. There was a significant change of shoulder stiffness with the improvement of local blood flow. The results indicated the observed fibrinolytic effect through oral administration of NKCP.

An improvement in shoulder stiffness was observed in the chronic study, suggesting an improved local circulation. While further studies are required to clarify NKCP's effect on the enhancement of fibrinolytic activity and its potential in preventing thrombosis. In the second study, where the active component in NKCP was identified as a 34 kDa protein, bacillopeptidase F (Omura *et al.*, 2005). The direct degradation of artificial blood clot in saline was observed using NKCP and the activity of protease was accounted for fibrinolytic effect. Prolonged PT (prothrombin time) and APTT (active partial thromboplastin time) were observed in rats models with intra-duodenum administration of dose-dependent study using NKCP. NKCP possessed both fibrinolytic and antithrombotic effect similar to heparin as observed in their *in vitro* and *in vivo* studies. The NKCP was derived from food and was considered as safe for clinical use based on animal experiments, along with preliminary clinical trials.

The *in vivo* acute toxicity was carried out by Yuan *et al.* (2012) with the fibrinolytic enzyme DFE from *B. subtilis* LD-8547 and results of the study indicated no obvious toxicity in mice model. The study employed carrageenan-induced thrombosis model. The tail thrombosis was effectively prevented by DFE and also thrombolytic activity of DFE was seen in carotid thrombosis *in vivo* model of rabbits, along with increase in bleeding and clotting time. Mukherjee *et al.* (2012) studied a direct acting serine protease with fibrinolytic activity for *Bacillus* sp. strain AS-S20-I, Bafibrinase. This was found to be non-toxic to HT29 cells or erythrocytes and did not act on collagen. There was no-toxic effect observed at a dose level of 2 mg/kg on mouse model and found to be safer.

A fibrinolytic enzyme from *B. amyloliquefaciens* FCF-11 was studied by Kotb *et al.* (2014). *In vitro* assays showed the direct action of enzyme on blood clots and also prolongation in clotting time of blood to 4.1-fold. The FCF-11 was found not to degrade collagen and also no-cytotoxic effect to HT29 cells or mammalian erythrocytes. The animal study using BALB/c mouse model showed no-toxic effect at a dose of 2 mg/kg as well as hemorrhagic activity. Even though large number of fibrinolytic enzymes have been studied from *Bacillus* spp. more thrust need to be given for their toxicity in order to make it safer for applications. In another study in which the natto preparation using *Bacillus subtilis natto* was fed to Spraque-Dawley male rats and inhibitory effect on platelet aggregation, along with shortening of euglobulin clot lysis time (ECLT) and prolongation in partial thromboplastin time (PATT) was found. The decrease in total cholesterol in serum was also observed in serum of the test animals (Park *et al*., 2012).

Biotechnological applications of fibrinolytic enzymes

The *Bacillus* species have a huge potential to secrete variety of proteases and most of these have the ability to degrade fibrin. Potent fibrinolytic enzyme have been expressed in other *Bacillus* spp. (Xiao *et al*., 2004) and *E. coli* (Kho *et al.*, 2005) for ease of purification, better yield, and in lactic acid bacterial system (Liang *et al.,* 2007) for possible starter cultures. Further, these fibrinolytic enzymes can be encapsulated in nano-capsules for higher stability and easy oral applications. Law and Zhang (2006) have encapsulated NKCP in Shellac, and about 60% retention in the enzyme activity after encapsulation. Shellac particles showed low permeability to acid. Ko *et al.* (2008) prepared alginate microparticles to assess the effect on fibrinolytic enzymes of Korean fermented soybean paste, Cheonggukjang (CGJ) and found that the encapsulated CGJ was stable at various range of pH and temperature compared to non-encapsulated CGJ. Similarly, Wei *et al.* (2012) have produced nattokinase from *B. subtilis* LSSE-22 on chickpeas as substrate and employed ethanol for extraction and precipitation of Nattokinase. Methacrylic acid –thylacrylate copolymer was used to encapsulate the nattokinase and to increase the stability at acidic pH. A variety of fibrinolytic enzymes have been reported from genus *Bacillus* that differ in their properties like molecular weight and substrate specificity and have the potential to become cost effective and orally administrable, direct acting thrombolytic agents. Experimental results on the effect of orally administered NK on canine (Sumi *et al*., 1990) and rat models (Suzuki *et al.*, 2003) as well as human trails (Sumi *et al.*, 1989; Omura *et al*., 2004) indicated their efficacy and safety and hence, NK has been commercially produced. These includes NSK-SD™, Cardiokinase™, Natto-K, Nattokinase NSK-SD, Orokinase, Nattozyme, Best – Nattokinase, Nattokinase- plus, Serracor-NK and Nattobiotic. Many *Bacillus* probiotics are available in the market and the ability of the *Bacillus* spp. to produce fibrinolytic enzymes is another property for probiotic bacteria which may add to its functionality. Hence, probiotic *Bacillus* strains need to be screened for fibrinolytic enzyme production ability as an additional beneficial attribute.

