Studies on fibrinolytic agent(s) from endophytic fungi

A

thesis submitted

in the partial fulfilment of the requirements for the award of degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

Submitted By

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(Reg. No. 900900010)

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June 2016

CERTIFICATE

Certified that the thesis entitled "Studies on fibrinolytic agent(s) from endophytic fungi" submitted by Mr. Vineet Meshram, Reg. no. 900900010 in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar University, Patiala, Punjab is a record of candidate's own independent and original research work carried out by himself under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "Studies on fibrinolytic agent(s) from endophytic fungi" in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy at Department of Biotechnology, Thapar University, Patiala is an authentic record of my own work during the period from January 2010 to June 2016, under the supervision of Prof. Sanjai Saxena, Professor, Department of Biotechnology, Thapar University. This report has not been submitted for the award of any degree or certificate in this or any other university.

Place: Patiala, Punjab

Date: Aug 10, 2016

Vineet Meshram

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ACKNOWLEDGMENT

In pursuit of this academic endeavour, I feel that I have been singularly fortunate because inspiration, guidance, direction, cooperation, love and care- all came in my way in abundance and it seems almost an impossible task for me to acknowledge the same in adequate term.

With profound sense of indebtedness and heartfelt gratitude, I thank **Prof. Sanjai Saxena**, Professor, Department of Biotechnology (DBT), Thapar University (TU), Patiala, Punjab (Pb) who guided and inspired me in pursuance of this work. His association with this endeavour of mine will remain a beacon light to me throughout my life. I sincerely thank him for making me a better person, guiding me and improving silliest of my mistakes. His presence was always there whenever I needed him whether it was professional or personal life.

My sincere gratitude to visionary director of TU, **Prof. Dr. Prakash Gopalan**, for providing advanced infrastructure and excellent research environment. I thank **Prof. Maneek Kumar**, Dean, Student affairs and **Prof. Ajay Batish**, Dean, Partnership and accreditations, TU, Patiala for their precious guidance and constant motivation.

Constructive suggestions and critical remarks of **Prof. Dinesh Goyal**, Head, DBT, **Prof. N. Tejo Prakash**, Head, School of energy and Environment, **Dr. Manmohan Chibber,** Associate professor, School of Chemistry and Biochemistry and **Dr. Manoj Baranwal**, Assistant Professor, DBT, TU, towards amelioration of this work has been of immense help in successfully achieving the objectives. I am highly obliged to them for their kind support in carrying out present research work.

A deep sense of gratitude to **Dr. Kamlesh Shukla**, Assistant Professor, SoS in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh who has been a source of inspiration for me. It's because of you sir that I have chosen research as a carrier option. I sincerely thank you for showing me the path. A special thanks to **Dr. Siddhardth Sharma**, Assistant Professor, DBT, TU, Dr. **Anita Rajor** and **Anoop Verma** for their suggestions and constant encouragement.

I am thankful to Prof. **Girish Sahni**, Director, CSIR for providing me with an opportunity to work in his lab at Institute of Microbial Technology, Chandigarh. I am thankful to **Mrs. Paramjit Kaur** and **Ms. Neha Rana**, Technical officer and **Ms. Navneet Kaur**, Research Scholar, IMTECH, Chandigarh for their kind cooperation in N-terminal sequencing and MALDI-TOF analysis.

I am highly indebt to **Dr. Avneet Pal Singh**, Asst. Professor, Department of Botany, Punjabi University, Patiala, Pb, **Dr. Riyaz Ul Hasan**, Scientist, IIIM, Jammu and Kashmir, **Dr. Dilip Hande**, Asst. Professor, Shri Shivaji Science College, Amravati and **Dr. Rajesh Kumar**, Scientist, NFCCI, Agharkar Research Institute, Pune, MS for their valuable suggestions on various aspects of mycology.

I am thankful to **University Grants Commission**, Government of India, New Delhi for providing me financial help through Rajiv Gandhi National Fellowship (F.16–1866(SC)/2010/SA–III). I

also thank **Department of Biotechnology**, Ministry of Science and Technology, Government of India for providing me fellowship under the sponsored project BT/PR/10083/NDB/52/95/2007. I appreciate the financial help received from **Department of Science and Technology**, Govt. of India for attending 17th Congress of European Mycologist at Madeira, Portugal. I also thank **Tel Aviv University**, Israel for granting VTAT fellowship to attend summer institute program.

I owe my heartfelt thank to **Dr. Karan Paul**, Assistant Professor, DAV University, Jalandhar, Pb for his kind guidance and cooperation in protein purification. I also thank him for a wonderful friendship cum brotherhood which I will cherish for lifetime. A special thanks to **Mr. Ram Naval**, **Mr. Surinder Pal** and other lab staff members of DBT, TU without whom's help many experiments would not be have been possible.

I am especially indebted to my seniors **Mr. Birinderjit singh**, **Dr. Charu Mutreja**, and **Gunjan Chopra** and for their constant love, cooperation and help. My special thanks to **Dr. Mahiti Gupta** and **Ms. Neha Kapoor**, Ph.D scholars for inspiring discussions while designing and planning experimental protocols and for providing a nice atmosphere in the lab. I thank them for supporting me in thick and thin phases of my life.

I acknowledge love, care, support and cooperation of my friends Vagish Dwibedi, Dr. Sumit Jaiswal, Dr. Lokesh Gambhir, Dr. Ravi Shukla, Dr. Nadeem Akhtar, Dr. Sukhdeep Kumar, Arkadeep Mukarjee, Rachan Karmakar, Rahul Rana, Anirudh Sharma, Labya Prabhas, Tikendra Verma, Megha Mishra, Late Shipra Mishra, Supriya Sachdeva, Anugya Shrivastava, Shruti Khare, Dr. Shelly Gupta, Noorpreet Dhanjal. I thank them all for wonderful moments and great company.

I sincerely thank Amma and Baba (**Shri Devendra Kumar Saxena** and **Smt. Savita Saxena**) and **Mrs. Merry Saxena** for their love, warmth and care. Discussion with Baba helped me in clearing the fundamental concepts about the subject. Love to my sister **Mahek.** I thank them all for making me feel like home.

I would like to thank my uncles **Mr. K.R. Rangari** & **Mr. Sanjay Meshram** without whom's motivation, co-operation and moral support I would never had completed this work.

Above all, it's my parents and sister who encouraged me to complete my Ph.D besides all odds and troughs of life. I owe my personal reckoning of gratitude and benevolence to them for their endless love, care, patience, inspiration, encouragement, motivation and constant driving force which has enabled me to complete my work.

Thanks to almighty for bestowing blessings on me.

Date: Aug 10, 2016

Place: Patiala **VINEET MESHRAM**

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S. no	Symbol	
1.	%	percentage
2.	′	minutes
3.	≥	equals to or greater than
4.	°	degree
5.	°C	degree celsius
6.	µg	microgram
7.	µl	microliter
8.	µm	micrometer
9.	cm	centimeter
10.	g	gram
11.	h	hour
12.	kDa	kilo Dalton
13.	K_m	Michaelis constant
14.	l	litre
15.	M	molar
16.	mA	milliampere
17.	mg	milligram
18.	min	minute
19.	ml	milliliter
20.	mm	millimeter
21.	mM	millimolar
22.	mPa	megapascel
23.	nm	nanometer
24.	psi	pounds per square inch
25.	rpm	revolutions per minute
26.	s	second
27.	U	unit (activity)
28.	v	volume
29.	v/v	volume by volume
30.	V_{max}	maximum reaction velocity
31.	w/v	weight by volume
32.	µg /ml	microgram per milligram
33.	µM	micromolar
34.	U/mg	units per milligram (Specific activity)
35.	U/ml	units per milliliter
36.	ng/µl	nanograms per microliter
37.	bp	base pair
38.	α	alpha
39.	β	beta
40.	γ	gamma
41.	±	plus minus
42.	MJ/Kg	millijoule per kiligram
43.	~	approximately
44.	mm^2	millimeter square
45.	mAU	milli Absorbance Unit

List of abbreviations

S. no	Abbreviations	Full form
1.	APSAC	Acylated Plasminogen–Streptokinase Activator Complex
2.	APTT	Activated Prothrombin Time
3.	BLAST	Basic Local Alignment Search Tool
4.	BSA	Bovine Serum Albumin
5.	CDB	Czapek Dox Broth
6.	CHN	Carbon Hydrogen Nitrogen
7.	CHO	Chinese Hamster Ovary
8.	CLA	Cathranthus Leaf Agar
9.	CMA	Corn Meal Agar
10.	CPA	Cathranthus Paste Agar
11.	CVDs	Cardiovascular disorders
12.	dNTP	Deoxy nucleotide triphosphate
13.	DVT	Deep Vein Thrombosis
14.	EC	Enzyme Commission
15.	EDTA	Ethylenediaminetetraacetic acid,
16.	EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
17.	HPLC	High Pressure Liquid Chromatography
18.	ITS	Internal Transcribed Spacer
19.	LSU	Large ribosomal subunit
20.	MALDI–ToF MS	Matrix–assisted Laser desorption/ionization–time of flight mass spectrometry
21.	MCL	Maximum Composite Likelihood
22.	MEA	Malt Extract Agar
23.	MEB	Malt Extract Broth
24.	MI	Myocardial Infarction
25.	MPLC	Medium Pressure Liquid Chromatography
26.	MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
27.	NIH	National Institute of Health, USA
28.	OD	Optical Density
29.	OMA	Oat Meal Agar
30.	PA	Plasminogen Activator
31.	PDA	Potato Dextrose Agar
32.	PDB	Potato Dextrose Broth
33.	PMSF	phenylmethane sulfonyl fluoride
34.	PT	Pro–thrombin time
35.	RB	Richard's Broth
36.	SDS–PAGE	Sodium dodecyl sulphate–Polyacrylamide gel electrophoresis
37.	SmF	Submerged Fermentation
38.	SNA	Synthetischer nahourstoffarmer agar
39.	SSF	Solid State Fermentation
40.	TAE	Tris acetate EDTA
41.	TCA	Trichloro acetic acid

42.	t–PA	Tissue Plasminogen Activator
43.	TPCK	Tosyl phenylalanyl chloromethyl ketone
44.	Tris	Tris(hydroxymethyl)aminomethane
45.	TSB	Tryptone Soya Broth
46.	TT	Thrombin Time
47.	U–PA	Urokinase Plasminogen Activator
48.	USFDA	United States Food and Drug Administrations
49.	VOCs	Volatile Organic Compounds
50.	WHO	World Health Organization
51.	WP	Wood Pieces
52.	YEPB	Yeast Extract Peptone Dextrose Broth
53.	YEPDA	Yeast Extract Peptone Dextrose Agar

Executive Summary

The present study was oriented towards exploration of potential endophytic fungi isolated from conserved rain forests of India for their fibrinolytic activity. Of the 211 isolates of endophytic fungi screened, culture filtrates of 67 isolates exhibited *in vitro* proteolytic activity while only 37 isolates displayed *in vitro* fibrinolytic activity. However, the direct *in vitro* fibrinolytic activity was only found in culture filtrates of six isolates. The endophytic fungal isolate #37 CRSTBRT exhibited maximum *in vitro* proteolytic as well as *in vitro* fibrinolytic activity. Using morphological as well as molecular taxonomic studies, #37 CRSTBRT was identified as *Xylaria curta*. The fibrinolytic moiety expressed in the culture filtrate of *Xylaria curta* was isolated using chromatographic techniques. Further, its purity, molecular mass and enzymatic activity was ascertained by using electrophoretic techniques, HPLC and MALDI–ToF MS analysis.

Xylarinase is a non–toxic, bi–functional monomeric fibrinolytic metalloprotease with a molecular mass of 33.76 kDa. The enzyme displayed both plasmin like and plasminogen activator like activity under *in vitro* conditions. It hydrolyses both Aα and Bβ chains of fibrin(ogen) displaying αβ fibrinogenase activity. Optimal fibrinolytic activity of xylarinase was observed at 35 °C, pH 8. EDTA and EGTA were the potent inhibitors of xylarinase suggesting that the enzyme was a metalloprotease having calcium dependence. However, Fe^{2+} and Zn^{2+} strongly inhibited the *in vitro* fibrinolytic activity of xylarinase. The K_m and V_{max} of xylarinase was 246 µM and 1.22 µM/min. The N-terminal sequence of xylarinase (SNGPLPGGVVWAG) did not show any homology with previously known fibrinolytic enzymes. Further, xylarinase was found to prolong the APTT and PT. Xylarinase exhibited no cytotoxic effect over RAW 264.7 murine macrophage cell.

The production yield of xylarinase was enhanced (8.5 % from 0.09 %) by producing it on rice chaff using solid state fermentation. The purified enzyme exhibited similar biochemical properties as that of xylarinase. The present study thus establishes endophytic fungi as a potential bio–resource of fibrinolytic agents. Xylarinase stands out as a potential candidate for further pre-clinical studies using animal models for development into a thrombolytic agent.

