Studies on a Fibrinolytic Enzyme Produced From Marine *Serratia marcescens* subsp. *sakuensis*

Thesis

Submitted in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby declare that the Research Thesis entitled "**Studies on a Fibrinolytic Enzyme Produced From Marine *Serratia marcescens* subsp. *sakuensis*"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal**, in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in the Department of Chemical Engineering, is a bonafide report of the research work carried out by me. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

Place: NITK-Surathkal
Date:

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CERTIFICATE

This is to certify that the Research Thesis entitled "**Studies on a Fibrinolytic Enzyme Produced From Marine *Serratia marcescens* subsp. *sakuensis*"** submitted by **Ms. Anusha Krishnamurthy (Register Number: 135034CH13F02)** as the record of the research work carried out by her, *is accepted as the Research Thesis submission* in partial fulfilment of the requirements for the award of degree of **Doctor of Philosophy**.

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ANUSHA

DEDICATED

TO

MY FAMILY

ABSTRACT

Fibrinolytic enzymes are agents/drugs that are responsible for the breakdown of fibrin in the blood clots. They find application in treatment of myocardial infarctions, ischemic strokes, cardiac and respiratory failure. This research work was aimed at isolating a fibrinolytic enzyme producing microorganism and to assess its suitability as a potential drug candidate for therapeutic applications. Six out of the eight bacteria isolated from the sea water sample tested positive for the fibrinolytic enzyme production during the initial screening experiments. Based on the results from screening experiments, one out of the six bacteria was chosen for further work and was identified as *Serratia marcescens* subsp. *sakuensis* (KU296189.1). The medium components were optimised by one-factor-at-a-time approach and Plackett-Burman design for enhanced production of fibrinolytic enzyme. A 3.4 fold increase in fibrinolytic enzyme activity was obtained with optimised production medium. The crude enzyme solution was then purified by a three step process involving ammonium sulphate precipitation, dialysis and size exclusion chromatography. Properties of the purified enzyme such as the molecular weight, optimum pH and temperature, stability at different pH and temperature, *in vitro* half-life, effect of metal ions and chemical reagents, *in vitro* clot lysing potential and thrombolytic mechanism, proteolytic activity against several substrates and its partial amino acid sequence were determined. Chemical modification of the purified enzyme was carried out using amino acid specific modifiers and modification with 2.5 mM EDAC resulted in a 9-fold increase in the fibrinolytic activity. The circular dichroism spectrum analysis of the modified and native enzyme revealed a similar structure except a few minor changes in a- helix and ß-sheet conformation of the enzymes. The findings suggest that the fibrinolytic enzyme produced in the present work could be considered as a potential candidate, safe for human use.

Keywords: Fibrinolytic, Marine microorganism, Plackett-Burman, *Serratia marcescens* subsp. *sakuensis.*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CCD	Central Composite Design
CD	Circular Dichroism
DEPC	Diethyl Pyrocarbonate
DTNB	5,5'- Dithiobis(2-Nitrobenzoic acid)
DTT	Dithiothreitol
EDAC	1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast Protein Liquid Chromatography
LC-MS/MS-TOF	Liquid Chromatography/Time-Of-Flight Mass Spectrometry
NBS	N-Bromosuccinimide
NCBI	National Centre for Biotechnology Information
PAGE	Polyacrylamide Gel Electrophoresis
PBD	Plackett-Burman Design
PMSF	Phenylmethylsulfonyl Fluoride
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic acid

NOMENCLATURE

Da	Daltons
°C	Degree Celsius
g	Acceleration due to gravity
g	Grams
L	Litre
mL	Millilitre
M	Molar
mM	Millimolar
rpm	Revolutions per minute
h	Hour
nm	Nanometer
U	Enzyme unit
[S]	Substrate concentration
[V]	Rate of reaction
K_m	Michaelis Menten constant
V_{max}	Maximum rate of reaction
k_{cat}	Turnover number
% w/v	Percentage weight by volume
% v/v	Percentage volume by volume

CHAPTER 1

INTRODUCTION

Cardiovascular diseases occur due to abnormal fibrin accumulation in the blood vessels resulting in the formation of thrombus. Deficiencies in coagulation inhibitors such as heparin cofactor II, antithrombin III, inadequate release of plasminogen activators and uncontrolled production of plasminogen activation inhibitors are few pathophysiological conditions that could lead to the formation of blood clots (Gallimore and Friberger 1991) inside the blood vessels, clinically termed as thrombosis. Thrombosis can restrict blood circulation in the arteries or veins, causing various medical conditions such as acute myocardial infarction, ischemic stroke, ischemic heart disease and high blood pressure (Lu and Chen 2012). However, under the normal physiological condition, there is a constant state of equilibrium maintained between clot formation and clot lysis. Haemostasis and fibrinolysis are the two biological processes that maintain proper blood flow and are a consequence of a complex series of cascading enzymatic reactions (Rau et al. 2007).

Haemostasis is the process by which bleeding is arrested when blood vessels are damaged. It occurs in three steps rapidly: vasoconstriction, platelet plug formation and coagulation (fibrin clot formation) (Marieb and Hoehn 2010). Vasoconstriction or vascular spasm involves the constriction of vessels in order to prevent the blood flow. This is followed by the aggregation of platelets, which form a temporary plug at the site of injury, releasing certain chemical messengers such as thromboxane A2 (TXA2), adenosine diphosphate (ADP) and serotonin. This causes more platelets to arrive at the site of injury, clump together leading to platelet plug formation and is termed as primary haemostasis. The final step of haemostasis (secondary haemostasis) is the conversion of fibrinogen to fibrin threads by the action of the enzyme thrombin (3.4.21.5). These fibrin threads along with platelet plug form a solid clot to seal the injured vessel wall to prevent blood loss (Porrett et al. 2005; Zdanowicz 2002).

Fibrinolysis is the process that involves the dissolution of the blood clot formed, restoring uniform blood flow within the blood vessels. The fibrinolytic system mainly comprises of a proenzyme plasminogen, enzymes that proteolytically activate plasminogen, several inhibitors that regulate the activation of plasminogen, activity of plasmin and consequential degradation of fibrin (Dobrovolsky and Titaeva 2002).

Plasmin (3.4.21.7) is the key enzyme present in the human body that is responsible for the degradation of fibrin into soluble fibrin degradation products (FDPs) (Blann et al. 2002). Regulation of the fibrinolytic system happens via a series of orchestrated interactions between several components involved in the cascade (Lu and Chen 2012).

Fibrinolytic agents used for clinical application fall under three categories based on their mode of action. The first category comprises of plasminogen activators that convert the proenzyme plasminogen to active plasmin and thereby facilitate fibrin clot lysis (Figure 1). The second category of fibrinolytic agents are plasmin like enzymes that have a similar role to that of the natural plasmin found within the body and therefore directly lyse the fibrin clots (Blann et al. 2002) (Figure 1). The third category of fibrinolytic agents consists of oral anticoagulants (OACs) which operate either as vitamin K antagonists or direct thrombin inhibitors and/or bind to factor X (Freedman 1992; Gonsalves et al. 2013). It is the *in vivo* action of fibrinolytic enzymes that has given rise to an enzyme based therapy for the lysis of fibrin clot.

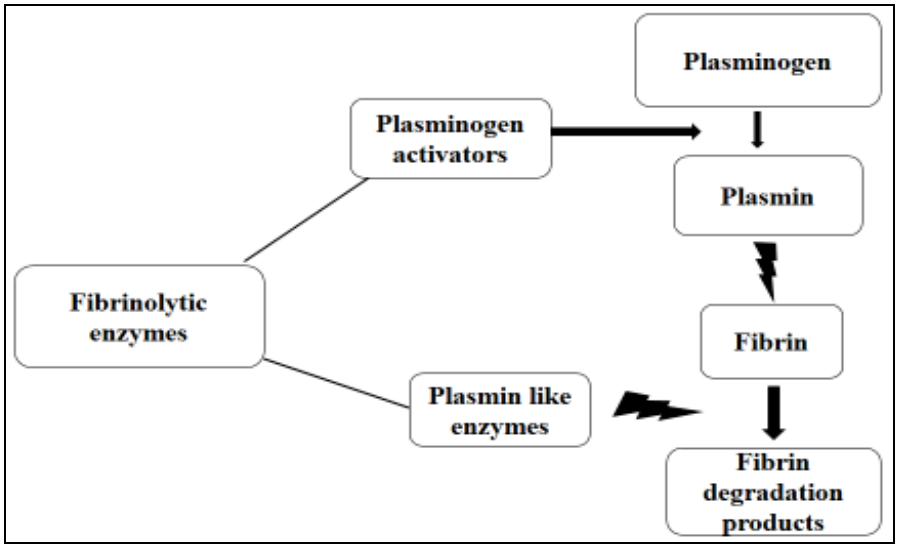


Figure 1.1 Mechanism of action of fibrinolytic enzymes (Source: Kotb 2013)

Owing to the advent of these fibrinolytic agents, three generations of thrombolytics emerged. The first generation of thrombolytic drugs included the fibrinolytic agents which were systemic activators that convert inactive plasminogen to active plasmin in circulation. The second generation of thrombolytics was produced with the help of

genetic engineering, composed of fibrin binding domains that target the activation of plasminogen resulting in the degradation of the fibrin clot. Examples of fibrinolytic agents belonging to this category include drugs that are produced by recombinant deoxyribonucleic acid technology (rDNA) (Longstaff and Thelwell 2005). Based on these, the third generation of thrombolytics was engineered, wherein the fibrin binding domains were modified with the aim of improved fibrin binding, zymogenecity, resistance to certain natural inhibitors, longer plasma half-life (Verstraete 2000). Reports suggest that the scientific data available on protein engineered second and third generation thrombolytic drugs, including it's *in vitro*, *in vivo* animal studies have not been translated into significant improvements in outcomes of clinical trials and thus, could be rendered as a failure (Longstaff and Thelwell 2005). The only milestone that could be achieved by these drugs is to do with its pharmacokinetics, with extended plasma half-life, but not fibrin specificity or inhibitor susceptibility (Longstaff and Thelwell 2005).

Some of the commercially available fibrinolytic agents are streptokinase (Streptase®), nattokinase, urokinase (Abbokinase®) and Alteplase (Activase®). In addition to these, there are certain oral anticoagulants (OACs) available for therapeutic use, namely heparin, warfarin (Coumadin®), dabigatran (Pradaxa®) and rivaroxaban (Xarelto®). Despite the availability of these varied fibrinolytic agents, not all of them have been put to therapeutic use owing to the drawbacks associated with them. Some of the demerits are inter and intrapatient variability of effective dose, food and drug interactions, intra-cranial haemorrhage, short half-lives, lower fibrin specificity, allergic reactions and high production costs (Blann et al. 2002; Turpie et al. 2002). Reports suggest the restricted use of these oral anticoagulants (OAC), especially in the aged populations, due to the intra-cranial bleeding associated with their administration (Miesbach and Seifried 2012). The OACs such as warfarin and heparin which are currently in use have delayed the onset of action and also exhibit certain food and drug interactions along with varied pharmacokinetics and pharmacodynamics property. Therefore, their usage requires routine laboratory monitoring and dosage adjustment in order to maintain the International Normalised Ratio (INR) within the therapeutic range (Bauer 2011). Particularly, warfarin shows

significant inter- and intrapatient variability of effective dose (Lip et al. 2011). Albeit the newer OACs have an edge over the well-established OACs in terms of immediate onset of action and absence of interactions with food, their renal elimination indicates the need for dosage reduction. In addition, the lack of availability of assays to measure the drug levels, high acquiring cost of the drug, short half-life which leads to a swift decrease in its anticoagulant effect, unavailability of specific antidote in case of excessive bleeding are some of the other drawbacks (Bauer 2011).

Hence, there arises a need for the development of novel fibrinolytic agents that are safe for therapeutic use with high efficacy and lower cost. This has paved way for the discovery of newer microbial sources for the production of fibrinolytic and/or thrombolytic agents with increased fibrin specificity and longer half-lives (Peng et al. 2005). Several attempts have been made to produce fibrinolytic enzymes by harnessing the natural resources, such as the microbial sources, medicinal mushrooms, earthworms and snakes (Lu and Chen 2012).

The marine habitat spanning almost 70% of the earth's surface is the largest reservoir of microorganisms, not just in the surface waters of the sea but also in greater depths from coastal regions to offshore regions, and specialised niches like hot thermal vents. Marine ecosystem provides a largely untapped resource for the isolation of novel microorganisms with the capacity to produce metabolites of therapeutic value (Das et al. 2006). Several unique and biologically active metabolites have been reported from marine microorganisms (Jensen and William 1994; Imada 2004; Zhang et al. 2005). They are mainly salt tolerant and have a diverse range of enzyme activity, capable of carrying out various biochemical reactions with the help of novel enzymes and their specialised mechanisms for adaptation to the extreme environments (Solingen et al. 2001). Since the sea water is saline in nature it is believed to provide a similar environment as that of the human blood plasma, hence the therapeutic enzymes of this origin particularly could be safer to use with less or no toxicity (Das et al. 2006).

The marine environment is characterised by hostile conditions such as low temperature, high pressure, salinity, the absence of light, etc. and the marine microbes adapt themselves to thrive in this environment. Salt tolerant marine microorganisms

provide an interesting alternative for therapeutic applications (Das et al. 2006). Till date, a number of reports suggest that the marine microorganisms are prolific producers of bioactive metabolites with varied action, such as anti-cancer, anti-inflammatory, antibiotic, antifungal, cytotoxic, neurotoxic, antimitotic, antiviral, antineoplastic, antiprotozoal and anti-proliferative properties. However, as compared to their terrestrial counterparts, there is very limited insight on the marine microbes reported so far. Despite the vast diversity of microbial consortium in deep oceans and the plethora of bioactivity that they display, very few drugs of marine microbial origin are in the market. With an integrated approach in microbiology, screening and natural product chemistry, the marine microbial metabolites are now advancing to be pharmaceutically important drug candidates (Bhatnagar and Kim 2010).

1.1 SCOPE OF THE RESEARCH WORK

Marine microorganisms are salt tolerant and have a diverse range of enzyme activity as opposed to their terrestrial counterparts. They are capable of carrying out various biochemical reactions with the help of novel enzymes and specialised mechanisms for adaptation to the extreme environments. Since the sea water is saline in nature it is believed to provide a similar environment as that of the human blood plasma, hence the therapeutic enzymes of this origin particularly could be safer to use with less or no toxicity. Very little work has been reported on the fibrinolytic enzyme producing capacity of marine microorganisms, particularly marine bacteria of the Arabian coastal origin.

The commercially available fibrinolytic agents suffer from several shortcomings such as (i) inter and intrapatient variability of effective dose (ii) short half-life span (iii) lesser fibrin specificity (iv) smaller therapeutic index (v) food and drug interactions (vi) allergic responses (vii) intra-cranial haemorrhage and/or substantial risk of bleeding complications. The scientific data available on most of the newer protein engineered thrombolytic drugs, including it's *in vitro*, *in vivo* studies have not been translated successfully into clinical trials, and hence could be rendered as a failure. This provides scope for exploring newer microbial sources for the production of fibrinolytic and/or thrombolytic agents.

In order to enhance the production of fibrinolytic enzyme, it is necessary to formulate an optimized media taking into account influential factors such as carbon source, nitrogen source, inducers and salts. Once the medium is optimised, the purified enzyme has to be characterised in order to determine the physicochemical characteristics of the test enzyme, which in turn would serve as a platform to evaluate its therapeutic potential. In addition, *in vitro* studies need to be performed to understand its clot lysis potential, the mechanism of clot lysis and to further strengthen its candidature as a potential candidate for clinical use. A comparison with the commercially available fibrinolytic agents could be made to provide insight on the efficacy of the test enzyme. The amino acid composition of the potential test candidate can be deciphered and would serve as an indispensable data to further understand its therapeutic role. Alternative methods to enhance the fibrinolytic enzyme activity can be explored, in turn increasing the specificity of the test enzyme towards the target protein.

1.2 OBJECTIVES

1. Isolation and screening of marine microorganisms for the production of fibrinolytic enzymes.
2. Optimisation of the medium for enhanced production of fibrinolytic enzyme.
3. Purification and characterisation of the enzyme.
4. *In vitro* clot lysing effect and the thrombolytic mechanism of the purified enzyme.
5. Chemical modification of the purified fibrinolytic enzyme.

1.3 ORGANIZATION OF THE THESIS

The stated objectives have been addressed in this doctoral work and are being discussed in the following chapters:

Chapter 1: Introduction

This chapter provides an introduction to the proposed research work by providing an insight on the clinical relevance of the target molecule. The chapter emphasizes the

need for a novel fibrinolytic drug candidate that would serve as a therapeutic agent in the clinical field. The scope and the rationale of the work have been mentioned and the formulated objectives have been presented.

Chapter 2: Review of literature

This chapter presents a comprehensive review of the literature pertaining to the proposed research work and the relevant published research articles have been summarised.

Chapter 3: Isolation and screening

This chapter gives an account of the isolation of marine microorganisms from seawater sample and the combination of screening strategies used to evaluate the fibrinolytic activity in the microorganisms isolated.

Chapter 4: Medium optimisation

This section reports the effect of medium components on the fibrinolytic enzyme production, both by the conventional and statistical approach. Further, an optimised medium was designed that resulted in enhanced fibrinolytic enzyme activity.

Chapter 5: Purification and characterisation of the enzyme

This chapter presents the experimental details and results pertaining to the purification of the fibrinolytic enzyme using methods such as ammonium sulphate precipitation, dialysis and fast protein liquid chromatography. Also detailed herein is the characterisation of the purified fibrinolytic enzyme along with it's *in vitro* applications.

Chapter 6: Chemical modification of the fibrinolytic enzyme

This chapter gives an account of the various amino acid specific modifiers employed to chemically modify the purified enzyme, so as to identify the amino acid residues that are essential for the catalytic activity of the fibrinolytic enzyme. This section also

reports the kinetics of the chemically modified enzyme, in addition to some of its characteristics with respect to the native unmodified enzyme.

Chapter 7: Summary and conclusions

This section offers a brief summary and conclusions of the findings of this research work with some recommendations for the future work.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Peptidases

Peptidases are a class of enzymes that catalyse the hydrolysis of peptide bonds in proteins. The hydrolysis mechanisms can be of two types, one being the specific hydrolysis where specific peptide bonds would be cleaved, and the other being the unspecific hydrolysis which involved the complete breakdown of protein to oligopeptides and or amino acid residues.

In general, peptidases can be grouped into two types, exopeptidases and the endopeptidases. Exopeptidases cleave near the ends of the polypeptide chain while the endopeptidases cleave within the polypeptide chain. The endopeptidases in turn divided into six major families based on their active site composition. The six major families are aspartic peptidases, serine peptidases, metallopeptidases, cysteine peptidases, glutamic peptidases and the threonine peptidases (Rawlings et al. 2010).

2.2 Sources of fibrinolytic enzymes

Based on the literature available, fibrinolytic enzymes were reported to have been produced from different microorganisms, such as bacteria of marine and food origin, actinomycetes, fungus, alga, snake venoms and earthworms, as shown in table 2.1.

Table 2.1 List of various sources for the production of a fibrinolytic enzyme

Source	Name	Reference
Bacteria	*Bacillus sphaericus*	Balaraman and Prabakaran 2007; Avhad et al. 2013
	Bacillus natto	Mahajan et al. 2010
	Bacillus subtilis	Jeong et al. 2004; Ko et al. 2004; Ashipala and He 2008; Deepak et al. 2008; Wu et a. 2010; Yeo et al. 2011; Raafat et al. 2012; Yuan et al. 2012;

Source	Name	Reference
Bacteria		Mahajan et al. 2012; Huang et al. 2013
	Bacillus amyloliquefaciens	Peng at al. 2003; Kotb 2014
	Bacillus vallismortis	Cheng et al. 2015
	Bacillus cereus	Narasimhan et al. 2015
	Bacillus circulans	Yogesh et al. 2015
	Bacillus megaterium	Kotb et al. 2015
	Bacillus halodurans	Vijayaraghavan et al. 2016
	Brevibacillus brevis	Majumdar et al. 2014
	Pseudomonas sp	Wang et al. 2009
	Pseudoalteromonas sp	Vijayaraghavan and Vincent 2014
	Vibrio vulnificus	Chang et al. 2005
	Streptococcus dysgalactiae	Bhardwaj et al. 2014
	Proteus penneri	Jhample et al. 2015
	Shewanella sp.	Vijayaraghavan and Vincent 2015
	Aeromonas	Cho et al. 2011

Source	Name	Reference
	Serratia sp.	Bhargavi et al. 2013
Actinomycetes	*Actinomyces thermovulgaris*	Egorov et al. 1976
	Streptomyces sp.	AbdeI-Naby et al. 1992; Wang et al. 1999; Simkhada et al. 2010; Chitte et al. 2002; Uesugi et al. 2011; Ju et al. 2012; Naveena et al. 2012; Cheng et al. 2015
Fungus	*Penicillium chrysogenum*	El-Aassar et al. 1990; Gopinath and Sneha 2012
	Fusarium pallidoroseum	El-Aassar 1995
	Fusarium oxysporum	Tao et al. 1997, 1998
	Aspergillus ochraceus	Batomunkueva and Egorov 2001
	Oidiodendrom flavum	Tharwat 2006
	Pleurotus ostreatus	Choi and Shin 1998
	Rhizopus chinensis	Xiao-Lan et al. 2005
	Cordyceps militaris	Cui et al 2008
	Paecilomyces tenuipes	Kim et al. 2011
	Micromycetes strains	Sharkova et al. 2015

Source	Name	Reference
Algae	*Codium intricatum*	Matsubara et al. 1998
	Codium divaricatum	Matsubara et al. 2000
	Costaria costata	Kim et al. 2013
Thermophiles	*Streptomyces megasporus*	Chitte and Dey 2000
	Microbiospora sp.	Goshev et al. 2005
	Thermoactinomyes	Goshev et al. 2005
Snakes	*Viperidae*	Markland 1998
	Elapidae	Markland 1998
	Crotalidae	Markland 1998
Earthworm	*Eisenia fetida*	Roch 1979

In addition, genetically engineered strains were also developed for increased enzyme yield. *Bacillus subtilis* was identified as a good host for the expression of the clot lysing enzymes because of its capacity to secrete functional extracellular proteins into the culture medium and its non-pathogenecity (Wong 1995). The protease deficient strain *Bacillus subtilis* WB600 could actively express subtilisin DFE (Peng et al. 2004). Xiao et al. (2004) replaced the native promoter of subtilisin DFE gene with the α-amylase gene from *Bacillus amyloliquefaciens* DC-4 which resulted in significant increase in the fibrinolytic activity from 80 U/ml to 200 U/ml. Owing to the

uniqueness of the source, enzymes can be expected to possess characteristics which are quite different from the ones that exist currently.

2.3 Fibrinolytic enzyme specificity towards fibrin and fibrinogen

Shivaprasad et al. (2009) reported that the cysteine proteases from asclepiadaceae plants exhibited both thrombin and plasmin like activities. Upon direct incubation of the plant latex enzyme fraction with the fibrinogen, they observed the formation of a fibrin clot similar to that formed by thrombin. In addition, on prolonged incubation, the degradation of the fibrin clot was observed suggesting a plasmin like activity. The enzyme fractions were found to hydrolyse the Aα and Bβ chains of the fibrinogen molecule to form a fibrin clot. These enzyme fractions later were also found to hydrolyse the subunits of fully cross linked fibrin α-polymer, α-chain, β-chain, γ-γ dimer, thereby degrading the clot. Similar observations were made by Bindhu et al. (2014) while working on the cysteine proteases present in the plant latex of *Calotropis gigantea.*

These activities could be due to the fact that the plant latexes have a single enzyme with a dual role of both clot forming and clot lysing, or that the plant latexes have two different enzymes one which is fibrinogenolytic in nature and the other being fibrinolytic in nature (Shivaprasad et al. 2009). In case of an enzyme performing a dual role, the affinity for its natural substrate matters. That enzyme might have the capability to utilise the available substrate or the provided substrate in the medium, thereby breaking it down to its natural substrate and eventually carrying out its catalytic function.

2.4 Production of microbial fibrinolytic enzyme

The two major obstacles against the successful application of the protease enzyme in the industry are the cost of enzyme production and its subsequent downstream processing. Many attempts have been made in laboratories to improve the expression of the fibrinolytic enzyme, which includes the selection of a culture medium ideal for fibrinolytic enzyme production, optimisation of the environmental conditions and lastly genetic modification of the microbial strain so as to achieve overexpression of the fibrinolytic enzyme (Peng et al 2005).

Selection of the medium components becomes critical for the fermentative production of fibrinolytic enzymes. As different microbes possess varied physiological characteristics, the need for optimising the nutrient components for cell growth and production of metabolites arises (Seo and Lee 2004). Mahajan et al. (2012) optimised the medium for enhancing the fibrinolytic enzyme production by *Bacillus subtilis*. They reported that $MgSO_4$, $CaCl_2$, soy peptone, yeast extract, maltose and NaCl had a major effect and pH had the least effect on the enzyme production. Similar results were reported by Liu et al. (2005) indicating that soy peptone, yeast extract and calcium chloride had a significant effect on the production of nattokinase. Agrebi et al. (2009) used hulled grains of wheat, casein peptone, $CaCl_2$, NaCl, $MgSO_4$, KH_2PO_4 to optimise the medium for the fibrinolytic enzyme production by *Bacillus subtilis*. Deepak et al. (2008) in their report optimised the medium for maximising the enzyme production. They could achieve maximum enzyme activity with the usage of glucose, peptone, $MgSO_4$ and $CaCl_2$ as media components. Vijayaraghavan and Vincent (2014) carried out solid-state fermentation of Pseudoalteromonas sp. by using cow dung as a substrate. They found the enzyme activity to be high with the supplementation of 1% (w/w) maltose and 0.1% (w/w) NaH_2PO_4 in the medium. It was suggested that fibrin when added to the production medium induced/activated the enzyme production (Chitte and Dey 2002; Peng and Zhang 2002). Although the conventional one factor at a time approach is necessary, it is not sufficient to achieve an optimised medium composition since it fails to take a comprehensive effect of all the factors into consideration and therefore is not sufficient to locate the region of optimal response (Peng et al. 2005). Hence, a number of statistical approaches such as fractional factorial design (FFD), central composite design (CCD) and so on are followed. Liu et al. (2005) had employed these statistical approaches and achieved an increase in the fibrinolytic enzyme activity by six times higher than the original medium.

Apart from the regular submerged state fermentation, another type of fermentation namely the solid-state fermentation (SSF) was employed for fungi particularly. SSF was found to be advantageous over the submerged state fermentation such as low operating costs and large productivity (Peng et al. 2005). Tao et al. (1997, 1998) had

studied the production of fibrinolytic enzyme from *Fusarium oxysporum* with different SSF methods thus enhancing the enzyme production largely and reducing the production costs.

2.5 Purification of enzymes

The purification of enzyme becomes the essential first step in understanding its function. A pure protein is used to determine its amino acid sequences, to understand the evolutionary relationships among protein of diverse organisms, and lastly to investigate its biological function. In general, proteins are purified in their active form based on characteristics such as size, solubility, charge and specific binding affinity. Protein mixtures are subjected to series of separation steps, each step exploiting a property of the protein to yield the desired protein of interest. The overall yield of the protein of interest is an important feature of a purification step.

Several purification steps are available, such as salting out, dialysis, gel-filtration chromatography, ion-exchange chromatography, affinity chromatography, high-pressure liquid chromatography etc (Berg et al. 2002). The underlying mechanism behind salting out is that with an increase in salt concentration there is a decrease in the solubility of proteins, thus, precipitating out the proteins. Ammonium sulphate is the most preferred choice of salt for salting out purpose with concentration varying from one protein to another. Salting out is followed by dialysis to remove the excess salt content in the concentrated protein sample via the pores of a semi-permeable membrane (Berg et al. 2002).

Gel filtration chromatography separates proteins based on the differences in their sizes. Larger molecules pass quickly through the column and elute first, followed by the smaller molecules that enter the beads of the packing material (stationary phase) and elute last. Ion exchange chromatography, on the other hand, separates proteins based on the difference in the net charge of the protein molecule. Cation exchange chromatography and anion exchange chromatography are the two types of ion exchange chromatography (Berg et al. 2002). Table 2.2 gives an account of reports that provide information on the steps adopted for purification of fibrinolytic enzyme

from different sources. Additionally, there are several other reports wherein a similar combination of steps have been employed for the purification of fibrinolytic enzymes.

Table 2.2 List of reports on purification of fibrinolytic enzyme

Source	Steps	Increase in enzyme activity	Reference
Bacillus subtilis	Hydrophobic chromatography, anion exchange and gel filtration chromatography	34.6 fold	Wang et al. (2006)
Fusarium sp.	ammonium sulphate precipitation, Mono-Q column, Superdex 75 column	158.49 fold	Wu et al. (2009)
Paecilomyces tenuipes	Ethanol precipitation, CM-cellulose column, Sephadex G-75 gel filtration column, POROS 20 HQ ion exchange column	4 fold	Kim et al. (2011)
Streptomyces sp.	80% ammonium sulphate precipitation, polyacrylamide gel column, DEAE-Sepharose fast flow anion exchange column	8.75 fold	Ju et al. (2012)
Bacillus subtilis	Partially purified, UnoQ Sepharose column, Butyl Sepharose FF hydrophobic column	32.42 fold	Mahajan et al. (2012)

Source	Steps	Increase in enzyme activity	Reference
Bacillus sp.	80% acetone precipitation, Sephacryl S-200 gel filtration column, Waters reverse-phase Nova-Pak C18 column	15.4 fold	Mukherjee et al. (2012)
Serratia marcescens	80% ammonium sulphate precipitation, Sephadex G-75 gel filtration column, DEAE-Cellulose column	15.2 fold	Bhargavi and Prakasham (2013)
Cirriformia tentaculata	40 - 60% ammonium sulphate precipitation, HiPrep Q column chromatography, Mono Q column chromatography, Superdex 75 column	2.8 fold	Park et al. (2013)
Brevibacillus brevis	Ammonium sulphate precipitation, Hi Prep Phenyl FF 16/10 and Hi Load Superdex 75 pg 16/60 FPLC column	12.4 fold	Majumdar et al. (2014)
Lyophyllum shimeji	Ethanol precipitation, Mono Q 5/5 anion exchange column, Superdex 200 100/300 size exclusion column	80.9 fold	Moon et al. (2014)

Source	Steps	Increase in enzyme activity	Reference
Bacillus circulans	45 – 80% ammonium sulphate precipitation, Sephadex G-75 gel filtration column	58.13 fold	Yogesh and Halami (2015)
Pheretima posthumous	70% ammonium sulphate precipitation, DEAE Cellulose column, Sephadex G-50 gel filtration column	8.01 fold	Verma and Pulicherla (2017)

2.6 Characterisation of enzymes

Enzyme characterisation studies are performed mainly for two reasons, one is to identify a special enzyme, to prove its presence or absence in a sample/specimen, such as an organism or tissue, and the other reason is to quantify the amount of enzyme present in the sample. The main factors that are involved in characterising an enzyme are pH, temperature, ionic strength, concentration of substrate and concentration of enzymes. Enzymes display their highest activity at their respective optimum conditions, while deviation from their optimum could lead to decrease in their activity, depending on the degree of deviation. Influence of pH on enzyme activity could be explained by the fact that, the activities of most enzymes follow a bell-shaped curve, increasing from zero in the strong acid region up to a maximum value, and decreasing to zero to the strong alkaline region. Two different effects are responsible for this behaviour: (i) the state of protonation of functional groups of amino acids and cofactors involved in the catalytic reaction and (ii) the native, three-dimensional protein structure of the enzyme. The temperature dependence of the activity of enzymes resembles in some respect the pH dependence: increasing with rising temperature, passing a maximum, followed by a decrease (Bisswanger 2011).

Metal ions are a part of almost all of the biological activities and hence it is necessary that the effect of metal ions on the enzyme activity be determined. Metal ions either directly participate in catalytic reaction or perform an auxiliary role in redox catalysis of substrates by enzymes (Tainer et al. 1983; Hart et al. 1999). Table 2.3 provides a list of reports describing the physicochemical properties of fibrinolytic enzymes. It can be observed from table 2.3 that the optimum pH for the fibrinolytic enzymes lie in the basic pH range and have an optimum temperature as high as 60 °C. Divalent cations show a stimulatory/inhibitory role on the fibrinolytic activity (table 2.3). It was suggested by Peng et al. (2005) that the influence of divalent metal ions on fibrinolytic activity in case of metallo fibrinolytic enzymes is a common phenomenon.

Table 2.3 List of reports on the properties of fibrinolytic enzymes

Optimum		**Effect of metal ions**	**Nature**	**Reference**
pH	**temp**			
7.8	55 °C	N.D*	Serine protease	Chang et al. (2000)
7.4	37 °C	Enhanced by Mg^{2+}, Ca^{2+}	Serine protease	Kim et al. (2006)
8	45 °C, 55 °C	Enhanced by Mg^{2+}, Ca^{2+}	N.D*	Tharwat 2006
8	55 °C	Inhibited by Fe^{3+}, Zn^{2+}	Serine protease	Wang et al. (2006)
9	60 °C	Inhibited by Ca^{2+},Zn^{2+},Cu^{2+},Ba^{2+}, Mg^{2+}	Serine protease	Agrebi et al. (2009)
7	50 °C	Enhanced by Fe^{2+} (5	Serine protease	Wang et al. (2009)

Optimum		Effect of metal ions	Nature	Reference
pH	**temp**			
		mM)		
8.5	45 °C	Enhanced by Ca^{2+}, Zn^{2+}	Serine metalloprotease	Wu et al. (2009)
4	40 °C	Inhibited by Cu^{2+}	Serine protease	Chung et al. (2010)
7.5	37 °C	Enhanced by Zn^{2+}, Fe^{2+}, Mg^{2+}	Serine protease	Lu et al. (2010)
7-8	45 °C	Inhibited by Co^{2+},Zn^{2+},Cu^{2+}, Fe^{2+}	Serine metalloprotease	Simkhada et al. (2010)
8	40 °C	N.D*	N.D*	Cho et al. (2011)
7	40 °C	Inhibited by Ba^{2+}, Cu^{2+}	Serine metalloprotease	Choi et al. (2011)
9.4	40 °C	Enhanced by Co^{2+}, Mg^{2+}	Metalloprotease	Hassanein et al. (2011)
5	35 °C	Enhanced by Ca^{2+}		Kim et al. (2011)
7	60 °C	N.D*	N.D*	Mander et al. (2011)
6 - 10	50 °C	Inhibited by Hg^{2+}	Serine protease	Yeo et al. (2011)
7.8	35 °C	Enhanced by Na^{+}, K^{+},	Serine	Ju et al. (2012)

Optimum		Effect of metal ions	Nature	Reference
pH	**temp**			
		Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}	metalloprotease	
9	50 °C	Enhanced by Ca^{2+}	Serine protease	Mahajan et al. (2012)
7.4	37 °C	N.D*	Serine protease	Mukherjee et al. (2012)
8	50-55 °C	Not effective	Serine protease	Simkhada et al. (2012)
9	37 °C	Enhanced by Ca^{2+}, Co^{2+}, Cu^{2+}, K^{+}, Mg^{2+}, Na^{+}, Zn^{2+}	Metalloprotease	Bhargavi and Prakasham (2013)
7	30 °C	Enhanced by Mg^{2+}, Ca^{2+}, Mn^{2+}	Metalloprotease	Choi et al. (2013)
8.5 - 9	50 – 60 °C	No significant effect with Ca^{2+}, Zn^{2+}	Serine protease	Park et al. (2013)
8	60 °C	Inhibited by Co^{2+}, Mn^{2+}	Serine protease	Choi et al. (2014)
8	40 °C	Enhanced by Fe^{2+}, Mn^{2+}	Serine protease	Kotb (2014)
7.4	37 °C	N.D*	Serine protease	Majumdar et al. (2014)
8	37 °C	Inhibited by Cu^{2+}, Co^{2+}	Serine	Moon et al. (2014)

Optimum		Effect of metal ions	Nature	Reference
pH	temp			
			metalloprotease	
6.5	50 °C	Inhibited by Cu^{2+}, Ca^{2+}, Mg^{2+}	Serine protease	Cheng et al. (2015)
8	50 °C	Enhanced by Zn^{2+}, Fe^{2+}	Serine protease	Kotb (2015)
8	50 °C	Enhanced by Ca^{2+}, Mg^{2+}	N.D*	Vijayaraghavan et al. (2015)
7.4	50 °C	Enhanced by Mg^{2+}, Mn^{2+}	Serine metalloprotease	Yogesh and Halami (2015)
9	60 °C	N.D*	N.D*	Vijayaraghavan et al. (2016)
8	40 °C	N.D*	Serine protease	Verma and Pulicherla (2017)

N.D* represents data not determined

2.6.1 Catalytic mechanism

Based on the catalytic mechanism, the fibrinolytic enzymes fall under three categories, such as the serine proteases, metalloproteases and the serine metalloproteases. Serine protease as the name suggests possess the nucleophilic ‘Ser' in the enzyme active site, which attacks the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate (Hedstrom 2002). Over one-third of all known proteolytic enzymes are serine protease (Cera 2009).Typically, the nucleophilicity of the catalytic ‘Ser' depends on a "catalytic triad" of Asp-His-Ser residues (Blow et al. 1969). The catalytic triad present within their structure also

known as the "charge relay" system usually consists of the following residues, Asp-His-Ser, Ser-His-Glu, Ser-Lys/His, His-Ser-His and N-terminal Ser (Blow 1997; Dodson and Wlodawr 1998). The four clans of serine proteases includes the chymotrypsin, subtilisin, carboxypeptidase Y and Clp protease (Dodson and Wlodawr 1998). Chymotrypsin-like proteases are the most abundant type serine protease present in nature. These are involved in many critical physiological processes such as digestion, haemostasis, apoptosis, signal transduction, reproduction and lastly the immune response (Neurath 1984; Johnson 2000; Joseph et al. 2001; Coughlin 2000; Barros et al. 1996). Series of sequential activation of serine proteases leads to blood coagulation, complement fixation and fibrinolysis (Davidson et al. 1990; Sim and Laich 2000; Collen and Lijnen 1986), wound healing, matrix remodelling (LeMosy et al. 1999; Van den Steen et al. 2001; Selvarajan et al. 2001). It has been reported that the rate of peptide hydrolysis by serine proteases are ~10^{10} fold greater than the uncatalysed reactions (Hedstrom 2002). The serine proteases generally follow three-step kinetic mechanism (a) formation of an enzyme-substrate (ES) complex (b) acylation of the active site serine and lastly (c) hydrolysis of the acyl-enzyme intermediate (Hedstrom 2002). These serine proteases catalyse the hydrolysis of ester or amide. Serine proteases are recognised by their irreversible by 3,4-dichloroisocoumarin (3,4-DCI), diisopropyl fluorophosphates (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-lysine chloromethyl ketone (TLCK) (Kotb 2012).