Genome sequence of *Bacillus subtilis*168 indicates the presence of genes which codes for several peptidases and proteases (Kunst *et al*., 1997). Thus, genomic comparison of related bacterial may help in analysing and exploration of new fibrinolytic enzymes. The N-terminal sequence of many fibrinolytic enzymes have showed many conserved amino acid sequences (Figure 2). However, significant changes are observed in their properties. Analysis of these sequences may help in understanding of mechanism and opportunities to improve specificity and potency of the enzyme through modification or replacement of amino acids sequences.

Conclusion

With the available information on fibrinolytic enzymes and as a future prospective, research need to be focussed on exploring novel fibrinolytic enzymes. A huge diversity is seen in species of *Bacillus* and also with their fibrinolytic enzymes. The detailed understanding of their sequences and properties may contribute to the development of novel affordable and safer thrombolytic agents to address the limitations of conventional thrombolytic such as

Cardiovascular diseases affects not only individual health but also the quality of life due to their high cost of treatment. Extensive studies on fibrinolytic enzymes promises to become a cost effective, safe and preventive solution for the management of heart diseases. The food-grade *Bacillus* spp. with the ability to produce fibrinolytic enzymes can be used for the formulation of functional foods and their enzyme preparation may become an alternative for conventional fibrinolytic molecules.

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References

Agrebi, R., Haddar, A., Hajji, M., Frikha, F., Mani, L., Jellouli, K. and Nasri, M. 2009. Fibrinolytic enzymes from a newly isolated marine bacterium *Bacillus subtilis* A26: characterization and statistical media optimization. Canadian Journal of Microbiology 55: 1049-1061.

Al-Juamily, E. F., and Al-Zaidy, B. H. 2012. Optimization conditions of production fibrinolytic enzyme from *Bacillus licheniformis* B4 Local Isolate. British Journal of Pharmacology and Toxicology 3(6): 289-295.

Anh, D.B.Q., Mi, N.T.T., Huy, D.N.A. and Hung, P.V. 2015. Isolation and optimization of growth condition of *Bacillus* sp. from fermented shrimp paste for high fibrinolytic enzyme production. Arabian Journal for Science and Engineering 40: 23–28.

Anson, M. L. 1938. The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. Journal of General Physiology 22: 79-89.

Astrup, T. And Mullertz, S. 1952. The fibrin plate method for estimating fibrinolytic activity. Archives of Biochemistry and Biophysics 40: 346-351.

Balaraman, K. and Prabhakaran, G. 2007. Production and purification of a fibrinolytic enzyme (thrombinase) from *Bacillus sphaericus.* Indian Journal of Medical Research 126: 459-464.

Barta, G. 1966. Dyed fibrin plate assay of fibrinolysis. Canadian Journal of Physiology and Pharmacology 44(2): 233–240.

Bode, C., Runge, M. and Smalling, R. W. 1996. The future of thrombolysis in the treatment of acute myocardial infarction. European Heart Journal 17: 55–60.

Chang, C. T., Fan, M.H., Kuo, F.C. and Sung, H. Y. 2000. Potent fibrinolytic enzyme from a mutant of *Bacillus subtilis* IMR-NK1. Journal of Agricultural and Food Chemistry 48: 3210-3216.