Chapter 1

Introduction

1 Background

Blood clots are formed as a preventive response of the human body to avoid excessive bleeding at the site of injuries and wounds (Raffat et al 2012). There are over twenty known enzymes that assist in the clot formation (Agrebi et al 2009; Kotb 2014a). Fibrin is the major protein involved in the clot formation which is formed from its inactive precursor fibrinogen via proteolytic action of thrombin (Peng et al 2005; Uesugi et al 2011). Fibrinogen is a soluble glycoprotein of 340 kDa consisting of two sets of three polypeptide chain designated as Aα, Bβ and γ (Choi et al 2013a). Fibrin monomers formed from fibrinogen polymerizes spontaneously and forms a fibrin gel network. Fibrin gel network provides strength and structure to the clot and also regulates the rate of clot formation and degradation (Silva et al 2013). Fibrin clot is hydrolysed by a serine protease, plasmin (EC 3.4.21.7) which is formed from plasminogen (Shirasaka et al 2012; Choi et al 2013b). Under normal conditions, blood clot formation and its dissolution is precisely regulated by the biological system via homeostasis. However, during an unbalanced condition, the insoluble blood clot does not get hydrolysed and adheres to the wall of the blood vessels resulting in thrombosis (Shimkhada et al 2012; Meshram and Saxena 2016). Thrombosis triggers venous and arterial thromboembolic events leading to various cardiovascular disorders (CVDs) which includes; myocardial infarction (MI), ischemic heart attack, arterial thrombosis, deep vein thrombosis (DVT) and pulmonary embolism (Baruh et al 2006; Farret et al 2014).

CVDs are burgeoning health concern across the globe affecting people socially and economically (Choi et al 2014; Bhardwaj and Angayarkanni 2015). CVDs are also the leading cause of morbidity and mortality worldwide responsible for over 18 million lives annually which represents 31 % of total global deaths. Of these deaths, around 7.4 million people died due to coronary heart disease whereas 6.7 million people succumbed to stroke. The scenario is expected to get worsened by 2030 where over 23.6 million people may get chronic CVDs, leading to death (Mander et al 2011; Ahmed et al 2014; Cheng et al 2015; WHO 2015). Eventually, over the last two decades there has been a gradual increase in number of deaths occurring due to CVD. Low and middle income group

countries are the severely affected by CVDs (Gupta et al 2013). Developing nations like India have now become a hub for CVDs with over three million deaths occurring every year which is equal to 25 % of all kinds of mortality occurring in the country. Almost all sections of the society, from young to old and rich to poor are now prone to CVDs. Current studies have shown an increased prevalence of CVDs in Indians as compared to other developing countries. Hence prevention, control, and treatment of CVDs is the need of the hour to minimize morbidity and mortality (Nag and Ghosh 2013; Chauhan and Aeri 2015).

Thrombolytic therapy is considered as the gold standard to obtain recanalization of blood flow in partially occluded veins and arteries (Ju et al 2012; Farret et al 2014). Thrombolytic therapy provides long term benefits with annual survival rate of 95 % (Ali et al 2014).Thrombotic disorders can be treated by thrombolytic agents like anti-coagulants, anti-platelets and direct thrombolytics (Choi et al 2013b). In earlier days, treatment of thromboembolic vascular disease was relied on the use of anti-coagulants, such as warfarin (coumarin) and heparin to inhibit the formation of fibrin clots. Eventually with the understanding of lysis of preformed fibrin which is accomplished *in vivo* by a process involving the conversion of inactive plasminogen to active plasmin enzyme led to an alternative enzyme based approach (Balaraman and Prabakaran 2007; Choi et al 2013a; Kotb 2014a). The current therapeutic modalities are enzyme based which involve intravenous administration of tissue plasminogen activator (t–PA), streptokinase and urokinase which instantly open up the blood flow caused due to fibrin blockage, thus reducing the chances of morbidity and mortality (Kim et al 1996; Mukherjee et al 2012; Raffat et al 2012; Choi et al 2014). Based on their mechanism of action, thrombolytic drugs are divided in two types viz. t-PA and plasmin like proteins. t-PA convert dormant plasminogen into plasmin which in turn hydrolyses the fibrin clot whereas plasmin like proteins directly hydrolyse the blood clots (Simkhada et al 2010; Mander et al 2011).

Yet another classification of thrombolytic agents is based on their origin wherein they are classified into three generations. First generation of thrombolytic agents are fibrin non–specific drugs whereas second generation drugs comprise of fibrin-specific t–PA and single–chain urokinase-

type plasminogen activators (u–PA) etc. Further, the third generation of thrombolytic agents mainly consists of recombinant versions of the first and second generations of thrombolytic agents. The third generation thrombolytic agents are more fibrin-specific with prolonged half-life which makes them suitable for single or double bolus administration (Ju et al 2012).

1.1 Fibrinolytic agent as antithrombotic drugs

Fibrinolytic agents are considered as promising and highly effective therapeutic agents in CVDs (Avhad et al 2013). They are intravenously administrated to recanalize the occluded arteries and veins (Mukherjee et al 2012). Streptokinase and urokinase are t–PA which function via conversion of plasminogen into plasmin to hydrolyse the fibrin clots while nattokinase and lumbrokinase possess plasmin like activity and hence they directly dissolve the blood clot (Mander et al 2011; Kotb 2013). Fibrin specific fibrinolytic enzymes are superior to non–specific fibrinolytic drugs as they do not deplete the systemic coagulation factors when compared to non-fibrin specific thrombolytic agents (Ghasemi et al 2012; Kotb 2013).

At present, there are four fibrinolytic drugs which are available in the market; alteplase, reteplase, streptokinase and tenecteplase (Kumar et al 2011). Streptokinase is the oldest and most commonly used fibrinolytic agent because of its cost effectiveness, short half–life and small duration of therapy (Keramati et al 2012). It was initially isolated in 1933 by strains of β-hemolytic *Streptococci* and used for treatment of peripheral arterial occlusive in 1947. It is a 47 kDa, non-fibrin specific extracellular enzyme that confers fibrinolytic activity by covalently binding with the circulatory plasminogen. It is included in the World Health Organization (WHO) model list of essential medicines (Banerjee et al 2004; Mahboubi et al 2012; Babu and Devi 2015). It has been used as a therapeutic agent in treatment of myocardial infarction following coronary thrombosis (Abdelghani et al 2005).

Streptokinase and urokinase belongs to the first generation of thrombolytic drugs while second generation of thrombolytic drugs includes alteplase and acylated plasminogen–streptokinase activator complex (APSAC). Alteplase is fibrin specific whereas APSAC is non–fibrin specific. The third

generation of thrombolytic drugs comprise of tenecteplase, reteplase, monoteplase which are highly fibrin specific and recombinant in nature (Kumar et al 2011). Alteplase has been approved for treatment of myocardial infarction in 1987 whereas tenceteplase got FDA approval in June 2000. In US, streptokinase, alteplase, reteplase and tenecteplase are used as thrombolytic drugs for treatment of MI while in Europe, streptokinase is the most commonly used fibrinolytic agent. In Canada also, streptokinase and t–PA are used as thrombolytic drugs whereas in India streptokinase, urokinase and t–PA are being used in therapy of CVDs (Baruah et al 2006).

Despite the widespread use of thrombolytic agents (fibrin specific and non-fibrin specific) they exhibit undesired attributes such as large therapeutic dose, low fibrin specificity, short plasma half–life, bleeding complications and are cost intensive making them out of reach for a common man (Peng et al 2005; Balaraman and Prabakaran 2007; Cui et al 2008). Streptokinase is a protein of non–human origin which triggers hypersensitivity reaction, therefore, a patient receiving streptokinase can only receive this thrombolytic agent once in its life time as they develop immunogenicity. It is also associated with serious risk of haemorrhage (Banerjee et al 2004). Urokinase is more expensive than streptokinase and is less effective than t–PA (Baruh et al 2006; Suri et al 2012). Alteplase is not antigenic but has higher risk of intracranial haemorrhage which may increase the chance of stroke (Hacke et al 2008). Further, continuous infusion of rt–PA is required due to its short plasma half–life (4–6 min). Reteplase has a lower affinity for fibrin and causes more fibrinogen depletion as compared to alteplase which increases the frequency of bleeding complications. Despite these traits of the fibrin-specific thrombolytic agents, clinical trials could not establish superiority of these agents over streptokinase with respect to decrease in mortality or the incidence of MI (Toombs 2001; Keramati et al 2012; Kotb 2014a; Bhardwaj and Angayarkanni 2015). Production of recombinant products like tenecteplase and reteplase is not cost effective (Kumar et al 2011). Such concerns call for an urgent need to explore new fibrinolytic agents which could have better efficacy, specificity and fewer side effects as compared to the existing thrombolytic drugs. There has been a continual search for exploring potential alternative fibrinolytic agents from different sources which includes

snake venom, centipede venom, earthworms, dung beetles, marine creatures, vampire bat, leeches, plants and microorganisms especially bacteria, actinomycetes, algae and fungi (Schleuning 2001; Liberatore et al 2003; Cho et al 2004; Peng et al 2005; Markland and Swenson 2010).

1.2 Microbes as a source of fibrinolytic agents

Recent advances in molecular biology and fermentation technology have attracted the attention of the research community to explore microbial metabolites as fibrinolytic agents (Avhad et al 2013). The major advantage with the microbial fibrinolytic agents is that the mass production of the fibrinolytic agent can be achieved to meet the market demand through large scale or industrial fermentations (Li et al 2007). Over the last few years' microbes have served as the fountainheads of different enzymes (Rovati et al 2010). The isolation of streptokinase from β-hemolytic *Streptococci* of Lancefield group A, C and G is considered as a landmark discovery in thrombolytic therapy which has paved the way for finding novel molecules from microbial resources. Streptokinase has been used clinically for many years now (Babu and Devi 2015; Bhardwaj and Angayarkanni 2015). Several other bacteria like *Staphylococcus aureus* produce staphylokinase, *Bacillus sphaericus* produce thrombinase, *Lactobacillus* and *Bacillus subtilis* produces enzanim which have strong fibrinolytic property (Balaraman and Prabakaran 2007; Agrebi et al 2009; Tamura et al 2011). Potent fibrinolytic agents have also been isolated from various other bacterial species including *Aeromonas* sp., *Escherichia coli*, *Pseudoalteromonas* sp., *Shewanella* sp., *Xenorhabdus indica* (Cho et al 2011; Pranaw et al 2014; Vijayaraghavan and Vincent 2015; Vijayraghvan et al 2015). Over the years, many novel fibrinolytic enzymes have been discovered from a range of microbial entities and many subsequent attempts have been made to increase the efficacy and specificity of fibrinolytic therapy (Peng et al 2005; Kotb 2014a). Microbial fibrinolytic enzymes especially those from the food grade microorganisms have also attracted much attention due to their potential to be developed as functional food additives and drugs to prevent or cure CVDs (Ghasemi et al 2012). Several bacterial species isolated from the fermented food were discovered to produce fibrinolytic enzymes. *Bacillus natto* obtained from Japanese soyabean fermented food natto, *Bacillus amyloliquefaciens* DC–4

isolated from the Chinese soybean–fermented food douche, *Bacillus* sp. CK from the Korean fermented-soybean sauce named chungkook-jang (Kim et al 1996), *Bacillus* sp. strains DJ–2 and DJ–4 from the Korean doen-jang (Kim and Choi 2000) and *Bacillus* sp. KA38 from the Korean salty fermented fish called jeot-gal are potential sources of fibrinolytic agents. *Bacillus subtilis* isolated from the Indonesian fermented food tempeh produces subtilisin which also possesses a strong fibrinolytic activity (Kim et al 2006). Subtilisin in the recent time has attracted medical interest due to its promising efficacy, specificity, safety, cost effectiveness, oral administration and stability in the gastrointestinal tract (Ghasemi et al 2012). The microbial fibrinolytic agents from food products can serve as nutraceutical agents and help to prevent or cure CVDs (Peng et al 2005; Dabbagh et al 2014).

Actinomycetes comprise of a special group of microbes, which have been a novel source of bioactive and structurally important metabolites to date (Ju et al 2012). Actinomycetes have also been bio–prospected in the quest for novel microbial fibrinolytic enzymes. Several *Streptomyces* species like *Streptomyces* sp. XZNUM 00004, *Streptomyces omiyaensis*, *Streptomyces* sp. CS684, *Streptomyces* sp. CS624, *Streptomyces* sp. NRC 411, *Streptomyces* sp. DPUA1576, *Streptomyces* sp. MCMB–379, *Streptomyces rimosus*, exhibited profound fibrinolytic activity (Abdel–Naby 1992; Shimkhada et al 2010; Chitte et al 2011; Mander et al 2011; Uesugi et al 2011; Ju et al 2012; Silva et al 2013). Actinokinase is a thermophilic serine endopeptidase produced by *S. megaspores* SD5 which possess fibrinolytic activity. Fibrin degradation potential of *S. megaspores* SD5, *Streptomyces* sp. XZNUM 00004, *Streptomyces* sp. CS684 and *Streptomyces omiyaensis* was many folds higher than that of plasmin (Chitte and Dey 2000, 2002; Ju et al 2012). Thus actinomycetes can be considered as an economically viable source of the production of fibrinolytic enzymes.

In addition to the above microorganisms, several algal species like *Codium divaricatum, Codium intricatum, Codium latum* were found to produce fibrinolytic enzyme (Matsubara et al 1998, 1999, 2000). Recently, novel bi–functional fibrinolytic enzymes like codiase, undariase and ulvease

were isolated from *Codium fragile, Undaria pinnatifida* and *Ulva pertusa* respectively (Choi et al 2013b; Choi et al 2014; Kang et al 2016).