Metalloproteases are a group of proteases that contain a metal ion at their active site, which acts as a catalyst in the hydrolysis of peptide bonds thereby causing protein degradation (Hooper 1994). Of the various metal ions, the most commonly used metal ion by a metalloprotease is a zinc ion (Zn^{2+}) (Hase and Finkelstein 1993). Apart from this, other transition metals such as Co^{2+} and Mn^{2+} have been reported to be utilised to restore function in a zinc-metalloprotease in which the Zn^{2+} core has been removed (Holmquist and Vallee 1974). Most of the metalloproteases require only one metal ion for catalysis, but some enzymes such as the cobalt and manganese metalloproteases possess two metal ions in the active site that act cocatalytically (Nagase 2001). Many at times, at the active site the metal ions are bound in a nearly tetrahedral

conformation. For hydrolysis, a total of three amino acid ligands (charged residues) along with a water molecule associate with the metal core (Vallee and Auld 1990). It has been reported that His, Glu, Asp or Lys are the metal ligands in a metallopeptidase, the occurrence of which in the catalytic site is His>>Glu>Asp>Lys (Hooper 1996). Depending on the region of hydrolysis of the targeted protein, at which the reaction takes place, the metalloproteases can be divided into two divisions: metalloendopeptidase and metalloexopeptidases (Barrett 1977; Garrett and Grisham 2010). These metalloproteases participate in a number of biological processes such as embryonic development, morphogenesis, the release of cytokines and growth factors, processing of peptide hormones, cell-cell fusion, cell adhesion and migration, viral polyprotein processing, bacterial cell wall biosynthesis, intestinal absorption of nutrient and in the metabolism of antibiotics. Unlike serine proteases, the metalloproteases do not form covalent intermediates during peptide bond hydrolysis (Nagase 2001). These metalloproteases exhibit inhibition with chelating agents such as ethylenediaminetetraacetic acid (EDTA) but not by sulfhydryl agents or DFP (Barett 1995). Literature reports of the various fibrinolytic metalloproteases are as shown in the table. Reports suggest that the protease produced by *Serratia* sps often referred to as serratiopeptidase/serralysin and is a metalloprotease that has a zinc atom (Bhagat et al. 2013; Bhargavi and Prakasham 2013).

Serine metalloproteases are a class of proteases that have combined characteristics of both the serine and metalloproteases. They show inhibition with the inhibitors of serine and metalloproteases.

2.6.2 Molecular weight of the fibrinolytic enzymes

Determination of molecular weight of an enzyme is an essential step in its characterisation as it provides information on the immunogenicity of the protein molecule. In general, larger the size of the molecule greater would be the immunogenicity exhibited by it and smaller the molecule lesser would be the immunogenicity displayed (Coico and Sunshine 2015). According to literature reports, the molecular weights of the fibrinolytic protease from bacterial sp. ranges from 14 KDa to 190 KDa (Wang et al. 2006; Agrebi et al. 2009; Lu et al. 2010; Hassanein et al. 2011; Yeo et al. 2011; Mahajan et al. 2012; Mukherjee et al. 2012;

Kotb 2014; Majumdar et al. 2014; Cheng et al. 2015; Kotb 2015; Yogesh and Halami 2015; Vijayaraghavan et al. 2015; Vijayaraghavan et al. 2016), actinomycetes and fungal sp. ranges from 21 KDa to 52 KDa (Simkhada et al. 2010; Choi et al. 2011; Mander et al. 2011; Ju et al. 2012; Choi et al. 2013; Moon et al. 2014), algae is 50 KDa (Choi et al. 2014). One such report by Bhargavi and Prakasham (2013) quoting the production of a protease from *Serratia* sps. that had a molecular weight of 50 KDa. Serratiopeptidase from *Serratia marcescens* was reported to have a molecular weight of 52 and 50 KDa (Mohankumar and Krishna Raj 2011; Devi et al. 2013).

2.6.3 Kinetic studies

Kinetic properties of a certain enzyme are required to identify the natural substrate of the enzyme, its affinity for a particular substrate amongst the other available substrates, its maximum reaction velocity. Majority of the literature available on kinetic studies of fibrinolytic enzymes reported the use of substrates such as casein, azocasein (Simkhada et al. 2010; Ju et al. 2012; Bhargavi and Prakasham 2013; Cheng et al. 2015; Verma and Pulicherla 2017) and synthetic peptide substrates (Chang et al. 2000; Lu et al. 2010; Choi et al. 2011; Kim et al. 2011; Mander et al. 2011; Simkhada et al. 2012; Choi et al. 2013; Park et al. 2013; Choi et al. 2014; Kotb 2014; Majumdar et al. 2014; Yogesh and Halami 2015). Mukherjee et al. (2012) had reported the use of fibrin as a substrate for the determination of the kinetic parameters of the fibrinolytic enzyme Bafibrinase from *Bacillus* sp.

2.7 Multispecific nature of fibrinolytic enzymes

Most of the fibrinolytic enzymes of microbial origin have been reported to exhibit multispecific nature for substrates. Wang et al. (2006) showed that the fibrinolytic enzyme, produced by *Bacillus subtilis* DC33, was capable of degrading the substrates such as fibrin, fibrinogen, casein, serum albumin and Na-benzoyl-L-arginine ethyl ester hydrochloride. In addition, the electrophoretic analysis of the fibrinogenolytic activity of the enzyme revealed that initially the Bβ-chains of fibrinogen and β-chains of fibrin were cleaved, followed by the cleaving of other chains of the fibrinogen and fibrin. A fibrinogenolytic enzyme produced by the *Streptomyces* sp. CS684, upon electrophoresis, hydrolysed the Bβ-chains of fibrinogen, leaving the Aα and ɣ chains

intact (Simkhada et al. 2010). Lu et al. (2010); Mander et al. (2011); Cho et al. (2011); Ju et al. (2012); Cheng et al. (2015) demonstrated the fibrinogenolysis pattern of the fibrinolytic enzyme, wherein the Aα, Bβ and ɣ-chains of fibrinogen were degraded. Serralysin produced by *Serratia* sps exhibited both fibrinolytic and fibrinogenolytic activities (Bhargavi and Prakasham 2013). Yogesh and Halami (2015) reported that the fibrinolytic serine metalloprotease produced by *Bacillus circulans* showed enzyme activity with several endogenous substrates such as fibrin, fibrinogen, casein, haemoglobin and bovine serum albumin. In their report, they also analysed the electrophoretic pattern of degradation of both fibrin and fibrinogen, in which case only the α-chain of fibrin, Aα, Bβ, partial ɣ chains of fibrinogen were degraded, there was no significant degradation observed with respect to the other chains of fibrin. However, this multispecific nature of the enzymes could be both beneficial as well as undesirable depending on the intended therapeutic purpose. In the event that the person has a pre-existing blood clot/thrombosis, the need for either a fibrinolytic enzyme with absolute specificity for fibrin clot is required or a fibrinolytic enzyme with multispecificity for substrates such as fibrin, fibrinogen is required, wherein the K_m and the K_{cat} values of the enzyme for the substrates would matter. Francis et al.(1984) had demonstrated the degradation of fibrin in pathological thrombi *in situ*, in patients suffering from acute vascular obstruction, there was a minimal degree of fibrinolysis observed, due to the presence of cross-linked ɣ-ɣ' dimers which weren't degraded, unlike in patients suffering from the chronic condition. The study reflected the variability in the haemostatic balance between blood coagulation and fibrinolysis *in vivo* due to the polypeptide chain composition of the cross-linked fibrin, indicating that this haemostatic balance may be evidently different in separate areas of a single blood clot. In such cases, the ability of the fibrinolytic enzyme to act upon both fibrin as well as fibrinogen would be desirable, so as to completely clear out the clot formed.

2.8 Protein Sequencing

It is the process of determining the amino acids present in the target protein entirely or of a part of the target protein or peptide. This helps in identifying, characterising the protein and/or study its post-translational modifications.

In general, there are two ways of protein sequencing, mass spectrometry and Edman degradation using a sequencer. Mass spectrometry of proteins requires the protein molecules present in the solution/solid state to be turned into an ionised form in the gas phase prior to being injected and accelerated in an electric or magnetic field for analysis. Tandem mass spectrometry (MS/MS) is used to measure the fragmentation spectra and identify the protein of interest at high speed and accuracy. The underlying principle involves the collision-induced dissociation to generate a set of fragments from a specific peptide ion. The fragmentation would primarily lead to the cleavage products that break along the peptide bonds. The observed fragment masses are then matched with the database of predicted masses for one or many given peptide sequences. There are two different methods for the ionisation of protein in mass spectrometry, namely the electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI). These ionisation techniques are used in conjugation with the mass analysers, known as the tandem mass spectrometry (Chait 2011). In ESI, the ions are created from the protein molecules present in solution and allow the fragile molecules to be ionised intact while preserving non-covalent interactions at times. On the other hand, in MALDI, the target proteins are embedded within a matrix which is usually in solid form and ions are generated by pulses of laser light. ESI produces more charged ions in comparison to that obtained from MALDI paving way for the measurement of high mass proteins. Likewise, MALDI is less likely to be affected by contaminants, buffers and additives in addition to being a faster technique. Proteins are characterised either by a top-down approach or a bottom-up approach, wherein a top-down approach refers to the proteins being analysed intact and bottom-up approach refers to the method in which the proteins are subjected to fragmentation prior to analysis. Apart from this an intermediate middle-down approach also exists wherein larger peptide fragments are analysed (Chait 2011). Typically the protocol consists of the following steps: the target protein molecules are digested with an enzyme to produce peptides, the charged/ionised peptides are then separated based on their different m/z ratios, later each of these peptides get fragmented into ions with the help of the tandem mass spectrometer and the m/z values of fragment ions are measured and the spectrum generated is used to determine the sequence. Tandem

mass spectrometry (MS/MS) often results in partial N- and C- terminal peptides (Barrera and Robinson 2011).

2.8.1 Partial amino acid sequence

In case of partial sequencing of a protein, sufficient information is generated for the partial sequence of the target protein to be compared with the reference database of several protein sequences (Barrera and Robinson 2011). Table 2.4 gives an account of the partial amino acid sequences of proteins from various sources.

Table 2.4 List of reports on the partial amino acid sequences of proteins

Source	Amino acid sequences	Reference
Neanthes japonica	RGVTDHLYN, RSPGWL, RSQVDGVMWDLGDLLGA	Deng et al. 2010
Pleurotus ostreatus	ATFVGCSATR, GGTLIHESSHFTR, YTTWFGTFVTSR	Liu et al. 2014
Streptomyces sp.	MARIGDGGDLLK, SNILLLGPTGSGKTLLAQTLAR, AGAKGIGFGATIRSK	Cheng et al. 2015
Cordyceps militaris	IEDFPYQVDLR, ANCGGTVISEK, YVLTAGHCAEGYTGLNIR, TNYASVTPITADMICAGFPEGK, KDSCSGDSGGPLVTGGK, VVGIVSFGTGCAR, ANKPGVYSSVASAEIR	Liu et al. 2015

2.9 *In vitro* assays

In vitro studies become the indispensable preliminary step in the evaluation of a microbial enzyme that has the potential to be developed into a drug. The in vitro

assays discussed herewith may provide critical inputs before proceeding to in vivo testing using animal models and clinical studies.

2.9.1 Fibrinolytic assays

Several fibrinolytic assays are available (Table 2.5) to assess the fibrinolytic potential of an enzyme of microbial origin. These estimation methods have been reviewed by (Kotb 2013). The assays comprise of either fibrin being used as the substrate directly or fibrin clot being produced by the addition of thrombin to fibrinogen. The endpoint of lysis resulting in the fibrin degradation products is determined by measuring the zone of lysis, colorimetry and nephelometric method. However, these assays alone do not represent fibrinolysis *in vivo* and hence will not be sufficient to evaluate the efficacy of the test compound. Therefore, *in vivo* studies have to be carried out taking into account the physiological conditions.

Table 2.5 List of various fibrinolytic assays

Fibrinolytic assays	Characteristics	References
Fibrin plate method	• The long incubation period • Uncertainty in the determination of lysis zones	(Fossum and Hoem 1996; Jespersen and Astrup 1983)
Dyed fibrin plate assay	• Lesser incubation period making it rapid • Better accuracy since it is based on colorimetry and not on the zone of fibrinolysis • No requirement for elaborate equipment • Uses untreated natural human fibrin. • Requires relatively larger volume of enzyme solution.	(Barta 1966)

Fibrinolytic assays	Characteristics	References
Rapid fibrin plate assay	• Aimed at exploring the plasminogen-enrichment so as to shorten the incubation period • Requires 3h of incubation as compared to the fibrin plate method that requires 16-20h of incubation.	(Marsh and Gaffney 1977)
Solid-phase fibrin plate assay	• 2h of incubation period required • Multiple samples can be assayed • Highly sensitive	(Millar and Smith 1983)
Fibrin microplate assay	• Better reliability • Applicable for several analytical tests • One of the possible extension of this method is that the cells that express plasminogen activator or fibrinolytic activity can be applied directly to the gel • Removal of proteinase inhibitors becomes essential for plasma samples	(Fossum and Hoem 1996)
Plasma Streptokinase lysis time	• Streptokinase can be replaced by the test enzyme. • This assay reflects the interplay of all components in the fibrinolytic system with the exception of the activators that are in excess.	(Gidron et al. 1978)

Fibrinolytic assays	Characteristics	References
	• A sensitive screening assay for deficient fibrinolytic activity *in vivo*.	
Streptokinase activated lysis time	• Depends on variation in the levels of various components of the fibrinolytic system.	(Gidron et al. 1978)
Nephelometric method for recording lysis time	• Alternate for Streptokinase activated lysis time	(Gidron et al. 1978)
Partial clot lysis assay	• Easier method wherein upon clot lysis, the red cells released and the amount of lysis that occurred at a given time can be determined by measuring the number of cells released.	(Howell 1964)
Hawkey and Stafford assay	• Relatively shorter and simpler assay for quantitative determinations.	(Hawkey and Stafford 1964)
Esterolytic assays	• Based on hydrolysis of synthetic peptide esters • Very sensitive and suitable for kinetic studies	(Kotb 2013)

However, the assays listed in the table 2.5 do not help in determining the multispecific nature of fibrinolytic enzymes towards various substrates. Thus, in order to determine the type of specificity exhibited by the test enzyme, tests such as protease assay, the amidolytic assay with various blood/plasma proteins as substrates can be performed

(Kotb 2013). The results obtained could further be substantiated by kinetic studies, wherein parameters such as the Michaelis constant (K_m), the rate of reaction (V_{max}) and the turn over number (K_{cat}) of the test enzyme for various substrates could be evaluated. These parameters would help to determine the affinity and specificity of the test enzyme for a particular substrate. Amidolytic assays involve synthetic peptides (specific peptides for a particular substrate), which are mostly tripeptides or quadrapeptides linked via an amide bond to a fluorophore/chromophore such as para-nitroaniline (pNA). This pNA is later cleaved at the C-terminal amide bond, liberating the fluorophore/chromophore, the fluorescence/absorbance of which can be measured spectrophotometrically. Amidolytic activity is expressed as the amount of p-nitroaniline released per minute by the enzyme. This assay is commonly carried out to evaluate the most sensitive/specific substrate for the test enzyme (Kotb 2013).

2.9.2 Anticoagulant and thrombolytic activity

This assay involves the visualisation of the blood clot formation in presence of the test compound, indicating its anticoagulant activity. On the other hand, dissolution of the pre-formed clot would indicate the thrombolytic activity of the test compound. According to this assay, fresh blood is collected and incubated with the test compound, along with a negative (saline) and positive control (such as streptokinase) respectively. The mixture is then allowed to stand at physiological temperature (37 ºC) for a fixed time interval and the clot formation and its subsequent dissolution is observed (Lu et al. 2010; Ju et al. 2012).

In addition to the mere visualisation, the percentage of clot lysis can also be determined based on the weight difference between formed clot versus lysed clot (Prasad et al. 2006; Narasimhan et al. 2015). This would indicate the anticoagulant nature of the test compound. On the other hand, to examine the thrombolytic property of a potential drug candidate, fresh blood could be replaced by pre-formed blood clot with the other steps maintained the same as above.

2.9.3 Clot formation and lysis assay (CloFAL)

It is a global assay of haemostasis widely used in the management of thrombosis and thrombophilia, wherein the formation of the fibrin clot and its subsequent dissolution

can be determined by turbidity measurements. This assay is used clinically to evaluate the net haemostatic balance which is unique to an individual at any given time (Goldenberg et al. 2008). It was reported to be used as a pre-operative screening test in which the clot formation, as well as fibrinolysis, would be measured simultaneously. In addition, it was used to evaluate both clotting and bleeding disorders in children and adults (Chitlur and Simpson 2015).

The assay is performed using platelet poor plasma (PPP), to which either calcium or tissue factor or phospholipid is added to initiate the process of coagulation, while tPA is added to begin fibrinolysis. tPA could be replaced by the test compound for which the fibrinolytic activity has to be measured. The absorbance of the resulting mixture is read at a dual wavelength of 405nm and 605nm for a period of 3 hours using a spectrophotometer. Here an increase in turbidity would indicate the increase in the aggregation of fibrin fibres. A waveform is generated describing the coagulation and fibrinolysis with information regarding the coagulation index, fibrinolytic index and maximum amplitude (Peng and Zhao 2009).

This assay has been used as a rapid adjunctive test for the routine measurement of factor VIII (anti-haemophilic factor) (AHF)/factor IX (Christmas factor) activity in the treatment of haemophilia A/B. It has been shown to be analytically sensitive to the components of coagulation and fibrinolytic systems, sensitive to physiological changes in haemostasis. Some of the demerits of this assay are reduced sensitivity in presence of platelet poor plasma, incapability to detect the mild deficiency in factor VIII (Chitlur and Simpson 2015).

2.10 Other fibrinolytic enzymes

Streptokinase was first produced in 1933 and found its application for clinical use in the mid-1940s (Feied and Handler 2004). It is a plasminogen activator that does not occur naturally in human circulation. The extracellular enzyme streptokinase (EC 3.4.99.22) is produced by various strains of β-haemolytic streptococci. The complete amino acid sequence of the streptokinase was first determined by Jackson and Tang (1982). In general, streptokinase has a molecular weight of 47 kDa and is made of 414 amino acid residues (Malke and Ferretti 1984). Among the streptokinases produced

by the different groups of streptococci, a considerable degree of heterogeneity exists (Banerjee et al. 2004). Streptokinase just like the tissue plasminogen activator and urokinase plasminogen activator do not have a direct fibrinolytic activity and exhibit their therapeutic action by complexing with the circulatory plasminogen forming a streptokinase-plasminogen activator complex, thereby activating the blood plasminogen to the clot lysing plasmin (Banerjee et al. 2004). Streptokinase is known to activate plasminogen both by fibrin-dependent and fibrin-independent mechanisms (Reed at al. 1998). This high-affinity 1:1 stoichiometric complex (streptokinase-plasminogen activator complex) is a highly specific protease that activates other plasminogen molecules to plasmin proteolytically (Bajaj and Castellino 1977; Castellino 1981). Streptokinase consists of multiple structural domains (α-, β- and ɣ-domains) that possess functional properties. The C-terminal domain of streptokinase is involved in plasminogen substrate recognition and activation (Kim et al. 1996, 2002; Zhai et al. 2003). Likewise, the Asp41-His48 region of streptokinase is necessary for its binding to the substrate plasminogen (Kim et al. 2000). Additionally, the role of the residues belonging to the adjacent region on the plasminogen activation has been discussed (Wakeham et al. 2002). It is said that the coiled region of the streptokinase ɣ-domain is essential for the activation of plasminogen molecule (Wu et al. 2001). Robinson et al. (2000) have found that the β-domain of streptokinase is involved in forming the streptokinase-plasminogen complex, required for plasminogen activation. Streptokinase preferentially binds to the extended conformation of plasminogen via the lysine binding site to trigger conformational activation of plasminogen (Boxrud and Bock 2000; Boxrud et al. 2001). The streptokinase-plasminogen activator complex interacts with the plasminogen through long-range protein-protein interactions to enhance the catalytic turnover (Sundram et al. 2003). Without the N-terminal residues, streptokinase is shown to have an unstable secondary structure (Banerjee et al. 2004).

The presence of fibrinolytic activity in human urine was first described in 1947 (Baruah et al. 2006). The active molecule responsible for the fibrinolytic activity was extracted and named urokinase in 1952 (Ouriel 2002). Urokinase is a naturally occurring serine protease (Ouriel 2002) that was originally produced from human

urine. It was also recently obtained by culturing human renal cells (Satoshkar et al. 1998). Structurally, it is composed of two polypeptide chains of 32 and 54 KDa (Baruah et al. 2006). The 54 KDa (high molecular weight form) is predominant in the urokinase from urine and the 32 KDa (low molecular weight form) is majorly found in the urokinase obtained from the tissue culture of kidney cells (Ouriel 2002). Urokinase is found to have a half-life of approximately 15 minutes (Feied and Handler 2004). Human urokinase is not antigenic, but its recovery from urine is expensive (Rouf et al. 1996). Similar to streptokinase, urokinase activates both the circulating plasminogen and the clot-bound plasminogen. However, their use has been found to be associated with a serious risk of haemorrhage (Rouf et al. 1996). Another variant of this streptokinase is the acylated plasminogen streptokinase activator complex (APSAC), which is a complex of bacterial streptokinase with an acylated active site of human plasminogen (Banerjee et al. 2004). It has a molecular weight of 131 KDa (Deitcher and Jaff 2002; Iqbal et al. 2002) with the proteolytic sites being inactivated with an anisoic acid (Nguyen and Samama 1987). These do not require free circulating plasminogen in order to be effective, has a longer half-life of 40 to 90 minutes as compared to that of streptokinase (Feied and Handler 2004; Nguyen and Samama 1987; Banerjee et al. 2004; Deitcher and Daff 2002).

Of the three available plasminogen activators (tPA, uPA, sPA), streptokinase of microbial origin is the least expensive, but immunogenic. Both tPA and uPA are glycoproteins that are trypsin-like serine proteases that activate plasminogen directly. uPA is produced by the kidneys and is secreted into the urine (Barlow 1976; Paoletti and Sherry 1977), while tPA is synthesised by the vascular endothelial cells (Camiolo et al. 1971; Strassburger et al. 1983). tPA has a high affinity for the fibrin clot, activates the clot-bound plasminogen 100-fold more effectively than the circulatory plasminogen (Banerjee et al. 2004). It has been reported that human tPA and uPA are immunogenically benign, but have short-lives *in vivo* and hence are of a limited therapeutic effectiveness. It is said that on comparing the various clinical trials and cost-effectiveness, streptokinase was suggested as the drug of choice for thrombolytic therapy (Mucklow 1995; Gillis and Goa 1996), however, this is debatable (Rouf et al. 1996; Sane and Little 1998; Werf 1999). Researchers believe that since streptokinase

is not a human origin, its introduction into the circulatory systems can lead to a severe anaphylactic response. This immune response depends on the level of antibodies present in circulation against streptokinase (Lee 1995; Jennings 1996). It is observed that the plasmin produced through the streptokinase mediated activation of plasminogen, in turn, lyses the streptokinase, thus limiting the *in vivo* half-life of streptokinase to about 30 minutes. Although streptokinase survives in the circulation for a longer time as compared to the tPA which has a half-life of about 5 minutes, it still is significantly less to exhibit an efficient therapy (Wu et al. 1998).

Staphylokinase (SAK) produced by the Staphylococcus sp. is also a potential alternative plasminogen activator (Matsuo et al. 1990; Okada et al. 2001). Attempts have been made to produce recombinant staphylokinase from Escherichia coli (Sako 1985; Collen et al. 1992; Schlott et al. 1994) and have been demonstrated to induce a fibrin-specific clot lysis in vitro (Matsuo et al. 1990; Lijnen et al. 1991). Several studies were conducted to compare the fibrinolytic properties of staphylokinase and streptokinase using animal models (Lijnen et al. 1991).

Apart from these, other plasminogen activators produced by recombinant DNA technology exist, such as alteplase, reteplase, tenecteplase. Alteplase is a serine protease which is produced by inserting a complementary DNA (cDNA) encoding for tPA and expressed in Chinese hamster ovary (CHO) cells (Rho and Louie 2004). It was the first recombinant tissue-type plasminogen activator found to be identical to native plasminogen activator that cleaves the ‘Arg-Val' bond present on the plasminogen thus resulting in the conversion of plasminogen to plasmin (Feied and Handler 2004). However, in the absence of fibrin, tPA results in a limited conversion of plasminogen unlike streptokinase or urokinase (Rho and Louie 2004). Ross (1999) and Ouriel (2002) had reported the half-life of alteplase to be 4-6 minutes.

Reteplase is a second generation recombinant tissue-type plasminogen activator (Feied and Handler 2004), with a molecular weight of 39 KDa (Ouriel 2002). Reteplase activates plasminogen directly and hence does not involve plasminogen complexing (Baruah et al. 2006). It has been reported that reteplase has a longer half-life of about 14 minutes and higher thrombolytic potency as compared to that of Alteplase (Caridi et al. 2005; Rho and Louie 2004).

Tenecteplase is a bioengineered variant of tPA (Ouriel 2002), wherein the three amino acids, threonine, asparagine and lysine are changed from the native tPA. Multiple point mutations in tenecteplase resulted in enlargement of the molecule resulting in a longer half-life of 18 minutes (Baruah et al. 2006), 25 minutes (Ross 1999).

Nattokinase, an active ingredient extracted from natto is a naturally occurring fibrinolytic enzyme. Natto is a traditional food made from soybeans subjected to fermentation by the bacterium, *Bacillus subtilis*. Nattokinase is known to have properties similar to that of plasmin found within the human body. It lyses fibrin directly and also increases the t-PA levels along with converting prourokinase to urokinase. By reducing the thrombus formation, nattokinase slows down the plaque formation and reverses the occurrence of atherosclerotic lesions (Milner and Makise 2004). Reports suggest the successful application of nattokinase in rats (Ohkuro et al. 1981; Fujita et al. 1995a; Fujita et al. 1995b). In 1993, nattokinase was characterised as a subtilisin-like serine protease, based on its increased sensitivity to protein substrate Suc-Ala-Ala-Pro-Phe-pNA for subtilisin (Fujita et al. 1993). A novel nattokinase protein (NKCP) with both antithrombotic and fibrinolytic effect was discovered by Omura et al. (2005). Wang et al. (2009) have produced nattokinase from *Pseudomonas* sp. with shrimp shells as substrate. An attempt was also made by Wu et al. (2010) to enhance the nattokinase production in *Bacillus subtilis* by altering the promoter of nattokinase gene (Papr N). The *in vivo* effect of nattokinase on carrageenan-induced tail thrombosis in a rat model was evaluated by Kamiya et al. (2010).

2.11 Chemical modification of amino acid residues

In general, chemical modification of the fibrinolytic enzymes is performed using amino-acid specific reagents, which mainly involves modification of side chains of the amino acids (Davis 2003). This, in turn, serves to enhance the functionality of the enzyme of interest (DeSantis and Jones 1999). Some of the commonly adopted strategies to modify enzymes include (i) chemical modification methods comprises of crosslinking using glutaraldehyde, 1,3-dibromoacetate or modification by amphipathic compounds such as PEG (polyethylene glycol) (Eyzaguirre 1996; Ogino and Ishikawa

2001) (ii) physical modification methods such as coating, entrapment/immobilization of the enzyme (iii) molecular biology techniques which encompass site-directed mutagenesis and/or directed evolution (DeSantis and Jones 1999; Davis 2003).

Recent reports suggest the use of a relatively new technique for the chemical modification of the enzymes. The technique makes use of specific amino-acid modifiers that target single/group of amino acid residues. This technique has been reported to overcome the shortcomings of both genetic as well as conventional chemical modification (Cowan and Lafuente 2011).

Chemical modification when performed using amino acid specific reagents solves the problems pertaining to site-directed modification, unlike the conventional chemical modification. The improvised chemical modification not only enhances the specific activity of the enzyme of interest but also increases its specificity towards the desired substrate (Rodrı´guez and Davis 2011). In addition, it gives an insight on the amino acids that are essential for the enzyme activity, in other words, amino acids that are present at or near the active site of the enzyme (Hu and Guo 2009). It is also advantageous over the genetic modification as the risk of inappropriate folding of the enzyme does not arise (Cowan and Lafuente 2011).

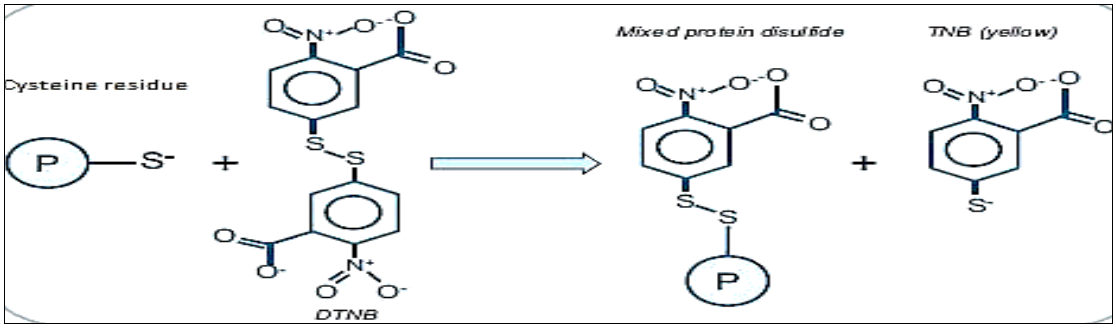


Figure 2.1 Reaction of DTNB with cysteine (adapted from Rudyk and Eaton 2014)

Among the 20 standard amino acids, only a few amino acid side chains can be modified easily and they are classified into three groups on the basis of their reactivity: (a) High reactivity exhibited by aspartate, glutamate, lysine, arginine, tyrosine, methionine, tryptophan, cysteine and histidine amino acids residues- (b) Medium

reactivity is shown by serine , threonine, asparagine and glutamine amino acid residues (c) Low reactivity is demonstrated by glycine, alanine, valine, leucine, isoleucine, phenylalanine and proline amino acid residues (Jorgensen 1983).

DTNB [5,5-Dithiobis-(2-Nitrobenzoic Acid)] is used commonly to modify the nucleophilic group or sulfhydryl group (cysteine) in the amino acids. According to Rudyk and Eaton (2014), DTNB acts on the sulphur atom present in the cysteine residue and forms a disulfide, leaving TNB (5-Thio-2-NitroBenzoicacid) (Figure 2.1).

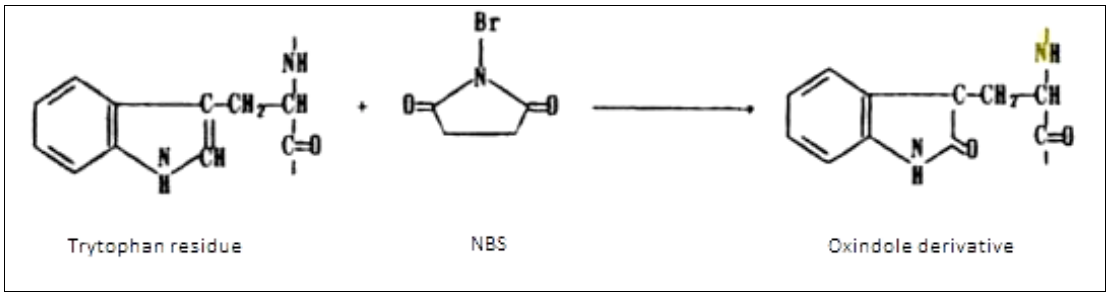


Figure 2.2 Reaction of NBS with tryptophan (adapted from Lundblad 1995)

NBS [N-Bromosuccinimide] is commonly used for modifying tryptophan residues. It is said to act on the second position of the indole ring which is the part of the tryptophan residue and produces an oxindole derivative (Lundblad 1995), the reaction is as shown in figure 2.2.

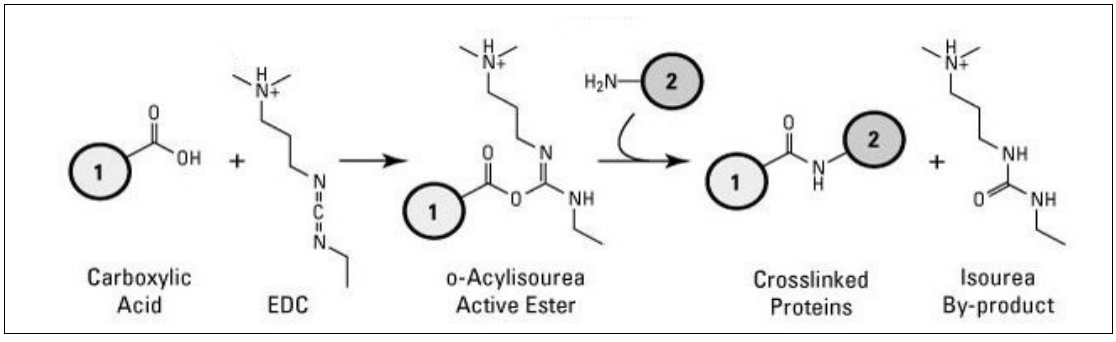


Figure 2.3 Reaction of EDAC with carboxylic acid (adapted from Hermanson 2013)

EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] is used to modify the carboxylate group residues in the amino acids. According to Hermanson (2013), EDAC reacts with carboxylic acid groups present in aspartic and glutamic acid to form

an active O-acylisourea intermediate that is easily displaced by a nucleophilic attack from free amino groups of the protein molecules present in the reaction mixture (Figure 2.3). The amino group forms an amide bond with the original carboxyl group and releases an EDC by-product which is a soluble urea derivative. The O-acylisourea intermediate is unstable in aqueous solutions, and thus get hydrolysed which leads to crosslinking between protein molecules and the release of an N-unsubstituted urea (Hermanson 2013).