Chen, P.T., Shaw, J.F, Chao, Y. P., Ho, T.H.D. and Yu, S. M. 2010. Construction of chromosomally located T7 expression system for production of heterologous secreted proteins in *Bacillus subtilis.* Journal of Agricultural and Food Chemistry 58: 5392–5399.

Choi, N.S., Min, C.D., Park, C.S., Ahn, K.H., Kim, J.S., Song, J.J., Kim, S.H., Yoon, B.D. and Kim, M.S. 2010. Expression and identification of a minor extracellular fibrinolytic enzyme (Vpr) from *Bacillus subtilis* KCTC 3014. Biotechnology and Bioprocess Engineering 15: 446-452.

Choi, N.S., Yoo, K.H., Hahm, J.H., Yoon, K.S., Chang, K.T., Hyun, B.H., Maeng, P.J. and Kim, S.H. 2005. Purification and characterization of a new peptidase, bacillopeptidase DJ-2, having fibrinolytic activity: produced by *Bacillus* sp. DJ-2 from Doen-Jang. Journal of Microbiology and Biotechnology 15(1): 72–79.

Deepak, V., Ilangovan, S., Sampathkumar, M.V., Victoria, M.J., Pasha, S., Pandian, S. and Gurunathan, S. 2010. Medium optimization and immobilization of purified fibrinolytic enzyme URAK from *Bacillus cereus* NK1 on PHB nanoparticles. Enzyme and Microbial Technology 47: 297-304.

Deepak, V., Kalishwaralal, K., Ramkumarpandian, S., Venkatesh-babu, S., Senthilkumar, S.R. and Sangiliyandi, G. 2008. Optimization of media composition for nattokinase production by *Bacillus subtilis* using response surface methodology. Bioresource Technology 99: 8170-8174.

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

Ghasemi, Y., Dabbagh, F. and Ghasemian, A. 2012. Cloning of a fibrinolytic enzyme (subtilisin) gene from *Bacillus subtilis* in *Escherichia coli*. Molecular Biotechnology 52: 1–7.

Hassanein, W. A., Kotb, E., Awny, N. M. and El-Zawahry, Y.A. 2011. Fibrinolysis and anticoagulant potential of a metallo protease produced by *Bacillus subtilis* K42. Journal of Biosciences 36: 773–779.

Hummel, B.C.W., Schor, J. M., Buck, F.F., Boggiano, E. and Derenzo, E.C. 1965. Quantitative enzyme assay of human plasminogen and plasmin with azocasein as substrate. Analytical Biochemistry 11: 532-547.

Hwang, K. J., Choi, K.H., Kim, M.J., Park, C.S. and Cha, J. 2007. Purification and characterization of a new fibrinolytic enzyme of *Bacillus licheniformis* KJ-31 isolated from Korean traditional jeot-gal. Journal of Microbiology and Biotechnology 17: 1469-1476.

Jeong, S. J., Heo, K., Park, J. Y., Lee, K.W., Park. J.Y., Joo, S. H. and Kim J. H. 2015. Characterization of AprE176, a fibrinolytic enzyme from *B. subtilis* HK176. Journal of Microbiology and Biotechnology 25(1): 89–97.

Jeong, Y.K., Kim, J.H., Gal, S.W., Kim, J.E., Park, S.S., Chung, K.T., Kim, Y.H., Kim, B.W. and Joo, W.H. 2004. Molecular cloning and characterization of the gene encoding a fibrinolytic enzyme from *Bacillus subtilis* strain A1. World Journal of Microbiology and Biotechnology 20: 711–717.

Jeong, Y.K., Park, J.U., Baek, H., Park, S.H, Kong, I.S., Kim, D.W. and Joo, W.H. 2001. Purification and biochemical characterization of a fibrinolytic enzyme from *Bacillus subtilis* BK-17. World Journal of Microbiology and Biotechnology 17: 89–92.

Jespersen, J. and Astrup, T. 1983. A study of the fibrin plate assay of fibrinolytic agents. Optimal conditions, reproducibility and precision. Haemostasis 13: 301–315.