1.3 Fungi as a source of fibrinolytic agent

The discovery of miracle drug Penicillin from *Penicillium notatum* heralded the golden era of antibiotics. Since then, fungi have significantly contributed in the welfare of mankind by producing bioactive compounds which have been used as anti–bacterial, anti–cancer, anti–oxidant and immunomodulatory agents (Berdy 2005). Fungi also offer themselves as a source of extracellular fibrinolytic enzymes (Amatayakul 1955). Several fungal species like *Aspergillus ochraceus* 513, *Aspergillus oryzae* KSK-3*, Fusarium oxysporum*, *Penicillium chrysogenum, Rhizopus chinensis* have been found to produce proteases which confer fibrinolytic activity (El–Assar et al 1990; Tao et al 1998; Batomunkueva and Egorov 2001; Xiao–lan et al 2005; Shirasaka et al 2012). Fungal species like *Fusarium* sp. BLB and *Rhizopus* sp. isolated from the Indonesian fermented food tempeh exhibited strong fibrinolytic activity. Fungal protease FP produced by *Fusarium* sp. BLB exhibited very strong fibrin degradation when compared with nattokinase (Sugimoto et al 2007). Apart from filamentous fungi, fibrinolytic enzymes have been successfully isolated and characterised from edible or medicinal mushrooms like *Armillariella mellea, Cordyceps sinensis, Flammulina velutipes*, *Formitella fraxinea, Ganoderma lucidum*, *Pleurotus eryngii*, *Pleurotus ostreatus*, *Paecilomyces tenuipes*, *Schizophyllum commune, Tricholoma saponaceum* (Shen et al 2007; Cha et al 2010; Lu et al 2010; Kim et al 2011; Kumaran et al 2011). *Cordyceps militaris,* a traditional Chinese mushroom possess various medicinal properties such as anti–microbial, anti–cancer and immunomodulatory. It has recently been found to produce a novel enzyme CMAse which is fibrinolytic in nature (Cui et al 2008). Herinase, a novel bi–functional fibrinolytic enzyme has been isolated from monkey head mushrooms, *Hericium erinaceum* (Choi et al 2013a).

1.4 Endophytic fungi as a source of fibrinolytic agents

Endophytic fungi are a group of highly diverse, polyphyletic microorganisms which colonize the intra or intercellular parts of the plant for at least a portion of their lives without causing obvious

infections (Petrini 1991; Bacon and White 2000; Strobel and Daisy 2003; Kusari et al 2013). They are ubiquitous in nature and occupy all the niches on the earth. Once the endophyte enters the internal tissue of the host they assume the latent phase for their entire life cycle or for an extended duration (Aly et al 2011; Kaul et al 2012). Their relationship with the host plant ranges from symbiotic, benign commensels, decomposers to latent pathogens (Promputha et al 2007). Endophytic fungi are hyperdiverse; it is estimated that more than 1.5 million species may exist (Arnold et al 2000). Endophytic fungi produce an array of metabolites which may defend the host plant against different stress conditions like pathogenic invasions and drought (Aly et al 2013; Meshram et al 2013; Qadri et al 2013). During the course of evolution, endophytes acquire the properties of their host and start producing putative phytochemicals some of which have medicinal importance. This hypothesis can be authenticated by considering the example of paclitaxel produced by *Taxomyces andrenae* an endophyte of *Taxus brevifolia* contributing to the billion dollar anticancer drug market (Strobel et al 1996). This holds a promise for their possible mass production through fermentation (Aly et al 2010; Zhao et al 2011; Kusari et al 2014). Many biologically active metabolites like campothecin, oomycidin, cytosporone, isopectacin etc. have been successfully isolated from the endophytic fungi possessing anti–cancer, anti–bacterial, anti–fungal and anti–oxidant activities (Firakova et al 2007; Elsebai et al 2014). Endophytic fungi produce various industrially important enzymes like amylase, cellulose, laccase, lipase, protease etc (Correa et al 2014). Thus, endophytic microorganisms are considered as a warehouse of biologically active metabolites possessing promising applications in agrochemical and pharmaceutical industries (Strobel and Daisy 2003; Kaul et al 2012; Zilla et al 2013; Zhang et al 2015).

Though endophytic fungi have been frequently exploited for pharmaceutical applications, there exists very preliminary and limited work on isolation and characterization of fibrinolytic agents from endophytic fungi (Meshram et al 2016a). Verticase, a serine protease that possesses fibrinolytic potential has been isolated from the endophytic *Verticillum* species (Li et al 2007). Apart from this, endophytic *Fusarium* CPCC 480097 and *Fusarium* BLB sp. produces fibrinolytic enzyme with higher

fibrinolytic activity than plasmin (Wu et al 2009b; Ueda et al 2007). *Bionectria* sp. from Yungas Pedemontana forest range in Argentina has been reported to be a potential source of direct (plasminogen–independent) fibrinolytic enzymes for different therapeutic purposes (Rovati et al 2010). It may be hypothesized that the production of these fibrinolytic enzymes (proteases) from endophytic fungi might be required for their survival and reproduction inside the host plant (Reddy et al 1996). Fungal fibrinolytic enzymes may have potential application in the pharmaceutical industry as thrombolytic agents with probably less antigenicity as compared to their bacterial counterpart's, streptokinase and staphylokinase. Some enzymes of fungal origin such as diastase, sarcosidase, haloperoxidase and superoxide dismutase have already been exploited as therapeutic agents (Saxena 2015). Thus, in the present study we have systematically screened endophytic fungi for their potential to produce extracellular fibrinolytic enzymes for its possible use as a thrombolytic agent.

Chapter 2

Present Approach

Plants harbour a suite of microorganisms which can be classified as mycorrhiza, saprophytes, pathogens, epiphytes and endophytes. Endophytes are extremely diverse and ubiquitous group of microorganisms that colonize the internal tissue of a host plant without showing any obvious signature of their existence. The predominant class of microbes which resides as endophytes belong to the fungal kingdom (Schulz et al 1999**;** Bacon and White 2000; Strobel and Daisy 2003; Aly et al 2011; Porras-Alfaro and Bayman 2011). In contrast to their pathogenic fungal counterparts, endophytic fungi exist in a mutualistic association with their host plants and provide a variety of fitness enhancements to host plants including increased tolerance to heavy metals, drought resistance, reduced herbivory, defence against pathogens, or enhanced growth and competitive ability (Saikkonen et al 1998; Tan and Zou 2001; Arnold et al 2003; Bae et al 2008). Endophytic fungi communicate with their host plant through metabolic interactions which enables them to produce signal molecules having interesting biological activities (Krohn et al 2007). Under selective evolutionary pressure, endophytes developed the ability to imbibe genetic information of the plant (horizontal gene transfer) and starts expressing them which results in production of the chemical moieties which were produced by plants originally. Thus, they start mimicking the host plant phytochemicals when cultured independently (Firakova et al 2007; Kusari et al 2012).

The serendipitous discovery of penicillin from the fungus *Penicillium chrysogenum* led the foundation for exploiting fungi as a novel source of biologically active metabolites. Since then, many fungal metabolites have been developed into important drugs such as lovastatin, cephalosporin and griseofulvin (Seenivasan et al 2008; Wainwright 2008). Ever since the discovery of “taxol” producing endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia*, endophytes have been immensely explored and exploited for the production of putative phytochemicals such as camptothecin, podophyllotoxin, huperzine, vincristine and vinblastine (Puri et al 2005, 2006; Li et al 2007; Kour et al 2008; Kusari et al 2009; Kumar et al 2013; Jia et al 2016).

Apart from being a potential source of putative phytochemicals, endophytic fungi also produce an array of bioactive moieties including enzymes which are expressed in response to biotic

and abiotic stresses to which plant is exposed from time to time (Kusari et al 2013). Endophytic fungi produce a plethora of biologically active metabolites which possess anti–microbial, anti–oxidant, anti–cancer, anti–inflammatory, insecticidal and immunomodulatory activities (Strobel and Daisy 2003; Tejasvi et al 2007; Kaul et al 2013). These bioactive compounds generally are structurally novel and may possess potential to contribute in development of new drugs directly or indirectly (Gao et al 2005; Strobel 2006)

Enzymes possessing novel activities are classical targets for microbial screening that aim for pharmaceutical, agrochemical or other industrial applications. However, the microorganisms for enzyme screening were predominantly explored from soil metagenomic libraries, marine environment or human gut. Some recent studies have indicated that endophytic fungi have the potential to produce enzymes such as proteases, lipases, L-asparaginases, cellulases, phytases which have applications in the pharmaceutical, agrochemical and other industries (Borges et al 2009; Lisboa et al 2013; Zaferanloo et al 2014; Krishnapura and Belur 2016; Muller et al 2016; Uzma et al 2016).

It is been enunciated that endophytic fungi elaborate a variety of enzymes such as proteases, amylases, cellulases, chitinases, laccases, lipases, pectinases as a mechanism to breach the barriers of plants and to counter the defense molecules elaborated by the plants (Suryanarayanan et al 2012). However, despite the above articulation, the endophytic fungi represent an underexplored ecological niche for novel biocatalysts or enzymes.

2.1 Hypothesis

Plant proteases play pivotal role in the plant physiology and development. It has been well documented that plant proteases play a significant role in plant defence (Van der Hoorn 2008). Serine protease contains a serine residue in its active site and is predominantly of two types; the chymotrypsin–like, the subtilisin–like (Maurer et al 2001; Gonzalez-Rabade et al 2011).

Endophytic fungi have been reported to produce some specific enzymes (hydrolases) such proteases, cellulases, chitinases and β–1, 3–glucanases during their establishment within the host as

well as in the process of providing fitness to the plant in terms of conferring resistance towards other plant pathogens, insects and nematodes (Gao et al 2010; Dutta et al 2014; Alvin et al 2014). Expression of specific proteases such as proteinase *At* has been well established during symbiotic / endophytic infection within the culture as well as in the plant tissue indicating that proteases play a role in establishment of symbiotic interaction between the plant and fungus (Lindstorm and Belanger 1994; Reddy et al 1996). It was also reported that *Fusarium oxysporum* f. sp. *lycopersici* did not exhibit a significant change in mycelial growth, sporulation and pathogenicity to tomato plants when the expression of proteinase *Prt1* was inactivated (Pietro et al 2001). In another study, it was confirmed that proteases play a significant role in *Trichoderma asperellum* as a mycoparasite as well as a plant opportunistic symbiont (Viterbo et al 2004). However, in majority of studies on protease expression by the fungus have been oriented towards understanding plant pathogenesis instead of symbiosis (Mosolov and Valueva 2006).

Fibrinolytic enzymes reported to date, predominantly belong to serine protease class. Thus the hypothesis of screening fibrinolytic activity of endophytic fungi is based on the premise that they produce an array of proteases for their survival and mutualistic interactions with plants which in turn could possibly possess fibrinolytic activity and thus could be used as a therapeutic alternative for busting internal blood clots which is responsible for many diseases in humans.

Hence based on the above hypothesis, the objectives of present study are as under:

I. Isolation and screening of endophytic fungi for fibrinolytic activity
II. Characterization of fibrinolytic agent(s) from selected endophytic fungi
III. To examine the mechanism of fibrinolytic activity by *in vitro* assay

Chapter 3

Review of Literature

One of the most astonishing features of blood is its clotting mechanism**.** The major purpose of this mechanism is to cease the blood loss from the damaged vessel. The clotting process involves regimented service of dozens of proteins, enzymes (which forms the clotting cascade) and vitamins and this is the reason why coagulation process is considered as an example of flawless planning.

3.1 Hemostasis

The blood clot formation following an injury begins with platelets which are activated and adhere to the macromolecules in the sub-endothelial tissues at the site of injury. These platelets then aggregate to form a primary haemostatic plug which reduces or temporarily stops the loss of blood. Platelets mediate the activation of coagulation factors which leads to the formation of an insoluble fibrin clot that strengthen the platelet aggregation. The three basic components of hemostasis are the vascular wall, platelets and coagulation cascade. Two models have been proposed for coagulation till date, the cascade model by Macfarlane (1964) and the waterfall model by Davie and Ratnoff (1964). Both the models described each clotting factor as a dormant proenzyme which is converted into an active enzyme. Both the theories suggested that clotting sequences were divided into two pathways: intrinsic and extrinsic pathway.

The intrinsic pathway consists of a series of protease reactions instigated by factors that are present within the blood. The intrinsic pathway involves prekallikrien, high molecular weight kininogen and factors XII, XI, IX, VIII and X. When the blood comes in contact of a negatively charged surface like membrane of activated platelets, the Hegeman factor known as factor XII is converted to its active form factor XIIa. High molecular weight kininogen act as cofactor and anchors the conversion of factor XII into its active form. Once a small quantity of activated Hegeman factor is accumulated, it converts prekallikrein to kallikrien which accelerates the conversion of factor XII to XIIa. Further factor XIIa together with high molecular weight kininogen cleaves factor XI into its active form XIa. Factor XIa proteolytically hydrolyses factor IX to IXa. Factor IXa further transit factor VIII into VIIIa. Factor IXa and VIIIa along with calcium ion and negatively charged phospholipid form a trimolecular complex ‘tenase’ which convert factor X into Xa. Factor Xa unite with factor Va to form

prothrombinase to convert dormant prothrombin into thrombin. Thrombin catalyses the formation of fibrin from fibrinogen (Fig 3.1).