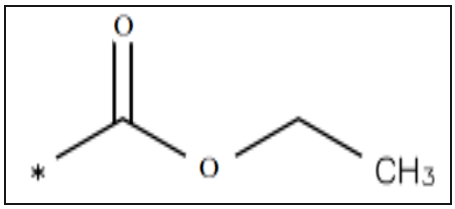


Figure 2.4 Carbethoxylated residues

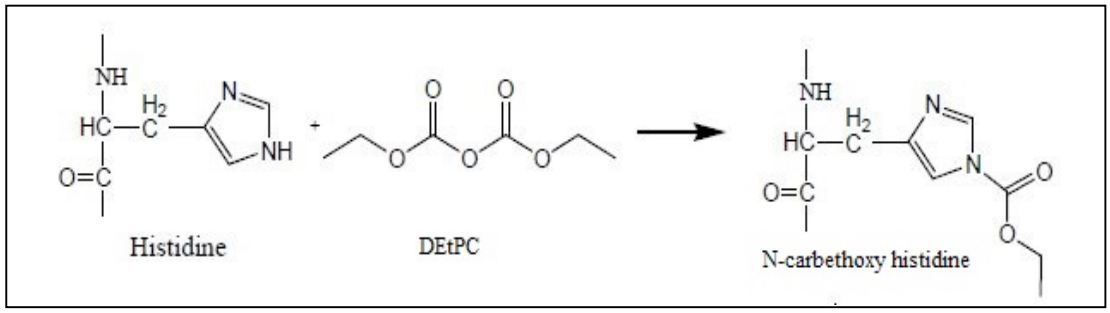


Figure 2.5 Reaction of DEPC with histidine (adapted from Zhou 2014)

DEPC [Diethyl Pyrocarbonate] is typically used to modify histidine residues. Zhou (2014) had reported that DEPC reacts with imidazole ring of histidine to produce carbethoxylated residues (Figure 2.4), called as N-carbethoxy histidine (Figure 2.5).

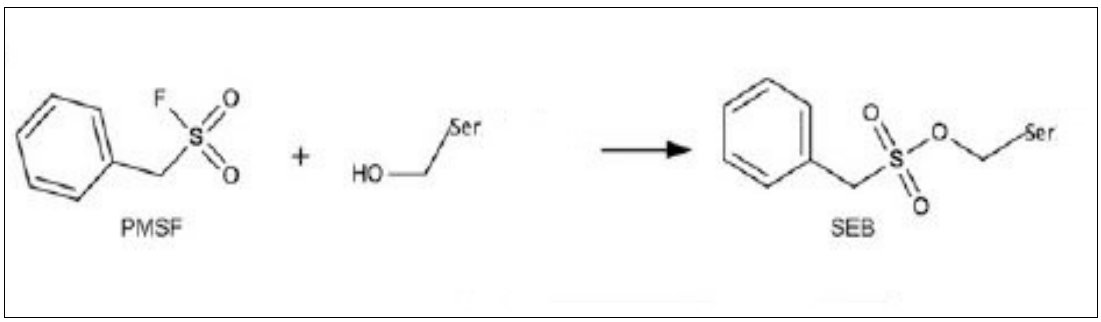


Figure 2.6 Reaction of PMSF with serine (adapted from Han et al. 2012)

Serine residues present in the amino acids are usually modified using PMSF [Phenylmethylsulfonyl fluoride]. PMSF acts on the hydroxyl group of the serine and forms O-benzylsulfonyl serine (SEB) as shown in figure 2.6. (Han et al. 2012).

Apart from the ones mentioned above, certain other reagents used are TNBS (2, 4, 6-Trinitrobenzene sulfonic acid) for modifying lysine residues, pCMB (para-chloromercuribenzoate) and IAM (iodoacetamide) to modify the thiol groups. Arginine, tyrosine and tryptophan residues are modified by using reagents such as CHD (1, 2-cyclohexanedione) and BD (2, 3-butanedione), TNM (tetranitromethane), NAcI (N-acetylimidazole); HNBB (2-hydro-5-nitrobenzyl bromide) (Hu and Guo 2009; Kotsira and Clonis 1998; Wen et al. 1999).

Till date, various enzymes such as the oxalate oxidase, xylanase, L-asparaginase, bromoperoxidase have been modified chemically using amino-acid specific reagents (Hu and Guo 2009; Wen et al. 1999; Pritsa et al. 2001; Kongkiattikajorn and Panijpan 2006). Oxalate oxidase (OxO), extracted and purified from wheat seedlings was chemically modified using reagents such as DTNB and NEM (N-ethylmaleimide). It was observed that the enzyme activity decreased with increasing concentrations of DTNB, NEM, EDAC and TNBS thereby indicating the need for cysteine, carboxylate, lysine residues for OxO activity. However, reagents such as DEPC, PMSF and NBS did not affect the enzyme activity, thus suggesting that histidine, serine and tryptophan residues are not essential for OxO activity (Hu and Guo 2009). Another such enzyme, xylanase from *Trichosporon cutaneum*, when modified with EDAC, Woodward's Reagent K, pCMB, IAM, substantial loss in the enzyme activity was observed, denoting that both carboxylate, cysteine residues and thiol groups are essential for the xylanase activity (Wen et al. 1999). On the other hand, when treated with reagents such as CHD, BD, TNBS, TNM, NAcI, DEPC, NBS and HNBB, no significant loss in the xylanase activity was seen, thus suggesting that arginine, lysine, tyrosine, histidine and tryptophan residues do not contribute to the enzyme activity (Wen et al. 1999). Pritsa et al. (2001) had reported the chemical modification of the enzyme L-asparaginase from *Thermus thermophiles* using DEPC, BD, EDAC, PMSF and NEM. In case of DEPC, BD and EDAC, the enzyme activity decreased with

increase in the concentration of the modifiers, thereby indicating that histidine, arginine and carboxylate residues may be present at or near the active site of the enzyme and would be essential for its activity. In presence of PMSF and NEM, there was partial or no inactivation of the enzyme. Kongkiattikajorn and Panijpan in 2006 reported the chemical modification of bromoperoxidase from the red alga *Gracilaria tenuistipitata*, wherein a 5 fold increase in the enzyme activity was observed when modified with TNBS and IAM.

CHAPTER 3

ISOLATION AND SCREENING OF MARINE MICROORGANISMS

FOR FIBRINOLYTIC ENZYME

This chapter describes the isolation of marine microorganisms from seawater sample. All isolates were screened for fibrinolytic enzyme production using both qualitative and quantitative approaches. Based on the results obtained from screening studies, one isolate was chosen, identified and taken forth for further studies as described in the subsequent chapters.

3.1 MATERIALS AND METHODS

Fibrin, thrombin, fibrinogen (human origin), bovine serum albumin powder and Sephadex G-100 were purchased from Sigma-Aldrich, India. Folin & Ciocalteu's phenol reagent and trichloroacetic acid were from LOBA Chemie, India. Starch casein agar, agar, asparagine, Luria Bertani broth, yeast extract powder and dialysis membranes-150 were purchased from HiMedia laboratories, India. Dextrose was obtained from Merck, India. K_2HPO_4, $MgSO_4 \cdot H_2O$, $CaCl_2 \cdot 2H_2O$ were purchased from Spectrum, Nice chemicals, India. All other chemicals and reagents used were of analytical grade.

3.1.1 Isolation and maintenance of microorganisms

Seawater from a depth of 10 m, 5 km away from Surathkal Coast in the Arabian Sea, was collected using an automated depth water sampler and was used for the isolation of microorganisms. Water samples were serially diluted (10^{-6} - 10^{-9}) and were plated on both starch casein agar and glucose asparagine agar and incubated at 37 °C for 48 h. Composition of glucose asparagine agar (g/L): glucose, 10; asparagine, 0.5; K_2HPO_4, 0.5; agar, 15 (pH 7) and starch casein agar (g/L) was: casein, 1; starch, 10; sea water, 37; agar, 15 (pH 7.2). Individual colonies after 48 hours of incubation were picked and transferred onto starch casein agar plates with amphotericin (50µg/mL) as the antifungal agent. Microorganisms were subcultured periodically every two months on starch casein agar and stored for use. Cultures were lyophilised using a combination of 10% (w/v) sucrose and 10% (w/v) skimmed milk as cryoprotectants and stored in the refrigerator for long-term use.

3.1.2 Qualitative screening

Qualitative screening was carried out according to the method described by Astrup and Mullertz (1952) with minor modifications. The cultures were plated on modified fibrin agar screening medium with a composition of 1.2% (w/v) agarose solution, 40 mg/mL human fibrinogen solution (prepared in 0.9% (w/v) saline) and 100 U/mL human thrombin solution. These plates were then incubated at 37 °C for 7 days.

The isolates positive for the fibrinolytic enzyme production were inoculated onto a sterile unoptimised medium having a composition (g/L): glucose, 20; peptone, 10; yeast extract, 10 ; $KH_2PO_4 \cdot 3H_2O$, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.5 g/L and pH 7, incubated at 37 °C for 48 h. The cell-free supernatant obtained by centrifuging the fermentation broth at 12,000 × *g* at 4 ºC for 10 min, was taken to confirm the presence of fibrinogenolytic and fibrinolytic activities according to the method described by Shivaprasad *et al.* (2009) with minor modifications. The cell-free broth was mixed with an equal volume of fibrinogen solution (prepared by dissolving 10 mg in 50 mL of 0.9% w/v NaCl solution). Absorbance of the mixture was read at 540 nm in UV/Vis spectrophotometer (LabIndia) every 15 min, starting from 0^{th} min to 210^{th} min against a suitable blank. The experiments were carried out in duplicates and the mean values were plotted. Thrombin (100 U/mL) was used as the control.

3.1.3 Assay for fibrinolytic activity

To estimate fibrinolytic activity, method described by Agrebi *et al.* (2009) was followed using fibrin as substrate. Substrate solution was prepared by mixing 10 mg of fibrin in 100 mL of 0.1 M glycine-NaOH buffer (pH 9). One millilitre enzyme solution was mixed with equal volume of substrate solution, incubated at 55 °C for 15 min. This was followed by the addition of 0.2 M TCA solution (1 mL) to the reaction mixture and incubated at room temperature for 15 min. The contents were centrifuged at 12,000 × *g* for 15 min at 4 °C to remove the precipitate. Absorbance of the supernatant was read at 280 nm against a suitable blank. A standard graph was constructed using different concentrations of tyrosine, ranging between 0 - 100 µg/mL and the absorbance was read at 280 nm (Appendix I). One unit of fibrinolytic enzyme

activity was expressed as 1 µg of tyrosine liberated per min, under standard assay conditions.

3.1.4 Assay for fibrinogenolytic activity

The fibrinogenolytic assay was carried out as described by Wang *et al.* (2009) with slight modifications. In brief, substrate solution was prepared by mixing 10 mg of fibrinogen in 50 mL of 0.9% (w/v) NaCl solution. One millilitre of the substrate solution was mixed with 0.5 mL of 245 mM phosphate buffer (pH 7) and incubated at 37 °C for 5 min. As our aim was to estimate the fibrinogenolytic activity, addition of thrombin was avoided, since thrombin converts fibrinogen to fibrin. One millilitre of enzyme solution was added to the reaction mixture, followed by mixing for 5 min at 30 °C. This was followed by the addition of 1 mL of 0.2M TCA solution to the reaction mixture. The reaction mixture was then centrifuged at 10,000 × *g* for 10 min at 4 °C and the absorbance of the supernatant was read at 280 nm. One unit of fibrinogenolytic enzyme activity is expressed as 0.01 per min increase in absorbance at 280 nm.

3.1.5 Anticoagulant activity

In vitro anticoagulant activity of the fibrinolytic enzymes were examined by the method described by Lu *et al.* (2010) with minor modifications. One millilitre of fresh human whole blood (healthy male volunteers) was added to equal volume of cell-free crude enzyme solutions (suitably diluted with 0.9 % NaCl, pH 7.4), in a sterile glass test tube, mixed gently and incubated at 37 ºC for 6 h. Filter sterilised saline (0.9 % NaCl, pH 7.4) was used as negative control. Coagulation of the blood was monitored with respect to control. Upon incubation for 6 h, the supernatant liquid was decanted and the extent of clot formed was observed visually.

3.1.6 Thrombolytic activity

The thrombolytic effect of the fibrinolytic enzymes was assessed according to the method described by Lu *et al.* (2010) with minor modifications. Briefly, one millilitre of fresh human whole blood (healthy male volunteers) was allowed to clot upon

incubation at 37 ºC for 1 h. Subsequently, to the solid clot formed, 1mL of cell-free crude enzyme solutions (suitably diluted with 0.9 % NaCl, pH 7.4) were added and incubated at 37 ºC for 6 h. Filter sterilised saline (0.9 % NaCl, pH 7.4) was used as negative control. Upon incubation for 6 h, the supernatant liquid was decanted and the extent of clot lysed was observed visually.

3.1.7 Partial purification of the fibrinolytic enzyme

Four isolates selected based on the initial screening studies were cultured in the previously mentioned unoptimised medium (3.1.2). The cell-free crude enzyme solutions of cultures C1, C3, C5 and C7 were then subjected to ammonium sulphate salt precipitation under ice-cold conditions. Initial trials were conducted by varying the concentration of ammonium sulphate (40%, 50%, 60%, 70%, and 80% saturation), in order to determine an optimal percentage saturation of the salt required for precipitation of the fibrinolytic enzyme. Care was taken to ensure that the desired final concentration of ammonium sulphate was achieved by gradually adding increasing concentrations of the salt at each step, following which the samples were centrifuged. The precipitated proteins were later dialysed using a dialysis membrane (molecular weight cut off of 12 – 14 KDa) against 20 mM sodium phosphate buffer of pH 7 overnight under ice-cold conditions.

3.1.8 Protein concentration

Protein concentration was determined using a method as described previously by Lowry *et al.* (1951), using BSA (Bovine Serum Albumin) as standard, measuring the absorbance at 660 nm (Appendix II).

3.1.9 Effect of pH on the partially purified enzyme

The partially purified enzyme solutions were incubated with the respective buffers (Appendix III) ranging from pH 4.0 to 9.0 for 30 min at room temperature along with the substrate and fibrinolytic enzyme activities were determined upon completion of the incubation period, under standard assay conditions. All experiments were carried in duplicates and average values were calculated.

3.1.10 Identification of the isolate

Out of all the isolates obtained, only one microorganism was chosen based on aforesaid mentioned criteria and was characterised by 16S rRNA gene sequencing and the phylogenetic analysis was carried out (by Microbial Culture Collection, National Centre for Cell Science, Pune, India), the report was generated using EZ Taxon Database (Krishnamurthy et al., 2017).

3.2 RESULTS AND DISCUSSION

3.2.1 Isolation and screening

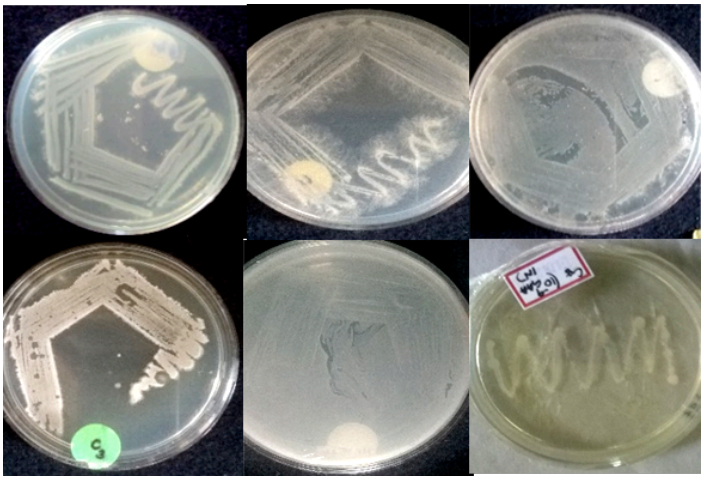


Figure 3.1 Six isolates on starch casein agar plate

In this study, eight bacterial strains designated as C1, C2, C3, C4, C5, C6, C7 and C8 were isolated from seawater sample. During the initial screening experiments that were performed using modified fibrin agar plates, only six isolates tested positive for fibrinogenolytic and fibrinolytic enzyme production (Figure 3.1). Appearance of turbid-zone during initial 48 h of incubation indicates the conversion of fibrinogen to fibrin due to fibrinogenolytic activity, followed by the appearance of a clear zone within the turbid zone indicating the presence of fibrinolytic activity after prolonged incubation for 7 days (Figure 3.2A). Colony morphologies of the 6 isolates that tested positive for the production of fibrinogenolytic/fibrinolytic enzyme were studied (Appendix IV).

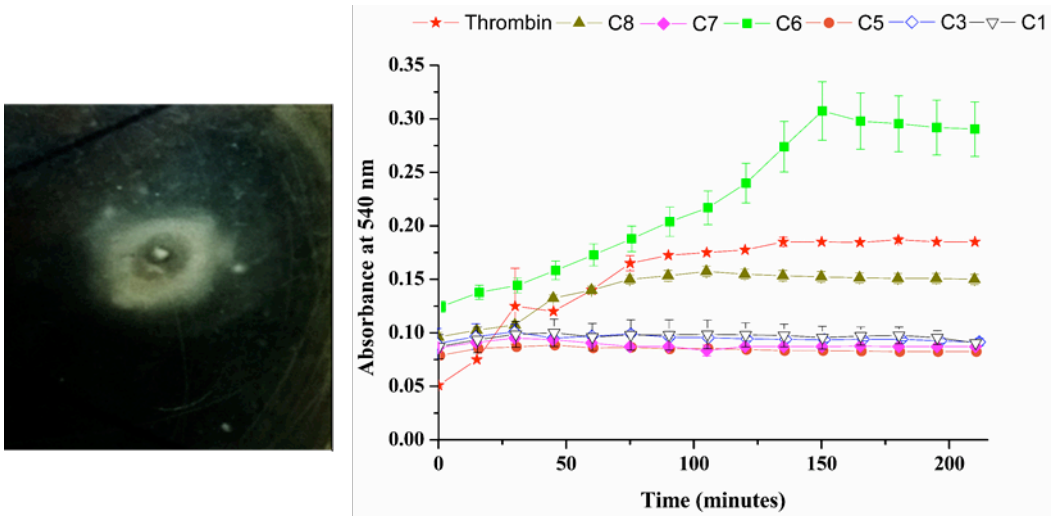


Figure 3.2 Screening A) Qualitative screening (appearance of clear zone within the turbid zone on modified fibrin agar plate), B) Spectrophotometric analysis for the confirmation of presence of fibrinogenolytic and fibrinolytic activities of the cell-free crude enzyme solutions (C1, C3, C5, C6, C7, C8), thrombin as control. Bars represent standard deviation (n = 2).

Qualitative measurement of fibrinolytic/fibrinogenolytic activity of cell-free broth showed that all the six isolates showed an instant increase in the absorbance when cell-free broth was mixed with fibrinogen solution (Figure 3.2B). On the contrary, thrombin solution, which was used as the control, took almost 50 min to show the same degree of absorbance. In case of isolates C8 and C6, the absorbance showed an increasing trend up to 105 min and 150 min respectively, whereas other isolates did not show any further increase in absorbance. However, the duration of both fibrinogenolytic and fibrinolytic activities observed varied among isolates. Here, the increase in absorbance is due to the increase in turbidity as a result of fibrinogenolytic activity involving the conversion of fibrinogen to fibrin clot. After reaching the peak absorbance, a gradual decrease was observed in all the cases, perhaps due to the breakdown of the fibrin clot into the fibrin degradation products.

A quick review of existing literature showed that most of the microorganisms capable of producing a fibrinolytic enzyme, also produce fibrinogenolytic enzyme (Mander *et al.*, 2011; Yogesh and Halami, 2015). In these reports, time-dependent degradation of

fibrinogen by the enzyme has been demonstrated by loading on to SDS-PAGE, but the fibrinogenolytic activities have not been quantified.

In the present study, as qualitative screening could only confirm the presence of fibrinolytic/fibrinogenolytic activity of the selected isolates, accurate determination of fibrinolytic activity and fibrinogenolytic activity of each individual isolate was necessary to select the most desired one. Using specific estimation methods, fibrinogenolytic and fibrinolytic activities for the cell-free crude enzyme extracts of all 6 isolates were determined, and the simplified ratios of fibrinolytic to fibrinogenolytic activity were calculated accordingly, as seen in table 3.1.

Table 3.1 Fibrinolytic and fibrinogenolytic activities of the cell-free crude enzyme solutions of the six isolates

Isolates	**Total Protein (mg)**	**Total Fibrinolytic Enzyme Activity (U/ml)**	**Specific Fibrinolytic Enzyme Activity* (U/mg protein)**	**Specific Fibrinogenolytic Enzyme Activity* (U/mg protein)**	**Fibrinolytic: Fibrinogenolytic ratio**
C7	2.51	191.65	76.26 ± 0.03	9.93 ± 0.01	8:1
C5	2.19	195.67	88.98 ± 0.07	12.70 ± 0.07	7:1
C3	2.31	181.28	78.44 ± 0.28	12.46 ± 0.02	7:1
C1	2.76	158.40	57.49 ± 0.01	10.12 ± 0.04	6:1
C6	2.47	163.25	66.01 ± 0.0	11.26 ± 0.01	6:1
C8	2.73	186.97	68.51 ± 0.10	9.95 ± 0.0	7:1

*Data represents mean ± SD (n = 2).

The total protein concentration of cell-free broth of all the 6 isolates were determined and were used to calculate specific fibrinolytic and fibrinogenolytic activities. The isolate C5 showed the highest specific fibrinolytic and fibrinogenolytic activity (Table 3.1). However, the ratio of specific fibrinolytic to specific fibrinogenolytic activity was 7:1 for C5 isolate while it was 8:1 for C7 isolate. This indicated that the enzyme produced by the isolate C7 has relatively increased specificity for fibrin substrate as compared to fibrinogen substrate. Similar results were reported in the aqueous

extracts of *Moringa oleifera* (Moringaceae) leaves (Satish *et al.*, 2012), root and latex extracts of *Asclepiadaceae* plants (Bindhu and Singh, 2014), and *Calotropis gigantea* (Shivaprasad *et al.*, 2009).

3.2.2 Anticoagulant activity

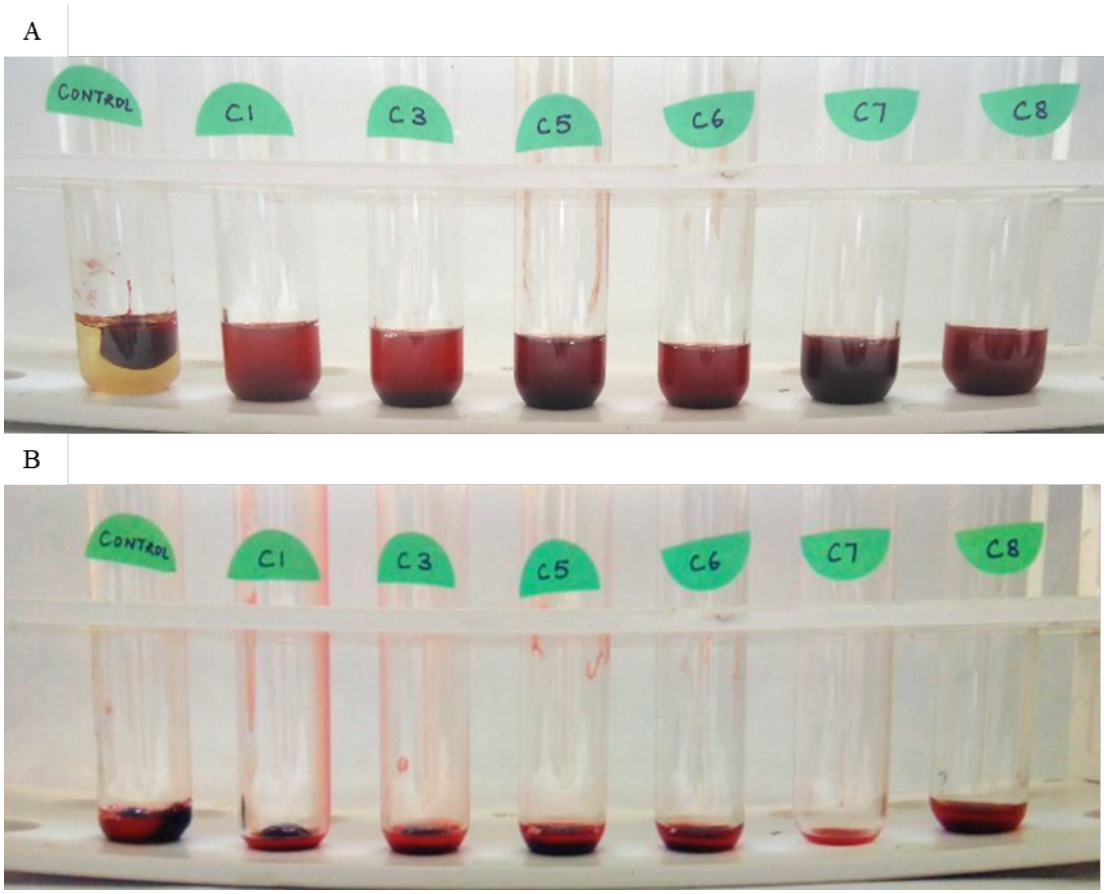


Figure 3.3 *In vitro* assay - Visualisation of anticoagulant effect of the cell-free crude enzyme solutions on human blood *in vitro* (A) formation of blood clots after incubation with the cell-free crude enzyme solutions for 6 h (B) the plasma decanted.

As shown in figure 3.3, clotting of blood had occurred in presence of normal saline (control) after 6 h whereas, in presence of crude enzyme solutions, clot formation was reduced. Particularly, in the test tube containing crude enzyme extract produced by C7 isolate, a minuscule amount of blood clot was formed after 6 h. These results indicate

that the crude enzyme extract of isolate C7 showed significant anticoagulant activity *in vitro* as compared to the control.

3.2.3 Thrombolytic activity

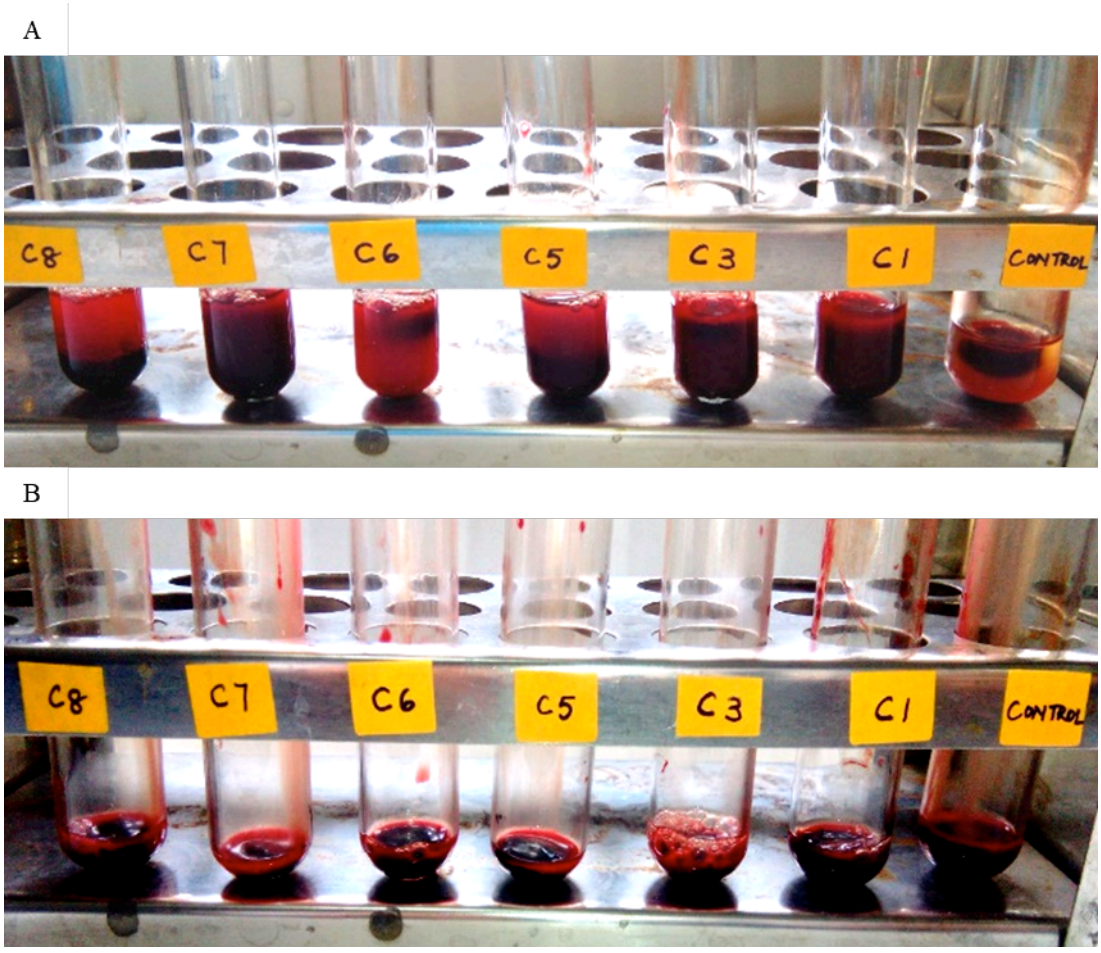


Figure 3.4 *In vitro* assay - Visualisation of lysis of pre-formed blood clots by the cell-free crude enzyme solutions *in vitro* (A) clot lysis observed upon 6 h of incubation of blood clots with the cell-free crude enzyme solutions (B) the plasma decanted.

Thrombolytic activity of the crude enzymes were indicated by the blood clot dissolution upon incubation for 6 h (Figure 3.4). However, in the control tubes (normal saline) there was no clot dissolution observed. Enzymes produced by the isolates C5 and C7 showed relatively greater clot lysis in comparison to enzymes produced by the other isolates. Similar results were obtained by Ju *et al.* (2012) and

Lu *et al.* (2010) with the bacterial strains *Streptomyces* sp. XZNUM 00004 and *Paenibacillus polymyxa* EJS-3 respectively.

3.2.4 Partial purification and effect of pH

Four isolates (C1, C3, C5 and C7) were selected based on the initial screening studies. Although C1 showed a relatively lesser fibrinolytic activity (Table 3.1), it showed reasonable clot lysis during *in vitro* trials (Figure 3.3 and 3.4). Further, the cell-free supernatants of these isolates were partially purified and their fibrinolytic activities were estimated (Table 3.2).

Table 3.2 Fibrinolytic activity upon partial purification

Isolate	Specific Fibrinolytic Activity* (U/mg)	Isolate	Specific Fibrinolytic Activity* (U/mg)
C1	10.91 ± 0.02	C5	64.02 ± 0.12
C3	24.46 ± 0.24	C7	90.30 ± 0.1

*Data represents mean ± SD (n = 2).

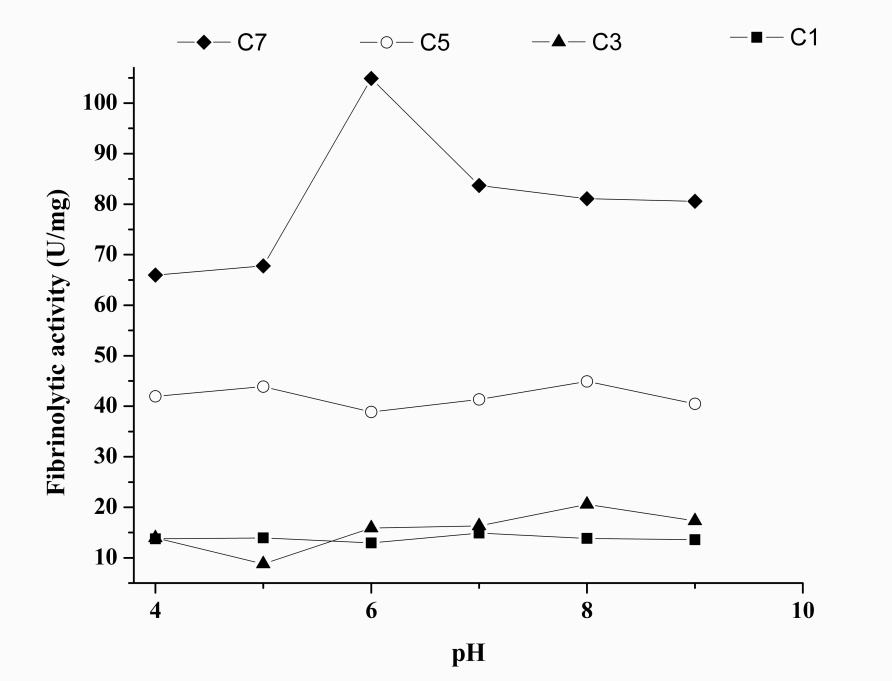


Figure 3.5 Effect of pH on fibrinolytic activity of partially purified fibrinolytic enzyme of the four isolates: C1, C3, C5, C7.

Effect of varying pH on the partially purified enzymes was also studied (Figure 3.5). Isolate C7 showed a maximum specific activity of 104.89 U/mg at pH 6.0 and 80.58 U/mg at pH 7.0. Thus, as compared to the other isolates, the isolate C7 showed maximum fibrinolytic activity at the human physiological pH range.

Based on the results obtained from screening experiments, the enzyme produced by C7 isolate showed significant anticoagulant and thrombolytic effect *in vitro*, relatively higher ratio of fibrinolytic/ fibrinogenolytic activity (8:1) with maximum fibrinolytic activity at the physiological pH range. Thus, the isolate C7 which showed superior performance in every aspect was chosen for further studies.

3.2.5 Identification of isolate C7

The organism was identified as *Serratia marcescens* subsp. *sakuensis* by 16S rRNA gene sequencing.

5’

TGGCTCAGATTGAACGCTGGCGGCAGGGCTTAACACATGCAAGTCGAGCGGTAG
CACAGGGGAAGCTTGCTCCCCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCT
GGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCA
TAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTCCC
AGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA
TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGA
AGGTGGTGAGCTTAATACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGG
CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCG
GGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGG
GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGT
GGCGAAGGCGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGG
TTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGG
AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACAT

CCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCC
AGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA
GTAGGGCTACACACGTGCTACAAGGCGTATACAAAGAGAAGCGACCTCGCGAGA
GCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAG
TAGGTAGCTTAACCTTCGGGGA 3’

Table 3.3 Molecular identification results

Sr. No.	Closest Neighbour Strain	Accession No.	Pairwise Similarity (%)	Diff/Total nucleotides
1.	*Serratia marcescens* subsp. *sakuensis*	AB061685	99.72	4/1438

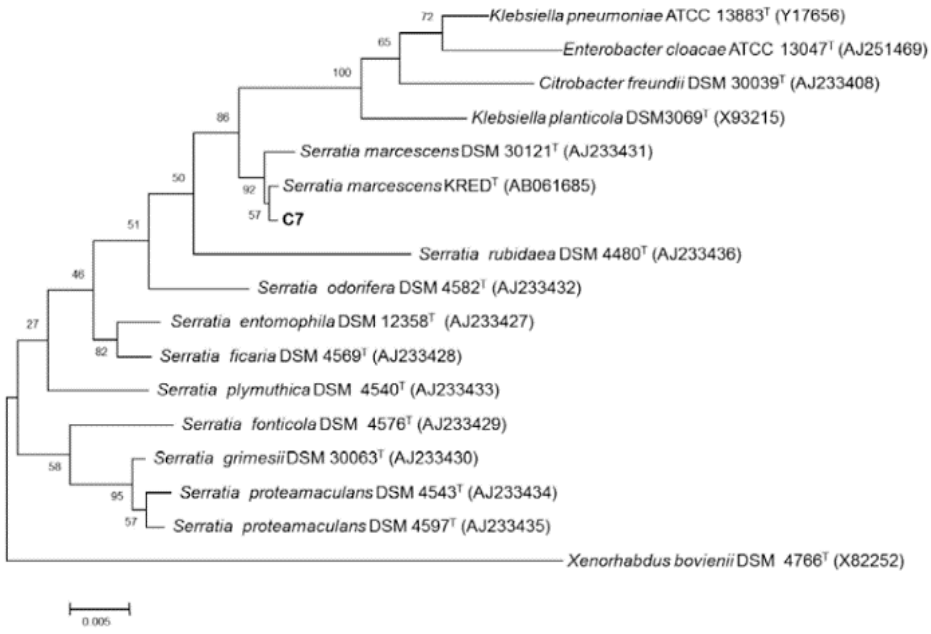


Figure 3.6 Phylogenetic tree of the isolate C7

The sequence data showed a pairwise sequence similarity of 99.72% with *Serratia marcescens* subsp. *sakuensis* (Ajithkumar *et al.*, 2003) (Table 3.3). The phylogenetic

tree of C7 is shown in figure 3.6, and the 16S rRNA sequence was submitted to GenBank database at NCBI with the accession number KU 296189.1.