Jo, H.D., Lee, H.A., Jeong, S.J. and Kim, J.H. 2011. Purification and characterization of a major

fibrinolytic enzyme from *Bacillus amyloliquefaciens* MJ5-41 isolated from Meju. Journal of Microbiology and Biotechnology 21: 1166-1173.

Jo, H.D., Kwon, G.H., Park J.Y., Cha, J., Song, Y.S. and Kim, J.H. 2011. Cloning and over expression of apre3-17 encoding the major fibrinolytic protease of *Bacillus licheniformis* CH 3-17. Biotechnology and Bioprocess Engineering 16: 352-359.

Ju, J.S., Kwon, G.H., Chun, J., Kim, J.S., Park, C.S., Kwon, D.Y. and Kim, J.H. 2007.Cloning of fibrinolytic enzyme gene from Bacillus subtilis isolated from Cheonggukjang and Its expression in protease-deficient *Bacillus subtilis* strains. Journal of Microbiology Biotechnology 17(6): 1018–1023.

Kho, C.W., Park, S.G., Cho, S., Lee, D.H. and Myung, P.K. 2005. Confirmation of Vpr as a fibrinolytic enzyme present in extracellular proteins of *Bacillus subtilis.* Protein Expression and Purification 39: 1-7.

Kim, W., Choi, K. and Kim, Y. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK11-4 screened from Chungkook-Jang. Applied Environmental Microbiology 62: 2482–2488.

Kim, H.K., Kim, G.T., Kim, D.K., Choi, W.A., Park, S.H., Jeong, Y.K. and Kong, I.S. 1997. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. KA38 originated from fermented fish. Journal of Fermentation and Bioengineering 84(4): 307–312.

Kim, S.H., Choi, N.S. and Lee, W.Y. 1998. Fibrin Zymography: A Direct analysis of fibrinolytic enzyme on gels. Analytical Biochemistry 263: 115-116.

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

Kim, S.B., Lee, D.W., Cheigh, C. I., Choe, E. A., Lee, S.J.,Hong, Y.H., Choi, H.J. and Pyun, Y.R. 2006. Purification and characterization of fibrinolytic subtilisin like protease of *Bacillus subtilis* TP-6 from an Indonesian fermented soybean Tempeh. Journal of Industrial Microbiology and Biotechnology 33: 436-444.

Kim, J B., Jung, W.H., Ryu, J. M., Lee, Y.J., Jung, J. K., Jang, H.W. and Kim, S.W. 2007. Identification of a fibrinolytic enzyme by *Bacillus vallismortis* and its potential as a bacteriolytic enzyme against Streptococcus mutans. Biotechnology Letters 29: 605-610.

Kim, G. M., Ae, R. L., Kang, W. L., Jae, Y. P., Jiyeon, C., Jaeho, C., Young, S. S. and Jeong, H. K. 2009. Characterization of a 27 kDa fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH51 isolated from Cheonggukjang. Journal of Microbiology and Biotechnology 19(9): 997-1004.

Ko, J.H., Yan, J.P., Zhu, L. and Qi, Y.P. 2004. Identification of two novel fibrinolytic enzymes from *Bacillus subtilis* QK02. Comparative Biochemistry and Physiology C- Toxicology and Pharmacology 137: 65–74.

Ko, J.A., Koo, S.Y. and Park, H.J. 2008. Effects of alginate microencapsulation on the fibrinolytic activity of fermented soybean paste (Cheonggukjang) extract. Food Chemistry 111: 921–924.

Kotb, E. 2012. Fibrinolytic bacterial enzymes with thrombolytic activity. Berlin: Springer

Kotb, E. 2013. Activity assessment of microbial fibrinolytic enzymes. Applied Microbiology and Biotechnology 97: 6647-6665.

Kotb, E. 2014. Purification and partial characterization of a chymotrypsin -like serine fibrinolytic enzyme from *Bacillus amyloliquefaciens* FCF-11 using corn husk as a novel substrate. World Journal of Microbiology and Biotechnology 30: 2071–2080.