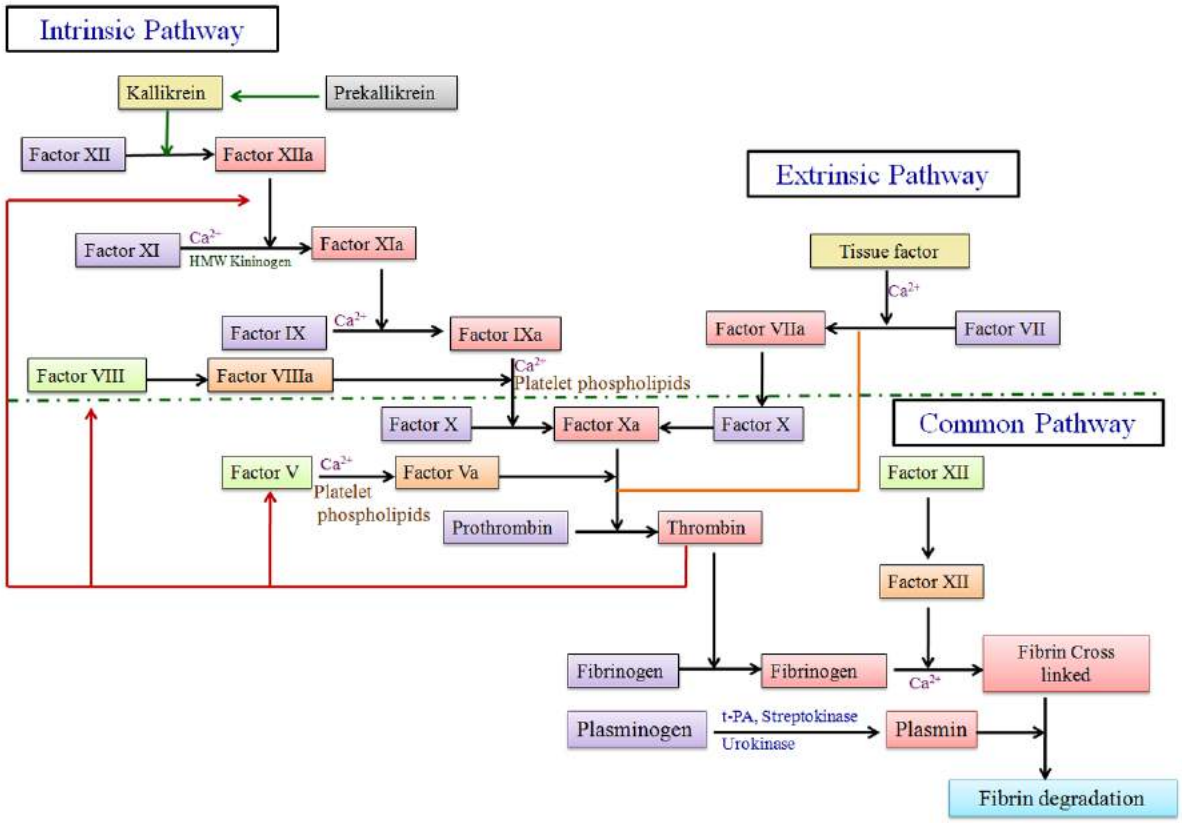


Fig 3.1: Clotting cascade mechanism

The extrinsic pathway requires tissue factor for its initiation which is located at tissue adventitia and comes in contact with blood only after following a vessel injury. When there is a vascular injury, the two proteins form a one to one complex in presence of calcium ion which catalyses the conversion of factor VII to a serine protease factor VIIa. Factor VIIa along with tissue factor complex facilitates the conversion of factor X to factor Xa by cleaving the Arg $_{52}$-Ile peptide bond at the amino acid terminal of the heavy chain. Factor Xa then combines with factor Va in presence of calcium and phospholipid to form prothrombinase which convert prothrombin into thrombin. Prothrombin is activated by hydrolysis of two internal peptide bonds i.e Arg_{271}-Thr and Arg_{320}-Ile. Factor Va act as a cofactor in prothrombin activation by increasing the velocity of the reaction by 100 fold. Thrombin then regulates the conversion of fibrinogen into fibrin. Fibrin formation takes place due to hydrolysis of peptide bond in each of the two α chains (Arg_{18}-Gly) and β

chains (Arg_{16}-Gly). This hydrolysis results in formation of four fibrinopeptides which give rise to fibrin monomers with a new amino acid terminal sequence of Gly-Pro-Arg in α chain and Gly-His-Arg at the β chain. This newly formed Gly-Pro-Arg residues of one monomer binds with the D domain of the neighbouring fibrin monomer leading to polymerisation of fibrin. Similar is the case with β chain. This linear polymerization reaction forms insoluble clot. Once the fibrin is forms, it also stimulates the conversion of factor XII to factor XIIa by thrombin in presence of calcium ions (Fig 3.1). Factor XIIa also polymerizes α_2–anti–plasmin and fibronectin to α chain of fibrin resulting in their incorporation into the fibrin clot (stormorken and Owren 1971; Rosenberg and Rosenberg 1984; Davie et al 1991).

Under normal physiological conditions, hemostasis is followed by fibrinolysis. So, once a clot has served its hemostatic function, it gets degraded by another protein known as plasmin. Plasmin is produced by proteolytic cleavage of circulating plasminogen by PA which ultimately degrades insoluble fibrin clot into soluble fibrin degradation product (Joshi and Sahni 2010) (Fig 3.2).

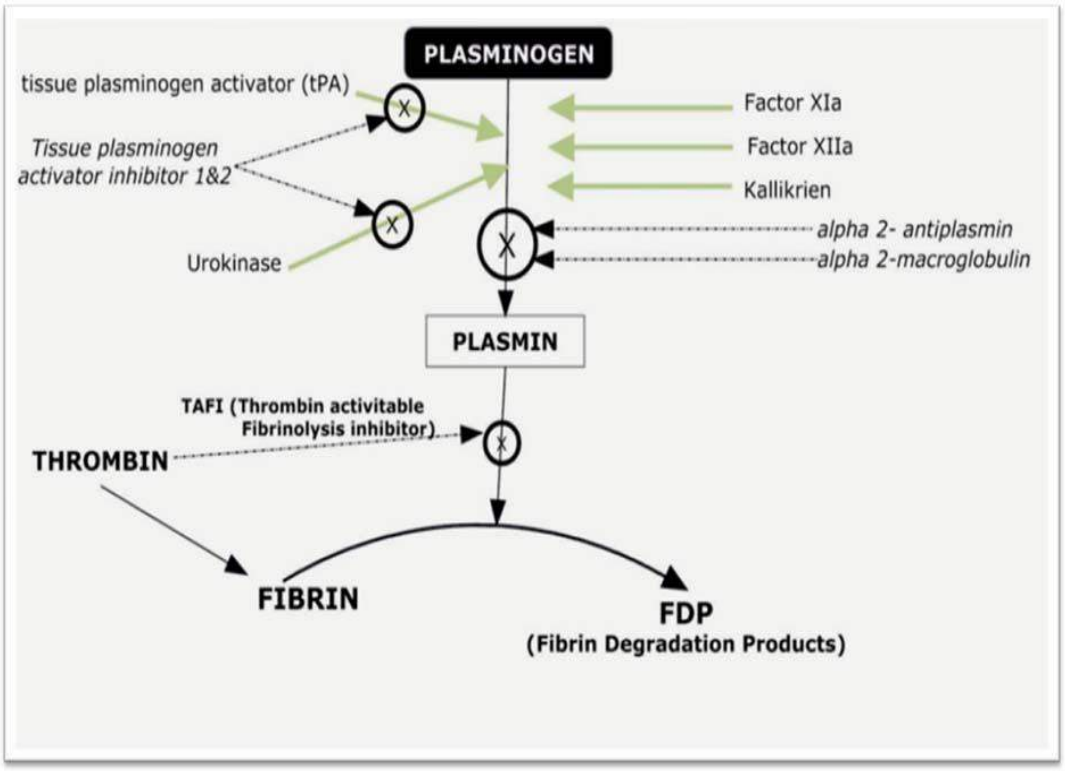


Fig 3.2: Mechanism of fibrinolysis

There are two types of PA: t–PA and u–PA. t–PA is secreted from the endothelial cell in response to thrombin or venous occlusion whereas u-PA is produced in its dormant form prourokinase which is activated by plasmin, kallikrein and factor XIIa. Fibrinolysis is regulated by plasma inhibitor α_2– anti–plasmin, PA inhibitor and thrombin activatable fibrinolysis inhibitor also called as carboxypeptidase U (Thelwell 2010; Fuentes et al 2014).

3.2 Evolutionary trend in thrombolytic therapy

Based on their mechanism of action, thrombolytic drugs are divided in two types: the former is PA which converts dormant plasminogen into active plasmin that hydrolyses the fibrin clot and the later one is plasmin like proteins that directly hydrolyse the blood clots (Simkhada et al 2010). Further, the thrombolytic agents are classified on the basis of their origin and mode of action (Table 3.1).

Table 3.1 Classification of thrombolytic agent

Specificity	Generations of thrombolytic drugs		
	First	**Second**	**Third**
Non fibrin specific	Streptokinase*	Pro–urokinase	–
	Urokinase*	APSAC*	–
Fibrin specific	–	–	Reteplase*
	–	–	Tenecteplase*
	–	–	Monteplase
	–	–	Lantoteplase
	–	–	Monteplase
			**Approved for clinical use*

3.2.1 First generation of thrombolytic drugs

The first generation of thrombolytic drugs include streptokinase and urokinase. Both streptokinase and urokinase are non-fibrin specific in nature. Streptokinase (EC 3.4.99.22) is an extracellular enzyme produced by many strains of β-hemolytic *Streptococci* of Lancefield group A, C and G (Banerjee et al 2004; Yadav et al 2008; Bhardwaj and Angayarkanni 2015). This enzyme was serendipitously discovered by William Smith Tillett in 1933 in β-hemolytic *Streptococci*. However streptokinase production was mostly preferred from group C S*treptococci* because they lack erythrogenic toxins (Babu and Devi 2015). Streptokinase is a non-fibrin specific enzyme that brings

about its fibrinolytic action by activating the free circulatory plasminogen. It binds non–covalently to the inactive plasminogen forming a 1:1 equimolar complex of plasminogen and Streptokinase which act upon other circulatory plasminogen molecule to cleave Arg-Val 561 bond to generate plasmin which mediates fibrinolysis (Chaudhary et al 1999; Baruh et al 2004; Kumar et al 2011; Bandehpour et al 2012; Mahboubi et al 2012; Keramati et al 2012; Kotb 2013).

Urokinase is a strong plasminogen activator originally isolated from human urine. It is produced by kidneys and is secreted in urine. Urokinase exists in two forms: a high molecular weight of 55 kDa and a low molecular weight of 33 kDa. Urokinase is a trypsin like serine protease that is capable of hydrolysing the L-lysine and L-arginine bond which exclusively catalyses the cleavage of Arg-Val bond in plasminogen by first order reaction to form active plasmin. The active plasmin degrades the fibrin mesh in the blood clot (Baruah et al 2006; kumar et al 2011).

Urokinase is less fibrin specific and less effective thrombolytic drug as compared to streptokinase. The isolation procedure is complicated, lengthy and costly. The yield is also very poor. About 1500 litres of urine is required for producing one clinical dose of urokinase which makes it quite expensive. To overcome these limitations, human cell cultures are being used for production of urokinase. Urokinase is now being produced from human embryo kidney cells, human lung edenoma carcinoma cell lines, human fibroblast, human umbilical vein endothelial cells, human myeloma cells and BHK-21/N cells. Cultured cells produces higher quantity of urokinase (50–100 ng/ml) as compared to that obtained from urine (10-15 ng/ml) (Vassalli et al 1985; Banerjee et al 2004; Baruah et al 2006; Roychoudhury et al 2006; Bansal and Roychoudhury 2006; Kunamneni et al 2008; Kumar et al 2011; Kotb 2013; Collen and Lijnen 2015).

3.2.2 Second generation of thrombolytic drugs

The limitations encountered by streptokinase and urokinase have prompted researcher to look for an ideal thrombolytic drug with improved efficacy, less immunogenicity and cost effectiveness. This led to the development of second generation of thrombolytic agents. The second generation of thrombolytic drugs comprised of anistreplase, pro–urokinase and alteplase. The former two

thrombolytic drugs are fibrin non–specific whereas the later is fibrin specific. Anistreplase (acylated plasminogen–streptokinase activator complex) is a complex in which a human plasminogen with acylated active site is coupled with bacterial streptokinase. Pro-urokinase is a second generation thrombolytic drug produced from a naturally occurring physiologic protease. It is composed of a single polypeptide chain of 414 amino acids with molecular weight of 49 kDa (Banerjee et al 2004; Baruah et al 2006; Kumar et al 2011; Collen and Lijnen 2015).

Alteplase is a fibrin specific recombinant tissue plasminogen activator produced by using recombinant DNA technology. The complementary DNA encoding for t-PA was expressed in Chinese hamster ovary (CHO) cells which produced a t-PA identical to native plasminogen. It is a single polypeptide chain of 70 kDa with 527 amino acids. It has been approved for use in USA and Europe. Alteplase has a high specificity and affinity for fibrin but it is associated with higher bleeding risk and short plasma half life (Baruah et al 2006; Kumar et al 2011; Dhillon 2012; Cohen and Macdonald 2014).