3.3 SUMMARY AND CONCLUSIONS

- Eight bacterial strains (C1, C2, C3, C4, C5, C6, C7, and C8) were isolated from the seawater sample.
- Out of these eight isolates, only six cultures tested positive for both fibrinolytic and fibrinogenolytic activities when plated on modified fibrin plate agar during the initial screening experiments.
- Further, screening studies comprised of a rational approach which included both qualitative and quantitative studies. Qualitative studies involved the visualisation of the presence of both fibrinolytic and fibrinogenolytic activities of the cell-free broths of the six isolates. It was seen that the duration of both fibrinogenolytic and fibrinolytic activities varied among the isolates.
- Quantitative studies included specific estimation methods that were used to measure fibrinolytic activity and fibrinogenolytic activity of the six isolates. The various assays included the measurement of the ratio of fibrinolytic and fibrinogenolytic activities and *in vitro* anticoagulant, thrombolytic activities of the crude enzyme solutions of the six isolates followed by evaluating the specific fibrinolytic activities of the four isolates and determining the optimum pH range for maximum fibrinolytic activity.
- It was found that the isolate C7 exhibited the highest ratio of fibrinolytic to fibrinogenolytic activity (8:1) as compared to the ratio displayed by the other isolates, thus, indicating a higher specificity towards fibrin.
- For a period of 6 h, there was reduced clot formation in the tubes containing the crude enzyme solutions as compared to the clot formed in control having saline. However, in the tube containing crude enzyme solution of C7, a minuscule amount of blood clot was formed, suggesting that fibrinolytic enzyme produced by the isolate C7 showed significant anticoagulant activity *in vitro*.

- In addition, the fibrinolytic enzyme produced by the isolates C7 and C5 showed relatively greater clot lysis as compared to the enzyme produced by the other isolates, indicating a higher thrombolytic potential.
- Based on the results obtained from the previous studies, four isolates (C1, C3, C5, and C7) were selected for further trials. Their cell-free supernatants were partially purified and the fibrinolytic activities were estimated.
- Effect of varying pH on the fibrinolytic enzyme activity was studied, it was found that C7 showed a specific fibrinolytic activity of 80.58 U/mg at pH 7.0 which was relatively higher when compared to other isolates. This result particularly is favourable as pH 7.0 is very close to the human physiological pH.
- Since the isolate C7 showed superior performance in all the screening experiments it was chosen for further studies.
- The organism was identified as *Serratia marcescens* subsp. *sakuensis* based on 16s rRNA gene sequencing was submitted to GenBank database at NCBI accession number (KU296189.1).

CHAPTER 4

MEDIUM OPTIMISATION FOR ENHANCED FIBRINOLYTIC ENZYME PRODUCTION

In this chapter growth profile of the organism was determined and effect of several nutritional components on the fibrinolytic enzyme production were evaluated using both conventional as well as statistical approach. Attempts were made to enhance the fibrinolytic enzyme production by varying the concentration of nutritional factors that were found to significantly influence the enzyme production.

4.1 MATERIALS AND METHODS

Fibrin (human origin) was purchased from Sigma-Aldrich, India. Starch casein agar, agar, asparagine, Luria Bertani broth, maltose, D-(+)-galactose, soya peptone, yeast extract powder and dialysis membranes-150 were purchased from HiMedia laboratories, India. Dextrose was obtained from Merck, India. NaCl, K_2HPO_4, $MgSO_4 \cdot H_2O$, $CaCO_3$, $CaCl_2 \cdot 2H_2O$, $FeSO_4$ were purchased from Spectrum, Nice chemicals, India. All other chemicals and reagents used were of analytical grade.

4.1.1 Growth profile of the microorganism

The growth profile of the organism was studied by culturing the microorganism in LB broth and incubating at 37 °C, 150 rpm. The absorbance of the broth was measured at 600 nm every two hours up till 74 h against a suitable blank. The data obtained was plotted with the absorbance values representing the cell density versus the time interval.

4.1.2 One factor trials

A total of 12 factors, 3 carbon sources, 3 nitrogen sources, 3 salts and 3 inducers were selected for the study.

For screening of carbon sources, shake flask experiments were conducted by altering 3 different carbon sources, glucose, galactose and maltose with their respective concentrations being 20 g/L, while maintaining the other medium components and their respective concentrations same as in the unoptimised medium (yeast extract, peptone, K_2HPO_4,$MgSO_4.7H_2O$ and $CaCl_2.2H_2O$; chapter 3).

For screening of nitrogen sources, shake flask experiments were conducted by altering 3 different nitrogen sources, soya peptone, yeast extract and tryptone with their

respective concentrations being 20 g/L, while maintaining the other medium components and their respective concentrations same as in the unoptimised medium (glucose, K_2HPO_4, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$; chapter 3).

For screening of salts, shake flask experiments were conducted by altering 3 different salts, K_2HPO_4, NaCl and $MgSO_4.7H_2O$ with their respective concentrations being 0.5 g/L, while maintaining the other medium components and their respective concentrations same as in the unoptimised medium (glucose, yeast extract, peptone and $CaCl_2.2H_2O$; chapter 3).

For screening of inducers, shake flask experiments were conducted by altering 3 different inducers, $CaCO_3$, $CaCl_2.2H_2O$ and $FeSO_4.7H_2O$ with their respective concentrations being 0.5 g/L, while maintaining the other medium components and their respective concentrations same as in the unoptimised medium (glucose, yeast extract, peptone, K_2HPO_4 and $MgSO_4.7H_2O$; chapter 3).

For all of the above trials, initial medium pH was set between 7.0-7.2. Two sets of flasks were prepared for each trial and 5% (v/v) of the active log phase culture was inoculated, followed by incubating at 37 °C, 150 rpm. Samples were withdrawn every 8 hours starting from the 16^{th} h till the 72^{nd} h to determine the time interval at which there is maximum desired enzyme activity. Care was taken to limit the sampling from each flask to a maximum of 4 times so as to minimise the changes in the medium volume and loss of biomass, which in turn could lead to a false decrease in desired enzyme activity. The samples were centrifuged at 10000 rpm for 5 min to separate out the cell debris. The crude culture supernatant was subjected to fibrinolytic enzyme assay and the total fibrinolytic enzyme activity was calculated for each trial (in duplicates), the average values of which were calculated and plotted.

As the organism was isolated from seawater, its effect on enzyme production was considered as a factor for the study. Experiments were designed with seawater as a medium component. The enzyme activity was estimated by incorporating different concentrations of seawater in the production medium along with the other components as shown in table 4.1. A control was maintained with no sea water being added. The flasks were incubated for 32 h at 37 °C, 150 rpm, following which samples were withdrawn and assayed for fibrinolytic enzyme activity.

Table 4.1 Effect of seawater on enzyme production

Expt	Galactose	Yeast Extract	NaCl	K_2HPO_4	$MgSO_4$	$FeSO_4$	$CaCO_3$	Seawater
	← % (w/v) →							% (v/v)
Control	2	2	0.05	0.01	0.1	0.01	0.1	-
1	2	2	-	-	-	-	-	1
2	2	2	-	-	-	-	-	5
3	2	2	-	-	-	-	-	10
4	2	2	-	-	-	-	-	15
5	2	2	0.05	0.01	0.1	0.01	0.1	5

4.1.3 Statistical optimisation by Plackett-Burman design

In order to determine the factors having a major effect on the fibrinolytic enzyme production, Plackett-Burman design matrix was constructed using Stat-Ease Design Expert software v7.0.0 (Table 4.2). A total of nineteen (n) variables comprising nutritional factors such as dextrose, galactose, maltose, yeast extract, soya peptone, NaCl, K_2HPO_4, $MgSO_4$, $CaCO_3$, $FeSO_4$, sea water (passed through 0.22 µm filter) and eight dummy variables were screened in twenty (n+1) experiments. Each variable was examined at low (-1) and high (+1) level of concentrations. Concentrations of the factors chosen were: dextrose (1-4% w/v), galactose (1- 4% w/v), maltose (1 - 4% w/v), yeast extract (0.5 - 2% w/v), soya peptone (0.5 - 2% w/v), NaCl (0.01 - 0.05% w/v), K_2HPO_4 (0.01 - 0.05% w/v), $MgSO_4$ (0.05 - 0.15% w/v), $CaCO_3$ (0.05 - 0.15% w/v), $FeSO_4$ (0.01 - 0.05% w/v) and sea water (1 - 5% v/v). All experiments were conducted in 250 mL Erlenmeyer flasks, with a medium volume of 100 mL and 5 % (v/v) inoculum was used. The initial medium pH was 7.0 to 7.2, the flasks were incubated at 37 ºC for 48 h, 150 rpm. Samples were withdrawn after every 8 h

starting from the 16th h. Experiments were carried out in duplicates, the average of enzyme activities weas calculated and taken as the response.

Table 4.2 Plackett-Burman design matrix generated by Design Expert 7.0

Std	Run	Block	Factors*										
			1 A	2 B	3 C	4 D	5 E	6 F	7 G	8 H	9 J	10 K	11 L
1	1	Block 1	1	1	-1	-1	1	1	1	1	-1	1	-1
16	2	Block 1	1	1	1	1	-1	1	-1	1	-1	-1	-1
3	3	Block 1	1	-1	1	1	-1	-1	1	1	1	1	-1
5	4	Block 1	-1	1	1	-1	1	1	-1	-1	1	1	1
15	5	Block 1	1	1	1	-1	1	-1	1	-1	-1	-1	-1
13	6	Block 1	1	-1	1	-1	1	-1	-1	-1	-1	1	1
7	7	Block 1	-1	-1	-1	1	1	-1	1	1	-1	-1	1
9	8	Block 1	1	-1	-1	-1	-1	1	1	-1	1	1	-1
11	9	Block 1	1	-1	1	-1	-1	-1	-1	1	1	-1	1
19	10	Block 1	1	-1	-1	1	1	1	1	-1	1	-1	1
2	11	Block 1	-1	1	1	-1	-1	1	1	1	1	-1	1
17	12	Block 1	-1	1	1	1	1	-1	1	-1	1	-1	-1
6	13	Block 1	-1	-1	1	1	-1	1	1	-1	-1	1	1
20	14	Block 1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
14	15	Block 1	1	1	-1	1	-1	1	-1	-1	-1	-1	1

Std	Run	Block	Factors*										
			1 A	2 B	3 C	4 D	5 E	6 F	7 G	8 H	9 J	10 K	11 L
4	16	Block 1	1	1	-1	1	1	-1	-1	1	1	1	1
12	17	Block 1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1
18	18	Block 1	-1	-1	1	1	1	1	-1	1	-1	1	-1
10	19	Block 1	-1	1	-1	-1	-1	-1	1	1	-1	1	1
8	20	Block 1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1

*A:Dextrose g/L, B:Galactose g/L, C:Maltose g/L, D:Yeast Extract g/L, E:Soya Peptone g/L, F:NaCl g/L, G:K_2HPO_4 g/L, H:$MgSO_4$ g/L, J:$CaCO_3$ g/L, K:$FeSO_4$ g/L, L:Sea Water mL/L

The data obtained was analysed with the help of Design Expert v7.0.0. Pareto chart and normal plot were constructed to determine the most significant factors that influence the fibrinolytic enzyme production.

4.1.4 Additional trials

Experiments were conducted to ascertain the effect of carbon sources on fibrinolytic enzyme production. A flask with no carbon source having the following components (% w/v): yeast extract 2, soya peptone 2, NaCl 0.05, $CaCO_3$ 0.15, $FeSO_4$ 0.05 and seawater 5% v/v was used as control. Different carbon sources such as dextrose, galactose, maltose and starch, with their respective concentrations maintained constant at 1% w/v were added one at a time, while the other components remained same as that in control.

Based on the results obtained from Plackett-Burman design of experiments, a separate set of experiments were conducted to determine the effect of various nitrogen sources, which were found to be highly significant. Concentration of nitrogen sources was varied in the range of 3% (w/v) to 9% (w/v) as shown in table 4.5. Additionally, the

effect of various inorganic nitrogen sources such as NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$ and NH_4NO_3 on the fibrinolytic enzyme production was determined (Table 4.6). Rest of the medium components consisted of the most significant components as observed from our previous trials. Flasks were incubated for 32 h at 37 °C, 150 rpm, samples were then withdrawn and the fibrinolytic enzyme activity was calculated.

Further, attempt was made to study if combination of yeast extract (12% w/v and 15% w/v) and NH_4Cl (3% w/v and 4% w/v) can augment the fibrinolytic enzyme production, while maintaining the other components same as that in control (Table 4.7). The flasks were later incubated for 32 h at 37 °C, 150 rpm. Samples were withdrawn and the fibrinolytic enzyme activity was calculated.

4.1.5 Fibrinolytic enzyme production in the optimised medium

The microorganism was then grown in final optimised medium with the composition (% w/v): yeast extract, 12 soya peptone, 1.25 NH_4Cl, 4 and $CaCO_3$, 0.075. Initial pH of the medium was maintained 7.0 - 7.2 and flasks were incubated for 32 h at 37 °C, 150 rpm, upon which the samples were analysed for total fibrinolytic activity (Krishnamurthy et al., 2017). Control consisted of fibrinolytic enzyme production with the unoptimised medium under the same fermentation conditions.

4.2 RESULTS AND DISCUSSION

4.2.1 Growth curve

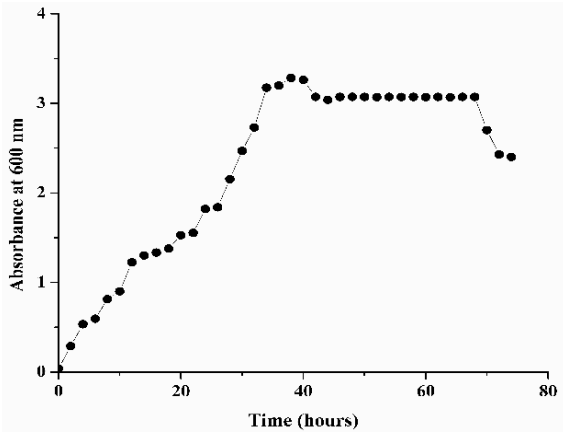


Figure 4.1 Growth profile of *Serratia marcescens* subsp. *sakuensis*

The growth pattern of *Serratia marcescens* subsp. *sakuensis* was monitored for a period of 3 days as shown in figure 4.1. The exponential phase lasted up to 40th h, after which the cells entered the stationary phase that lasted for 68 hours until the cells started to die.

4.2.2 Results of trials with one factor at a time approach

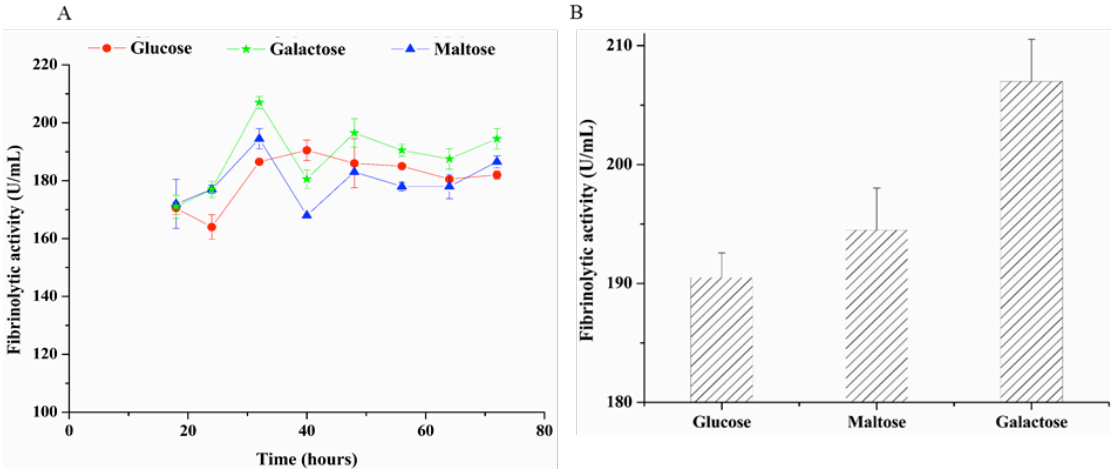


Figure 4.2 Effect of carbon sources on the enzyme production (A) time course variation (B) maximum enzyme obtained with each carbon source. Bars represent standard deviation (n = 2).

One factor at a time approach was followed for optimising the medium components for the enhanced production of the fibrinolytic enzyme. In case of effect of the three carbon sources on enzyme production by *Serratia marcescens* subsp. *sakuensis*, a maximum activity of 207 U/mL was observed when galactose was used as the sole carbon source (Figure 4.2). However, the ability of the microorganism used in the present work, to utilise dextrose, maltose and galactose, equally has been reported in Bergey's Manual of Systemic Bacteriology (2007). Time course variation studies revealed that enzyme production peaked at 32 h with galactose as the carbon source (Figure 4.2).

Studies on the effect of nitrogen sources on the enzyme production resulted in a maximum activity of 250.5 U/mL with yeast extract (Figure 4.3). It was reported that

incorporation of organic nitrogen sources in the medium resulted in an increase in the enzyme activity (Liu et al. 2005; Avhad et al. 2013).

Effect of the salts on the enzyme production resulted in a maximum activity of approximately 176 U/mL with each of the salt (Figure 4.4). Marginal difference in the enzyme activites in presence of the salts was observed.

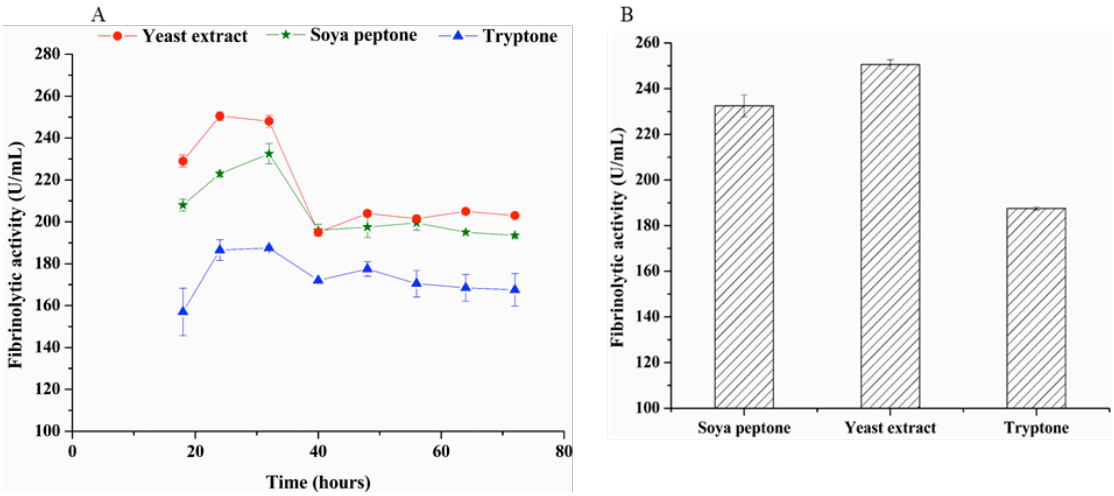


Figure 4.3 Effect of nitrogen sources on the enzyme production (A) time course variation (B) maximum enzyme obtained with each nitrogen source. Bars represent standard deviation (n = 2).

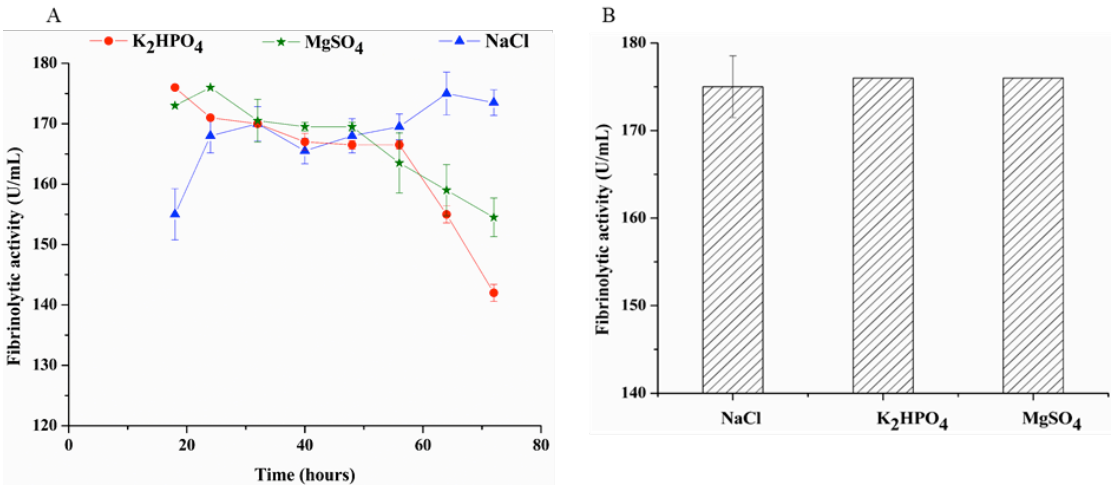


Figure 4.4 Effect of salts on the enzyme production (A) time course variation (B) maximum enzyme obtained with each salt. Bars represent standard deviation (n = 2).

Among the inducers studied, maximum enzyme activity of 179 U/mL was obtained in presence of both $FeSO_4$ and $CaCO_3$ (Figure 4.5). Presence of these in seawater has long been documented (Emerson et al. 2003). It has been reported that around 20% to 30% of the carbonates produced in the surfaces of the ocean, is preserved in marine sediments. These carbonates are mainly in the form of calcium carbonates, abundantly available in the sedimentary rocks and the ocean, wherein the topographic rises in the ocean floor are $CaCO_3$ rich, while the abyssal planes have traces of $CaCO_3$.These sedimentary calcium carbonates get deposited, form shells of marine plants and animals. It is this calcium carbonate that mainly contributes to the alkalinity of seawater, preventing drastic changes in pH values upon acid contamination (Emerson et al. 2003). Most of the iron redox cycling takes place within the sediments, due to the relatively rapid oxidation kinetics of ferrous ions, which is soluble in water. This iron redox cycling, wherein the electrons shuttle between the oxidised, reduced states and the subsequent adsorption of the reduced form of iron onto the sediment surfaces, is an important phenomenon commonly observed during processes such as bioturbation and irrigation (Emerson et al. 2003).

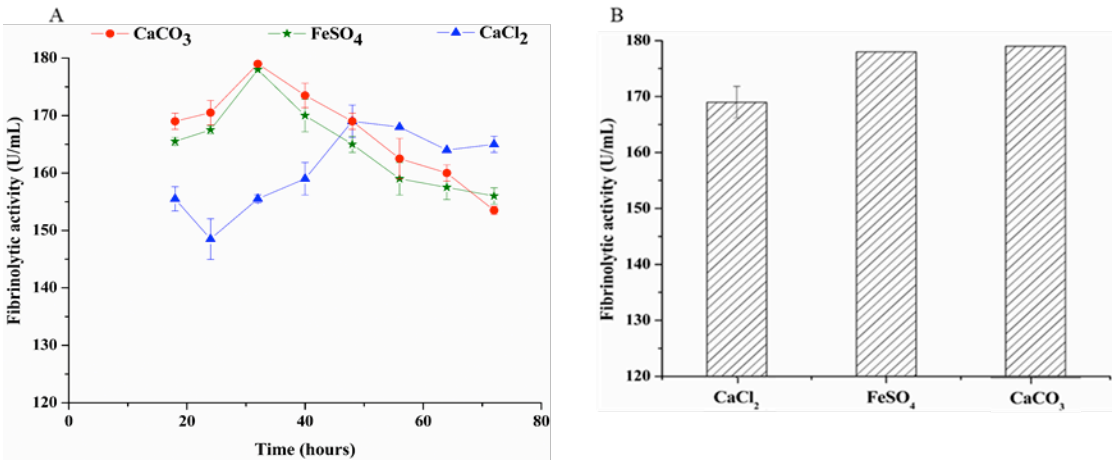


Figure 4.5 Effect of inducers on the enzyme production (A) time course variation (B) maximum enzyme obtained with each inducer. Bars represent standard deviation (n = 2).

It was observed that the addition of seawater caused a marginal increase in the enzyme productivity as compared to the control (Table 4.3). A maximum enzyme

activity of 193.75 U/mL was obtained with 1% (v/v) seawater in the medium. Kato et al. (1972) had studied the protease production by a marine psychrophilic bacterium using artificial sea water as a medium component. It was observed that both growth and enzyme activity was maximum in the medium containing seawater at the highest concentration. However, it was reported by Chandrasekaran and Dhar (1983) that increase in salt concentration leads to a change in lipid composition of the cell membrane, thereby decreasing growth rate and also reducing enzyme production.

Table 4.3 Effect of seawater on enzyme production

Expt	Total Enzyme Activity (U/mL)
Control	184.29 ± 1.79
1	193.75 ± 2.21
2	188.32 ± 1.32
3	189.93 ± 2.14
4	182.03 ± 0.56
5	186.51 ± 1.62

*Data represents mean ± SD (n = 2).

4.2.3 Results of Plackett-Burman design of experiments

Selection of the most significant factor was made using Plackett-Burman design. The design matrix (Table 4.2) was generated by Design Expert 7.0 (Stat ease, Inc.). The mean square value and F test value for yeast extract (D) and soya peptone (E) was higher than that for other nutritional and dummy variables indicating that the two are important nutritional components affecting the fibrinolytic enzyme production. Larger F value of 22.79 and *p* value of 0.0001 implied that the model was significant. The coefficient of determination, R^2 for the model was 0.9691 showing the goodness of fit

of the model. The adjusted R^2 was 0.9265. These values indicate good adequacy of the model.

Pareto chart offers a convenient way to view the results obtained. It shows the significance of variables affecting the enzyme production. From the Pareto chart (Figure 4.6A), it can be observed that only two factors namely yeast extract and soya peptone out of eleven nutritional factors had a large positive influence on the fibrinolytic enzyme production. Nevertheless, galactose, $FeSO_4$, sea water, $CaCO_3$ and NaCl showed a minimal positive effect on the enzyme production. Additionally, the normal probability plot of the effects was constructed to identify the most significant factors and confirm the previously obtained results. On the normal probability plot, the effect coefficients namely yeast extract and soya peptone showed large deviations (Figure 4.6B) from the line, as they do not come from the normal distribution and hence were considered significant. On the other hand, all non-significant effect coefficients will fall along the straight line. The results observed from normal plot lie on par with that observed in Pareto chart.

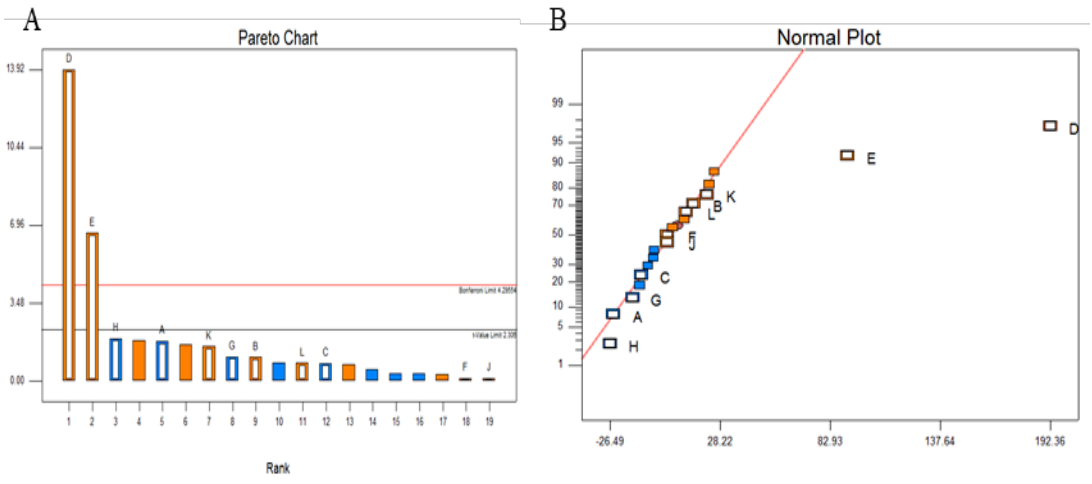


Figure 4.6 Effect of medium components using Plackett-Burman design (A) Pareto chart (B) Normal Probability plot. (A-Dextrose, B-Galactose, C-Maltose, D-Yeast Extract, E-Soya Peptone, F- NaCl, G- K_2HPO_4, H- $MgSO_4$, J- $CaCO_3$, K- $FeSO_4$, L-Sea water)

4.2.4 Additional trials

Further, to substantiate the results obtained from Plackett-Burman, separate set of experiments were performed to determine the contribution of carbon sources on the enzyme production when incorporated in the fermentation medium. From table 4.4, it was observed that there was a marginal change in fibrinolytic enzyme activity as compared to that in the control where no carbon source was incorporated. Thus, the results obtained indicated that the inclusion of carbon source does not enhance the fibrinolytic enzyme production significantly.

Table 4.4 Effect of carbon source on fibrinolytic enzyme production

Carbon Source	Total Enzyme Activity* (U/mL)
None (Control)	229 ± 3.28
Dextrose	239.93 ± 0.71
Galactose	231.79 ± 3.88
Maltose	228.74 ± 1.98
Starch	214.26 ± 4.24

*Data represents mean ± SD (n = 2).

Also, since it was shown previously that the addition of nitrogen source contributed significantly to the production of fibrinolytic enzyme, separate set of experiments were conducted by varying the concentration of most significant factor (yeast extract) and the fibrinolytic enzyme activity was determined (Table 4.5). Results obtained suggested that with an increase in the concentration of yeast extract, enzyme activity also increased. A maximum of 487.29 U/mL of fibrinolytic activity was obtained with 9% (w/v) of yeast extract, which is a 2.5 fold increase in the enzyme activity as compared to that in unoptimised medium (191.65 U/mL) (Table 3.4), therefore signifying that the organism has a remarkably high nitrogen demand. This could be explained by the fact that marine microbial communities utilise the sinking organic carbon as an energy source because often such microbes are found in nitrogen limiting conditions, hence they prefer a nitrogen-rich environment for their

growth (Emerson and Hedges, 2003). Deepak *et al.* (2008) showed that only peptone had a significant effect on nattokinase production as compared to the other medium components. Likewise, Liu *et al.* (2005) had reported that nitrogen sources such as soya peptone and yeast extract had a significant effect on nattokinase production. Thus, the results obtained in this study were consistent with the reported literature.

Table 4.5 Effect of varying concentration of yeast extract on fibrinolytic enzyme production

Yeast Extract	Soya Peptone	$CaCO_3$	$FeSO_4$	Sea Water	Total Enzyme Activity* (U/mL)
← % (w/v) →				% (v/v)	
3	1.25	0.075	0.125	1.25	381.85 ± 0.73
3	1.25	-	0.125	1.25	370.13 ± 2.51
6	1.25	0.075	-	-	448.15 ± 0.45
9	1.25	0.075	-	-	487.29 ± 1.00

*Data represents mean ± SD (n = 2).

Table 4.6 Total enzyme activity with different inorganic nitrogen sources

Concentration % (w/v)	0.5	0.75	1	1.5	2
NH_4Cl	306.29 ± 0.66	305.02 ± 1.87	341.63 ± 1.09	350.79 ± 1.93	356.38 ± 2.64
$(NH_4)_2SO_4$	388.41 ± 3.40	342.4 ± 1.62	362.73 ± 0.01	323.33 ± 1.59	314.68 ± 0.22
$NaNO_3$	356.58 ± 0.09	310.87 ± 2.11	348.24 ± 2.93	367.56 ± 0.15	324.09 ± 0.15
NH_4NO_3	335.78 ± 1.24	345.19 ± 0.12	328.41 ± 3.47	338.07 ± 0.45	398.33 ± 3.96

*Data represents mean ± SD (n = 2).

Due to high nitrogen requirement of the organism for fibrinolytic enzyme production, various inorganic nitrogen sources were incorporated in the medium and its effect on enzyme production was determined by estimating the total enzyme activity (Table 4.6). A linear increase in enzyme activity was observed with increasing concentration of NH_4Cl, resulting in an enzyme activity of 356.38 U/mL at 2% (w/v) concentration (Table 4.6). Jayalakshmi et al. (2012) had reported a maximum enzyme activity of 1.6 U/mL in presence of NH_4Cl as compared the enzyme activity with other inorganic nitrogen sources.

Combination of yeast extract (12% w/v) and NH_4Cl (4% w/v) in the production medium, gave a maximum enzyme activity of 656.88 U/mL (Table 4.7).

Table 4.7 Total enzyme activity using a combination of yeast extract and NH_4Cl

Expt	Yeast Extract	Soya Peptone	NH_4Cl	$CaCO_3$	Total Enzyme Activity
	←	% (w/v)	→		(U/mL)
Control	9	1.25	2	0.075	492.65 ± 1.58
A	12	1.25	3	0.075	656.55 ± 0.01
B	12	1.25	4	0.075	656.88 ± 0.11
C	15	1.25	3	0.075	648.41 ± 2.03
D	15	1.25	4	0.075	654.86 ± 0.96

*Data represents mean ± SD (n = 2).

4.2.5 Fibrinolytic enzyme production in the optimised medium

Maximum enzyme activity of 661.14 U/mL was achieved with the optimised medium, which was 3.4 times enhanced enzyme activity as compared to the control (191.65 U/mL) (Table 3.4).

4.3 SUMMARY AND CONCLUSIONS

- Growth pattern of *Serratia marcescens* subsp. *sakuensis* was studied for a period of 3 days.
- One factor at a time approach was followed to optimise the medium components for enhanced production of fibrinolytic enzyme.
- Among the various carbon and nitrogen sources employed, galactose and yeast extract resulted in a relatively higher enzyme activity. Salts such as NaCl, K_2HPO_4, $MgSO_4$ and inducers such as $FeSO_4$ and $CaCO_3$ when used gave an increased fibrinolytic activity.
- Incorporation of seawater in the production medium resulted in a marginal increase in the enzyme activity as compared to the control with no sea water.
- The results obtained from the Plackett-Burman design of experiments showed that yeast extract and soya peptone were the two major factors that displayed positive influence on the fibrinolytic enzyme production. Other factors such as galactose, $FeSO_4$, sea water, $CaCO_3$ and NaCl showed minimal positive effect/no significant contribution to the enzyme production.
- Additional set of experiments were conducted to ascertain the role of carbon sources on the fibrinolytic enzyme production and only a marginal change in the enzyme activity was observed when compared to the control.
- Various inorganic nitrogen sources were chosen for studying their effect on the enzyme production. A linear increase in the fibrinolytic activity (356.38 U/mL) was observed with increasing concentration of 2% (w/v) ammonium chloride.
- Since yeast extract was found to play a major part in the fibrinolytic enzyme production, the concentration of yeast extract was varied and at 9% (w/v) of yeast extract, 2.5 fold increase in the enzyme production was achieved as compared to that in the control.
- A combination of 12% (w/v) yeast extract and 4% (w/v) NH_4Cl in the production medium gave a maximum enzyme activity of 656.88 U/mL.