Kunst, F., Ogasawara, N., Moszer, I. et al. 1997. The complete genome sequence of Gram-positive bacterium Bacillus subtilis. Nature 390: 249-266.

Kwon, G.H., Park, J.Y., Kim, J.S., Lim, J., Park, C.S., Kwon, D.Y. and Kim, J.H. 2011. Cloning and expression of a bpr gene encoding bacillopeptidase F from *Bacillus amyloliquefaciens* CH86-1. Journal of Microbiology and Biotechnology 21(5): 515–518.

Lassen, N. 1953. Heat denaturation of plasminogen in the fibrin plate method. Acta Physiologica Scandinavica 27: 371.

Law, D. and Zhang, Z. 2006. Novel Encapsulation of a Fibrinolytic Enzyme (Nattokinase) by Shellac. XIVth International Workshop on Bioencapsulation, Lausanne.

Lee, S.K., Bae, D.H., Kwon, T.J., Lee, S.B., Lee, H.H. and Park, J.H. 2001. Purification and characterization of a fibrinolytic enzyme from *Bacillus* sp. KDO-13 isolated from soybean paste. Journal of Microbiology and Biotechnology 11: 845–52.

Li, X., Wang, X., Xiong, S., Zhang, J., Cai, L. and Yang, Y. 2007. Expression and purification of recombinant nattokinase in *Spodoptera frugiperda* cells. Biotechnology Letters 29: 1459–1464.

Liu, J., Xing, J., Chang, T., Ma, Z. and Liu, H. 2005. Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods. Process Biochemistry 40: 2757-2762.

Liang, X., Zhang, L., Zhong, J. and Huan, L. 2007. Secretoryexpression of a heterologous nattokinase in *Lactococcus lactis*. Applied Microbiology and Biotechnology 75: 95-101.

Lopez-Sendon, J., De Lopez, S.E., Bobadilla, J.F., Rubio, R., Bermejo, J. and Delcan, J.L.1995. The efficacy of different thrombolytic drugs in the treatment of acute myocardial infarction. Revista Española de Cardiología 48: 407-439.

Mahajan, P.M., Gokhale, S.V. and Lele, S.S. 2010. Production of nattokinase using *B. natto* NRRL 3666: media optimization, scale up and kinetic modelling. Food Science and Biotechnology 19: 1593–1603.

Mahajan, P. M., Nayak, S. and Lele, S. S. 2012. Fibrinolytic enzyme from newly isolated marine bacterium *Bacillus subtilis* ICTF-1: Media optimization, purification and characterization, Journal of Bioscience and

Bioengineering 113: 307–314.

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

Mahmoud, M. G., Ghazy, I. A., Ibrahim, S., Fahmy, A. S., El-Badry, M. O. and Abdel-Aty, A.M. 2011. Purification and characterization of a new fibrinolytic enzyme of *Bacillus polymaxa* NRC-A. International Journal of Academic Research 3: 542-547.

Mukherjee, A.K., Rai, S. K. Thakur, R., Chattopadhyay, P. and Kar, S. K. 2012. 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 94: 1300-1308.

NCMH, 2005. National Commission on macroeconomics and Health, Estimations and causal analysis, Burden of Diseases, Background paper in India.1-5.

Omura, K., Kaketani, K., Maeda, H. and Hitosugi, M. 2004. Fibrinolytic and anti-thrombotic effect of the protein from *Bacillus subtilis* (natto) by the oral administration. Journal of Japanese Society of Biorheology 18: 44–51.

Omura, K., Hitosugi, M., Zhu, X., Ikeda, M., Maeda, H and Tokudome, S. 2005. A newly derived protein from *Bacillus subtilis natto* with both antithrombotic and fibrinolytic effects. Journal of Pharmaceutical Science 99: 247 – 251.

Paik, H.D., Lee, S.K., Heo, S., Kim, S.Y., Lee, H and Kwon, T.J. 2004. Purification and characterization of the fibrinolytic enzyme produced by *Bacillus subtilis* KCK-7 from Chungkookjang. Journal of Microbiology and Biotechnology 14(4): 829–835.