3.2.3 Third generation of thrombolytic drugs

The third generation thrombolytic drugs were developed with an aim to overcome the shortcomings in the previous drugs including efficacy, specificity, safety and ease of administration. The third generation thrombolytic agents are more fibrin specific with prolong half life which make it suitable for single or double bolus administration. Reteplase is a single chain recombinant plasminogen activator produced as a deletion mutant of alteplase that is expressed in *E.coli* whereas tenecteplase is a multiple point (three) mutant of alteplase that have been specifically bioengineered to have extended fibrin specificity and half-life allowing convenient single bolus dosing (Kumar et al 2011). A summary of thrombolytic drugs has been given in table 3.2

Table 3.2 Comparative summary of different thrombolytic drugs

Thrombolytic drug	**Features**							
	Molecular Weight (kDa)	Plasma half life (min)	Fibrin specificity	Plasminogen activation	Dose	Plasma clearance	Patency rate	Development phase
Streptokinase	47	18	(-)	Indirect	1.5 mU/hr	10.8±8.8	30 % at 90 min	MI approved
Anistreplase	131	90-112	(-)	Indirect	-	65±25	50 % at 90 min	MI approved
Urokinase	32-54	15	(-)	Direct	3m U/hr	-		MI approved
Saruplace	47	6-9	(-)	Direct	20 mg bolus + 60 mg/6 min	594±60	71.8 % at 60 min	Stroke –Proof of concept
Alteplase	70	4-8	(+)	Direct	15 mg bolus +3 h reinfusion upto 85 mg	572±32	46-75 at 90 min	Stroke, MI approved
Reteplase	40	11-14	(+)	Direct	Double bolus 10 U + 10 U in 30 min apart	103±138	60-63 % at 90 min	MI approved, Stroke- Concept of proof
Tenecteplase	70	20	(++)	Direct	0.5 mg/kg single bolus	105	63 % at 90 min	MI approved Stroke preclinical
Lantoteplase	54	37	(-)	Direct	120 kU/kg single bolus	51±16	57-83 % at 90 min	MI proof of concept
Monteplase		23	(-)	Direct	0.22 mg/kg single bolus	-	53-69 % at 60 min	Preclinical phase
Pamiteplase		30-47		Direct	0.1 g/kg single bolus	135±10	50-54 % at 60 min	Preclinical phase

3.3 Fibrinolytic agents from plants

Since time immemorial, plant based natural products have been used as a source of medicine to treat various diseases. All traditional system of medicines such as Ayurveda, Siddha, Unani and Chinese involved the use of medicinal plants. Natural products offer a pool of fibrinolytic enzymes which could be developed into clinically useful products (Mannan et al 2011; Elumalai et al 2012; Choi et al 2014). Sterols isolated from *Bacopa monnieri Linn* enhance the fibrinolytic activity of the fibrinolytic activity of endothelial cells (Kojima et al 1986). Garlic capsules significantly reduce total serum cholesterol and triglycerides, and increased fibrinolytic activity in patients with coronary heart disease (Bordia et al 1998). Verma and Bordia (2001) also studied the effect of ginger on fibrinolytic activity. A fibrinolytic protease was purified and characterised from the Chinese herb *Spirodela polyrhiza*. The purified protease is a dimer which produces strong fibrinolytic activity with specific activity of 27998 U/mg (Choi and Sa 2001).

Polyphenols and terpenoids isolated from *Ginkgo biloba* have been found to possess fibrinolytic activity. Further, *Ginkgo biloba* also exhibited cardioprotective activity in rats with isoproterenol-induced myocardial necrosis (Pietri et al 1997; Naderi et al 2005). The methanolic extract of aged sorghum vinegar protected mice against thrombotic death when administrated orally at >10 mg/Kg of body weight. The low molecular weight fraction of the extract also exhibited strong fibrinolytic activity (Fan et al 2009). Bromelain obtained from pineapple exhibited *in vitro* and *in vivo* thrombolytic and fibrinolytic properties (Maurer 2001). Seed oil extract of pan tropical shrub, *Cassia alata* has been reported to possess thrombolytic activity (Mannan et al 2011). Rutin is a citrus flavonoid glycoside found in *Fagopyrum esculentum* plant that showed excellent thrombolytic activity by blocking the enzyme protein disulfide isomerase found in all cells involved in blood clotting. It is approved safe by USFDA (Dar and Tabassum 2012). Klafke et al (2012) found that the extract of *Campomanesia xanthocarpa* holds anti–platelet, anti–thrombotic and fibrinolytic activities. Thrombolytic property was observed in peel and aerial part of pomegranate (Kumar and Pushpa 2012). The methanolic extract of *Aponogeton undulates*, *Cucumis sativus*, *Protium serratum*

and *Tinospora crispa*were also found to possess clot lysis activity (Chowdhury et al 2011; Islam et al 2013; Sayeed et al 2014). Similarly, the methanolic leaf extract of *Bougainvillea glabra* was found to possess significant thrombolytic property at a dose of 800 µg/ml (Elumalai et al 2012). Plant extracts of *Gynocardia odorata* enumerate basic thrombolytic activity of 10–20% (Asif et al 2014).

3.4 Fibrinolytic agents from worms, animals and marine creatures

Lumbrokinase is direct acting fibrinolytic enzymes isolated from earthworm, *Lumbricus rubellus*. Lumbrokinase is used as fibrinolytic agent in China, Korea and Japan (Park et al 1998; Verma et al 2011). Yang and Ru (1997) isolated a fibrinolytic enzyme from earthworm *Eisenia fetida*. A glycosylated fibrinolytic enzyme was also obtained from same earthworm species which digested fibrin (Li et al 2003).

Basilase (22kDa) is a direct acting fibrinolytic enzyme produced from the venom of *Crotalus basilicsus basilicsus* (Datta et al 1995). Fibrolase is zinc metalloproteinase isolated from the venom of a copper head snake *Agkistrodon contortrix contortrix*. Desmoteplase is a plasminogen activator fibrinolytic enzyme isolated from the saliva of vampire bat *Desmodus rotundus* (Liberatore et al 2003).

A novel serine protease NJF was isolated from *Neanthes japonica*. The enzyme exhibited a strong specific activity of 11,232 U/mg (Deng et al 2010). Fibrinolytic enzymes were also isolated from the stingray's venomous extract of *Dasyatis sephen* and *Aetobatis narinari*. The enzymes are biochemically characterised as serine and metalloproteases. *D. sephen* significantly extend the coagulation of plasma as compared to *A. narinari* (Rajesh Kumar et al 2011). UFEIII is a novel bi-functional chymotrypsin–like serine protease purified and characterised from the marine invertebrate *Urechis unicinctus* (Bi et al 2013 a, b). Apart from this, the whole body extract of caterpillar *Lonomia achelous* also exhibited fibrinolytic activity. It has lower activity than plasmin (Coll-Sangrona and Arocha-Pinango 1998).

3.5 Fibrinolytic agents from bacteria

The isolation of streptokinase from the β-hemolytic *Streptococci* heralded a new era of exploring microbes as a putative source of fibrinolytic agent. Streptokinase is the most sought fibrinolytic agent (Banerjee et al 2004). Streptokinase production has also been reported from different streptococcal species including *Streptococcus dysgalactiae, S. equinus* and *S*. *equisimilis* (Keramati et al 2012; Babu and Devi 2015; Bhardwaj and Angayarkanni 2015)

Staphylokinase, a strong fibrin specific plasminogen activator was isolated from lysogenic strains of S*taphylococcus aureus*. Staphylokinase lacks fibrin binding and thrombin inhibitor property which ultimately improves its thrombolytic potential (Collen and Lijnen 1994; Szarka et al 1999).

Bacillus species amongst the bacteria have been the most prolific producers of fibrinolytic agent. *Bacillus* species produces an array of intra and extracellular protease enzymes which possesses fibrinolytic property (Heo et al 2013; Majumdar et al 2014). *Bacillus subtilis* strain A1 isolated from soil sediments in Korea produced a bi–functional fibrinolytic enzyme with a specific activity of 1632.2 U/mg (Yeo et al 2001). Bacillokinase (BK II) is another fibrinolytic enzyme produced by *Bacillus subtilis* A1 strain (Jeong et al 2004). Thrombinase, a t–PA was isolated from the fermented broth of *Bacillus sphaericus* with a specific activity of 4258 U/mg (Balaraman and Prabakaran 2007). BSF1 is a 28 kDa fibrin specific serine protease isolated from the marine bacterium *B. subtilis* A 26. (Agrebi et al 2009). Fibrinolytic enzymes produced by non-food grade bacteria are listed in table 3.3.

Further, *Bacillus* sp. strain AS-S20-I produces a non–toxic, non–hemorrhagic, direct–acting fibrinolytic serine enzyme called Bafibrinase (Mukherjee et al 2012). A novel direct acting fibrinolytic was isolated from *Bacillus sphaericus* MTCC 3672 displaying similar clot dissolving capacity like Nattolife (nattokinase) (Avhad et al 2013). A chymotrypsin-like serine protease (18.2 kDa) was produced by *Bacillus amyloliquefaciens* FCF-11 over corn husk. The enzyme did not degrade collagen and was also non-toxic to the mammalian erythrocytes HT29 cells. It was also found not to have any toxicity and haemorrhagic activity on BALB/c mouse model (Kotb 2014).

Table 3.3 Bacteria from non-food sources producing fibrinolytic enzymes

Bacteria	Source	enzyme	Reference
Streptococcus hemolyticus	exudates of infected wound	Streptokinase	Tillet 1933, Babu and Devi 2015
Staphylococcus aureus	human skin	Staphylokinase	Gerheim 1948
Bacillus subtilis BK-17	decaying rice plant	Bacillokinase	Jeong et al 2001
Bacillus subtilis A1 strain	Soil	Bacillokinase II	Jeong et al 2004
Vibrio vulnificus	ATCC 29307	vEP	Chang et al 2005
Bacillus sphaericus	Vector Control Research Centre Puducherry	Thrombinase	Balaraman and Prabakaran 2007
Paenibacillus polymyxa EJS-3	Root tissue of *S. japonica*	-	Lu et al 2010
B. subtilis A 26	marine water	BSF1	Agrebi et al 2009
Bacillus subtilis K42	soyabean flour	metalloprotease	Hassanein et al 2011
Bacillus subtilis AK	-	Enzamin	Tamura et al 2011
Lactobacillus sp.	-	Enzamin	Tamura et al 2011
Bacillus subtilis HQS-3	marine water	metalloprotease	Huang et al 2013
Bacillus Sphaericus MTCC 3672	pond soil, Goa		Avhad et al 2013
Serratia marcescens	Soil	Serratiopeptidase	Mohankumar and Hari Krishna Raj 2011
Aeromonas sp. JH 1	gut of earthworm	-	Cho et al 2011
Bacillus sp. strain AS-S20-I	Soil, Assam	Bafibrinase	Mukherjee et al 2012
Bacillus amyloliquefaciens FCF-11	food samples	chymotrypsin-like serine protease	Kotb 2014
Xenorhabdus indica KB-3	*Steinernema thermophilum*	alkaline metalloprotease	Pranaw et al 2014
Pseudoalteromonas sp., IND11	Fishiries, Kanyakumari	-	Vijayraghvan and Vincent 2014
Shewanella sp. IND20	Fishiries, Kanyakumari	-	Vijayraghvan et al 2015

An endophytic bacterium, *Paenibacillus polymyxa EJS-3* produced a 63.3 kDa chymotrypsin-like serine protease which possesses profound fibrinolytic activity (Lu et al 2010). *Xenorhabdus indica* KB-3 isolated from entomopathogenic nematode symbiont *Steinernema thermophilum* produced an alkaline metalloprotease enzyme which depicted fibrinolytic activity (Pranaw et al. 2014).

Fibrinolytic enzymes viz nattokinase and subtilisin from food grade microorganisms have been isolated in the recent past and are much in demand due to their fibrin specificity, ease in administration, cost effectiveness and increased plasma half life (Sumi et al 1987; Mine et al 2004;

Dabbagh et al 2014; Kotb et al 2013). Fibrinolytic enzymes from food grade microorganisms are listed in table 3.4.

Table 3.4 Fibrinolytic enzyme from food grade bacteria

Bacteria	Source	Enzyme	Reference
Bacillus subtilis natto	Natto, fermented soybean, Japan	Nattokinase	Sumi et al 1987
Bacillus sp. strain CK 11-4	Korean fermented-soybean sauce, Chungkook-Jang	CK	Kim et al 1996
Bacillus. sp. strain DJ-4	Korean fermented food 'Doen-Jang	subtilisins BPN0	Kim and Choi 2000
B. subtilis		subtilisin NAT	Urano et al 2001
B. subtilis TP-6	Indonesian fermented soybean, Tempeh	subtilisin-like protease	Kim et al 2006
B. subtilis DC33	Chinese soybean-fermented food Douchi	subtilisin	Wang et al 2006
B. vallismortis	Korean condiment Chungkook-jang	subtilisin	Kim et al 2007
B. subtilis LD-8547	Chinese soybean-fermented food douche	--	Wang et al 2008
B. amyloliquefaciens CB1	Korean fermented soy food cheongguk-jang	Protease	Heo et al 2013
B. subtilis HK176	Korean fermented soy food cheongguk-jang	--	Jeong et al 2015

3.6 Fibrinolytic agents from a*lgae*

Marine algae are prolific producers of novel bioactive compounds. Various studies have shown that algae produce numerous compounds with excellent biological activities like anticoagulant, antiviral, anti-inflammatory, and antibacterial (Choi et al 2013b, Kim et al 2013). The anticoagulant property of algae has been discovered several decades ago but very little work exist on their exploration for fibrinolytic property. The first attempts were made by Matsubura et al (1998) where they harness the fibrin degrading potential of five algal species *viz. Codium fragile*, *C. divaricatum*, *C. pugniformis C. cylindricum* and *C. intricatum.* Among them, *C. intricatum* exhibited highest fibrinolytic activity

(Matsubara et al 1998). *Codium divaricatum,* marine green algae from the coast of Hiroshima Prefecture, Japan exhibit fibrinolytic activity by the virtue of a serine protease of 31 kDa. The enzyme showed similarity with snake venom in degradation of fibrinogen (Matsubara et al 2000).

A serine protease (60.54 kDa) was purified from brown seaweed *Costaria costata*. The enzyme exerted direct thrombolytic activity. The enzyme also prevented the thrombus formation in carrageenan-induced mice tail model. It also extends prolonged activated partial thromboplastin time whereas had very mild effect on prothrombin time (Kim et al 2013).