- The results obtained indicate that the organism *Serratia marcescens* subsp. *sakuensis* has a remarkably high nitrogen demand for the production of fibrinolytic enzyme.
- Experiments performed with optimised medium resulted in a 3.4 fold increase in the fibrinolytic activity (661.14 U/mL).

CHAPTER 5

PURIFICATION AND CHARACTERISATION OF THE FIBRINOLYTIC ENZYME

In this chapter, the fibrinolytic enzyme produced using the optimised medium (chapter 4) was purified. Several physicochemical characteristics of the purified enzyme were studied in addition to determining its *in vitro* clot lysing potential in comparison to the commercially available fibrinolytic agents. The aim of this work was to examine the applicability of this fibrinolytic enzyme as a potential candidate for therapeutic use.

5.1 MATERIALS AND METHODS

Human fibrin, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), ɣ-globulin, holo-transferrin and serum albumin of bovine origin were purchased from Sigma-Aldrich, India. ENrich SEC 650 column was procured from BIO RAD, India. Standard protein marker was purchased from Merck Millipore, India. Bovine haemoglobin was purchased from HiMedia, India. Streptokinase (≥ 3500 U/mg) and Heparin (140 U/mg) were purchased from Sigma-Aldrich, India. Other chemicals used were of analytical grade.

5.1.1 Enzyme purification

Fibrinolytic enzyme was produced by culturing *Serratia marcescens* subsp. *sakuensis* in the optimised medium having the composition yeast extract (12% w/v), soya peptone (1.25% w/v), NH_4Cl (3% w/v) and $CaCO_3$ (0.075% w/v) for 32 h at 37 °C, 150 rpm (Krishnamurthy et al., 2017). The fermentation broth was centrifuged at 12000 × g for 15 min to obtain the cell-free crude enzyme solution, which was then subjected to ammonium sulphate precipitation (40% saturation) under ice-cold conditions. The precipitated protein sample was then dialysed using a dialysis membrane (molecular weight cut off of 12 – 14 KDa) against 20 mM sodium phosphate buffer of pH 7 overnight under ice-cold conditions.

The dialysed enzyme samples were applied to ENrich SEC 650 column in FPLC system (BIO RAD NGC Chromatography System) of dimensions (10 mm x 300 mm) pre-equilibrated with 20 mM phosphate buffer saline pH 7. A constant flow rate of 1 mL/min was maintained with 20 mM phosphate buffer saline of pH 7. Active fractions of 1 mL each were collected and pooled together and were lyophilised.

These were subjected to standard fibrinolytic assay in addition to total protein estimation.

5.1.2 Molecular mass estimation

The molecular weight of the fibrinolytic enzyme was determined using 12% polyacrylamide SDS-PAGE as described previously by Laemmli (1970) with a broad range protein ladder. Samples for SDS PAGE were run under reducing and denaturing conditions and were stained with silver nitrate staining method (Chevallet et al. 2006).

5.1.3 Effect of pH on fibrinolytic enzyme activity and stability

Optimum pH for the enzyme activity was determined using fibrin as a substrate. Fibrinolytic activity was assayed over a pH range of 3-11 at 55 °C. Buffer systems such as acetate buffer solutions (pH 3.0 - 6.0) and phosphate buffer solutions (pH 7.0 - 11.0) were used. Activity at optimum pH was considered to be 100%.

In order to determine the stability of the purified enzyme at different pH, the purified enzyme was pre-incubated in the respective buffer solutions (pH 3 to pH 11) at room temperature for one hour and the residual fibrinolytic enzyme activity of the samples were determined under standard assay conditions. Activity of the unincubated enzyme was taken as 100%.

5.1.4 Effect of temperature on fibrinolytic enzyme activity, stability and *in vitro* half-life

The effect of temperature on the fibrinolytic enzyme activity was studied over temperatures ranging from 25 ºC to 85 ºC during the reaction assay in 20 mM phosphate buffer of pH 7.0, using fibrin as substrate. Activity at optimum temperature was considered to be 100%.

The thermal stability profile of the purified enzyme was investigated by incubating the enzyme at different temperatures, 25 ºC, 30 ºC, 37 ºC, 45 ºC, 55 ºC, 65 ºC, 75 ºC and 85 ºC in the 20 mM phosphate buffer solution of pH 7.0, for a maximum of 2 h. Aliquots were withdrawn at different time intervals and the residual fibrinolytic enzyme activities were determined under standard assay conditions. Activity of non-heated enzyme was considered as 100%.

The *in vitro* half-life of the fibrinolytic enzyme was determined using the thermal stability profile exhibited by the enzyme at both its optimum temperature and at

physiological temperature for different time intervals. The half-life for the fibrinolytic enzyme was calculated using a graph that was plotted with relative enzyme activity versus time.

5.1.5 Effect of metal ions and chemical reagents on enzyme activity

The effects of various metal ions (Co^{2+}, Cu^{2+}, Pb^{2+}, Li^{+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, K^{+}, Na^{+}) and chemical reagents (PMSF, EDTA and DTT) on the fibrinolytic enzyme activity were investigated. The concentration of these metal ions (table 5.2) was chosen based on their respective concentration range usually found in human blood. The purified enzyme sample was pre-incubated with these metal ions and chemical reagents at 37 ºC for 1 h in 20 mM phosphate buffer (pH 7.0), followed by which the samples were subjected to fibrinolytic enzyme assay and the residual enzyme activity was determined. Here, the activity of enzyme assayed in the absence of metal ions/chemical reagents was defined as 100%.

5.1.6 Kinetic studies

Different concentrations of fibrin substrate (0.1 mg/mL to 5 mg/mL) were used to determine the initial reaction rates. The assay was conducted under optimum conditions (pH 7.0 and 55 ºC). The Michaelis-Menten constant (K_m) and maximum rate of reaction (V_{max}) values were determined according to Lineweaver-Burk double reciprocal plot upon plotting the respective $1/[S_0]$ and $1/[V_0]$ values (Nelson and Cox 2005). Subsequently, the K_{cat} and the catalytic efficiency (K_{cat}/K_m) of the enzyme were also calculated.

5.1.7 Proteolytic activity with different substrates

The proteolytic activity of the fibrinolytic enzyme was measured using fibrinolytic enzyme assay with minor modifications. Fibrin was replaced with other protein substrates such as haemoglobin, ɣ-globulin, transferrin, serum albumin and casein at 1% (w/v) concentration. The purified enzyme sample was incubated with each of these substrates for 15 min at 37 ºC and the remaining assay procedure was carried out similar to the standard fibrinolytic enzyme assay. Control was maintained with fibrin as substrate. One unit of proteolytic activity was expressed as 1µg of tyrosine liberated per min at 37 ºC. Proteolytic activity was expressed as relative activity (%)

with the enzyme activity in control considered as 100%. In addition, the F/C ratio (ratio of fibrinolytic activity/caseinolytic activity) was calculated.

5.1.8 *In vitro* trials with purified enzyme

5.1.8.1 Percentage clot lysis

The clot lysis activity of the fibrinolytic enzyme was estimated by the method of Prasad et al. (2006) with minor modifications. Informed consent was obtained for experimentation with human subjects. Fresh whole blood is collected from healthy volunteers and was immediately citrated using 3.2% of 105 mM trisodium citrate (pH 7.4) in the ratio of 9 parts venous blood to 1 part of the anticoagulant solution. From this 0.5 mL of blood is added into pre-weighed Eppendorf tubes (W_1), followed by the addition of 0.2 mL of 2% $CaCl_2$ and the mixture is incubated at 37 ºC in the water bath for an hour to induce clot formation. The tubes are then centrifuged at 5000 rpm at 0 ºC for 10 min and the serum was aspirated out. Each of the tubes was weighed again to determine the weight of the clot (W_2) following the addition of 0.5 mL of test enzyme (29 U and 38 U) to these clots. These tubes were then incubated at 37 ºC for a period of 3 h after which the fluid formed due to clot lysis is carefully removed and the tubes with the remnants of the clot are weighed (W_3). 0.5 mL of 0.9% (w/v) saline was used as negative control and 0.5 mL of streptokinase and heparin each was used as positive control. Percentage clot lysis was expressed as the difference in the weight before and after clot lysis, i.e.

$$\frac{(W_2\text{-}W_3)}{(W_2\text{-}W_1)} \times 100$$

5.1.8.2 Determination of *in vitro* clot lysis mechanism of the enzyme

The thrombolytic mechanism of the purified enzyme was determined by the method described by Ju et al. (2012) with minor modifications. Informed consent was obtained for experimentation with human subjects. Fresh whole blood is collected from healthy volunteers and is allowed to form a clot followed by the removal of serum as described above. One set of tubes containing the clot formed were incubated in water bath at 85 ºC for 30 min in order to denature plasminogen, while the other set of tubes containing the clot formed were kept unheated. The test enzyme was then

added to the heated and unheated blood clots and incubated at 37 ºC for a period of 3 h, the clot dissolution rates were evaluated at different time intervals.

5.1.9 Identification of partial amino acid sequence

The purified enzyme (lyophilised form) was submitted for partial amino acid sequence analysis to National Chemical Laboratories, Pune, India. The sample was subjected to trypsin digestion and sequenced using TripleTOF 5600 (LC-MS/MS-TOF analysis). AB SCIEX Protein pilot software database was used for identification of the peptide sequence. The peptide sequences were then analysed using NCBI-BLAST database for determining the homologous sequences.

5.2 RESULTS AND DISCUSSION

5.2.1 Purification of enzyme

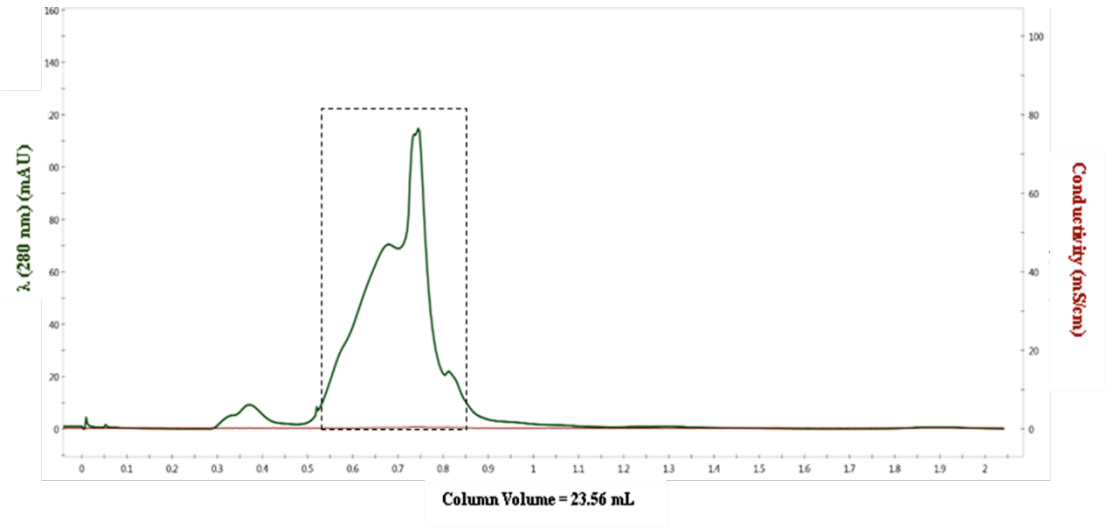


Figure 5.1 Gel filtration chromatography on an ENrich SEC 650 FPLC column, the highlighted region comprises of the active fractions of the fibrinolytic enzyme

The fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* was purified from the cell-free fermentation broth using a three-step purification process. The cell-free broth was subjected to ammonium sulphate precipitation (40% saturation) followed by dialysis and the dialysed enzyme solution was then passed through the ENrich SEC 650 column (BIO RAD NGC Chromatography System). The resulting FPLC

chromatogram is as shown in figure 5.1. Purified fibrinolytic enzyme showed an increased specific activity (1033 U/mg) by 21.08 fold with a 19.38% recovery (Table 5.1) (Krishnamurthy et al., 2018). The enzyme activity obtained in the present study is significantly higher than the fibrinolytic enzymes from *Pheretima posthumous*, *Brevibacillus brevis* strain FF02B, *Lyophyllum shimeji*, with a maximum specific activity of 18.92 U/mg, 64.9 U/mg and 469.3 U/mg respectively (Verma and Pulicherla 2017; Majumdar et al. 2014; Moon et al. 2014).

Table 5.1 Summary of steps involved in purification

Purification Steps	**Total Protein (mg)[a]**	**Total Activity (U/mL)[b]**	**Specific Activity (U/mg)[c]**	**Recovery (%)**	**Purification fold**
Crude enzyme	3.26	160	49 ± 7	100	1
Dialysis	0.78	59	76 ± 13	36.87	1.55
FPLC SEC 650	0.03	31	1033 ± 23	19.38	21.08

[a] Protein concentration was estimated by standard Lowry's
[b] One unit of fibrinolytic enzyme activity was defined as 1µg of tyrosine liberated per min under the experimental conditions used.
[c] Values represent the mean of three independent experiments and ± standard errors are reported.

5.2.2 Molecular mass estimation

SDS-PAGE analysis was performed in order to determine the molecular weight of the purified fibrinolytic enzyme using 12% polyacrylamide gel under reducing and denaturing conditions. As seen in figure 5.2, the purified enzyme resulted in a single band corresponding to a molecular weight of 43 KDa. The fibrinolytic enzyme produced in the present work is a smaller molecule in comparison to streptokinase, fibrinolytic enzyme from *Serratia* sp. RSPB 11 and Serratiopeptidase from *Serratia marcescens* that have a molecular weight of 47 KDa, 50 KDa and 52 KDa respectively (Bhardwaj and Angayarkanni 2015, Bhargavi and Prakasham 2013; Mohankumar and Raj 2011). As it is widely known, smaller the size of the molecule lesser will be the immunogenicity exhibited by the molecule, thereby rendering it relatively safe for human use.

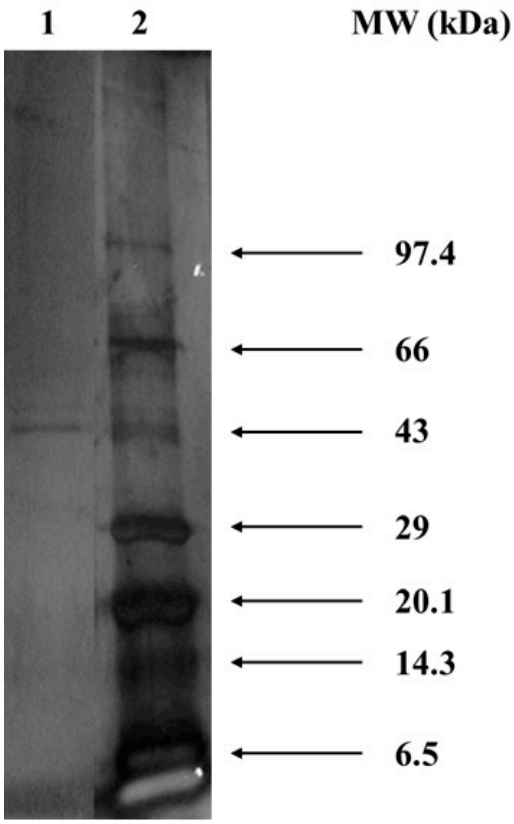


Figure 5.2 SDS-PAGE of the purified fibrinolytic enzyme. Lane 1, purified enzyme; Lane 2, protein markers

5.2.3 Effect of pH on enzyme activity and stability

The ability of an enzyme to function effectively in a broad range of pH is preferred when it has an intended therapeutic use. Thus, it is essential to evaluate the fibrinolytic activity of the enzyme at different pH, in addition to assesing its stability at different pH for a fixed time interval. Relative fibrinolytic activity (%) was calculated for pH 3 to pH 11 and the maximum activity (at optimum pH) shown by the fibrinolytic enzyme has been considered as 100%. As shown in figure 5.3, the optimum pH for the fibrinolytic enzyme activity was found to be 7, which is close to the human physiological pH, while being active over a wide range of pH between 5 and 10, exhibiting relative activity of 35% or more. For the pH stability studies, residual fibrinolytic activity (%) was determined and the fibrinolytic activity of the unincubated enzyme was taken as 100%. From the pH stability profile, it can be inferred that the enzyme was highly stable over a broad range of pH, maintaining 67%

of its original activity at pH values ranging between 3 and 11 for a period of one hour at room temperature (Figure 5.4). The results obtained were similar to the pH optimum of fibrinolytic enzyme produced by *Streptomyces* sp. XZNUM 00004, which was found to be 7.8 and displaying stability between the pH range 5 and 8 (Ju et al. 2012). Another such fibrinolytic enzyme produced from a marine *Bacillus subtilis* showed optimum pH at 9 with a broad pH stability between the pH range of 5 and 11 (Mahajan et al. 2012).

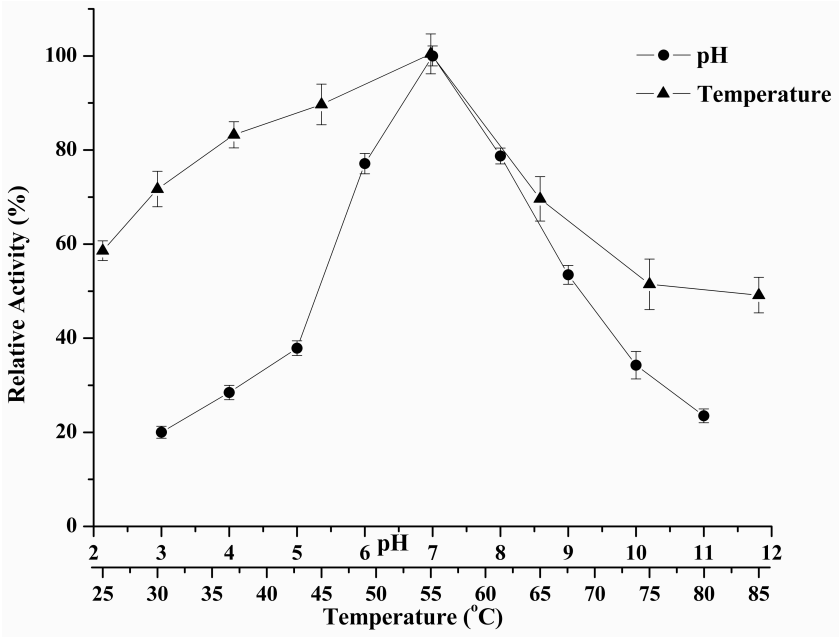


Figure 5.3 Effect of pH and temperature on the activity of the purified enzyme. Bars represent standard deviation (n = 3).

5.2.4 Effect of temperature on enzyme activity and stability at different temperatures, *in vitro* half-life

Relative fibrinolytic activity (%) was calculated for temperatures 25 ºC to 85 ºC and the maximum activity (at optimum temperature) shown by the fibrinolytic enzyme has been considered as 100%. The optimum temperature for the fibrinolytic enzyme activity was found to be 55 ºC (Figure 5.3), exhibiting more than 50% of the relative activity between the temperature range of 25 - 85 ºC. In order to determine the thermal stability profile of the fibrinolytic enzyme, residual fibrinolytic activity (%) was determined and the enzyme activity of the non-heated enzyme was taken as

100%. Uptill 65 ºC, the enzyme retained more than 50% of its activity for a period of 2 h, however, at 85 ºC, only 13.6% of the activity was retained for a period of 2 h (Figure 5.4). As shown in figure 5.4 at physiological temperature of 37 ºC, more than 80% of the enzyme activity was displayed and retained more than 50% of its activity for 2 h, rendering it favourable for human use.

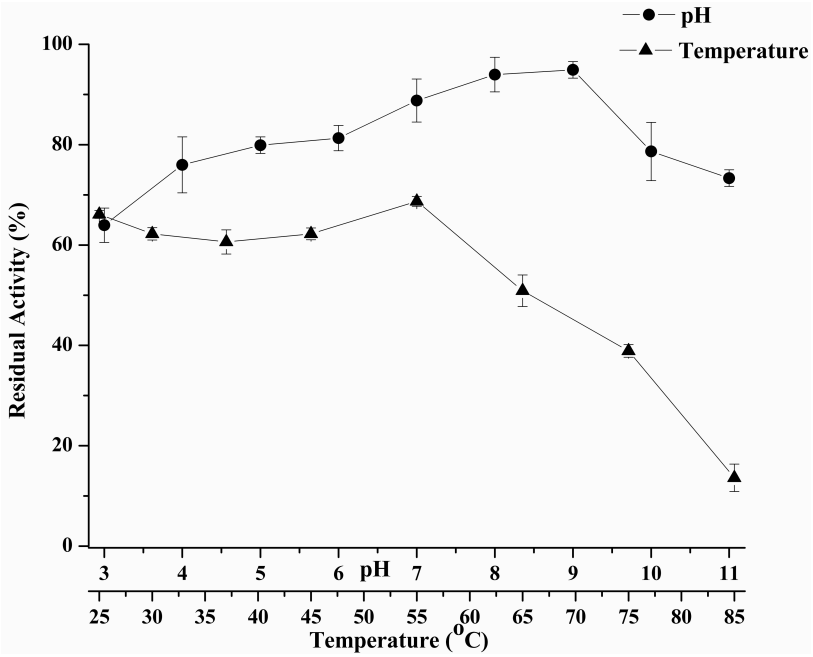


Figure 5.4 Effect of pH and temperature on the stability of the purified enzyme. Bars represent standard deviation (n = 3).

The results obtained differ from that reported by Mahajan et al. (2012), where the fibrinolytic enzyme produced by marine *Bacillus subtilis* showed an optimum temperature of 50 ºC, exhibiting a complete loss in enzyme activity at temperatures above 60 ºC for a period of 10 min. The fibrinolytic enzyme produced by *Streptomyces* sp. XZNUM 00004 showed optimum temperature of 35 ºC and remained active at temperatures below 65 ºC (Ju et al. 2012). The *in vitro* half-life of the fibrinolytic enzyme at physiological temperature (37 °C) and optimum temperature (55 °C) was found to be approximately 19 h and 29 h respectively (Figure 5.5). However, further studies need to be carried out to evaluate the same under *in vivo* conditions.

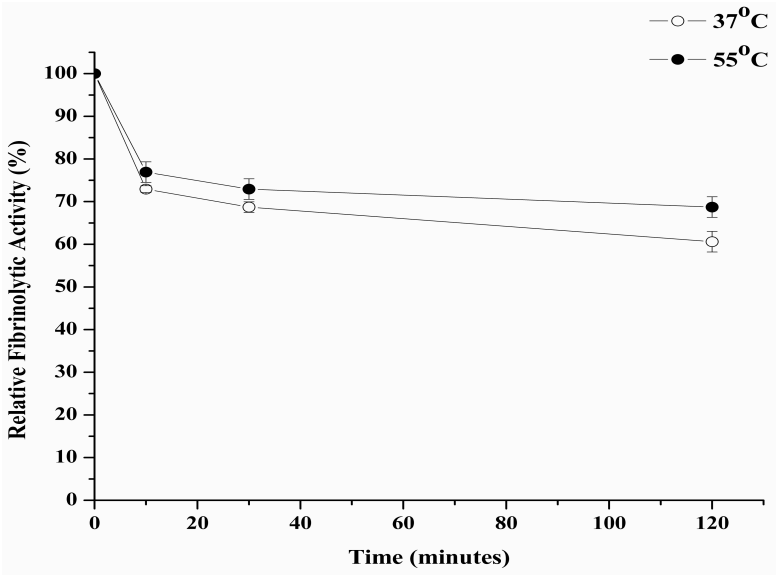


Figure 5.5 Comparative *in vitro* half-life of the fibrinolytic enzyme at 37 °C and 55 °C

5.2.5 Effect of metal ions and chemical reagents on enzyme activity

Blood is composed of several metal ions that have a significant role to play and thus, it becomes critical to determine the effect of metal ions on the catalytic activity of the fibrinolytic enzyme activity. Concentration of metal ions (Co^{2+}, Cu^{2+}, Pb^{2+}, Li^{+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, K^{+}, Na^{+}) chosen for study were as mentioned in table 5.2. As the control, fibrinolytic activity without the addition of any metal ion to the reaction mixture was considered as 100%. As shown in table 5.2, fibrinolytic activity enhanced in presence of divalent cations such as Mn^{2+}, Mg^{2+} and Zn^{2+} ions. Similar observations were reported by Yogesh and Halami (2015), wherein the fibrinolytic serine metalloprotease showed enhanced activity by more than two folds in the presence of Mn^{2+} and Mg^{2+}. Peng et al. (2005) had suggested that in case of metallo fibrinolytic enzymes, the influence of divalent metal ions on the enzyme activity is a common phenomenon.

Among the chemical reagents, both PMSF and EDTA which are well-known serine and metalloprotease inhibitors, inhibited the fibrinolytic enzyme activity as compared to the control, indicating the serine metalloprotease nature of the fibrinolytic enzyme. These findings were comparable to the results reported by Wu et al. (2009), Simkhada

et al. (2010), Mander et al. (2011), Choi et al. (2011), Moon et al. (2014) and Yogesh and Halami (2015). Reducing agent such as DTT showed an increase of 11% in the enzyme activity, perhaps due to its role in reducing the disulphide bonds in proteins and peptides, thereby preventing the formation of intermolecular and intramolecular disulphide bonds between the cysteine residues of proteins (AG Scientific 2013).

Table 5.2 Effect of metal ions and chemical reagents on the fibrinolytic activity

Chemicals	Concentration (mM)	Relative Activity (%)
Control	-	100
Co^{2+}	0.15	67 ± 2.87
Cu^{2+}	0.15	65.96 ± 0.5
Pb^{2+}	0.15	67.76 ± 1.79
Li^{+}	0.15	53.33 ± 0.49
Mn^{2+}	0.15	158.88 ± 4.71
Zn^{2+}	0.15	103.33 ± 1.57
Ca^{2+}	1	64.46 ± 2.86
Mg^{2+}	1	107.77 ± 0
Fe^{3+}	5	89.84 ± 1.43
Fe^{2+}	5	68.78 ± 1.79

Chemicals	Concentration (mM)	Relative Activity (%)
K^+	50	68.78 ± 1.79
Na^+	150	68.07 ± 3.47
PMSF	1	62.6 ± 1.11
EDTA	1	65.15 ± 0.45
DTT	1	111 ± 2.59

*Data represents mean ± SD (n = 3)

5.2.6 Kinetic studies

The kinetic parameters were determined under standard assay conditions (pH 7.0 and 55 ºC) using different concentrations of fibrin as substrate. The study revealed that the purified fibrinolytic enzyme obeys Michaelis-Menten kinetics (Figure 5.6 A). The Lineweaver-burk double reciprocal plot was constructed with $1/[S_0]$ values versus $1/[V_0]$ values using Origin Pro 8 (Figure 5.6 B). The Michaelis-Menten constant (K_m) was found to be 0.66 mg/mL and maximum rate of reaction (V_{max}) was estimated to be 158.73 U/mL respectively. The turnover number (K_{cat}) and catalytic efficiency of the fibrinolytic enzyme were found to be 12.21 min^{-1} and 18.32 mL/(mg min), respectively.

Most of the literature available on kinetic studies of fibrinolytic enzymes reported the use of substrates such as casein and azocasein. The K_m values reported by Simkhada et al. (2010), Ju et al. (2012), Bhargavi and Prakasham (2013), Verma and Pulicherla (2017) were 4.2 mg/mL, 0.96 mg/mL, 1.216 mg/mL, and 0.09 - 0.4 g/mL respectively, using casein/azocasein as substrate. On the contrary, kinetic parameters determined with casein/azocasein does not necessarily reflect a higher affinity for the substrate fibrin. K_m value of the enzyme studied in the present work was 0.66 mg/mL,

which is less than most of the enzymes reported in the literature, therefore, indicating a higher affinity for the substrate used. As the substrate used in the present work was fibrin, the result becomes even more significant.

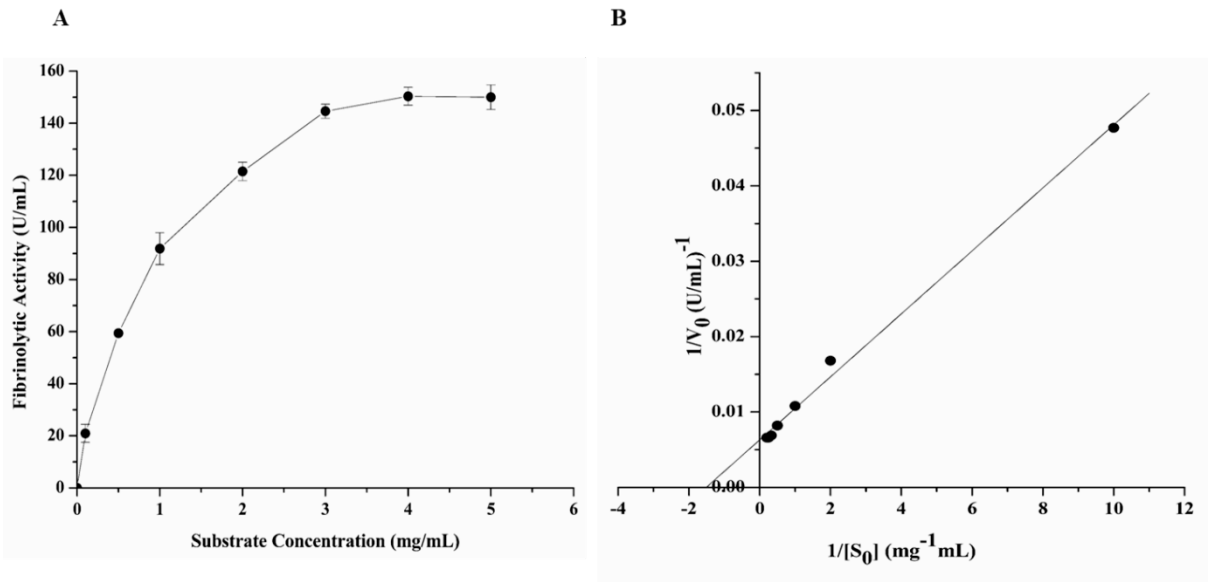


Figure 5.6 Enzyme kinetics of the fibrinolytic enzyme. (A) Michaelis-Menten curve for enzyme-substrate reaction (B) Lineweaver-Burk Plot. Bars represent standard deviation (n = 3).

5.2.7 Enzyme activity using several protein substrates

As the fibrinolytic enzyme would be delivered into the circulatory system, it is important to assess its proteolytic activity towards several blood proteins. The proteolytic activity of the fibrinolytic enzyme was evaluated using different substrates (1% w/v) such as haemoglobin, ɣ-globulin, transferrin, serum albumin and casein. Relative fibrinolytic activity (%) was calculated and the enzyme activity with fibrin substrate (control) was considered as 100%. From table 5.3, it can be observed that the proteolytic activity with casein and serum albumin was 3.2 times and 6.4 times higher, respectively in comparison to the control. This could be a result of relatively higher affinity of the enzyme towards albumin. Haemoglobin forms the major protein in the blood. Moreover, the concentration of albumin is much lesser than the concentration of haemoglobin in whole blood. However, no enzyme activity was evidenced with the blood proteins such as haemoglobin, ɣ-globulins and transferrin.

This could be because of the complex nature of the substrates, thereby rendering the enzyme incapable of breaking them down. A similar study on a fibrinolytic enzyme from *Bacillus megaterium* KSK-07 was reported by Kotb (2015) wherein, enzyme activity towards blood proteins such as fibrinogen, collagen, mucin, serum albumin, elastin and IgG was evaluated and mild proteolytic activity was observed with haemoglobin, mucin, ɣ-globulins, elastin and collagen.

Majority of the proteolytic enzymes in their native form do not show absolute specificity towards a single protein. Thus, alternative approaches such as chemical modification or genetic modification of the native enzyme could be explored to enhance its specificity towards the target protein.

Table 5.3 Enzyme activity against several protein substrates

Protein Substrates	Relative Activity (%)
Control (Fibrin)	100
Caesin	321.76 ± 0
Serum Albumin	644.11 ± 0
ɣ-Globulins	No Activity
Haemoglobin	No Activity
Transferrin	No Activity

*Data represents mean ± SD (n = 2)

The Fibrinolytic activity/Caseinolytic activity ratio (F/C ratio) for the enzyme was found to be 0.31 (Table 5.4). The F/C ratio obtained in the present work was closely similar to the F/C ratio of the commercially prepared protease Subtilisin BPN and was higher than that of proteases purified from *Streptomyces griseus*, *Aspergillus oryzae*, *Tritirachium album* and trypsin from bovine pancreas (Kim et al. 1996), while human

plasmin shows the maximum F/C ratio (Kotb 2015) (Table 5.4). Determination of F/C ratio helps in comparing the rate of reaction with different substrates, which gives an indication of the affinity of the enzyme for fibrin substrate. Since this work was aimed at characterising a fibrinolytic enzyme, it is important to understand the enzyme specificity towards the substrates fibrin and casein. A higher F/C ratio would indicate higher affinity towards fibrin and a lower F/C ratio would indicate higher affinity towards casein.

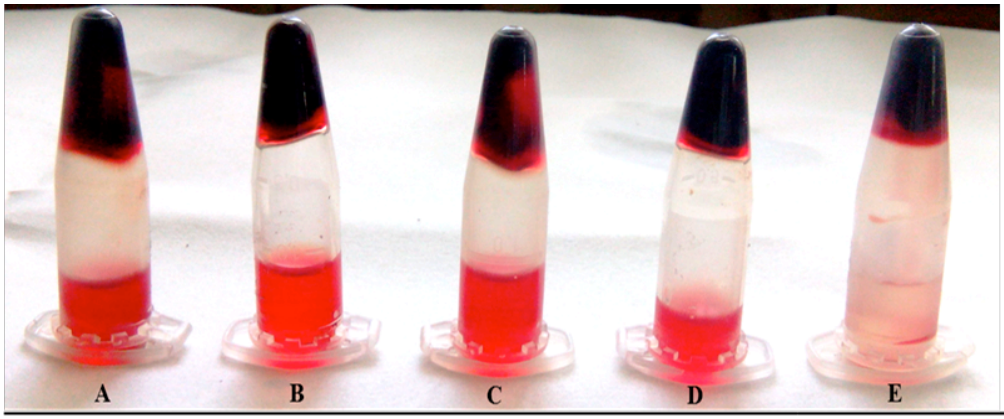
Table 5.4 Comparison of F/C ratios of the fibrinolytic enzyme produced in the present study with that of the other proteases

Protease source	F/C ratio	Reference
Streptomyces griseus (type XXI)	0.20	Kim et al. (1996)
Trypsin (bovine pancreases type I)	0.22	Kim et al. (1996)
Aspergillus oryzae (type XIII)	0.28	Kim et al. (1996)
Tritirachium album (type XXVIII)	0.30	Kim et al. (1996)
Serratia marcescens subsp. *sakuensis*	0.31	Present work
Subtilisin BPN (type XXVII)	0.32	Kim et al. (1996)
Plasmin (human plasma)	1.24	Kotb (2015)

5.2.8 *In vitro* clot lysis

The clot lysing ability of the fibrinolytic enzyme at different concentrations (29 U and 38 U) was evaluated *in vitro* using fresh whole blood collected from healthy volunteers. Saline (0.9% w/v) was used as the negative control. The *in vitro* clot lysis

study of the fibrinolytic enzyme (38 U) showed a maximum of 37.88% clot lysis, which was relatively higher than that exhibited by commercial streptokinase (248 U) and heparin (95 U) for a period of 3 h (Figure 5.7). This indicates that the fibrinolytic enzyme produced in the present work exhibits higher affinity towards the fibrin clot as compared to the two commercial fibrinolytic agents used as positive control. Similar results were obtained by Ju et al. (2012), where the test enzyme SFE1 (200 IU/mL) showed complete clot lysis as compared to their positive control urokinase (200 IU/mL) and negative control saline. Prasad et al. (2006) reported the *in vitro* dissolution of clots using four different dilutions of streptokinase and achieving a maximum clot lysis of 70.8% with 30,000 IU streptokinase.