Peng, Y., Huang, Q., Zhang, R. H. and Zhang, Y.Z. 2003. Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4. Comparative Biochemistry and. Physiology. B-Biochemistry and Molecular Biology 134: 45–52.

Peng,Y., Yang, X.J., Xiao, L. and Zhang, Y.Z. 2004. Cloning and expression of a fibrinolytic enzyme (subtilisin DFE) gene from *Bacillus amyloliquefaciens* DC-4 in Bacillus subtilis. Research in Microbiology 155(3): 167-173.

Peng, Y., Yong, X. and Zhang, Y. 2005. Microbial Fibrinolytic enzymes: an overview of source, production, properties and thrombolytic activity in vivo. Applied Microbiology and Biotechnology 69: 126-132.

Park, S.G., Kho, C.W., Cho, S., Lee, D.H., Kim, S.H. and Park, B.C. 2002. A functional proteomic analysis of secreted fibrinolytic enzymes from *Bacillus subtilis* 168 using a combined method of two-dimensional gel electrophoresis and zymography. Proteomics 2: 206-211.

Seo, J.H. and Lee, S.P. 2004. Production of fibrinolytic enzyme (KK) from soybean grits fermented by *Bacillus firmus* NA-1. Journal of Medicinal Food 7: 442–449.

Soni, S.K. 2007. Microbes: A source of energy for 21st century New India Publishing Agency. p. 79-81. New Delhi.

Soni, S., Sandhu, D., Vikhu, K. and Kamra. K. 1986. Microbiological Studies on dosa fermentation. Food Microbiology 3(1): 45-53.

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

Sumi, H., Hamada, H., Mihara, H., Nakanishi, K. and Hiratani, H. 1989. Fibrinolytic effect of the Japanese traditional food natto (nattokinase).Thrombosis and Haemostasis 62(1): 549.

Sumi, H., Hamada, H., Nakanishi, K. and Hiratani, H. 1990. Enhancement of fibrinolytic activity in plasma by oral administration of Nattokinase. Acta Haematologica 84: 139-143.

Suzuki, Y., Kondo, K., Ichise, H., Tsukamoto, Y., Urano, T. And Umemura, K. 2003. Dietary supplementation with fermented soybean suppresses intimal thickening. Nutrition 19: 261–264.

Tamang, J. P., Tamang, N., Thapa, S., Dewan, S., Tamang, B., Yonzan, H., Rai, A.M., Chettri, R., Chakrabarty, J. and Kharel, N. 2012. Microorganism and nutritional value of ethnic fermented foods and alcoholic beverages of north India. Indian Journal of Traditional knowledge 11: 7-25.

Van de Guchte, M., Kodde, J., Van der vossen, J. M. B. M., Kok, J. and Venema, G. 1990. Heterologous gene expression in *Lactococcus lactis* subsp. *lactis*: synthesis, secretion, and processing of the Bacillus subtilis. Neutral protease. Applied and Environmental Microbiology 56(9): 2606-2611.

Venkatanagaraju, E. and Divakar, G. 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(5): 81-93

Wang, C., Ji, B., Li, B. and Ji, H. 2006. Enzymatic properties and identification of a fibrinolytic serine protease purified from *Bacillus subtilis* DC33. World Journal of Microbiology and Biotechnology 22: 1365-1371.

Wang, S.H., Zhang, C., Yang, Y.L., Diao, M. and Bai, M.F. 2008. Screening of high fibrinolytic enzyme producing strain and characterization of fibrinolytic enzyme produced from *B. subtilis* LD-8547. World Journal of Microbiology and Biotechnology 24: 475-482.

Weng, M. Z., Zheng, Z. L., Bao, W., Cai, Y.J., Yin, Y. and Zou, G.L. 2009. Enhancement of oxidative stability of the subtilisin nattokinase by site-directed mutagenesis expressed in *Escherichia coli.* Biochimica Biophysica Acta (BBA) - Proteins and Proteomics 1794(11): 1566–1572.

Wei, X., Luo, M., Xie, Y., Yang, L., Li, H., Xu, L. and Liu, H. 2012. Strain screening, fermentation, separation, and encapsulation for production of nattokinase functional food. Applied Biochemistry and Biotechnology 168(7): 1753-1764.