Codiase is a bi–functional fibrinolytic enzyme produced from marine green alga *Codium fragile*. It is a 48.9 kDa serine protease having clot busting, anticoagulant, and antiplatelet properties (Choi et al 2013b). Similarly, a direct acting fibrinolytic enzyme undariase was isolated from *Undaria pinnatifida* which inhibits thrombolytic under *in vitro* conditions (Choi et al 2014). Recently, Ulvease (50 kDa), a bi–functional fibrinolytic enzyme was isolated from green alga *Ulva pertusa*. Ulvease exhibited higher fibrinolytic activity as compared to plasmin and urokinase. It also prolonged APTT and also protected mice from collagen and epinephrine simulated pulmonary thromboembolism (Kang et al 2016).

3.7 Fibrinolytic agents from actinomycetes

Actinomycetes are special group of microorganisms that are prolific producers of biologically active and structurally diverse metabolites (Ju et al 2012). A fibrinolytic enzyme was also isolated from *Streptomyces* sp. NRC 411. The enzyme exhibits a specific activity of 15 U/mg (Abdel-Nebi et al 1992). Chitte and Dey (2000) isolated a potent thermophilic chymotrypsin-like serine peptidase (35 kDa) with direct fibrinolytic activity from *Streptomyces megasporus* strain SD5 (Chitte and Dey 2000, 2002). Similarly, *Streptomyces* sp. CS684 produced a 35 kDa novel metalloprotease with better clot busting property than plasmin under *in vitro* conditions (Simkhada et al 2010).

A serine protease FP28 (17.6 kDa) having thrombolytic potential was produced by *Streptomyces* sp. CS628. When tested for carrageenan induced tail-thrombosis model, the enzyme lowers the frequency of thrombus formation inside the rat tail. FP28 did not extend bleeding time

thus was considered that the enzyme activity is not associated with bleeding complications (Simkhada et al 2012).

A trypsin like bi-functional serine protease was purified from *Streptomyces omiyaensis* which possesses eighteen fold higher activity than plasmin. The molecular weight and specific activity of the enzyme was 27 kDa and 136.2 U/mg (Uesugi et al 2011).

Actinokinase is a serine endopeptidase type fibrinolytic enzyme produced by thermophilic *Streptomyces* sp. MCMB-379. Actinokinase (500 IU) rapidly dissolved clot within 20 min of administration as compared to urokinase and streptokinase which require around 40 and 80 min to dissolve the clot (Chitte et al 2011). Ju et al (2012) purified and characterised a direct acting fibrinolytic enzyme (20 kDa) from *Streptomyces* sp. XZNUM 00004.

A novel bi-functional 44 kDa serine protease was isolated from *Streptomyces* sp. P3. The enzyme exhibits higher fibrinolytic activity as compared to urokinase with specific activity of 2,563.6 U/mg. The enzyme did not prolong the bleeding time suggesting that the fibrinolytic activity was not coupled with any risk of bleeding side effects (Cheng et al 2015).

3.8 Fibrinolytic agents from fungi

Fibrinolytic enzymes have been predominantly reported from macrofungi (Table 3.5). A potential fibrinolytic enzyme CMase (27.3 kDa) was isolated from the Chinese traditional mushrooms *Cordyceps militaris* (Cui et al 2008). Similarly, a chymotrypsin-like serine metalloprotease (34 kDa) was isolated from the fruiting bodies of Korean *Cordyceps militaris* (Choi et al 2011). Further, a thermophilic acid-stable fibrinolytic enzyme was also isolated from *Cordyceps militaris* cultivated over rice bran (Kim et al 2011).

Schizophyllum commune produces a 21.32 kDa serine protease which exhibited fibrinolytic activity higher than plasmin under tested conditions with specific activity of 145.8 U/mg. The fibrinolytic activity was highly improved by magnesium ion (Lu and Chen 2010; Lu et al 2010). Park et al (2010) also purified a 17 kDa bi-functional fibrinolytic enzyme from *S. commune*. A potent fibrinolytic enzyme was isolated from the oyster mushroom *Pleurotus ostreatus* (Li et al 2014).

Table 3.5 Fibrinolytic agents from mushrooms

Source	**Source**	**enzyme**	**Reference**
Grifola frondosa	Mushroom farm		Nonaka et al 1997
Pleurotus ostreatus	Culture collection, Seoul	metalloprotease	Shin and Choi 1998
Armillaria mella	Mushroom, Korean Agricultural Culture Collection	*A. mellea* metalloprotease (AMMP)	Lee et al 2005
Flammulina velutipes	Department of industrial crop production and precession, Korea	chymotrypsin-like serine metalloprotease	Park et al 2007
Pleurotus ostreatus	Department of industrial crop production and proecession, Korea	POFE, metalloprotease	Shen et al 2007
Cordyceps militaris		CMase	Cui et al 2008
Schizophyllum commune	Bioresource Collection and Research Center (BCRC, Taiwan, R.O.C.).		Lu et al 2010
S. commune KCTC 6482	Korean Agricultural Culture Collection (KACC), Suwon, Korea		Park et al 2010
Pleurotus eryngii	Hampyoung Mushroom Co., Korea	subtilisin-like serine proteases	Cha et al 2010
Ganoderma lucidum Vk12	ecological niches Southern India		Kumaran et al 2011
Hericium erinaceum	Korean Mushroom Company (Suwon, Republic of Korea	Herinase (metalloprotease)	Choi et al 2013
Pleurotus ostreatus 4241	China General Microbiological Culture Collection Center		Liu et al 2014

Subtilisin-like serine proteases with fibrinolytic property was purified from *Pleurotus eryngii* cultivated over corn hub. The molecular weight and specific activity of the purified enzyme was 14

kDa and 52.8 U/mg (Cha et al 2010). Intracellular fibrinolytic enzyme was isolated from *Ganoderma Lucidum* Vk12. The specific activity of the enzyme was 2279.1 U/mg (Kumaran et al 2011). Herinase, a novel bifunctional fibrinolytic enzyme was obtained from the fruiting bodies of monkey head mushrooms*, Hericium erinaceum*. Herinase is a metalloprotease in nature with high specificity for substrates like t-PA and plasmin. The specific activity of the purified enzyme was 220.65 U/mg (Choi et al 2013a).

Fibrinolytic enzymes have also been successfully purified and characterised from several filamentous fungi (Table 3.6). El-Aassar (1990) synthesised proteases enzyme with fibrinolytic activity using immobilised cultures of *Penicillium chrysogenum* H9. Further, El-Aassar et al (1995) screened ten fungal species for production of fibrinolytic enzyme using solid state fermentation procedure. *Fusarium pallidoroseum* showed maximum production of fibrinolytic enzyme. *Fusarium oxysporum* also produced fibrinolytic enzyme when cultivated over rice chaff (Tao et al 1997).

Fibrinolytic enzyme purified from *Aspergillus ochraceus* had molecular weight of 36.5 kDa and the activity showed by it had a close relatedness to *Agkistrodon* snake venom (Batomunkueva and Egorov 2001). Fibrinolytic enzyme isolated from the thermophilic fungi *Oidiodendron flavum* exhibited a specific activity of 2.5 U/mg (Tharwat et al 2006). *Rhizomucor miehei* isolated from the butcheries at Zagazig, Egypt also produced fibrinolytic enzyme over optimized Czapek Dox medium with a specific activity of 88.22 U/mg (Ali and Ibrahim 2008). Entomopathogenic fungus, *Paecilomyces tenuipes* also produced a new fibrinolytic enzyme whose molecular mass was 14 kDa and showed specific activity of 1413.81 U/mg (Kim et al 2011). *Aspergillus oryzae* KSK-3 isolated from rice produced a strong fibrinolytic enzyme (MW: 30 kDa) with specific activity of 1005 U/mg (Shiraska et al 2012).

Table 3.6 Fibrinolytic agents from filamentous fungi

Source	Source	Reference
Fusarium semitectum		Fayee et al 1976
Penicillium chrysogenum H9	Local soil, Egypt	El-Aassar et al 1990
Fusarium oxysporum		Abdel-Fattah et al 1993
Fusarium pallidoroseum	Local soil, Egypt	El-Aassar 1995
Fusarium oxysporum	Culture collection	Tao et al 1997
Aspergillus ochraceus 513	Culture collection	Batomunkueva and Egorov 2001
Rhizopus chinensis 12	Starter for brewing rice wine, China	Xiao-Lan et al 2005
Oidiodendron flavum		Tharwat et al 2006
Paecilomyces tenuipes	Culture Collection of DNA Bank	Kim et al 2011
Aspergillus oryzae KSK-3		
Fusarium sp.BLB	Indonesian fermented food 'Temp'	Sugimoto et al 2007
Mucor subtillissimus	Caatinga soil, Serra Talhada, PE-Brazil	Nascimento et al 2015

3.9 Fibrinolytic agents from endophytic fungi

Endophytic fungi are considered as a lucrative source of bioactive metabolites. Many medicinally important compounds have been isolated from endophytic fungi. Preliminary and limited work has been done to isolate fibrinolytic enzymes from endophytic fungi. It stills remains an unexplored ecosystem for thrombolytic agents. Verticase (31 kDa) is a fibrinolytic enzyme isolated from *Verticillium* sp. Tj33, an endophyte of *Trachelospermum jasminoides* (Li et al 2007). *Fusarium* sp. BLB was isolated as an endophyte from leaf of *Hibiscus* plant produced alkaline protease with fibrinolytic activity. The molecular mass and specific activity of the enzyme was 27 kDa and 665 U/mg (Ueda et al 2007). Fu-P, a novel fibrinolytic enzyme (28 kDa) was purified and characterised from endophytic *Fusarium* sp. CPCC 480097. Fu-P was characterised to be chymotrypsin-like serine metalloprotease. It displayed a dose dependent hydrolysis of blood clot. 0.3 mg/ml solution of enzyme dissolved 36.5% of the thrombus under specified conditions (Wu et al 2009 a, b). Over two hundred endophytic fungi were isolated from Las Yungas Pedemontana forest, Argentina and were screened for their fibrinolytic potential. Only *Bionectria ochroleuca* LY 4.1 and LY 4.4 and *Cladosporium cladosporioides* LY 4.2 showed fibrinolytic activity. The enzymes produced were able to degrade fibrin in a direct way (Rovati et al 2010).

3.10 Endophytic fungi: A potential bio–resource of industrially important enzymes

Endophytes comprise of an extremely diverse group of microorganisms that are ubiquitous in plants and maintain a symptomless and unobtrusive union with their hosts for at least a period of their life cycle (Stone et al 2000; Saxena et al 2015). During the alliance, none of the interacting partner is harmed and the benefits obtained are solely dependent on the interacting partners. Thus endophytism is a novel, cost effective plant-microbe association driven by location and not by function (Kusari et al 2012). Endophyte produces several metabolites to cross talk with its host. These metabolites include enzymes which are produced in order to acquire nutrient and colonisation inside the plant tissue and to provide defence against microbial infection (Borges et al 2005). Several enzymes have been isolated and purified from various endophytic which include amylase, cellulose, chitinase, protease, lipase etc (Table 3.7) (Correa et al 2014). *Fusicoccum* sp. BCC 4124 produces thermo and glucose tolerant amylase enzyme (Champreda et al 2007). Amylase has also been isolated from endophytic *Cylindrocephalum* sp, *Gibberella pulicaris*, *Acremonium* sp., *Synnematous* sp. and *Nodilusporium* sp.bysubmerged fermentation. Similarly, *Rhizopus oryzae, Cercospora kikuchii, Fusarium oxysporum* were found to produce lipase enzyme (Costa-Silva et al 2011; Correa et al 2014). Rajulu et al (2011) screened 162 endophytic fungal isolated for their chitinase producing ability. Out of 162, thirty one isolates were found to be producing chitinase enzyme of which *Aureobasidium pullulans* and *Lasiodiplodia theobromae* exhibit maximum production. Patil et al (2012) isolated asparaginase producing endophytic fungi from leaf and bark of *Aegle marmelos.*

Protease enzyme catalyses the hydrolysis of peptide bonds of proteins. They are generally referred to as proteolytic enzymes or proteinases. They are largely used in leather, detergent, food and pharmaceutical industries. Fibrinolytic enzymes generally are serine proteinases in nature. However, reports on the production of fibrinolytic agents/enzymes biosynthesized by endophytic fungi are scarce. Very limited work exists on screening endophytic fungi possessing proteolytic as well as fibrinolytic activity.