	Clot lysing agents	% Clot lysis*
A	Positive Control (Heparin 95 U)	14.2±2.27
B	Positive Control (Streptokinase 248 U)	13.17±0.58
C	Test Enzyme (38 U)	37.88±4.16
D	Test Enzyme (29 U)	15.57±1.17
E	Negative Control	1.55±0.48

Figure 5.7 Percentage clot lysis observed with various fibrinolytic agents. Data represents mean ± SD (n = 2).

An attempt was made to understand *in vitro* thrombolytic mechanism of the purified fibrinolytic enzyme. Clot lysis was observed under both heated and unheated conditions. It is presumed that plasminogen molecule present in the blood clot gets denatured when the clot is incubated at 85 ºC and hence the clot lysis activity

observed under heated condition would indicate a plasmin-like activity. However, if clot lysis is observed under unheated condition, the enzyme might either act as plasminogen activator that converts plasminogen to plasmin and thereby lyse the clot or as a plasmin-like enzyme. Interestingly, percentage clot lysis achieved in case of unheated blood clots was almost double than that obtained with blood clots that were heated (Table 5.5). Results obtained thus, indicate that the fibrinolytic enzyme lysed the blood clot by both direct, indirect clot lysis mechanism and might perhaps function better as a plasminogen activator. These results differ with the only existing report which had made a similar attempt to determine the *in vitro* thrombolytic mechanism of fibrinolytic enzyme from *Streptomyces* sp. XZNUM 00004 (Ju et al. 2012). However, further studies are required to elucidate precise mechanism of clot lysis exhibited by the enzyme in question.

Table 5.5 Percentage of clot lysis under heated and unheated conditions

Experimental conditions	**Clot lysis* (%)**
Heated	18 ±1.47
Unheated	34 ±1.13

*Data represents mean ± SD (n = 2)

5.2.9 Identification of partial amino acid sequence

A total of seven peptide sequences were obtained as shown in the MS-TOF Chromatogram (Figure 5.8). The amino acid sequences of the identified peptide fragments were FSAEQQQQAK, GIDKIDLSFFNK, INLNEK, SFSDVGGLK, DQSYNGFTINAK, SLGTDGAVNTSSFK and YGNWTQNER. Further, these peptides were analysed using the NCBI BLAST database for sequence similarity with the previously reported similar enzymes (Table 5). The four former peptide sequences showed a high degree of homology with peptidase M10 (also known as metalloprotease) from *Yersinia frederiksenii*, serine 3-dehydrogenase from *Serratia grimesii* and serralysin from *Serratia* (Table 5.6). These represent the conserved regions of the protein sequence. However, the three latter peptide sequences did not

show any homology with the sequences available on the NCBI-BLAST database, thus, indicating that the fibrinolytic enzyme produced in the present study could be a unique enzyme (Krishnamurthy et al., 2018).

Table 5.6 Comparison of peptide sequences of the fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* with earlier reported similar enzymes

Name of the source	Sequence[a]	Identity (%)	Positives (%)	Accession number
Peptidase M10 *Yersinia frederiksenii*	**Peptide 1:** FSAEQQQQAK AG **Peptide 2:** GIDKIDLSFFNK GTDTFDFSGYSQNQK **Peptide 3:** INLNEK INLNEK **Peptide 4:** SFSDVGGLK SFSDVGGLKG	67%	72%	WP 50099498.1
Serine 3-dehydrogenase *Serratia grimesii*	**Peptide 1:** FSAEQQQQAK AG **Peptide 2:** GIDKIDLSFFNK GNDTFDFSGFSQNQR **Peptide 3:** INLNEK INLNEK **Peptide 4:** SFSDVGGLK SFSDVGGLK	67%	72%	WP 037422473.1
Serralysin *Serratia*	**Peptide 1:** FSAEQQQQAK AG **Peptide 2:** GIDKIDLSFFNK GNDTFDFSGYTANQR **Peptide 3:** INLNEK INLNEK **Peptide 4:** SFSDVGGLK SFSDVGGLK	67%	72%	CUW14522.1

[a]Identical amino acid sequences are underlined, peptide sequence of the fibrinolytic enzyme (present work) is in red colour.

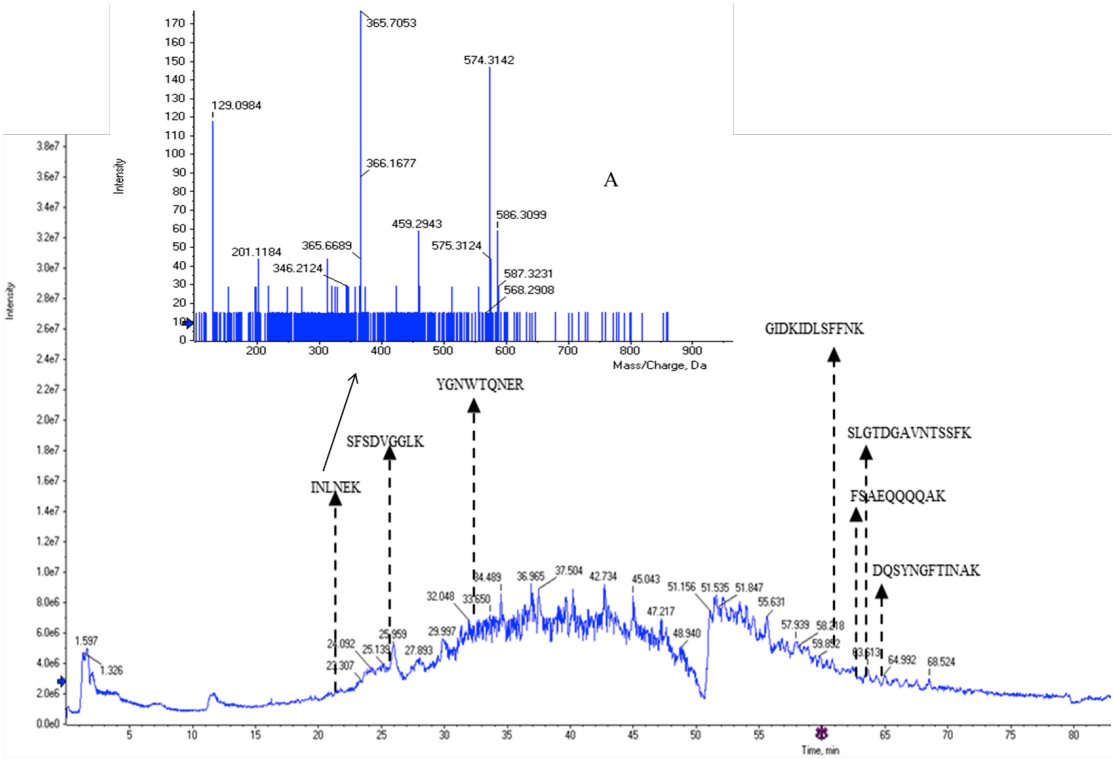


Figure 5.8 MS-TOF Chromatogram showing the identified peptide sequences with their retention time, (A) MS spectrum of the peptide sequence INLNEK

5.3 SUMMARY AND CONCLUSIONS

- In the current study, we have purified and biochemically characterised a novel fibrinolytic enzyme from marine *Serratia marcescens* subsp. *sakuensis* (KU296189.1).
- The purified enzyme had a molecular weight of 43 KDa and showed a specific activity of 1033 U/mg with 19.38% recovery and 21.08 purification fold.
- The optimum pH and temperature of the enzyme were found to be 7 and 55 °C. It was highly stable over a broad range of pH, maintaining 67% of its original activity at pH values ranging from 3 to 11 for a period of one hour at room temperature. For temperatures up to 65 °C, the enzyme retained more than 50% of its activity for a period of 2 h.
- The *in vitro* half-life of the fibrinolytic enzyme at physiological temperature was found to be 19 h.

- The enzyme activity increased in presence of divalent cations such as Mg^{2+}, Mn^{2+}, Zn^{2+} and DTT. However, decrease in fibrinolytic activity with PMSF and EDTA indicates the serine metalloprotease nature of the enzyme.
- The apparent K_m and V_{max} values of the purified enzyme were 0.66 mg/mL and 158.73 U/mL respectively, while the K_{cat} and catalytic efficiency values were found to be 12.21 min^{-1} and 18.32 mL/(mg min) respectively.
- The fibrinolytic enzyme exhibited proteolytic activity with casein and albumin with F/C ratio of 0.33, but not with blood proteins such as haemoglobin, ɣ-globulins and transferrin.
- Purified fibrinolytic enzyme (38 U) showed 38% clot lysis which was significantly higher in comparison to that of streptokinase and heparin for a period of 3 h during *in vitro* studies.
- In addition, clot lysis was observed under both heated and unheated conditions, suggesting that thrombolytic mechanism of the enzyme could be by both direct and indirect mechanism of clot lysis.
- LC-MS/MS-TOF analysis of the purified protein resulted in a total of 7 peptide sequences, of which only four showed 67% homology with the sequences of other proteases. The other three peptide fragments did not display any homology with any of the protein sequences present in the database.
- The results obtained in this work indicates that the fibrinolytic enzyme produced could be a novel enzyme with several therapeutically favourable characteristics and can be further explored to assess its suitability as a potential candidate for therapeutic use.

CHAPTER 6

CHEMICAL MODIFICATION OF THE FIBRINOLYTIC ENZYME

Chemical modification of the purified enzyme using amino acid specific modifiers was attempted to determine the significant amino acid residues present in the active site of the fibrinolytic enzyme. Also, chemical modification of the enzyme might improve its characteristics. The kinetic parameters of the chemically modified enzyme was evaluated and its secondary structure was determined by CD spectrum analysis. In addition, the effect of several metal ions on the fibrinolytic activity of the modified enzyme was studied.

6.1 MATERIALS AND METHODS

Fibrin and fibrinogen (human origin) were purchased from Sigma. DEPC, NBS and PMSF were procured from Sigma-aldrich (India) while DTNB and EDAC were procured from Hi-media. All other chemicals and reagents used were of analytical grade.

6.1.1 Chemical modification of fibrinolytic enzyme

The purified fibrinolytic enzyme was subjected to modification by different modifiers that target specific amino acid residues. The modifiers used were DTNB (5, 5-Dithio-bis-(2-nitrobenzoic) acid), NBS (N-bromosuccinimide), EDAC (Ethyl-3-(3-dimethylaminopropyl) carbodiimide), DEPC (Diethylpyrocarbonate) and PMSF (Phenylmethanesulfonyl fluoride) which acts upon cysteine, tryptophan, carboxylates, histidine and serine residues respectively.

6.1.1.1 Modification of cysteine residues

Modification of cysteine residues was performed using DTNB according to the method followed by Hu and Guo (2009). 50 mM DTNB stock solution was prepared in 100 mM phosphate buffer (pH 8.0). Different concentrations of DTNB (10 mM, 15 mM, 20 mM and 30 mM) were taken to investigate its effect on the fibrinolytic activity. Equal volumes of DTNB and purified enzyme sample were added to the test tube and incubated at 37 °C in 20 mM phosphate buffer (pH 8.0) for 2 h. A control was maintained with distilled water instead of enzyme. Following incubation period,

samples were subjected to dialysis overnight against 20 mM phosphate buffer (pH 7). The samples were then tested for the fibrinolytic activity under standard assay conditions and results were expressed in terms of relative activity (%).

6.1.1.2 Modification of tryptophan residues

For modification of tryptophan residues, NBS was used and the reaction was carried out according to the method of Hu and Guo (2009). Stock solution of 5 mM NBS was prepared in 100 mM phosphate buffer (pH 8.0). The concentration of NBS was varied (0.01 mM, 0.05 mM, 1 mM), and equal volumes of NBS and the purified enzyme sample was added and incubated at 37 °C in 20 mM phosphate buffer (pH 8.0) for 2 h. Distilled water was added in place of enzyme solution and maintained as control. Samples were then dialysed overnight against 20 mM phosphate buffer (pH 7). This was followed by determining the fibrinolytic activity of the samples. Fibrinolytic activity was expressed in terms of relative activity (%).

6.1.1.3 Modification of histidine residues

Modification of histidine residues by DEPC was performed by the method described by Hu and Guo (2009). Stock solution of 30 mM DEPC was prepared in 99.9% (v/v) ethanol. Different concentrations of DEPC (1mM and 30 mM) were taken for the modification reaction. Equal volumes of purified enzyme sample and DEPC was added and incubated at 37 °C in 20 mM phosphate buffer (pH 8.0) for 2 h. Distilled water was added instead of enzyme and maintained as the control. Upon incubation, samples were subjected to dialysis overnight against 20 mM phosphate buffer (pH 7).The dialyzed enzyme sample was then tested for the fibrinolytic activity, under standard assay conditions and was expressed in terms of relative activity (%).

6.1.1.4 Modification of serine residues

The serine residues were modified using PMSF according to the method described by Hu and Guo (2009). Stock solution of 30 mM PMSF was prepared in 99.9% (v/v) ethanol. Different concentrations of PMSF (1 mM and 30 mM) were taken and the modification reaction was carried out at 37 °C in 20 mM phosphate buffer (pH 8.0)

for 2 h containing equal volumes of purified enzyme solution and PMSF. Following this, samples were subjected to dialysis overnight against 20 mM phosphate buffer (pH 7). Distilled water was added instead of enzyme solution and maintained as the control. The dialyzed samples were then tested for the fibrinolytic activity, under standard assay conditions and was expressed in terms of relative activity (%).

6.1.1.5 Modification of carboxylate residues

The carboxylates residues such as glutamate and aspartate were modified using EDAC according to the method of Hu and Guo (2009). Stock solution of 100 mM EDAC was prepared in 99.9% (v/v) ethanol. The concentration of EDAC (0.5 -100 mM) was varied and used for the modification reaction. Equal volume of enzyme sample and the EDAC was added and incubated at 37 °C in 10 mM potassium acid phthalate-NaOH buffer (pH 4.1) for 2 h. Distilled water was added instead of the enzyme sample and maintained as the control. Samples were then subjected to dialysis overnight against 20 mM phosphate buffer (pH 7).The dialyzed samples were then tested for the fibrinolytic activity, under standard assay conditions and was expressed in terms of relative activity (%).

6.1.2 Fibrinogenolytic activity assay

Fibrinogenolytic activity was determined according to the method of Wang et al. (2009) with minor modifications as described in chapter 3

6.1.3 Protein concentration

Protein concentration was determined by Lowry's method (1951), using bovine serum albumin (BSA) as standard as described in chapter 3

6.1.4 Kinetic parameters

Different substrate concentrations (0.004 mg/mL to 0.03 mg/mL) of both fibrin and fibrinogen were used to determine the initial reaction rates. The assay was conducted under optimum conditions (pH 7.0 and 55 ºC). The Michaelis-Menten constant (K_m) and maximum rate of reaction (V_{max}) values were determined according to

Lineweaver-Burk double reciprocal plot upon plotting the respective $1/[S_0]$ and $1/[V_0]$ values for each of the substrates (Nelson and Cox 2005). Subsequently, K_{cat} and catalytic efficiency (K_{cat}/K_m) of the enzyme for fibrin substrate were also calculated.

6.1.5 CD (Circular Dichroism) spectra

CD analysis for native and modified enzyme was performed using Jasco J-715 spectropolarimeter. The analysis was carried out using a cell of 1 mm path length with wavelength range 190-260 nm. CD spectra recorded in the wavelength region were an average of three scans at a scan speed of 50 nm/min, response time of 4 s and corrected by subtracting the appropriate blank runs of 20 mM sodium phosphate buffer (pH 7). CD values (mdeg) obtained were aligned with increasing wavelength (nm) and were plotted on a graph. The secondary structure percentage predictions were made using K2D2 software.

6.1.6 Effect of metal ions on modified enzyme

The concentration of metal ions chosen were similar to that present in human blood (Table). The chemically modified fibrinolytic enzyme (with 2.5 mM EDAC) was incubated at 37 °C for 1 h with metal ions such as Cu^{2+}, Mn^{2+}, Zn^{2+}, Li^{+}, Co^{2+}, Pb^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, K^{+} and Na^{+} at concentrations as mentioned in table. The samples were then subjected to fibrinolytic enzyme assay and the activity was expressed in terms of residual activity (%). The activity of the enzyme in the absence of metal ions was taken as 100%.

6.2 RESULTS AND DISCUSSION

6.2.1 Modification on cysteine residues

With increasing concentration of DTNB the fibrinolytic activity decreased and an enzyme activity of 7.22% was obtained with 30 mM DTNB (appendix V) (Figure 6.1A). According to Rudyk and Eaton (2014) the thiol side chain [-SH] present in the cysteine residues of enzyme acts a nucleophile thereby reducing DTNB into a mixed protein disulfide and 5-Thio-2-NitroBenzoicacid (TNB). Thus, results obtained in this

study indicates the need of intact cysteine residues for fibrinolytic activity as these cysteine residues are a part of the catalytic site. Most of the reports available suggest that DTNB has an inhibitory effect on proteases, such as that described by Park et al. (2013), wherein the addition of DTNB to the protease resulted in inhibition of almost 50% of the enzyme activity. Uchikoba et al. (2002) had reported that the addition of 1 mM DTNB to the cysteine protease resulted in about 85% loss in the enzyme activity.

6.2.2 Modification on tryptophan residues

It was observed that with increasing concentration of NBS the fibrinolytic enzyme activity decreased, 0.1 mM NBS resulted in complete loss of enzyme activity (Appendix VI) (Figure 6.1B). The results obtained suggest that tryptophan residues play an important role in the fibrinolytic enzyme catalysis and could be a part of the active site of the enzyme. Similar results were reported by Chang et al. (2012) wherein the fibrinolytic activity was found to be reduced to 1.5% with 0.5 mM NBS. They had stated in their report that NBS plays the role of a protein-oxidizing agent that particularly oxidizes tryptophan residues to produce oxindole derivative.

6.2.3 Modification of histidine residues

As shown in the appendix VII, at both high and low concentrations of DEPC, the fibrinolytic activity decreases considerably (Figure 6.1A). Hnizda et al. (2008) had quoted that higher concentrations of DEPC leads to the formation of products such as formyl-biscarbethoxy histidine and urethanecarbethoxy histidine that results in disruption of the imidazole ring that forms the active group of histidine, thereby rendering it unfit for the catalytic action of the enzyme (Hnizda et al. 2008). Thus, results obtained in this study indicate the requirement of intact histidine residues for fibrinolytic enzyme catalysis as they may be present in the catalytic site of the enzyme. These results are comparable to the findings reported by Chang et al. (2012), wherein, 85% loss in the enzyme activity was observed on modification with 2.5 mM DEPC.

6.2.4 Modification of serine residues

PMSF at both higher (1 mM) and lower (30 mM) concentration had an inhibitory effect on the fibrinolytic activity (Appendix VIII). Fibrinolytic enzyme activity was found to reduce to 2.23% with 30 mM PMSF when compared to that in the control (Figure 6.1A). The results obtained suggest the presence of serine residues in the catalytic site of the enzyme and thereby plays a role in the catalytic activity of the enzyme. These findings concur with the results reported in chapter 5, where the fibrinolytic enzyme was found to be a serine metalloprotease in nature. These results are similar to the results reported by Agrebi et al. (2009), Chang et al. (2012), Ju et al (2012) and Mahajan et al. (2012). Han et al. (2012) had outlined the mode of action of PMSF, it is said to react with hydroxyl group (-OH) present in the enzyme to produce o-benzyl sulfonyl-serine.

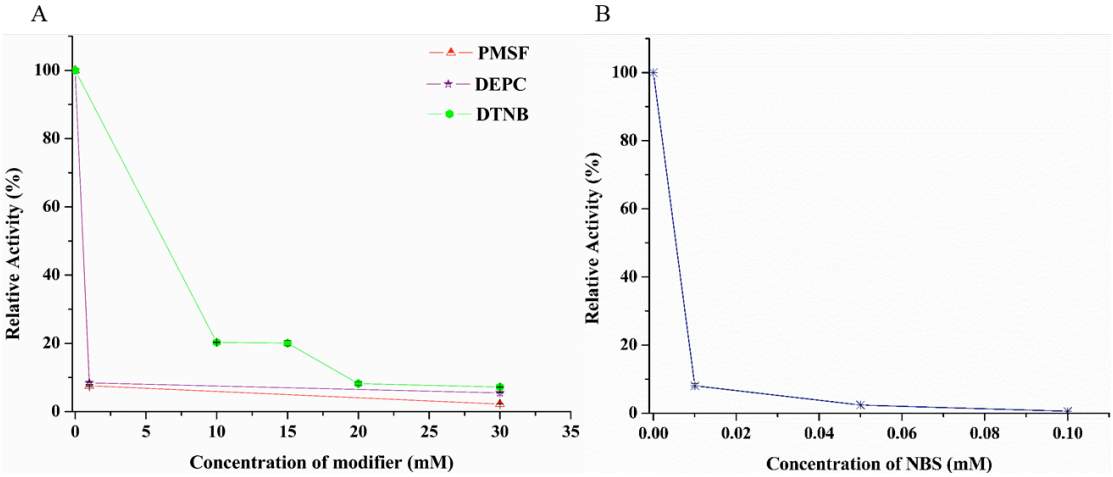


Figure 6.1 Comparison of the effect of modifiers on enzyme activity (A) Effect of PMSF, DEPC and DTNB on fibrinolytic activity (B) Effect of NBS on fibrinolytic activity. Bars represent standard deviation (n = 3).

6.2.5 Modification on carboxylate residues

Upon modification with EDAC, the fibrinolytic activity increased significantly, indicating that aspartic and/or glutamic residues are essential for the enzyme activity. From the initial trials with EDAC (0-100 mM) a relative enzyme activity of 273.08%

was achieved with 5 mM EDAC (Appendix IX). Further trials performed to ascertain the effect of EDAC on the fibrinolytic enzyme activity resulted in a 9 fold increase in the fibrinolytic activity (913%) with 2.5 mM EDAC as shown in appendix IX (Figure 6.2). The results suggest the presence of carboxylate residues in the catalytic site of the fibrinolytic enzyme. According to Wen et al. (1999) and Hu and Guo (2009), EDAC modifies the carboxylate residues by reacting with their side chains as well as with the C-terminal residues. Liu et al. (2013) had reported that EDAC is a carbodiimide that reacts with the carboxyl (-COOH) group to form O-acylisourea intermediate, this intermediate shows high reactivity towards amine and thus leads to the formation of an amide bond between two protein molecules. At 2.5 mM concentration, there might be sufficient EDAC molecules to react with the enzyme thereby forming an amide bond network among the enzyme molecules. This network might enhance the substrate accessibility to the active site of the enzyme. Moreover, Mosesson (2005) and Weisel (2007) reported that fibrin comprises a highly branched network with cross-linked γ-chains that facilitates interaction with bonded enzyme molecules. These findings concur with the results obtained in our study.

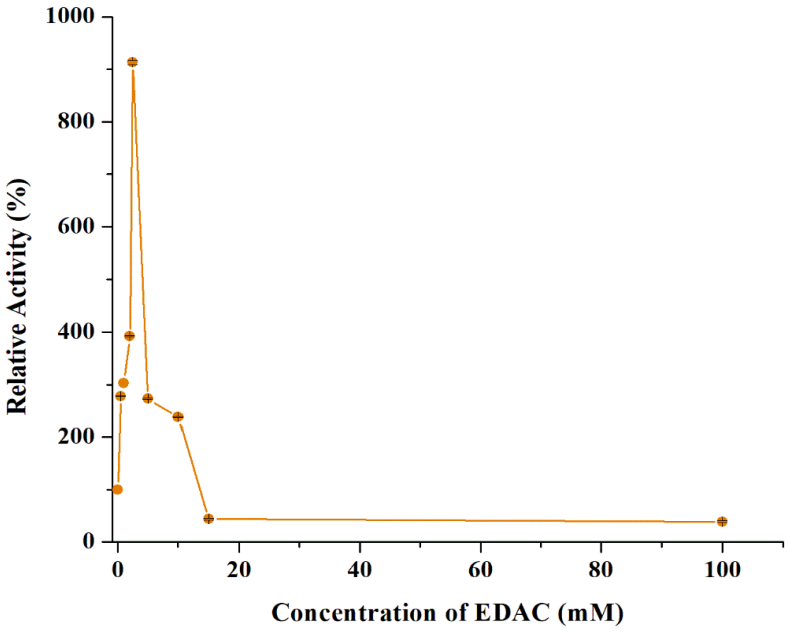


Figure 6.2 Effect of varying concentration of EDAC on fibrinolytic activity. Bars represent standard deviation (n = 3).

From all of the above modification reactions, only EDAC resulted in a 9 fold enhancement in the fibrinolytic activity, while other modifiers such as DEPC, DTNB, PMSF and NBS led to reduced fibrinolytic enzyme activity (Figure 6.1 and 6.2). In addition to these, experiments were conducted to investigate the specificity of the chemically modified enzyme (2.5 mM EDAC) towards the substrates fibrin and fibrinogen as compared to the unmodified enzyme (control) (table 6.1). The ratio of fibrinolytic/ fibrinogenolytic activity was calculated for both modified enzyme sample as well as for the control. As seen in table 6.1, the modified enzyme exhibited fibrinolytic activity of 7226.3 U/mg and fibrinogenolytic activity of 4707.22 U/mg. However, the fibrinolytic to fibrinogenolytic ratio was estimated to be 1.54:1, which is similar to that obtained in the control.

Table 6.1 Fibrinolytic and fibrinogenolytic activities of control and modified sample

	Specific enzyme activity* **(U/mg)**	**Fibrinolytic/Fibrinogenolytic activity**
Control (unmodified enzyme)		
Fibrinolytic	377.88 ± 0.87	1.69:1
Fibrinogenolytic	222.48 ± 8.69	
Modification with 2.5mM EDAC		1.54:1
Fibrinolytic	7226.30 ± 0.00	
Fibrinogenolytic	4707.22 ± 19.68	

*Data represents mean ± SD (n = 2)

6.2.6 Kinetic parameters

Kinetic parameters were determined under standard assay conditions using different concentrations of the substrates fibrin and fibrinogen. As shown in figure 6.3, the chemically modified fibrinolytic enzyme obeyed Michaelis-Menten kinetics with both fibrin and fibrinogen as substrate. From the Lineweaver-Burk plot (Figure 6.4A), the chemically modified enzyme for fibrin substrate showed Michaelis constant (K_m) of

0.0013 mg/mL, with a maximum rate (V_{max}) of 62.62 U/mL, turnover number (K_{cat}) of 5.22 min^{-1} and catalytic efficiency (K_{cat}/K_m) of 4015.38 mL/(mg min). While on the other hand, for fibrinogen substrate the modified enzyme displayed a K_m and V_{max} of 0.0016 mg/mL and 81.63 U/mL respectively (figure 6.4B).

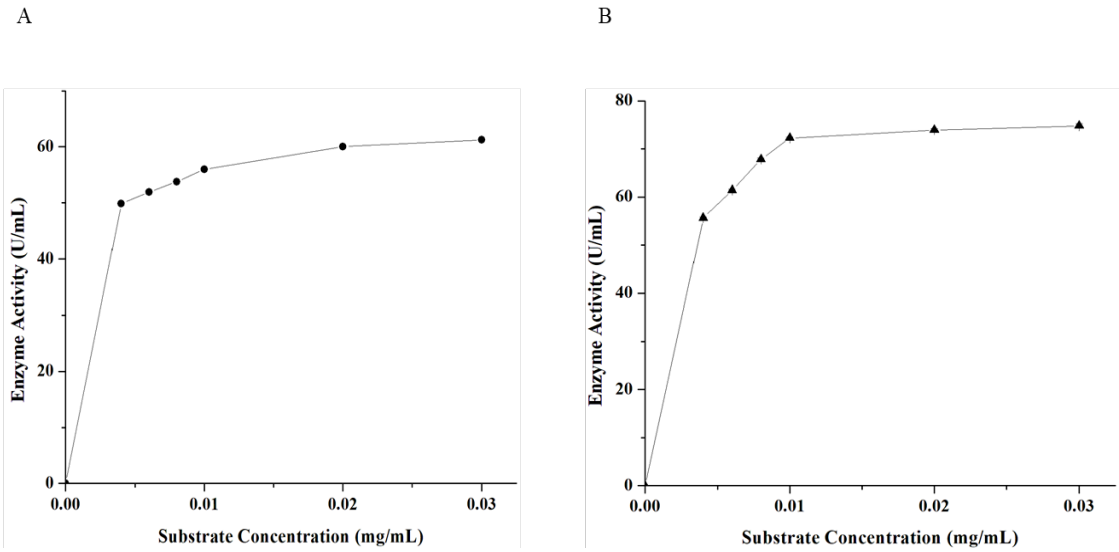


Figure 6.3 Michaelis-Menten plot for modified enzyme activity with (A) fibrin (B) fibrinogen. Bars represent standard deviation (n = 2).

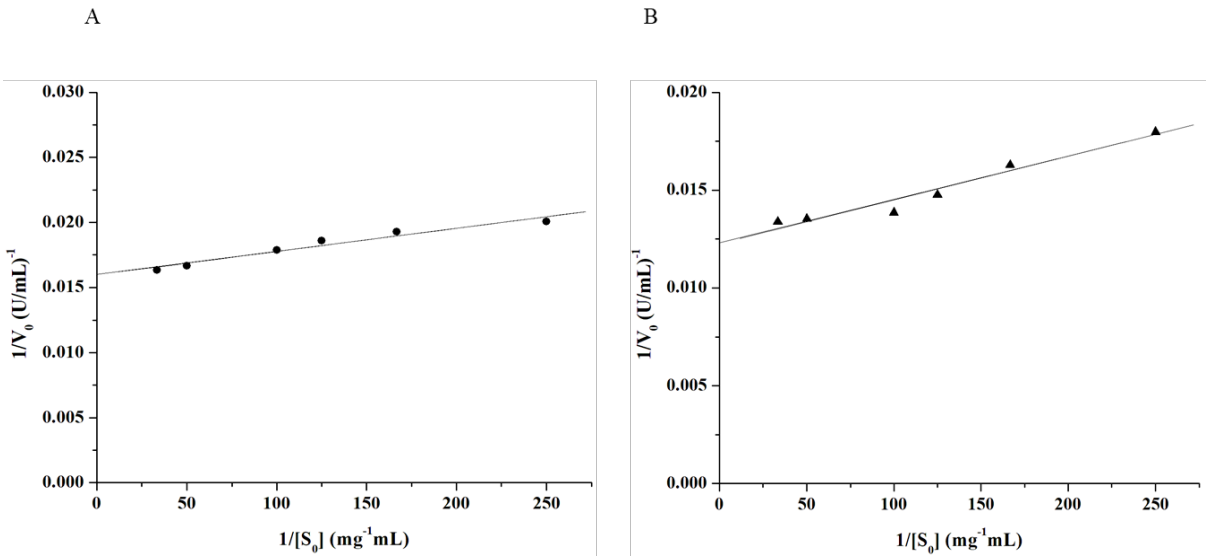


Figure 6.4 Lineweaver-Burk plot for modified enzyme activity with (A) fibrin (B) fibrinogen

Table 6.2 Comparison of kinetic parameters of the unmodified and chemically modified enzyme

Kinetic Parameters	Unmodified Enzyme	Chemically Modified Enzyme	
	Substrate used		
	Fibrin	Fibrin	Fibrinogen
K_m*	0.66 mg/mL	0.0013 mg/mL	0.0016 mg/mL
V_{max}*	158.73 U/mL	62.62 U/mL	81.63 U/mL
K_{cat}*	12.21 min^{-1}	5.22 min^{-1}	-
Catalytic efficiency*	18.32 mL/(mg min)	4015.38 mL/(mg min)	-

*Data represents mean ± SD (n = 2)

In comparison to the results obtained in chapter 5, as shown in table 6.2, the unmodified enzyme exhibited a K_m value of 0.66 mg/mL with fibrin substrate, K_m value of 0.0013 mg/mL obtained for the chemically modified enzyme with fibrin is significantly lesser, thus, indicating a higher affinity for substrate fibrin. Hence, on chemical modification, a significant increase in the enzyme specificity towards fibrin substrate has been achieved. Also, the K_m value obtained with fibrin is relatively lesser than that achieved with fibrinogen, indicating a relatively higher enzyme specificity towards fibrin. Increase in the catalytic efficiency was also observed on chemical modification of the enzyme as compared to catalytic efficiency observed in case of the unmodified enzyme (Table 6.2).

6.2.7 CD spectra analysis

The secondary structure of the modified and native unmodified fibrinolytic enzyme was determined by CD spectrum in the UV region. Identical spectra were obtained for both native and fibrinolytic enzyme (Figure 6.5A). The secondary structure of both the fibrinolytic enzymes (chemically modified and unmodified) were analysed using K2D2 software. No significant change was observed in the secondary structure of the modified enzyme as compared to that of the native enzyme. The percentages of

secondary structure elements calculated using K2D2 software showed that there were slight differences in α- helix and β-sheet conformations between native and modified enzyme. Native enzyme had 2.11% α- helix, 51.77% β-sheet and 46.12% random coil contents, while modified enzyme had 1.78% α- helix, 52.93% β-sheet and 45.29% random coil contents (figure 6.5B).

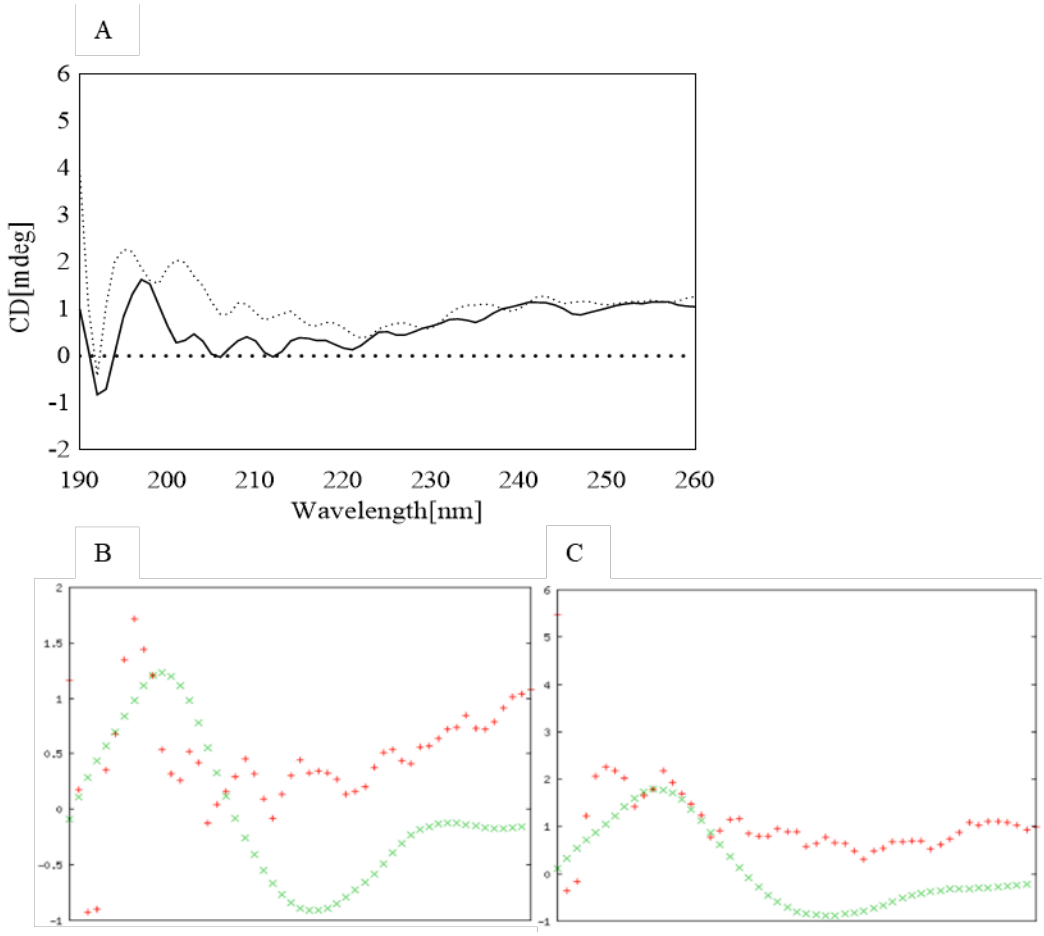


Figure 6.5 (A) CD spectra of native (smooth line) and modified (dashed line) fibrinolytic enzyme (B) K2D2 analysis: (+) input spectrum and (×): predicted spectrum of CD spectra of native enzyme (C) K2D2 analysis: (+) input spectrum and (×): predicted spectrum of CD spectra of modified enzyme

6.2.8 Effect of metal ions on modified enzyme

As shown in table 6.3, divalent metal ions such as Cu^{2+}, Mn^{2+}, Zn^{2+}, $Ca^{2+}$and Mg^{2+} enhanced the fibrinolytic activity of the chemically modified enzyme. The results

obtained are comparable to that reported in chapter 5, wherein the divalent metal ions such as Mn^{2+}, Mg^{2+} and Zn^{2+} increased the fibrinolytic activity of the unmodified enzyme (Table 6.3).