Wei, X., Luo, M., Xu, L., Zhang, Y., Lin, X., Kong P. and Liu, H. 2011. Production of fibrinolytic enzyme from *Bacillus amyloliquefaciens* by fermentation of chickpeas, with the evaluation of the anticoagulant and antioxidant properties of chickpeas. Journal of Agricultural and Food Chemistry 59: 3957–3963.

WHO. 2013. Downloaded from *–http://www.who.int/mediacentre/facts heets/fs317/en/-* on 26/04/2014.

World life expectancy data. 2014. Downloaded from-*http://www.world lifeexpectancy.com/world-rankings-total-deaths* -on 29/10/2015.

Wong, S.L., Price, C.W., Goldfarb, D.S. and Doi, R.H. 1984. The subtilisin E gene of *Bacillus subtilis* is transcribed from a sigma-37 promoter *in vivo.* Proceedings of National Academy of Science USA 81: 1184–1188.

Wu, S.M., Feng, C. Zhong, J. and Huan, L.D. 2011. Enhanced production of recombinant nattokinase in *Bacillus subtilis* by promoter optimization. World Journal of Microbiology and Biotechnology 27: 99–106.

Xiao, L., Zhang, R.H., Peng, Y. and Zhang, Y.Z. 2004. Highly efficient gene expression of a fibrinolytic enzyme (subtilisin DFE) in Bacillus subtilis mediated by the promoter of α-amylase gene from *Bacillus amyloliquefaciens*, Biotechnology Letters 26: 1365-1369.

Yin, L. J., Lin, H.H. and Jiang, S.T. 2010. Bio-properties of potent nattokinase from *Bacillus subtilis* YJ. Journal of Agricultural and Food Chemistry 58: 5737-5742.

Yogesh, D. and Halami, P. M. 2015a. Evidence that multiple proteases of *Bacillus subtilis* can degrade fibrin and fibrinogen. International Food Research Journal 22(4): 1662-1667.

Yogesh, D. and Halami, P. M. 2015b. A fibrin degrading serine- metallo-protease of *Bacillus circulans* with α-chain specificity, Food Bioscience11: 72-78.

Yongjun, C., Wei, B., Shujun, J., Meizhi, W., Yan, J., Yan, Y., Zhongliang, Z. and Goulin, Z. 2011. Direct evolution improves fibrinolytic activity of nattokinase from *Bacillus natto.* FEMS Microbiology Letters 325: 155–161.

Yuan, J., Yang, J., Zhuang, Z., Yang, Y., Lin, L. and Wang, S. 2012. Thrombolytic effect of Douchi fibrinolytic enzyme from *Bacillus subtilis* LD-8547 in vitro and in vivo. BMC Biotechnology 10: 12-36.

Yanagisawa, Y., Chatake, T., Chiba- Kamoshida, K., Naito, S.,Ohsugi, T., Sumi, H., Yasuda, I. and Morimoto, Y. 2010. Purification, crystallization and preliminary X-ray diffraction experiment of nattokinase from *Bacillus subtilis natto.* Acta Crystallographica Section F Structural Biology and Crystallization Communications 66: 1670–1673.

Yanagisawa, Y., Chatake, T., Naito, S., Ohsugi, T. Yatagai, C., Sumi, H., Kawaguchi, A., Chiba-Kamosida, K., Ogawa, M., Adachi, T. and Morimoto, Y. 2013. Structure determination and deuteration of Nattokinase. Journal Synchrotron Radiation 20: 875–879.

Zhang, R.H., Xiao, L., Peng, Y., Wang, H.Y., Bai, F. and Zhang Y.Z. 2005. Gene expression and characteristics of a novel fibrinolytic enzyme (subtilisin DFE) in Escherichia coli. Letters in Applied Microbiology 41: 190–195.

Zhang, Z. L., Ye, M, Q., Zuo, Z.Y., Liu, Z.G., Tai, K. C. and Zou, G.L. 2006. Probing the importance of H-Bond in active site of the subtilisin nattokinase by site directed mutagenesis and molecular dynamics simulation. Biochemical Journal 395: 509-515.