Table 3.7 Enzymes produced by various endophytic fungi

Endophytic fungus	**Host plant**	**Enzyme**	**References**
Alternaria sp.	*Eremophilia longifolia*	Amylase	Zhang et al 2010
Preussia minima	*Eremophilia longifolia*	Amylase	Zhang et al 2010
Nigrospora sphaerica	*Costus igneus*	Amylase	Amirita et al 2012
Drechslera hawaiiensis	*Adathoda vasica*	amylase, lipase, protease	Amirita et al 2012
Curvularia vermiformis	*Coleus aromaticus*	cellulase, lipase, protease	Amirita et al 2012
Colletotrichum sp	*Cinnamomum iners, Camellia sinensis*	Cellulase, mannanase, protease, xylanase	Moy et al 2002
Xylaria sp.	*Trichilla connaroides*	Cellulase, mannanase, xylanase, protease	Moy et al 2002
Pestalotiopsis sp.	*Manglietia garrettii*	Cellulase, mannanase	Moy et al 2002
Acremonium sp.	*Acrostichum aureum*	amylase, cellulase, lipase	Maria et al 2005
Alternaria chlamydosporus	*Acanthus ilicifolius*	cellulase, lipase, protease	Maria et al 2005
Aspergillus sp.	*Acrostichum aureum*	cellulase, lipase, protease	Maria et al 2005
Paecilomyces variabilis	*Osbeckia chinensis*	amylase, lipase, protease, xylanase	Bhagobaty and Joshi 2012
Penicillium sp.	*Camellia caduca*	cellulase, lipase, protease, xylanase	Bhagobaty and Joshi 2012
Pestalotiopsis guepinii	*Opuntia ficus-indica*	cellulase, protease, xylanase	Bezerra et al 2012
Fusarium oxysporum	*Musa* sp.	Protease	Ng'ang'a et al 2011
Colletotrichum carssipes	*Lawsonia inerims*	amylase, protease	Amirita et al 2012
Xylaria sp.	*Coleus aromaticus*	amylase, laccase, protease	Amirita et al 2012
Chaetominum globosum	*Glinus lotoides*	Laccase	El-Zayat 2008
Acephala applanata	Conifer roots	Amylases, laccases, proteinases	Reddy et al 1996
Mycelia sterilia	*Trichilla connaroides*	Cellulase, mannanase, xylanase	Moy et al 2002

Verticase, fibrinolytic enzyme has been isolated from endophytic *Verticillium* spp. and was found to be a serine protease (Li et al 2007). An endophytic *Fusarium* CPCC 480097 produces a fibrinolytic enzyme with higher fibrinolytic activity than plasmin (Wu et al 2009b). *Bionectria* sp., from Yungas Pedemontana forest range in Argentina has been reported to be a potential source of direct (plasminogen-independent) fibrinolytic enzymes for different therapeutic purposes (Rovati et al 2010).

Fibrinolytic enzymes isolated from endophytic fungi may act as a t-PA or directly evoke the fibrinolytic activity. Thus proteases from endophytic fungi have a great promise for development of new fibrinolytic agents with least side effects in thrombolytic therapy (Li et al 2007; Wu et al 2009a,b; Ueda et al 2007; Correa et al 2014).

Chapter 4

Materials & Methods

4.1 Plant sample collection

Healthy parts (leaves, stems and bark) of plants belonging to four families (*Apocynaceae, Lauraceae, Rutaceae* and *Taxaceae*) were collected from the conserved rainforest areas of the biodiversity hotspots of India. Six regions from the conserved rain forest areas across the country were chosen for collection of plant samples (Table 4.1). Plants parts of *Aegle marmelos, Cinnamomum malabaricum, Cinnamomum zeylanicum, Tabernaemontana divaricata, Catharanthus roseus* and *Rauwolfia serpentina* were collected from the Western Ghats of India during July 2009. Healthy twigs of *Cinnamomum camphora* were collected from North–eastern Himalayan region and bark of *Taxus baccata* was collected during March 2011 from Almora region in Uttarakhand in the Northern Himalayan ranges. From each plant, five leaves, three stems (3 × 6 cm) and two bark pieces were collected. Plant samples were kept in sterile packets and stored at 4 °C till further use.

Table 4.1 : Information summary on the plant sampling sites

Biodiversity hot spot	Sampling site	State	Geographical coordinates
Western Ghats	BRT wildlife Sanctuary*	Karnataka	11°59′38″N 77°8′26″E
	Yelandur wildlife Sanctuary	Karnataka	12.07°N 77.03°E
	Wayanad wildlife Sanctuary	Kerala	11°38′46″N 76°21′50″E
	Neyyar wildlife Sanctuary	Kerala	8°24′N 77°10′E
North–eastern Himalayas	Darjeeling	West Bengal	27°3′N 88°16′E
North Himalayas	Almora	Uttarakhand	29.62°N 79.67°E

*BRT: Biligiri Ranganatha Swamy Temple Wildlife Sanctuary

4.2 Isolation of endophytic fungi

Plant samples (leaves, bark and stems) were thoroughly washed under running tap water, air dried and cut into small segments of 1 cm. The plant samples were then surface sterilized using 2 % (v/v) sodium hypochlorite (HiMedia, India) for 5 min followed by 70 % ethanol (v/v) for 2 min and 30 % ethanol (v/v) for 1 min. These were then finally rinsed with sterile distilled water and allowed to surface dry under aseptic conditions. The surface sterilized samples were then cut into 2–3 mm segments with the help of sterile blade and placed on potato dextrose agar (PDA, HiMedia, India) with ventral side facing the medium. PDA plates were supplemented with 100 µl of streptomycin (100 mg/l, HiMedia, India) to

check bacterial growth. The plates were then incubated at 26 ± 1 °C for 10–15 days with 12 h light/dark cycles. The efficiency of surface sterilization was confirmed by imprinting the surface sterilised plant part on the PDA plate. The absence of fungal growth on the respective medium portion confirmed the efficacy of surface sterilization. Individual colonies after proper incubation were picked from the edge of an advancing colony with sterile fine needle and transferred onto fresh PDA plates. These cultures were considered as pure isolates of endophytic fungi (Schluz et al 1993; Naik et al 2008; Zhang et al 2010). The endophytic fungal isolates were tentatively identified using various mycological keys (Booth 1971; Alexopoulos et al 1996; Seifert et al 1996; Barnett and Hunter 1998; Abdollahzadeh 2010). Further, the endophytic fungal cultures were encoded based on the plant isolated, its part and the place from where it was collected. For example #37 CRSTBRT (where #37 refers to segment number; CR stands for host plant *Cathranthus roseus*; ST refers to stem, BRT refers to BRT Wild Life Sanctuary, Karnataka (Place of sample collection) (Fig 4.1). The endophytic fungi were maintained on glycerol–PDA slants (Composition Glycerol–PDA: 39 g of premade PDA (HiMedia, India dissolved in 1000 ml of lukewarm distilled water and supplemented with 10 % glycerol; autoclaved at 121 °C, 15 psi for 15 min). For each isolate, three slants were prepared. The isolates were further stored at 4 °C (Ezra et al 2004).

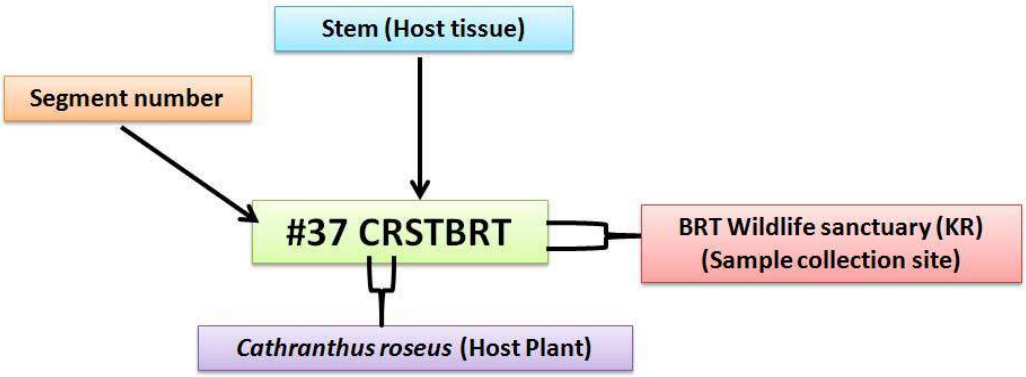


Fig 4.1: Method of coding the pure isolates of endophytic fungi

4.3 Production of culture filtrates

The endophytic fungi were subjected for production of culture filtrate in a chemically defined medium. Briefly, the method comprised of aseptic inoculation of 5 mm mycelial disc of a 7–day old culture of

endophytic fungus in 25 ml pre–sterilized Czapek Dox broth (CDB, HiMedia, India) followed by incubation at 26 ± 1 °C, 130 revolutions per minutes (rpm) for 15 days. After completion of the incubation period, mycelial mass was separated using Whatman filter paper no. 4 (GE Healthcare Life Sciences, USA), followed by centrifugation at 8,000 ×g (Hitachi RX II series, Japan) for 10 min at room temperature. The supernatant was then passed through 0.22 µm nitrocellulose membrane (GE Healthcare Life Sciences, USA) to make it cell free (Ali and Ibrahim 2008).

4.4 Qualitative plate assays for enzyme activities

Two *in vitro* qualitative plate assays viz. proteolytic and fibrinolytic were employed for preliminary screening of culture filtrate for its proteolytic and fibrinolytic potential.

4.4.1 *In vitro* proteolytic assay

The assay comprised of preparation of 1 % skimmed milk plates dissolved in 20 mM Tris–HCl buffer, pH 7.8) (HiMedia, India) as a substrate together with 1 % w/v agar (HiMedia, India) (Rovati et al 2010). After solidification of the medium, 5 mm wells were scooped in which 20 µl of culture filtrate of endophytic fungi were dispensed aseptically, sealed and incubated at 37 °C for 18–24 h. Un–inoculated CDB medium served as control. After culmination of the incubation, the proteolytic activity was assessed by measuring diameter of the halo formed around the wells due to hydrolysis of the milk protein, casein. All the tests were performed in triplicates and their mean and standard deviation was calculated.

4.4.2 *In vitro* fibrinolytic assay

Fibrinolytic activity was determined by a modified procedure described by Astrup and Mullertz (1952) which involved preparation of plasminogen rich and plasminogen free fibrin plates. Plasminogen free fibrin plates were prepared by mixing 5 ml of 0.5 % w/v bovine fibrinogen (Calbiochem, Darmstadt, Germany) in 20 mM Tris–HCl buffer (pH 7.8) with 10 ml of 1 % agarose (HiMedia, India) and 100 µl of thrombin (100 NIH U/ml, Sigma Aldrich, USA). Plasminogen rich plates were supplemented with 5 U of plasminogen (Calbiochem, Darmstadt, Germany). Plates were allowed to stand for 30 min at room

temperature to form fibrin clot. Subsequently, 20 µl of culture filtrate was dispensed into 5 mm wells previously made with a sterile cork borer. Plates were then incubated at 37 °C for 24 h. After incubation, the diameter of the clear zone was measured and number of units was determined by using a standard curve of plasmin (human plasma, Calbiochem, Darmstadt, Germany). The diameter of the clear zone was directly proportional to the potency of the fibrinolytic activity (Cui et al 2008; Choi et al 2013a, b).

4.4.2.1 *Standard curve of plasmin*

Plasminogen free fibrin plates were prepared as described in the section 4.4.2. Briefly, 5 mm wells were punched and 20 µl of plasmin at varying concentrations (10 U, 5 U, 2.5 U, 1.25 U, and 0.625 U) (from human plasma, Calbiochem, Darmstadt, Germany) was dispensed in each well. The plates were then incubated at 37 °C for 24 h and zone of clearance around each well was recorded. Diameter of halo was calculated by diagonally measuring the clear zone around the well. Each concentration of plasmin was tested in triplicates and their mean and standard deviation was calculated (Rovati et al 2010; Mahboubi et al 2011). Subsequently, a standard curve was plotted between the concentration of plasmin and diameter of halo formed for correlation of plasmin like fibrinolytic activity of culture broth, crude and pure enzyme.

4.5 Protein precipitation from culture broth of selected isolates

The endophytic fungal isolates exhibiting both plasmin like direct fibrinolytic activity and t-PA like indirect fibrinolytic activities in the *in vitro* fibrinolytic assay were selected for further characterization. The crude enzyme extract of these selected isolates was obtained by using cold acetone as well as ammonium sulphate precipitation methods.

4.5.1 Cold acetone precipitation method

To one volume of the culture broth, four volumes of chilled acetone (Merck, AR grade) was slowly added and stored at 4 °C for overnight. The next day, protein precipitate was collected by centrifugation at 13,000 ×g (Hitachi RX II series, Japan) for 15 min at 4 °C. The precipitate was then dissolved in minimum

volume of 20 mM Tris–HCl buffer (pH 7.8) (Mukherjee et al 2012; Kotb 2014b). The precipitate so obtained was tested for its *in vitro* fibrinolytic activity *via* fibrin plate assay as described previously (Section 4.4.2) while the protein content was determined by Bradford's method (Bradford 1976).

4.5.2 Ammonium sulphate precipitation method

Solid ammonium sulphate (HiMedia, India) was slowly added to the culture broth to achieve saturation with slow and continuous stirring at 4 °C. The mixture was then incubated overnight at 4 °C and the next day protein precipitate was collected by centrifugation at 13,000 ×g (Hitachi RX II series, Japan) for 15 min at 4 °C. The precipitate was then dissolved in minimum volume of 20 mM Tris–HCl buffer (pH 7.8) and further dialysed against the same buffer overnight. Subsequently, the dialysed fraction was recovered after centrifugation at 13,000 ×g for 15 min at 4 °C. The dialysed precipitate so obtained was tested for *in vitro* fibrinolytic activity via fibrin plate assay as described previously (Section 4.4.2), while the protein content was determined by Bradford's method (Ueda et al 2007; Wu et al 2009b; Shirasaka et al 2012)

4.6 Determination of protein content of crude enzyme extract

Protein content of crude enzyme extract was determined by using Bradford's method. Briefly, 10 µl of the protein solution was mixed with 90 µl of Bradford's reagent (Sigma Aldrich, USA) and incubated at 37 °C for 30 min and absorbance was measured at 595 nm using a BIOTEK® Powerwave 340 plate reader. The protein content of crude enzyme extract was determined against a reference curve of bovine serum albumin (BSA) (HiMedia, India) (Bradford 1976; Wu et al 2009b).