Table 6.3 Comparison of the effect of various metal ions on fibrinolytic activity of both chemically modified and unmodified enzyme

Metal ion	Concentration (mM)	Residual activity (%)	Residual activity (%)
		Chemically modified enzyme	Unmodified enzyme
Control	-	100	100
Cu^{2+}	0.15	108.80 ± 0.74	65.96 ± 0.5
Mn^{2+}	0.15	107.78 ± 0.73	158.88 ± 4.71
Zn^{2+}	0.15	126.39 ± 0.73	103.33 ± 1.57
Li^{+}	0.15	72.57 ± 0.73	53.33 ± 0.49
Co^{2+}	0.15	89.13 ± 0.75	67 ± 2.87
Pb^{2+}	0.15	77.75 ± 0.71	67.76 ± 1.79
Ca^{2+}	1	104.66 ± 0.74	64.46 ± 2.86
Mg^{2+}	1	118.11 ± 0.75	107.77 ± 0
Fe^{3+}	5	96.38 ± 0.73	89.84 ± 1.43
Fe^{2+}	5	86.55 ± 1.46	68.78 ± 1.79
K^{+}	50	83.96 ± 0.73	68.78 ± 1.79
Na^{+}	150	93.27 ± 0.74	68.07 ± 3.47

*Data represents mean ± SD (n = 2)

6.3 SUMMARY AND CONCLUSIONS

- Different amino acid specific modifiers such as DTNB, NBS, DEPC, EDAC and PMSF were used to chemically modify the fibrinolytic enzyme.
- On modification with DTNB, NBS, DEPC and PMSF, the fibrinolytic enzyme activity reduced, thus, denoting that cysteine, histidine, tryptophan and serine residues are essential for fibrinolytic activity and are present at the active site of the enzyme.
- Modification with 2.5 mM EDAC resulted in a 19 fold enhancement in the fibrinolytic enzyme activity (7226 U/mg) as compared to that of the unmodified enzyme (378 U/mg), signifying the role and presence of carboxylic acid residues in the catalytic site of the enzyme.
- The ratio of fibrinolytic to fibrinogenolytic activity for the modified enzyme was found to be 1.54:1 which was closely similar to that of the native enzyme (1.69:1).
- The kinetic parameters such as K_m, V_{max}, K_{cat} and catalytic efficiency of the chemically modified enzyme was found to be 0.0013 mg/mL, 62.62 U/mL, 5.22 min^{-1}, 4015.38 mL/(mg min) for fibrin substrate. Thus, on chemical modification, affinity of the enzyme for fibrin substrate increased notably resulting in an enhancement of catalytic efficiency by 219 times. For fibrinogen as substrate, the K_m and V_{max} were estimated to be 0.0016 mg/mL and 81.63 U/mL respectively.
- The CD spectrum of both the modified and native enzyme was analysed. It was found that secondary structure of the chemically modified enzyme was similar to that of the unmodified enzyme except for a few minor changes in the percentages of secondary structure elements. K2D2 analysis showed that the native enzyme had 2.11% α- helix, 51.77% β-sheet and 46.12% random coil contents, while modified enzyme had 1.78% α- helix, 52.93% β-sheet and 45.29% random coil contents.

- The effect of metal ions on the fibrinolytic activity of the modified enzyme was studied. Divalent cations such as Cu^{2+}, Mn^{2+}, Zn^{2+}, $Ca^{2+}$and Mg^{2+} led to enhanced fibrinolytic activity.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Regardless of the availability of fibrinolytic agents not many have been put to use in the medical field owing to the drawbacks associated with them. The preclinical studies on most of the newer protein engineered fibrinolytic agents have not been translated successfully into clinical trials. Due to this, age-old fibrinolytic drugs such as heparin and warfarin are still in use despite their drawbacks. Hence, there is a constant impetus for the development of a novel fibrinolytic candidate that is safe for therapeutic use. On this context, present work was taken up to isolate a marine microorganism capable of producing a fibrinolytic enzyme with therapeutically favourable characteristics that would make it a potential candidate for clinical use.

This doctoral work on "Studies on a Fibrinolytic Enzyme Produced From Marine *Serratia marcescens* subsp. *sakuensis*" has been discussed in the thesis in six chapters. Following is the summary of the research work.

7.1 SUMMARY

A total of eight bacteria were isolated from the sea water sample collected from Surathkal Coast in the Arabian Sea. These isolates were screened for their ability to produce fibrinolytic enzyme by adopting a screening strategy that provided insight on the specificity towards fibrin, activity at the physiological pH range, anticoagulant and thrombolytic effect *in vitro*. Based on the results obtained from qualitative and quantitative screening studies, the isolate C7 showed superior performance in every aspect and thus, was chosen for further studies. It was identified as *Serratia marcescens* subsp. *sakuensis* (KU296189.1) on 16S rRNA gene sequencing and phylogenetic analysis. Experiments were conducted to enhance the production of fibrinolytic enzyme through medium optimisation using one-factor-at-a-time approach and Plackett-Burman design. The results obtained demonstrated that nitrogen sources had a profound effect on fibrinolytic enzyme production. A 3.4 fold increase in the fibrinolytic activity was achieved with the optimised medium composition as compared to that in the unoptimised medium.

Further, the enzyme was purified by salt precipitation, dialysis followed by size exclusion chromatography. Characterisation studies of the serine metalloprotease

fibrinolytic enzyme revealed some therapeutically important characteristics such as (i) having an optimum pH close to the human physiological pH (ii) having a K_m of 0.66 mg/mL for fibrin substrate (iii) absence of proteolytic activity with the blood proteins such as haemoglobin, ɣ-globulins and transferrin (iv) exhibiting higher clot lysis *in vitro* than commercial streptokinase and heparin for a period of 3 h. LC-MS/MS-TOF analysis of the purified protein resulted in a total of 7 peptide sequences, of which three peptide fragments failed to show any homology with any of the protein sequences present in the database, indicating the uniqueness of the fibrinolytic enzyme produced in the present work. Chemical modification of the fibrinolytic enzyme was attempted using certain amino acid specific modifiers. Upon modification with 2.5 mM EDAC, a 9 fold increase in the fibrinolytic activity was observed as compared to the activity of the native unmodified enzyme. For the chemically modified enzyme, K_m obtained with fibrin was lesser than the K_m with fibrinogen, indicating a relatively higher affinity towards fibrin substrate. The secondary structure of both the modified and unmodified enzyme determined by CD spectrum analysis was similar except a few minor changes in the percentages of secondary structure elements.

7.2 SIGNIFICANT FINDINGS

- Studies on medium optimisation for enhancing the fibrinolytic enzyme production indicated a remarkably high nitrogen demand by the marine *Serratia marcescens* subsp. *sakuensis*.
- The fibrinolytic enzyme showed many intriguing characteristics such as its optimum pH being close to the human physiological pH, thermostable nature withstanding temperatures upto 85 °C for 2 h, absence of proteolytic activity with blood proteins such as haemoglobin, ɣ-globulins and transferrins.
- The *in vitro* clot lysing ability of the fibrinolytic enzyme was significantly higher than that displayed by commercial fibrinolytic agents such as heparin and streptokinase.
- LC-MS/MS-TOF analysis of the protein resulted in a total of 7 peptide fragments, of which 3 fragments failed to show any homology with the protein

sequences in the database, indicating the uniqueness of the enzyme produced in the present work.

- A 19-fold enhancement in the fibrinolytic activity was observed on modification of the enzyme with 2.5 mM EDAC, suggesting the presence of carboxylate residues such as glutamate and aspartate in the catalytic site of the enzyme and therefore having a role in enzyme catalysis.
- Kinetic studies for both unmodified and chemically modified fibrinolytic enzyme were carried out using fibrin substrate and the K_m value obtained for the modified enzyme was significantly lesser than the K_m value achieved for the unmodified enzyme. These results indicate that affinity of the chemically modified enzyme for fibrin substrate increased notably resulting in an enhancement of catalytic efficiency by 219 times.
- CD spectrum analysis of the unmodified and modified fibrinolytic enzyme revealed similar secondary structures except minor changes in α- helix and β-sheet conformation of the enzymes.
- Therefore, results obtained in the present study advocate the suitability of the fibrinolytic enzyme produced as a potential candidate for medical applications.

7.3 CONCLUSIONS

- *Serratia marcescens* subsp. *sakuensis* isolated from marine habitat was found to be a potential source for the production of fibrinolytic enzyme.
- The fibrinolytic enzyme produced showed therapeutically favourable characteristics and hence could be rendered favourable for human use.
- In comparison to the commercial fibrinolytic agents such as streptokinase and heparin, the *in vitro* clot lysis (%) potential exhibited by fibrinolytic enzyme produced in the present work was significantly higher and thus, could be considered as a potential candidate for clinical application.
- Chemical modification of the fibrinolytic enzyme showed that carboxylate residues are a part of active site and modification of these residues led to a significant increase in the fibrinolytic activity.

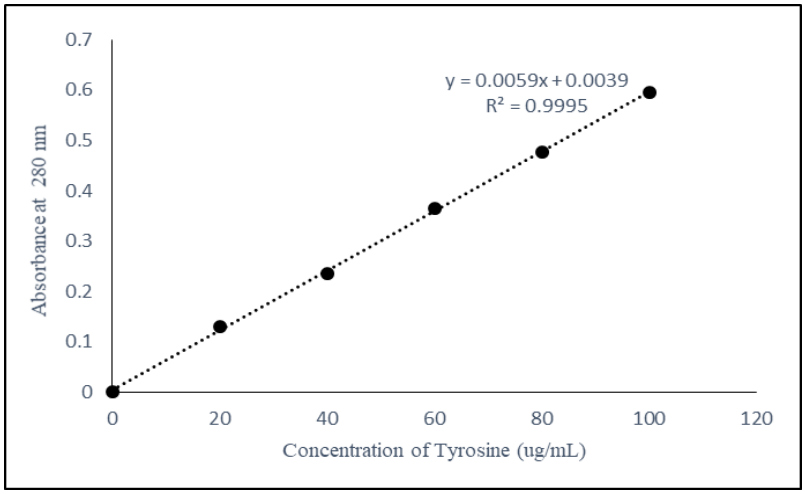
7.4 SCOPE FOR FUTURE WORK

- *In vivo* studies could be performed in order to evaluate the therapeutic potential of the enzyme in animal models.
- The complete protein structure could be determined by proteomic studies, which could further be used for *in silico* studies for drug designing applications.

APPENDICES

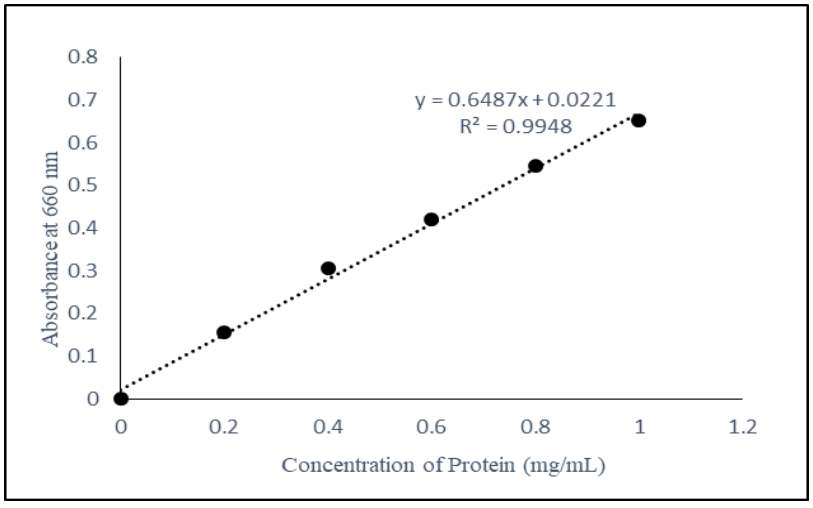
APPENDIX I

Standard graph for fibrinolytic enzyme activity



APPENDIX II

Standard protein curve for total protein estimation



APPENDIX III

Acetate buffer composition

pH	Vol of 0.1M acetic acid (mL per L)	Vol of 0.1M sodium acetate (mL per L)
3.0	982.3	**17.7**
4.0	847	153
5.0	357	643
6.0	52.2	947.8

Phosphate buffer composition

pH	Vol of 0.1M disodium hydrogen phosphate (mL per L)	Vol of 0.1M HCl (mL per L)	Vol of 0.1M NaOH (mL per L)
7.0	756	244	
8.0	955.1	44.9	
9.0	955	45	
10.0	966.4		33.6
11.0	965.3		34.7

APPENDIX IV

Colony morphologies and gram staining properties of the six isolates

Isolates	**Colour**	**Shape**	**Elevation**	**Margin**	**Surface**	**Density, Consistency**	**Gram stain**
C1	White	Filamentous	Flat	Filamentous	Smooth	Translucent, Hard to get off	+ve
C3	Grey	Irregular	Raised	Undulate	Rough	Opaque, Dry	+ve
C5	White	Spindle, filamentous	Flat	Entire	Smooth	Transparent, Hard to get off	+ve
C6	Orange	Circular	Convex	Entire	Glistening	Opaque, Mucoid	+ve
C7	White	Filamentous	Convex	Entire	Smooth	Translucent, Viscid	-ve
C8	Creamish white	Circular	Convex	Entire	Smooth	Opaque, Butyrous	+ve

APPENDIX V

Effect of DTNB on fibrinolytic activity

DTNB (mM)	Relative activity (%)
0	100±0
10	20.28±0.15
15	20.06±0.76
20	8.21±0.45
30	7.22±0.1

*Data represents mean ± SD (n = 2)

APPENDIX VI

Effect of NBS on fibrinolytic activity

NBS (mM)	Relative activity (%)
0	100 ± 0
0.01	8.09 ± 0.61
0.05	2.42 ± 0.02
0.1	0.61 ± 0.06

*Data represents mean ± SD (n = 2)

APPENDIX VII

Effect of DEPC on fibrinolytic activity

DEPC (mM)	Relative activity (%)
0	100 ± 0.00
1	8.42 ± 0.46
30	5.49 ± 0.04

*Data represents mean ± SD (n = 2)

APPENDIX VIII

Effect of PMSF on fibrinolytic activity

PMSF (mM)	Relative activity (%)
0	100 ± 0.00
1	7.58 ± 0.24
30	2.23 ± 0.02

*Data represents mean ± SD (n = 2)

APPENDIX IX

Effect of EDAC on fibrinolytic activity

EDAC (mM)	Relative activity (%)
0	100 ± 0
0.5	278.28 ± 0.81
1	303.21 ± 0.0
2	392.29 ± 1.03
2.5	913.01 ± 3.51
5	273.08 ± 1.11
10	238.45 ± 1.34
15	43.59 ± 1.72
100	39.12 ± 1.59

*Data represents mean ± SD (n = 2)

REFERENCES

Abdel-Naby, M.A., EI-Diwany, A.I., Shaker, H.M. and Ismail, A-M.S. (1992). "Production and properties of fibrinolytic enzyme from *Streptomyces* sp. NRC 411." *World J. Microbiol. Biotechnol.*, 8, 267-269.

Agrebi, R., Haddar, A., Hajji, M., Frikha, F., Manni, L., Jellouli, K. and Nasri, M. (2009). "Fibrinolytic enzymes from a newly isolated marine bacterium *Bacillus subtilis* A26 characterization and statistical media optimization." *Can. J. Microbiol.*, 55(9), 1049–1061.

AG Scientific,Inc., Dithiothreitol (DTT) Application You Must Know. agscientific.com/blog/2013/01/dithiothreitoldtt-application-you-must-know/, 2013.

Ajithkumar, B., Ajithkumar, V., Iriye, R., Doi, Y. and Sakai, T. (2003). "Spore-forming *Serratia marcescens* subsp. *sakuensis* subsp. nov., isolated from a domestic wastewater treatment tank." *Int. J. Syst. Evol. Microbiol.*, 53, 253-258.

Ashipala, O.K. and He, Q. (2008). "Optimisation of fibrinolytic enzyme production by *Bacillus subtilis* DC-2 in aqueous two-phase system (poly-ethylene glycol 4000 and sodium sulphate)." *Bioresource Technol*., 99, 4112-4119.

Astrup, T. and Mullertz, S. (1952). "The fibrin plate method of estimating of fibrinolytic activity." *Arch. Biochem. Biophys.*, 40, 346-51.

Avhad, D.N., Vanjari, S.S and Rathod, V.K. (2013). "A Novel Fibrinolytic Enzyme from *Bacillus Sphaericus* MTCC 3672: Optimisation and Purification Studies." *Amer J Curr Microbiol*., 1, 1-13.

Bajaj, A.P. and Castellino, F.J. (1977). "Activation of human plasminogen by equimolar levels of streptokinase." *J. Biol. Chem.*, 252, 492-498.

Balaraman, K. and Prabakaran, G. (2007). "Production and purification of a fibrinolytic enzyme (thrombinase) from *Bacillus sphaericus*." *Indian J. Med. Res*., 126, 459-464.

Banerjee, A., Chisti, Y. and Banerjee, U.C. (2004). “Streptokinase-a clinically useful thrombolytic agent.” *Biotech. adv.*, 22(4), 287-307.

Barlow, G.H. (1976). “Urinary and kidney cell plasminogen activator (urokinase).” *Methods Enzymol.*, 45, 239-244.

Barrera, N.P. and Robinson, C.V. (2011). “Advances in the mass spectrometry of membrane proteins: from individual proteins to intact complexes.” *Annu. Rev. Biochem.*, 80, 247-271.

Barrett, A.J. (1977). “Proteinases in Mammalian Cells and Tissues.” Elsevier/North-Holland Biomedical Press, Amsterdam, Netherlands, pp 181-208.

Barett, A.J. (1995). “Proteolytic enzymes: aspartic and metallopeptidases.” *Methods Enzymol.*, 248, 183.

Barros, C., Crosby, J.A. and Moreno, R.D. (1996). “Early steps of sperm-egg interactions during mammalian fertilisation.” *Cell Biol. Int.*, 20(1), 33-39.

Barta, G. (1966). “Dyed fibrin plate assay of fibrinolysis.” *Can. J. Physiol. Pharmacol.*, 44(2), 233-240.

Baruah, D.B., Dash, R.N., Chaudhari, M.R. and Kadam, S.S. (2006). “Plasminogen activators: A comparison.” *Vascul. Pharmacol*., 44, 1-9.

Batomunkueva, B.P. and Egorov, N.S. (2001). “Isolation, purification and resolution of the extracellular proteinase complex of *Aspergillus ochraceus* 513 with fibrinolytic and anticoagulant activities.” *Microbiology*, 70(5), 519-522.

Bauer, K. (2011). “Recent Progress in anticoagulant therapy: Oral direct inhibitors of thrombin and factor Xa.” *J. Thromb. Haemost.*, 9, 12-19.

Berg, J.M., Tymoczko, J.L., Stryer, L. (2002). “Biochemistry”, W H Freeman, New York.

Brenner, D.J., Krieg, N.R., James R. and Staley. (2007). “Bergey's Manual of Systematic Bacteriology, Volume 2. In: The Proteobacteria, Part B: The Gammaproteobacteria.” Springer Science & Business Media.

Bhagat, S., Agarwal, M. and Roy, V. (2013). “Serratiopeptidase: A systematic review of the existing evidence.” *Int. J. Surg.*, 11(3), 209-217.

Bhardwaj, S. and Angayarkanni, J. (2014). “Streptokinase production from *Streptococcus dysgalactiae* subsp. *equisimilis* SK-6 the presence of surfactants, growth factors and trace elements.” 3 Biotech.

Bhargavi, P.L. and Prakasham, R.S. (2013). “A fibrinolytic, alkaline and thermostable metalloprotease from the newly isolated *Serratia* sp RSPB11.” *Int. J. Biol. Macromolec.*, 61, 479-486.

Bhatnagar, I. and Kim, S-K. (2010). “Immense Essence of Excellence: Marine Microbial Bioactive Compounds.” *Mar. Drugs.*, 8(10), 2673-2701.

Bindhu, O.S. and Singh, M.K. (2014). “Hemostatic, milk clotting and blood stain removal potential of cysteine proteases from *Calotropis gigantea* (L.) R. Br. Latex.” *Pharmacogn. Mag.*, 10(2), 350-356.

Bisswanger, H. (2011). “Practical Enzymology. In: General aspects of enzyme analysis.” Wiley-VCH Verlag GmbH & Co. KGaA., pp 5-91.

Blann, A.D., Landray, M.J. and Lip, G.Y.H. (2002). “ABC of antithrombotic therapy: An overview of antithrombotic therapy.” *BMJ*, 325, 762-765.

Blow, D.M., Birktoft, J.J. and Hartley, B.S. (1969). “Role of a buried acid group in the mechanism of action of chymotrypsin.” *Nature*, 221, 337-340.

Blow, D.M. (1997). “The tortuous story of Asp…His…Ser: structural analysis of alpha-chymotrypsin.” *Trends Biochem. Sci.*, 22(10), 405-408.

Boxrud, P.D. and Bock, P.E. (2000). "Streptokinase binds preferentially to the extended conformation of plasminogen through lysine binding site and catalytic domain interactions." *Biochemistry (US)*, 39, 13974-13981.

Boxrud, P.D., Verhamme, I.M.A., Fay, W.P. and Bock, P.E. (2001). "Streptokinase triggers conformational activation of plasminogen through specific interactions of the amino-terminal sequence and stabilizes the active zymogen conformation." *J. Biol. Chem.*, 276, 26084-26089.

Camiolo, S.M., Thorsen, S. and Astrup, T. (1971). "Fibrinogenolysis and fibrinolysis with tissue plasminogen activator, urokinase, streptokinase-activated human globulin, and plasmin." *Proc. Soc. Exp. Biol. Med.*, 138, 277-280.

Caridi, J.G. et al. (2005) Thrombolysis. www.venousaccess.com/thrombolysis.htm

Castellino, F.J. (1981). "Recent advances in the chemistry of the fibrinolytic system." *Chem Rev.*, 81, 431-446.

Cera, E.D. (2009). "Serine proteases." *IUBMB Life*, 61(5), 510-515.

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." *J. Agric. Food Chem.*, 48, 3210–3216.

Chait, B.T. (2011). "Mass Spectrometry in the Postgenomic Era." *Annu. Rev. Biochem.*, 80, 239-246.

Chandrasekaran, S. and Dhar, S.C. (1983). "A low-cost method for the production of extracellular alkaline protease using tapioca starch." *J. Ferment. Technol.*, 61, 511-514.

Chang, A.K., Kim, H.Y., Park, J.E., Acharya, P., Park, I.S., Yoon, S.M., You, H.J., Hahm, K.S., Park, J.K. and Lee, J.S. (2005). "*Vibrio vulnificus* secretes a broad-specificity metalloprotease capable of interfering with blood homeostasis through prothrombin activation and fibrinolysis." *J. Bacteriol.*, 187, 6909-6916.

Chang, C.T., Wang, P.M., Hung, Y.F. and Chung, Y.C. (2012).“Purification and biochemical properties of a fibrinolytic enzyme from *Bacillus subtilis*-fermented red bean.” *Food Chem.*, 133, 1611-1617.

Cheng, G., He, L., Sun, Z., Cui, Z., Du, Y. and Kong, Y. (2015). “Purification and Biochemical Characterization of a Novel Fibrinolytic Enzyme from *Streptomyces* sp. P3.” *J. Microbiol. Biotechnol.*, 25(9), 1449-1459.

Cheng, Q., Xu, F., Hu, N., Liu, X., Liu, Z. (2015). “A novel Ca^{2+} -dependent alkaline serine-protease (Bvsp) from *Bacillus* sp. with high fibrinolytic activity.” *J. Mol. Catal. B Enzym.*, 117, 69-74.

Chevallet, M.S., Luche, T. and Rabilloud. (2006). “Silver staining of proteins in polyacrylamide gels.” *Nat. Protoc.*, 1, 1852–1858.

Chitlur, M. and Simpson, M.L. (2015). “Pediatric Thrombotic Disorders. In: Goldenberg, N.A., Manco-Johnson, M.J. (eds), Role of global assays in thrombosis and thrombophilia.” Cambridge University Press, United Kingdom, pp. 142-157.

Chitte, R.R. and Dey, S. (2000). “Potent fibrinolytic enzyme from a thermophilic *Streptomyces megasporus* strain SD5.” *Lett. Appl. Microbiol.*, 31, 405–410.

Chitte, R.R. and Dey, S. (2002). “Production of a fibrinolytic enzyme by thermophilic *Streptomyces* species.” *World J. Microbiol. Biotechnol.*, 18(4), 289–294.

Cho, H-Y., Seo, M.J., Park, J.U., Kang, B.W., Kim, G-Y., Joo, W.H., Lee, Y-C., Cho, Y-S. and Jeong, Y.K. (2011). “Detection of a Unique Fibrinolytic Enzyme in *Aeromonas* sp. JH1.” *J. Microbiol.*, 49(6), 1018-1021.

Choi, H.S. and Shin, P.H. (1998). “Purification and partial characterization of a fibrinolytic protease in *Pleurotus ostreatus*.” *Mycologia*, 90(4), 674– 679.

Choi, D., Cha, W-S., Park, N., Kim, H-W., Lee, J.H., Park, J.S. and Park, S-S. (2011). “Purification and characterization of a novel fibrinolytic enzyme from fruiting bodies of Korean *Cordyceps militaris*.” *Bioresour. Technol.*, 102, 3279–3285.

Choi, B-S., Sapkota, K., Choi, J-H., Shin, C-H., Kim, S. and Kim, S-J. (2013). "Herinase: A Novel Bi-functional Fibrinolytic Protease from the Monkey Head Mushroom, *Hericium erinaceum*." *Appl. Biochem. Biotechnol.*, 170, 609-622.

Choi, J-H., Sapkota, K., Kim, M-K., Kim, S., Kim, S-J. (2014). "Undariase, a Direct-Acting Fibrin(ogen)olytic Enzyme from *Undaria pinnatifida*, Inhibits Thrombosis *In Vivo* and Exhibits *In Vitro* Thrombolytic Properties." *Appl. Biochem. Biotechnol.*, 173, 1985-2004.

Chung, D-M., Choi, N-S., Maeng, P.J., Chun, H.K. and Kim, S-H. (2010). "Purification and Characterisation of a Novel Fibrinolytic Enzyme from Chive (*Allium tuberosum*)." *Food Sci. Biotechnol.*, 19(3), 697-702.

Coico, R. and Sunshine, G. (2015). "Immunology: A Short Course" 6th edn., John Wiley & sons, New York.

Collen, D. and Lijnen, H.R. (1986). "The fibrinolytic system in man." *Crit. Rev. Oncol. Hematol.*, 249-301.

Collen, D., DeCock, F., Vanlinthout, I., Declerck, P.J., Lijnen, H.R. and Stassen, J.M. (1992). "Comparative thrombolytic and immunogenic properties of staphylokinase and streptokinase." *Fibrinolysis*, 6, 232-242.

Coughlin, S.R. (2000). "Thrombin signalling and protease-activated receptors." *Nature*, 407(6801), 258-264.

Cowan, D.A. and Lafuente, R.F. (2011). "Enhancing the functional properties of thermophilic enzymes by chemical modification and immobilization." *Enzyme Microb. Technol.*, 49, 326– 346.

Cui, L., Dong, M.S., Chen, X.H., Jiang, M., Lv, X. and Yan, G. (2008). "A novel fibrinolytic enzyme from *Cordyceps militaris*, a Chinese traditional medicinal mushroom." *World J. Microbiol. Biotechnol.*, 24, 483–489.

Das, S., Lyla, P.S. and Khan, S.A. (2006). “Marine microbial diversity and ecology: importance and future perspectives.” *Curr Sci India.*, 90 (10), 1325-1335.

Davidson, D.J., Higgins, D.L. and Castellino, F.J. (1990). “Plasminogen activator activities of equimolar complexes of streptokinase with variant recombinant plasminogens.” *Biochemistry*, 29, 3585-3590.

Davis, B.G. (2003). “Chemical modification of biocatalysts.” *Curr. Opin. Biotechnol.*, 14, 379–386.

Deepak, V., Kalishwaralal, K., Ramkumarpandian, S., Babu, S.V., Senthilkumar, S.R. and Sangiliyandi, G. (2008). “Optimisation of media composition for Nattokinase production by *Bacillus subtilis* using response surface methodology.” *Bioresource Technol*., 99, 8170-8174.

Deitcher, S.R. and Jaff, M.R. (2003) Pharmacologic and clinical characteristics of thrombolytic agents. www.medreviews.com/pdf/articles/RICM3Suppl2S25pdf.

Deng, Z., Wang, S., Li, Q., Ji, X., Zhang, L. and Hong, M. (2010). “Purification and characterization of a novel fibrinolytic enzyme from the polychaete, *Neanthes japonica* (Iznka).” *Bioresour. Technol.*, 101, 1954–1960.

DeSantis, G. and Jones, J.B. (1999). “Chemical modification of enzymes for enhanced functionality”. *Curr. Opin. Biotechnol.*, 10, 324-330.

Devi, C.S., Joseph, R.E., Saravanan, H., Naine, S.J. and Srinivansan, V.M. (2013). “Screening and molecular characterization of *Serratia marcescens* VITSD2: A strain producing optimum serratiopeptidase.” *Front. Biol.*, 8(6), 632-639.

Dobrovolsky, A.B. and Titaeva, E.V. (2002). “The Fibrinolysis System: Regulation of Activity and Physiologic Functions of Its Main Components.” *Biochemistry (Moscow)*, 67(1), 116-126.

Dodson, G. and Wlodawr, A. (1998). “Catalytic triads and their relatives.” *Trends Biochem. Sci.*, 23(9), 347-352.

Egorov, N.S., Kochetov, G.A. and Khaidarova, N.V. (1976). “Isolation and properties of the fibrinolytic enzyme from the *Actinomyces thermovulgaris* cultural broth.” *Mikrobiologiia*, 45, 455-459.

El-Aassar, S.A., El-Badry, H.M. and Abdel-Fattah, A.F. (1990). “The biosynthesis of proteases with fibrinolytic activity in immobilized cultures of *Penicillium chrysogenum* H9.” *Appl. Microbiol. Biotechnol.*, 33(1), 26– 30.

El-Aassar, S.A. (1995). “Production and properties enzyme in solid state cultures of *Fusarium pallidoroseum*.” *Biotechnol. Lett.*, 17(9), 943–948.

Emerson, S. and Hedges, J. (2003). “Sediment diagenesis and benthic flux. In: Holland, H.D., Turekian, K.K. (eds). Treatise on Geochemistry”, Elsevier, Amsterdam.

Eyzaguirre, J. (1996). “An overview on chemical modification of enzymes.The use of group-specific reagents.” *Biological Research*, 29, 1-11.

Feied, C. and Handler, J.A. (2004). Thrombolytic therapy.www.emedicine.com/emerg/topic831.htm.

Fossum, S. and Hoem, N.O. (1996). “Urokinase and non-urokinase fibrinolytic activity in protease-inhibitor-deprived plasma, assayed by a fibrin micro-plate method.” *Immuno. Pharmacol.*, 32, 119-121.

Francis, C.W., Markham, R.E. and Jr. Marder, V.J. (1984). “Demonstration of *in situ* fibrin degradation in pathologic thrombi.” *Blood*, 63, 1216-1224.

Freedman. (1992). “Oral anticoagulants: pharmacodynamics, clinical indications and adverse effects.” *J. Clin. Pharmacol.*, 32, 196-209.

Fujita, M., Hong, K., Ito, Y., Fujii, R., Kariya, K. and Nishimuro, S. (1995). “Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat.” *Biol. Pharm. Bull.*, 18(10), 1387-1391.

Fujita, M., Hong, K., Ito, Y., Misawa, S., Takeuchi, N., Kariya, K. and Nishimuro, S. (1995). "Transport of nattokinase across the rat intestinal tract." *Biol. Pharm. Bull.*, 18(9), 1194-1196.

Fujita, M., Nomura, K., Hong, K., Ito, Y., Asada, A. and Nishimuro, S. (1993). "Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan." *Biochem. Biophys. Res. Commun.*, 197(3), 1340-1347.

Gallimore, M.J. and Friberger, P. (1991). "Chromogenic Peptide Substrate Assays and their Clinical Applications." *Haemostasis and Thrombosis*, 5, 117-127

Garrett, R.H. and Grisham, C.M. (2010). Biochemistry, University of Virginia, United States, pp 103.

Gidron, E.R., Margalit, Y. and Shalitin. (1978). "A rapid screening test for reduced fibrinolytic activity of plasma: streptokinase activated lysis time." *J. Clin. Path.*, 31, 54–57.

Gillis, J.C. and Goa, K.L. (1996). "Streptokinase-A pharmacoeconomic appraisal of its use in the management of acute myocardial infarction." *PharmacoEconomics*, 10(3), 281-310.

Goldenberg, N.A., Hathaway, W.E., Manco-Johnson and M.J., Jacobson, L. (2008). "Methods for a Global Assay of Coagulation and Fibrinolysis." US Patent US20080268483 A1.

Gonsalves, W.I, Pruthi, R.K. and Patnaik, M.M. (2013). "The New Oral Anticoagulants in Clinial Practice." *Mayo Clin. Proc.*, 88, 495-511.

Gopinath, S.M. and Sneha, S.H. (2012). "SESSAMUMOIL CAKE A-NOVEL SUBSTRATE FOR PRODUCTION OF FIBRINOLYTIC ENZYMES ON SOLID STATE FERMENTATION BY *PENICILLIUM CHRYSOGENUM* AGSF16." *Adv. Pharmacol. Toxicol.*, 13(2), 13-17.

Goshev, I., Gousterova, A. and Vasileva-Tonkova, E. (2005). "Characterisation of the enzyme complexes produced by two newly isolated thermophylic actinomycete strains during growth on collagen-rich materials." *Process Biochem.*, 40(5), 1627-1631.

Han, Q., Robinson, H. and Li, J. (2012). "Biochemical identification and crystal structure of kynurenineformamidase from *Drosophila melanogaster*." *Biochem. J.*, 446, 253–260.

Hart, P.J., Balbirnie, M.M., Ogihara, N.L., Nersissian, A.M., Weiss, M.S., Valentine, J.S. and Eisenberg, D. (1999). "A structure-based mechanism for copper-zinc superoxide dismutase." *Biochemistry (Moscow)*, 38(7), 2167–2178.

Hase, C.C. and Finkelstein, R.A. (1993). "Bacterial extracellular zinc-containing metalloproteases." *Microbiol. Rev.*, 57, 823-837.

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." *J. Biosci.*, 36(5), 773-779.

Hawkey, C.M. and Stafford, J.L. (1964). "A standard clot method for the assay of plasminogen activators, anti-activators, and plasmin." *J. Clin. Path.*, 17, 175.

Hedstrom, L. (2002). "Serine Protease Mechanism and Specificity." *Chem. Rev.*, 102(12), 4501-4524.

Hermanson, G.T. (2013). "Bioconjugate Techniques" 3rd edn., Academic Press.

Holmquist, B. and Vallee, B.L. (1974). "Metal substitutions and inhibition of thermolysin: spectra of the cobalt enzyme." *J. Biol. Chem.*, 249, 4601-4607.

Hooper, N.M. (1994). "Families of zinc metalloproteases." *FEBS Lett.*, 354, 1-6.

Hooper, H.M. (1996). "Zinc metalloproteases in Health and Disease." CRC Press, London.

Howell, M. (1964). “A method for assessing clot lysis.” *J. Clin. Path*, 17, 310.

Hnizda, A., Santrucek, J., Sanda, M., Strohalm, M. and Kodicek, M. (2008). “Reactivity of histidine and lysine side-chains with diethylpyrocarbonate - A method to identify surface exposed residues in proteins.” *J. Biochem. Biophys. Methods*, 70, 1091–1097.

Hu, Y. and Guo, Z. (2009). “Purification and characterization of oxalate oxidase from wheat seedlings”. *ActaPhysiologiaePlantarum*, 31, 229–235.

Huang, S., Pan, S., Chen, G., Huang, S., Zhang, Z., Li, Y. and Liang, Z. (2013). “Biochemical characteristics of a fibrinolytic enzyme purified from a marine bacterium, *Bacillus subtilis* HQS-3.” *Int. J. Biol. Macromolec.*, 62, 124-130.

Imada, C. (2004). “Enzyme inhibitors of marine microbial origin with pharmaceutical importance.” *Mar Biotechnol*., 6, 193-198.

Iqbal, O., Tobu, M., Demir, M., Fareed, J., Aziz, S. and Messmore, H. (2002). “The role of thrombolytic drugs in the management of AMI and Stroke.” *Turk. J. Haematol.*, 19(2), 151-177.

Jackson, K.W. and Tang, J. (1982). “Complete amino acid sequence of streptokinase and its homology with serine proteases.” *Biochemistry*, 21, 6620-6625.

Jayalakshmi, T., Krishnamoorthy, P., Ramesh Babu, P.B., Vidhya, B. (2012). “Production, purification and biochemical characterization of alkaline fibrinolytic enzyme from *Bacillus subtilis* strain-GBRC1” *J. Chem. Pharm. Res.*, 4(12), 5027-5031.

Jennings, K. (1996). “Antibodies to streptokinase—once is enough.” *BMJ*, 4, 312-393.

Jensen, P. and William, F. (1994). “Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives.” *Annu. Rev. Microbiol*., 48, 559-584.

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 characterisation of the gene encoding a fibrinolytic enzyme from *Bacillus subtilis* strain A1." *World J Microbiol Biotechnol*., 20, 711-717.

Jespersen, J. and Astrup, T., (1983). "A study of the fibrin plate assay of the fibrinolytic agents. Optimal conditions, reproducibility and precision." *Haemost.*, 13, 301-315.

Jhample, S.B., Bhagwat, P.K. and Dandge, P.B. (2015). "Statistical media optimization for enhanced production of fibrinolytic enzyme from newly isolated *Proteus penneri* SP-20." *Biocatal. Agric. Biotechnol.*, 4, 370–379.

Ju, X., Cao, X., Sun, Y., Wang, Z., Cao, C., Liu, J. and Jiang, J. (2012). "Purification and characterisation of a fibrinolytic enzyme from *Streptomyces* sp. XZNUM 00004." *World J. Microbiol. Biotechnol.*, 28, 2479-2486.

Johnson, D.E. (2000). "Noncaspase proteases in apoptosis." *Leukemia*, 14(9), 1695-1703.

Jorgensen, S.B. (1983). "Practical approaches to chemical modification of enzymes for industrial use." *Biochem. Soc. Trans.*, 11, 20-21.

Joseph, K., Ghebrehiwet, B. and Kalpan, A.P. (2001). *Biol. Chem.*, 382, 71-75.

Kamiya, S., Hagimori, M., Ogasawara, M. and Arakawa, M. (2010). "*In vivo* evaluation method of the effect of nattokinase on carrageenan-induced tail thrombosis in a rat model." *Acta Haematol.*, 124, 218-224.

Kato, N., Nagasawa, T., Tani,.Y. and Ogata, K. (1972). "Protease Formation by a Marine Psychrophilic Bacterium." *Agric. Biol. Chem*., 36(7), 1177-1184.

Kim, W., Choi, K., Kim, Y., Park, H., Choi, J., Lee, Y., Oh, H., Kwon, I. and Lee, S. (1996). "Purification and characterization of a fibrinolytic enzyme produced from

Bacillus sp. strain CK 11-4 screened from Chungkook-Jang." *Appl. Environ. Microbiol.*, 62, 2482.

Kim, D.M., Lee, S.J., Kim, I.C., Kim, S.T. and Byun, S.M. (2000). "Asp41-His48 region of streptokinase is important in binding to a substrate plasminogen." *Thromb. Res.*, 99, 938.

Kim, J-S., Sapkota, K., Park, S-E., Choi, B-S., Kim, S., Hiep, N.T., Kim, C-S., Choi, H-S., Kim, M-K., Chun, H-S., Park, Y. and Kim, S-J. (2006). "A Fibrinolytic Enzyme from the Medicinal Mushroom *Cordyceps militaris*." *J. Microbiol.*, 44(6), 622-631.

Kim, H.C., Choi, B.S., Sapkota, K., Kim, S., Lee, H.J., Yoo, J.C. and Kim, S.J. (2011). "Purification and characterization of a novel, highly potent fibrinolytic enzyme from *Paecilomyces tenuipes*." *Process Biochem.*, 46, 1545–1553.

Kim, D-W., Sapkota, K., Choi, J-H., Kim, Y-S., Kim, S., Kim, S-J. (2013). "Direct acting anti-thrombotic serine protease from brown seaweed *Costaria costata*." *Process Biochem.*, 48(2), 340-350.

Ko, J.H., Yan, J.P., Zhu, L. and Qi, Y.P. (2004). "Identification of two novel fibrinolytic enzymes from *Bacillus subtilis* QK02." *Comp. Biochem. Physiol C Toxicol Pharmacol.*, 137(1), 65-74.

Kongkiattikajorn, J. and Panijpan, B. (2006). " Increased Thermostability and Activity of Chemically Modified Bromoperoxidase from the Red Alga *Gracilariatenuistipitata*.". *Science Asia*, 32 (1), 19-23.

Kotb. (2012). "Fibrinolytic Bacterial Enzymes with Thrombolytic Activity." SpringerBriefs in Microbiology, Springer-Verlag Berlin Heidelberg. isbn: 978-3-642-24979-2, 978-3-642-24980-8.

Kotb, E. (2013). "Activity assessment of microbial fibrinolytic enzymes." *Appl. Microbiol. Biotechnol.*, 97, 6647-6665.

Kotb, E. (2014). “Purification and partial characterisation of a chymotrypsin-like serine fibrinolytic enzyme from *Bacillus amyloliquefaciens* FCF-11 using corn husk as a novel substrate.” *World J. Microbiol. Biotechnol.*, 30, 2071-2080.

Kotb, E. (2015). “Purification and Partial Characterisation of Serine Fibrinolytic Enzyme from *Bacillus magaterium* KSK-07 Isolated from Kishk, a Traditional Egyptian Fermented Food.” *Appl. Biochem. Microbiol.*, 51(1), 34-43.

Kotsira, V.P. and Clonis, Y.D. (1998). “Chemical Modification of Barley Root Oxalate Oxidase Shows the Presence of a Lysine, a Carboxylate, and Disulfides, Essential for Enzyme Activity”. *Arch. Biochem. Biophys.*, 356 (2), 117–126.

Krishnamurthy, A., Belur, P.D., Rai, P. and Rekha, P.D. (2017). “Production of Fibrinolytic Enzyme by the Marine Isolate *Serratia marcescens* subsp. *sakuensis* and its *in vitro* Anticoagulant and Thrombolytic Potential.” *J. Pure Appl. Microbio.*, 11(4), 1987-1998.

Krishnamurthy, A., Belur, P.D. (2018). “A novel fibrinolytic serine metalloprotease from the marine *Serratia marcescens* subsp. *sakuensis*: Purification and characterization.” *Int. J. Biol. Macromolec.*, 112, 110-118.

Laemmli, U.K. (1970). “Cleavage of Structural Proteins during Assembly of Head of Bacteriophage- T4.” *Nature*, 227, 680–685.

Lee, H.S. (1995). “How safe is the readministration of streptokinase.” *Drug Saf.*, 13, 76-80.

LeMosy, E.K., Hong, C.C. and Hashimoto, C. (1999). “Signal transduction by a protease cascade.” *Trends Cell Biol.*, 9(3), 102-107.

Lijnen, H.R., Van Hoef, B., De Cock, F., Okada, K., Ueshima, S., Matsuo, O. and Collen, D. (1991). “On the mechanism of fibrin-specific plasminogen activation by staphylokinase.” *J. Biol. Chem.*, 266, 11826-11832.

Lip, G.F., Andreotti, L., Fauchier, L., Huber, K., Hylek, E., Knight, E., Lane, D.A., Levi, M., Marin, F., Palareti, G. and Kirchhof, P. (2011). "Bleeding risk assessment and management in atrial fibrillation patients." *Thromb. Haemost.*, 106, 997-1011.

Liu, J.G., Xing, J.M., Chang, T.S., Ma, Z.Y. and Liu, H.Z. (2005). "Optimization of nutritional conditions for nattokinase production by *Bacillus natto* NLSSE using statistical experimental methods." *Process Biochem.*, 40, 2757–2762.

Liu, L., Deng, D., Xing, Y., Li, S., Yuan, B., Chen, J. and Xia, N. (2013). "Activity analysis of the carbodiimide-mediated amine coupling reaction on self-assembled monolayers by cyclic voltammetry." *ElectrochimicaActa*, 89, 616– 622.

Liu, X.L., Zheng, X.Q., Qian, P.Z., Kopparapu, N.K., Deng, Y.P., Nonaka, M. and Harada, N. (2014). "Purification and characterization of a novel fibrinolytic enzyme from culture supernatant of *Pleurotus ostreatus*." *J. Microbiol. Biotechnol.*, 24(2), 245-53.

Liu, X., Kopparapu, N-K., Shi, X., Deng, Y., Zheng, X. and Wu, J. (2015). "Purification and Biochemical Characterisation of a Novel Fibrinolytic Enzyme from Culture Supernatant of *Cordyceps militaris*." *J. Agric. Food Chem.*, 63(8), 2215-2224.

Longstaff, C. and Thelwell, C. (2005). "Understanding the enzymology of fibrinolysis and improving thrombolytic therapy." *FEBS Letters*, 579, 3303-3309.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). "Protein measurement with the Folin phenol reagent." *J. Biol. Chem.*, 193(1), 265–275.

Lu, C-L. and Chen, S-N. (2012). "Fibrinolytic Enzymes from Medicinal Mushrooms In: Eshel, F. (eds) Protein Structure." *InTech*, Croatia, pp. 396.

Lu, F., Lu, Z., Bie, X., Yao, Z., Wang, Y., Lu, Y. and Guo, Y. (2010). "Purification and characterisation of a novel anticoagulant and fibrinolytic enzyme produced by endophytic bacterium *Paenibacillus polymyxa* EJS-3." *Thromb. Res.*, 126, 349-355.

Lundblad, R.L. (1995). “Techniques in protein modification In: Chemical modification of Tryptophan.” CRC Press LLC, Florida, pp 187-205.

Mahajan, P.M., Gokhale, S.V. and Lele, S.S. (2010). “Production of nattokinase using *Bacillus natto* NRRL 3666: media optimisation, scale up and kinetic modelling.” *Food. Sci. Biotechnol*., 19, 1593-1603.

Mahajan, P., Nayak, S. and Lele, S. (2012). “Fibrinolytic enzyme from newly isolated marine bacterium *Bacillus subtilis* ICTF-1: Media optimisation, purification and characterisation.” *J Biosci Bioeng*., 113(3), 307-314.

Majumdar, S., Sarmah, B., Gogoi, D., Banerjee, S., Ghosh, S.S., Banerjee, S., Chattopadhyay, P. and Mukherjee, A.K. (2014). “Characterisation, mechanism of anticoagulant action, and assessment of therapeutic potential of a fibrinolytic serine protease (Brevithrombolase) purified from *Brevibacillus brevis* strain FF02B.” *Biochimie*, 103, 50-60.

Malke, H., Ferretti, J.J. (1984). “Streptokinase: cloning, expression and excretion by *Escherichia coli*.” *Proc. Natl. Acad. Sci. USA*, 81, 3557-3561.

Mander, P., Cho, S.S., Simkhada, J.R., Choi, Y.H. and Yoo, J.C. (2011). “A low molecular weight chymotrypsin-like novel fibrinolytic enzyme from *Streptomyces* sp. CS624.” *Process Biochem.*, 46, 1449–1455.

Marieb, E.N. and Hoehn, K. (2010). “Human anatomy and physiology” 8th edn., Benjamin Cummings, San Francisco.

Markland, F.S. (1998). “Snake venoms and the hemostatic system.” *Toxicon*, 36, 1749–1800.

Marsh, N.A. and Gaffney, N.J. (1977). “The rapid fibrin plate—a method for plasminogen activator assay.” *Thromb. Haemostat.*, 38, 545–551.

Matsubara, K., Sumi, H., Hori, K. and Miyazawa, K. (1998). “Purification and characterization of two fibrinolytic enzymes from a marine green alga, *Codium intricatum*.” *Comp. Biochem. Physiol. Biochem. Mol. Biol.*, 119, 177–181.

Matsubara, K., Hori, K., Matsuura, Y. and Miyazawa, K. (2000). “Purification and characterization of a fibrinolytic enzyme and identification of fibrinogen clotting enzyme in a marine green alga, *Codium divaricatum*.” *Comp. Biochem. Physiol. Biochem. Mol. Biol.*, 125(1), 137–143.

Matsuo, O., Okada, K., Fukao, H., Tomioka, Y., Ueshima, S., Watanuki M. and Sakai, M. (1990). “Thrombolytic properties of staphylokinase.” *Blood*, 76, 925-929

Miesbach, W. and Seifried, E. (2012). “New direct oral anticoagulants-current therapeutic options and treatment recommendations for bleeding complications.” *Thromb. Haemost.*, 108, 625-632.

Millar, W.T. and Smith, J.F. (1983). “The comparison of solid phase and fibrin plate methods for the measurement of plasminogen activators.” *Thromb. Res.*, 30, 431–439.

Milner, M. and Makise, K. (2004). “Natto and Its Active Ingredient Nattokinase: A Potent and safe Thrombolytic Agent.” *Alternative and Complementary Therapies.*, 8(3), 157-164.

Mohankumar, A. and Krishna Raj, R.H. (2011). “Production and Characterization of Serratiopeptidase Enzyme from *Serratia marcescens*.” *Int. J. Biol.*, 3, 39–51.

Moon, S.J., Kim, H., Kim, M.S., Choi, B.R., Park, S., Kim, H., Ahn, H.S., Chun, Y.K., Shin, J., Kim, D.K., Kim, S., Lee, Y., Seo, Y.H., Kim, C.S., Kim. (2014). “Purification and characterization of a novel fibrinolytic a chymotrypsin like serine metalloprotease from the edible mushroom *Lyophyllum shimeji*.” *J. Biosci. Bioeng.*, 117, 544–550.

Mosesson, M.W. (2005). “Fibrinogen and fibrin structure and functions.” *J. Thromb. Haemost.*, 3, 1894–1904.

Mucklow, J.C. (1995). “Thrombolytic treatment-streptokinase is more economical than Alteplase.” *BMJ*, 311-1506.

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.

Nagase, H. (2001). “Current Protocols in Protein Science”, Chapter 21, John Wiley & Sons.

Narasimhan, M.K, Chandrasekaran, M. and Rajesh, M. (2015). “Fibrinolytic enzyme production by newly isolated *Bacillus cereus* SRM-001 with enhanced *in-vitro* clot lysis potential.” *J. Gen. Appl. Microbiol.*, 61, 157-164.

Naveena, B., Sakthiselvan, P., Elaiyaraju, P. and Partha, N. (2012). “Ultrasound induced production of thrombinase by marine actinomycetes: Kinetic and optimisation studies.” *Biochem. Eng. J.*, 61, 34-42.

Nelson, D.L and Cox, M.M. (2005). “Lehninger principles of biochemistry.” 4rth edn., W.H. Freeman and Company, New York.

Neurath, H. (1984). “Evolution of proteolytic enzymes.” *Science*, 224(4647), 350-357.

Nguyen, G. and Samama, M. (1987). “New thrombolytic agent.” *Ann. Biol. Clin. (Paris)*, 45(2), 212-218.

Ogino, H. and Ishikawa, H. (2001). “Enzymes Which Are Stable in the Presence of Organic Solvents”. *J. Biosci. Bioeng.*, 91(2), 109-116.

Ohkuro, I., Komatsuzuki, T., Kuriyama, S. and Kawashima, M. (1981). “The level of serum lysozyme activity in animals fed on a diet containing natto *Bacilli* [in Japanese].” *Med. Bio.*, 102, 335-337.

Okada, K., Ueshima, S., Fukao, H. and Matsuo, O. (2001). "Analysis of complex formation between plasminogen and staphylokinase or streptokinase." *Arch. Biochem. Biophys.*, 393, 339-341.

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." *J. Pharmacol. Sci.*, 99(3), 247-251.

Ouriel, K. (2002). Safety and efficacy of various thrombolytic agent. http://www.medreviews.com/pdfs/articles/RICM_3suppl2_s17.pdf.

Paoletti, R. and Sherry, S. (1977). "Proceedings of the serono symposia. Thrombosis and Urokinase." vol. 9, Academic Press, New York, pp. 257.

Park, J.W., Park, J.E., Choi, H.K., Jung, T.W., Yoon, S.M. and Lee, J.S. (2013). "Purification and characterisation of three thermostable alkaline fibrinolytic serine proteases from the polychaete *Cirriformia tentaculata*." *Process Biochem.*, 48, 979-987.

Peng, S.M. and Zhao. (2009). "Pharmaceutical Bioassays: Methods and Applications, Methods and Applications of Thrombus-Related Assays." Wiley, New Jersey, pp. 59.

Peng, Y., Zhang, Y.Z. (2002). "Optimization of fermentation conditions of douchi fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4." *Chin. Food Ferment. Ind.*, 28, 19–23

Peng, Y., Huang, Q., Zhang, R.H. and Zhang, Y.Z. (2003). "Purification and characterisation of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douche, a traditional Chinese soyabean food." *Comp. Biochem. Physiol. Biochem. Mol. Biol*., 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*." *Res. Microbiol.*, 155(3), 167–173.

Peng, Y.X., Yang, Y. and Zhang. (2005). "Microbial fibrinolytic enzymes: An overview of source, production, properties, and thrombolytic activity *in vivo*." *Appl. Microbiol. Biotechnol.*, 69, 126-132.

Porrett, P.M., Drebin, J.A., Atluri, P., Karakousis, M.D. and Roses, R.E. (2005). "The surgical review and subtitle: An Integrated Basic and Clinical Science Study Guide." Williams & Wilkins, Philadelphia.

Prasad, S., Kashyap, R.S., Deopujari, J.Y., Purohit, H.J., Taori, G.M. and Daginawala, H.F. (2006). "Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs." *Thromb. J.*, 4, 14

Pritsa, A.A. and Kyriakidis, D.A. (2001). "L-asparaginase of *Thermus thermophilus*: Purification, properties and identification of essential amino acids for its catalytic activity." *Mol. Cell. Biochem.*, 216, 93-101.

Raafat, A.I., Araby, E. and Lotfy, S. (2012). "Enhancement of fibrinolytic enzyme production from *Bacillus subtilis* via immobilisation process onto radiation synthesised starch/dimethylaminoethyl methacrylate hydrogel." *Carbohyd Polym*., 87, 1369-1374.

Rau, J.C., Beaulieu, L.M., Huntington, J.A. and Church, F.C. (2007). "Serpins in thrombosis, hemostasis and fibrinolysis." *J. Thromb. Haemost.*, 5(1), 102-115.

Rawlings, N.D., Barrett, A.J. and Bateman, A. (2010). "MEROPS: the peptidase database." *Nucleic Acids Res*., 38, 227-233.

Reed, G.L., Liu, L., Houng, A.K., Matsueda, L.H. and Lizbeth, H. (1998). "Mechanisms of fibrin independent and fibrin dependent plasminogen activation by streptokinase." *Circulation*, 98, 199.

Robinson, B.R., Liu, L., Houng, A.K., Sazanova, I.Y. and Reed, G.L. (2000). "The streptokinase beta domain plays a critical role in activator complex formation and substrate docking." *Circulation*, 102, 490.

Rho, J.P. and Louie, S.G. (2004). “Hand Book of Pharmaceutical Biotechnology.” Chapter 4, CRC Press, New York, pp 65-78.

Roch, P. (1979). “Protein analysis of earthworm coelomic fluid: I. Polymorphic system of the natural hemolysin of *Eisenia fetida andrei*.” *Devel. Comp. Immun.*, 3, 599–608.

Rodrı´guez, A.D. and Davis, B.G. (2011). “Chemical modification in the creation of novel biocatalysts”. *Curr. Opin Chem. Biol.*, 15, 211–219.

Ross, A.M. (1999). “New plasminogen activators – a clinical review.” *Clin. Cardiol.*, 22, 165-171.

Rouf, S.A., Moo-Young, M. and Chisti, Y. (1996). “Tissue-type plasminogen activator: characteristics, applications and production technology.” *Biotechnol. Adv.*, 14, 239-266.

Rudyk, O. and Eaton, P. (2014). “Biochemical methods for monitoring protein thiol redox states in biological systems.” *Redox Biol.*, 2, 803–813.

Sako, T. (1985). “Overproduction of staphylokinase in *Escherichia coli* and its characterization.” *Eur. J. Biochem.*, 149, 557-563.

Sane, D.C. and Little, W.C. (1998). “Is time running out on streptokinase?” *J. Am. Coll. Cardiol.*, 31, 780-782

Satish, A., Sairam, S., Ahmed, F. and Urooj, A. (2012). “Moringa oleifera Lam.: Protease activity against blood coagulation cascade.” *Pharmacognosy Res.*, 44, 9.

Satoshkar, R.S., Bhandarkar, S.D. and Ainapure, S.S. (1998). “Pharmacology and Therapeutics.” Popular prakashan press, Mumbai, India.

Schlott, B., Hartmann, M., Guhrs, K.H., Birchhirschfeid, E., Pohl, H.D. and Vanderschueren, S. (1994). “High yield production and purification of recombinant staphylokinase for thrombolytic therapy.” *Biotechnology*, 12, 185-189.

Selvarajan, S., Lund, L.R., Takeuch, T., Craik, C.S. and Werb, Z. (2001). "A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation." *Nat. Cell Biol.*, 3, 267-275.

Seo, J.H. and Lee, S.P. (2004). "Production of fibrinolytic enzyme from soybean grits fermented by *Bacillus firmus* NA-1." *J. Med. Food*, 7(4), 442–449.

Sharkova, T.S., Kuraov, A.V., Osmolovskiy, A.A., Matveeva, E.O., Kreyer, V.G., Baranova, N.A. and Egorov, N.S. (2015). "Screening of Producers of Proteinases with Fibrinolytic and Collagenolytic Activities among Micromycetes." *Microbiology*, 84(3), 359-364.

Shivaprasad, H.V., Riyaz, M., Kumar, R.V., Dharmappa, K.K., Tarannum, S., Siddesha, J.M., Rajesh, R. and Vishwanath, B.S. (2009). "Cysteine proteases from the Asclepiadaceae plant latex exhibited thrombin and plasmin like activities." *J. Thromb. Thrombolysis*, 28, 304–308.

Sim, R.B. and Laich, A. (2000). "Serine proteases of the complement system." *Biochem. Soc. Trans.*, 28, 545-550.

Simkhada, J.R., Mander, P., Cho, S.S. and Yoo, J.C. (2010). "A novel fibrinolytic protease from *Streptomyces* sp. CS684." *Process Biochem.*, 45, 88-93.

Simkhada, J.R., Cho, S.S., Mander, P., Choi, Y.H. and Yoo, J.C. (2012). "Purification, biochemical properties and antithrombotic effect of a novel *Streptomyces* enzyme on carrageenan-induced mice tail thrombosis model." *Thromb. Res.*, 129, 176-182.

Solingen, P., Dean, V.M., Wilhelmus, A.H.K., Christopher, B., Robertus, B., Scott, D.P. and Brian, E.J. (2001). "A novel *Streptomyces* isolated from an East African Soda Lake." *Extremophiles*, 5, 333-341.

Strassburger, W., Wollmer, A., Pitts, J.E., Glover, I.D., Tickle, I.J., Blundell, T.L., Steffens, G.J., Günzler, W.A., Otting, F. and Flohé, L. (1983). "Adaptation of

plasminogen activator sequences to known protease structures." *FEBS Lett.*, 157, 219-223.

Sundram, V., Nanda, J.S., Rajagopal, K., Dhar, J., Chaudhary, A. and Sahni, G. (2003). "Domain truncation studies reveal that the streptokinase-plasmin activator complex utilises long range protein-protein interactions with macromolecular substrate to maximise catalytic turnover." *J. Biol. Chem.*, 278, 30569-305677.

Tainer, J.A., Getzoff, E.D., Richardson, J.S. and Richardson, D.C. (1983). "Structure and mechanism of copper, zinc superoxide dismutase." *Nature*, 306(5940), 284–287.

Tao, S., Peng, L., Beihui, L., Deming, L. and Zuohu, L. (1997). "Solid state fermentation of rice chaff for fibrinolytic enzyme production by *Fusarium oxysporum*." *Biotechnol. Lett.*, 19(5), 465–467.

Tao, S., Peng, L., Beihui, L., Deming, L. and Zuohu, L. (1998). "Successive cultivation of *Fusarium oxysporum* on rice chaff for economic production of fibrinolytic enzyme." *Bioprocess Eng.*, 18(5), 379–381.

Tharwat, N.A. (2006). "Purification and Biochemical Characterisation of Fibrinolytic Enzyme Produced by Thermophilic Fungus *Oidiodendron flavum*." *Biotechnology*, 5(2), 160-165.

Turpie A.G.G, Chin, B.S.P. and Lip, G.Y.H. (2002). "Venous thromboembolism: pathophysiology, clinical features, and prevention." *BMJ*, 325, 887-890.

Uchikoba, T., Okubo, M., Arima, K. and Yonezawa, H. (2002). " Isolation and Characterization of a Cysteine Protease of Freesia Corms". *Biosci. Biotechnol. Biochem.*, 66 (2), 448-452.

Uesugi, Y., Usuki, H., Iwabuchi, M. and Hatanaka, T. (2011). "Highly potent fibrinolytic serine protease from *Streptomyces*." *Enzyme Microb. Technol.*, 48, 7–12.

Vallee, B.L. and Auld, D.S. (1990). "Zinc coordination, function, and structure of zinc enzymes and other proteins." *Biochemistry*, 29 5647-5659.

Van den Steen, P.E., Opdenakker, G., Wormald, M.R., Dwek, R.A. and Rudd, P.M. (2001). "Matrix remodelling enzymes, the protease cascade and glycosylation." *Biochim. Biophys. Acta.*, 1528(2-3), 61-73.

Verma, M.K. and Pulicherla, K.K. (2017). "Broad substrate affinity and catalytic diversity of fibrinolytic enzyme from *Pheretima posthumous*-Purification and molecular characterisation study." *Int. J. Biol. Macromolec.*, 95, 1011-1021.

Verstraete, M. (2000). "Third-generation thrombolytic drugs." *Am. J. Med.*,. 109, 52–58.

Vijayaraghavan, P. and Vincent, S.G.P. (2014). "Statistical optimisation of fibrinolytic enzyme production by *Pseudoalteromonas* sp. IND11 using cow dung substrate by response surface methodology." *SpringerPlus*, 3, 60.

Vijayaraghavan, P. and Vincent, S.G.P. (2015). "A low cost fermentation medium for potential fibrinolytic enzyme production by a newly isolated marine bacterium, *Shewanella* sp. IND20." *Biotechnology Reports*, 7, 135-142.

Vijayaraghavan, P. and Vincent, S.G.P., Arasu, M.V. and Al-Dhabi, N.A. (2016). "Bioconversion of agro-industrial wastes for the production of fibrinolytic enzyme from *Bacillus halodurans* IND18: Purification and biochemical characterisation." *Electron. J. Biotechnol.*, 20, 1-8.

Wakeham, N., Terzyan, S., Zhai, P.Z., Loy, J.A., Tang, J. and Zhang, X.C. (2002). "Effects of deletion of streptokinase residues 48-59 on plasminogen activation." *Protein Eng.*, 15, 753-761

Wang, J., Wang, M. and Wang, Y. (1999). "Purification and characterisation of a novel fibrinolytic enzyme from *Streptomyces* spp." *Chin. J. Biotechnol.*, 15(2), 83-89.

Wang, C.T., Ji, B.P., Nout, B.L.R., Li, P.L. and Chen, L.F. (2006). "Purification and characterisation of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional Douchi." *J. Ind. Microbiol. Biotechnol*., 33, 750-758.

Wang, S., Chen, H., Liang, T. and Lin, Y. (2009). "A novel nattokinase produced by *Pseudomonas* sp. TKU015 using shrimp shells as substrate." *Process Biochem.*, 44, 70-76.

Weisel, J.W. (2007). "Structure of fibrin: impact on clot stability". *J. Thromb. Haemost.*, 5 (1), 116–124.

Wen, L., Miao, Z.W. and Qing, W.D. (1999). "Chemical Modification of Xylanase from *Trichosporon cutaneum* shows the Presence of Carboxyl Groups and Cysteine Residues Essential for Enzyme Activity." *J. Protein Chem.*, 18(6), 677-686.

Werf, F.V.D. (1999). "Streptokinase…never again." *Eur. Heart. J.*, 20, 1215.

Wong, S.L. (1995). "Advances in the use of *Bacillus subtilis* for the expression and secretion of heterologous proteins." *Curr. Opin. Biotechnol.*, 6, 517–522.

Wu, X.C., Ye, R.Q., Duan, Y.J. and Wong, S-L. (1998). "Engineering of plasmin-resistant forms of streptokinase and their production in *Bacillus subtilis*: streptokinase with longer functional half-life." *Appl. Environ. Microbiol.*, 64, 824-829

Wu, D.H., Shi, G.Y., Chuang, W.J., Hsu, J.M., Young, K.C., Chang, C.W. and Wu, H.L. (2001). "Coiled coil region of streptokinase gamma-domain is essential for plasminogen activation." *J. Biol. Chem.*, 276, 15025-15033.

Wu, B., Wu, L., Chen, D., Yang, Z. and Luo, M. (2009). "Purification and characterisation of a novel fibrinolytic protease from *Fusarium* sp. CPCC 480097." *J. Ind. Microbiol. Biotechnol*., 36, 451-459.

Wu, S-M., Feng, C., Zhong, J. and Huan, L-D. (2010). "Enhanced production of recombinant nattokinase in *Bacillus subtilis* by promoter optimisation." *World J. Microbiol. Biotechnol*., 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*." *Biotechnol. Lett.*, 26, 1365–1369.

Xiao-Lan, L., Lian-Xiang, D., Fu-Ping, L., Xi-Qun, Z. and Jing, X. (2005). "Purification and characterization of a novel fibrinolytic enzyme from *Rhizopus chinensis* 12." *Appl. Microbiol. Biotechnol.*, 67(2), 209-214.

Yeo, W.S., Seo, M.J., Kim, M.J., Lee, H.H., Kang, B.W., Park, J.U., Choi, Y.H. and Jeong, Y.K. (2011). "Biochemical analysis of a fibrinolytic enzyme purified from *Bacillus subtilis* strain A1." *J. Microbiol.*, 49(3), 376-380.

Yogesh, D. and Halami, P.M. (2015). "A fibrin degrading serine metallo protease of *Bacillus circulans* with α-chain specificity." *Food Biosci.*, 11, 72-78.

Yuan, J., Yang, J., Zhuang, Z., Yang, Y., Lin, L. and Wang, S. (2012). "Thrombolytic effects of Douchi Fibrinolytic enzyme from *Bacillus subtilis* LD-8547 *in vitro* and *in vivo*." *BMC Biotechnol.*, 12, 1-9.

Zdanowicz, M.M. (2002). "Essentials of pathophysiology for pharmacy." CRC Press, Florida.

Zhai, P., Wakeham, N., Loy, J.A., Zhang, X.C. (2003). "Functional roles of streptokinase C-terminal flexible peptide in active site formation and substrate recognition in plasminogen activation." *Biochemistry*, 42, 114–20.

Zhang, L., An, R., Wang, J., Sun, N., Zhang, S., Hu, J. and Kuai, J. (2005). "Exploring novel bioactive compounds from marine microbes." *Curr Opinion Microbiol*., 8, 276-281.

Zhou, Y. (2014). "Structural analysis of proteins by covalentlabeling and mass spectrometric detection".Ph.D. thesis, University of Massachusetts Amherst, Amherst, MA, USA.

LIST OF PUBLICATIONS BASED ON THE CURRENT WORK

JOURNAL PUBLICATIONS

- Krishnamurthy, A., Belur, P.D., Rai, P. and Rekha, P.D. (2017). "Production of Fibrinolytic Enzyme by the Marine Isolate *Serratia marcescens* subsp. *sakuensis* and its *in vitro* Anticoagulant and Thrombolytic Potential." *J. Pure Appl. Microbio.*, 11(4), 1987-1998.
- Krishnamurthy, A., Belur, P.D. (2018). "A novel fibrinolytic serine metalloprotease from the marine *Serratia marcescens* subsp. *sakuensis*: Purification and characterisation." *Int. J. Biol. Macromolec.*, 112, 110-118.

PAPER ACCEPTED

- Krishnamurthy, A., Belur, P.D. and Subramanya, S.B. (2018). "Methods available to assess therapeutic potential of fibrinolytic enzymes of microbial origin: A review." *Journal of Analytical Science and Technology.*

PAPER UNDER REVIEW

- Krishnamurthy, A., Mundra, S., Belur, P.D. (2018). "Improving the catalytic efficiency of fibrinolytic enzyme from *Serratia marcescens* subsp. *sakuensis* by chemical modification." *Process Biochem.*

CONFERENCE PUBLICATIONS

- Krishnamurthy, A. and Prasanna, B.D. (2017). "Studies on fibrinolytic enzyme from marine *Serratia marcescens* subsp. *sakuensis* (KU296189.1)." International Conference on Advances in Biotechnology and Biotherapeutics (ICABBS -2017) Sathyabama University, Chennai, Paper No. 5033R, March 08-10. (Published as journal article in *Int. J. Appl. Bioeng*.)
- Krishnamurthy, A. and Prasanna, B.D. (2015). "Marine microorganisms -A Source for the Production of Fibrinolytic Enzymes." National Conference on Recent Trends in Microbial Biotechnology (RTMB)-2015 Hyderabad, Telangana, India, Paper No. BEET-033, February 26-28. (Won the "Best Poster presentation" award).

❖ Krishnamurthy, A. and Prasanna, B.D. (2014). “Production of Fibrinolytic Enzyme from Marine Microorganisms.” 55th Annual Conference. National Conference on Empowering Mankind with Microbial Technologies (AMI-EMMT) Coimbatore, India, Paper No. MD-08, November12-14.

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- 2012-13: A project on "Extraction and Purification of Industrially Important Enzyme from Fruit Waste and Clinical Wastes by Submerged Fermentation" at Mount Carmel College, Bangalore, Karnataka, India.
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- 2011-12: A project on "Assessment of mitogenecity of sugar binding lectins from *Eudrilus eugineae* on invertebrate and vertebrate cells and Characterization of sugar binding lectins isolated from different parts of *Eudrilus eugineae*" at Mount Carmel College, Bangalore, Karnataka, India.
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