4.7 Optimization of suitable culture conditions

Culture conditions of the selected endophytic isolate (#37 CRSTBRT) exhibiting maximum extracellular *in vitro* fibrinolytic activity during preliminary screening were further optimized.

4.7.1 Selection of suitable medium for optimal *in vitro* fibrinolytic activity

Six broths viz. CDB (HiMedia, India), malt extract broth (MEB, HiMedia, India), potato dextrose broth (PDB, HiMedia, India), Richard's broth (HiMedia, India), tryptone soya broth (TSB, HiMedia, India) and yeast extract peptone dextrose broth (YEPB, HiMedia, India) were inoculated with the selected endophytic isolate to identify the culture broth inducing maximum *in vitro* fibrinolytic activity by fibrin plate assay. Briefly, 50 ml of pre–sterilized broth was inoculated with 5 mm mycelial plug of 7-day old culture of #37 CRSTBRT and then incubated at 26 ± 1 °C, 130 rpm for 15 days (Park et al 2007; Ali and Ibrahim 2008; Kumaran et al 2011). After the culmination of incubation period, the spent broths were separated from the fungal biomass by filtration to obtain the culture filtrates. Subsequently, the culture filtrates were precipitated with ammonium sulphate as described in section 4.5.2 and the protein precipitate so obtained was tested as a crude enzyme for its *in vitro* fibrinolytic activity using plasminogen free fibrin plate method as described previously in section 4.4.2.

4.7.2 Evaluation of *in vitro* fibrinolytic activity of intracellular proteins

To ascertain that the fibrinolytic activity was due to extracellular protein produced by #37 CRSTBRT, culture filtrate was produced using CDB as described in section 4.3. The fungal biomass was separated from the broth using Whatman filter paper no 4. Approximately, 10 g of the fungal biomass was homogenized using 20 mM Tris–HCl buffer (pH 7.8) and subsequently centrifuged at 10,000 ×g for 10 min to separate cellular debris from the homogenate. The homogenate was then subjected to ammonium sulphate precipitation method and the precipitate so obtained was tested for its *in vitro* fibrinolytic activity as described in section 4.4.2 (Kim and Kim 1999; Ali et al 2014).

4.7.3 Selection of optimal ammonium sulphate concentration for protein precipitation

Culture broth of endophytic isolate #37 CRSTBRT was prepared as described in the section 4.3. Solid ammonium sulphate (HiMedia, India) was slowly added to the culture broth to achieve 20–80 % saturation and incubated at 4 °C overnight. The amount of ammonium sulphate to be added to achieve

desired saturation percentage was calculated using an online ammonium sulphate calculator (http://www.encorbio.com/protocols/AM-SO4.htm). The next day protein precipitate was collected by centrifugation at 13,000 ×g (Hitachi RX II series, Japan) for 15 min at 4 °C (Green and Hugans 1954; Cha et al 2010; Huang et al 2013). The precipitate was then dissolved in minimum volume of 20 mM Tris–HCl buffer (pH 7.8). The fractions so obtained were tested for *in vitro* fibrinolytic activity as described previously in section 4.4.2.

4.7.4 Selection of optimal buffer concentration for *in vitro* fibrinolytic activity

To ascertain optimal buffer concentration for maximum *in vitro* fibrinolytic activity, the crude enzyme extract was prepared as described in section 4.5.2. The precipitate was then dissolved in minimum volume of Tris–HCl having varying concentrations within the range of 10 mM to 60 mM buffer (pH 7.8). The precipitates were then tested for *in vitro* fibrinolytic activity as previously described in section 4.4.2.

4.8 Identification of endophytic fungus exhibiting potential in *vitro* fibrinolytic activity

Endophytic isolate #37 CRSTBRT which exhibited the highest *in vitro* fibrinolytic activity was identified using classical morphological and molecular taxonomic tools.

4.8.1 Morphotaxonomy

The endophytic isolate #37 CRSTBRT was first identified using morphological characters. The culture was grown on 11 different media including PDA, 2 % malt extract agar (MEA, HiMedia, India) , 2 % Corn meal agar (CMA, HiMedia India), 2 % oat meal agar (OMA), synthetischer nahourstoffarmer agar (SNA), 2 % yeast extract peptone dextrose agar (YEPDA, HiMedia, India), 1 % water agar (WA), pine leaf agar (PLA), cathranthus leaf agar (CLA), cathranthus paste agar (CPA) to induce sporulation. These were incubated at 28 °C for six–eight weeks under different culture conditions viz. light, dark, alternate light and dark cycles and UV radiations. The fungus was also grown directly on wood turnings at 28 °C. The morphological characters including colony size, texture and color were taken into account for the

identification of the fungus (Petrini and Petrini 1985; Guo et al 1998; Fournier et al 2011; Chen et al 2013; Sanchez–Ortiz et al 2016).

4.8.1.1 *Microscopic identification*

For microscopic studies, mycelium was picked with a sharp needle and was placed over clean glass slide containing a drop of water. The mycelial mass was teased and separated into fine filaments with help of needle. A drop of lactophenol cotton blue (HiMedia, India) was added and then the cover slip was placed avoiding trapping of any air bubble. The glass slide was gently tapped twice. The slide was mounted using DPX (SD fine chemicals, India) and observed under different magnifications (40X–1000X) using a Nikon eclipse 50i microscope coupled with CCD camera and measurements carried out using NIS element software (Stadler et al 2005; 2008).

4.8.2 Molecular taxonomy

For precise delineation of the endophytic fungus #37 CRSTBRT, molecular taxonomic tools like genomic DNA isolation, PCR amplification, sequencing and its phylogenetic placement was carried out.

4.8.2.1 *Genomic DNA isolation*

Genomic DNA extraction was done by using the Wizard Genomic DNA purification kit (Promega, Madison, USA). About 0.1–0.2 g of cultured mycelia were scrapped off from 3 to 4 days old culture with sterile inoculation loop and crushed to very fine powder in a pestle and mortar using liquid nitrogen. Subsequently, 600 µl of nuclei lysis buffer was added and crushed gently. The content was then transferred into fresh microcentrifuge tube (Tarsons, India), vortexed and incubated at 65 °C for 15 min in water bath (Stuart Water Bath SBS40, NJ, USA), with mixing after every 5 min. After incubation in water bath for 15 min, the content was centrifuged at 10,000 ×g for 5 min. The supernatant was then transferred to fresh microcentrifuge tube which was followed by addition of 5 µl of RNase (10 mg/ml) and further incubation at 37 °C for 15 min. Subsequently, 200 µl of protein precipitating solution was added, vortexed for 20 s and centrifuged at 12,000 ×g for 3 min. The supernatant again transferred into

fresh microcentrifuge tubes containing 600 µl of isopropanol and mixed gently by inverting the tubes. Initially, the threads of DNA were seen in the tubes which were then centrifuged at 12,000 ×g for 3 min for precipitation of the DNA. The obtained DNA pellet was washed with 70 % ethanol (AR grade, Merck, USA) followed by centrifugation at 12,000 ×g for 1 min. The pellet was air dried, dissolved in 50 µl of DNA rehydration buffer and was kept at 4 °C overnight (Kapoor and Saxena 2014; Gupta et al 2015a, b).

4.8.2.2 *Qualitative and quantitative estimation of genomic DNA*

Agarose gel (0.8 %, HiMedia, India) was prepared in 1X Tris acetate EDTA and 0.5 µg /ml of ethidium bromide (HiMedia, India) was added to it. The gel was casted in a BioRad mini gel electrophoresis chamber and allowed to solidify. After solidification, the comb was carefully removed and running buffer (1X TAE) was poured into the electrophoretic tank so that the gel remained fully immersed in the buffer. The DNA samples were then mixed with 5X loading buffer and loaded into the wells. A constant voltage of 50 V was maintained till the tracking dye reached three fourth of the gel during the electrophoretic separation. The gel was then observed under UV transilluminator to confirm the presence of DNA. Further, the gel imaging was performed under UV light in Bio–Rad gel documentation system using Quantity–1–D analysis software. Quantitative estimation of the genomic DNA was done by spectrophotometric analysis of the sample. The absorbance of the sample was taken at 260 nm, to determine the concentration of the sample. 1 OD is equivalent to 50 µg/ml DNA sample. The concentration of the DNA was calculated by using the following formula

$$\text{DNA Concentration (µg/ml)} = OD_{260nm} \times 50\ \mu g/ml \times \text{Dilution factor}$$

The purity and quantity of DNA was checked using NanoDrop 2000 (Thermo Scientific™, USA) at 260 nm and 280 nm (Kapoor and Saxena 2014; Gupta et al 2015a, b).

4.8.2.3 *PCR amplification*

Three nuclear gene fragments viz. internal transcribed spacer (ITS) region, RNA polymerase subunit II (RPBII) and large ribosomal subunit (LSU) were amplified for species level identification of #37 CRSTBRT

(Table 4.2). The PCR reaction mixture composition for amplification of ITS and RPBII region was same. Amplification was performed in a 25 µl reaction mixture volume comprising of 1µl of extracted genomic DNA, 0.8 µM of each primer pair (Xcleris labs Pvt. Ltd, Gujarat, India), 2.5 mM of dNTP (Genei, Bangalore, India), 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 1.5 U of *Taq* DNA polymerase (Genei, Bangalore, India).

Table 4.2 Primer sequences used for the amplification of genomic DNA

Primer	**Sequence (5'–3')**	**Reference**
ITS1	TCCGTAGGTGAACCTGCGG	White et al 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al 1990
RPBII–5f	GAYGAYMGWGATCAYTTYGG	Liu et al 1999
RPBII–7cr	CCCATRGCTTGYTTRCCCAT	Liu et al 1999
LROR	ACCCGCTGAACTTAAGC	Vilgalys and Hester 1990
LR5	TCCTGAGGGAAACTTCG	Vilgalys and Hester 1990

The thermal cycling parameters for ITS and RPBII region amplification was as follows: initial denaturation at 96 °C for 5 min followed by 39 cycles of 95 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s followed by final extension at 72 °C for 5 min (O'Donnell et al 1998). Whereas, for amplification of LSU region, the reaction mixture comprised of 1 µl of extracted genomic DNA, 0.6 µM of each primer pair (Xcleris labs Pvt. Ltd, Gujarat, India), 2.5 mM of each dNTP (Genei, Bangalore, India), 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 1.5 U of *Taq* DNA polymerase (Genei, Bangalore, India). The cycling parameters were: initial denaturation at 95 °C for 3 min followed by 35 cycles of 94 °C for 60 s, 56 °C for 90 s, 72 °C for 70 s followed by final extension at 72 °C for 5 min (Rovati et al 2010; Shirasaka et al 2012). The amplified products of ITS–rDNA, RPBII and LSU regions were sequenced at Xcleris Lab, Ahmedabad, Gujarat, India.

4.8.2.4 *Sequence assembly and phylogenetic placement*

The chromatograms obtained after sequencing were checked for their purity (≥ 95%) using Sequencher ver 5.0 (www.genecodes.com). Further, the sequences were assembled to generate final consensus

sequence. The final consensus sequences were then submitted to GenBank. The ITS, RPBII and LSU sequences of #37 CRSTBRT were subjected to BLAST similarity search at the NCBI website to ascertain its homology with the closely related organisms.

Three phylogenetic trees based on ITS, RPBII, and LSU were constructed using MEGA 5.0. Each phylogenetic tree comprised of sequence under study, the reference taxa obtained from BLAST similarity search and one out group (Tamura et al 2011). The sequences were then aligned with CLUSTALW in MEGA 5.0. The aligned sequences were subsequently edited so as to make alignment uniform. The aligned sequence file was then exported to FASTA and MEGA format. The evolutionary history was inferred using the Neighbor–Joining method (Saitou and Nei 1987). 1000 bootstrap replicates were taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The percentage of replicate trees in which the closely related taxa were clustered together in the bootstrap test was shown next to the branches. The evolutionary distances were calculated by employing the Maximum Composite Likelihood (MCL) method and represented in the units of the number of base substitutions per site. Gamma distribution (shape parameter = 5) was used to deduce rate variation among sites. Gaps were treated as missing data (Tamura et al 2004).

4.9 Enzyme purification

For purification of fibrinolytic enzyme, the selected organism was grown on CDB to obtain 10 l culture filtrate which was then subjected to ammonium sulphate precipitation followed by chromatographic and electrophoretic techniques to isolate the pure protein possessing fibrinolytic activity (Fig 4.2).

4.9.1 Ammonium sulphate precipitation

Solid ammonium sulphate (HiMedia, India) was slowly added to the culture broth to achieve 60 % saturation. The next day, protein precipitate was collected by centrifugation at 13,000 ×g (Hitachi RX II series, Japan) for 15 min at 4°C. The precipitate was dissolved in minimum volume of 20 mM Tris–HCl

buffer (pH 7.8). The crude enzyme extract was dialysed against the same buffer overnight at 4 °C using a 10 kDa dialysis membrane (HiMedia, India). Further, the enzyme solution was concentrated using amicon filters (10 kDa, Sigma Aldrich, USA) (Cui et al 2008; Wu et al 2009b; Simkhada et al 2010).

4.9.2 Ion exchange chromatography

The protein solution (2 ml) was loaded onto column containing anion exchanger (Q–Sepharose, Sigma Aldrich, USA) with a bed volume of 3 cm equilibrated with 20 mM Tris–HCl, pH 7.8. The bound proteins were eluted with a linear gradient of 0-0.5 mM NaCl (HiMedia, India). The fractions were pooled and analysed by Sodium dodecyl sulphate–Polyacrylamide gel electrophoresis (SDS–PAGE). Protein content was analysed at 280 nm (Bi et al 2013a; Choi et al 2013a).

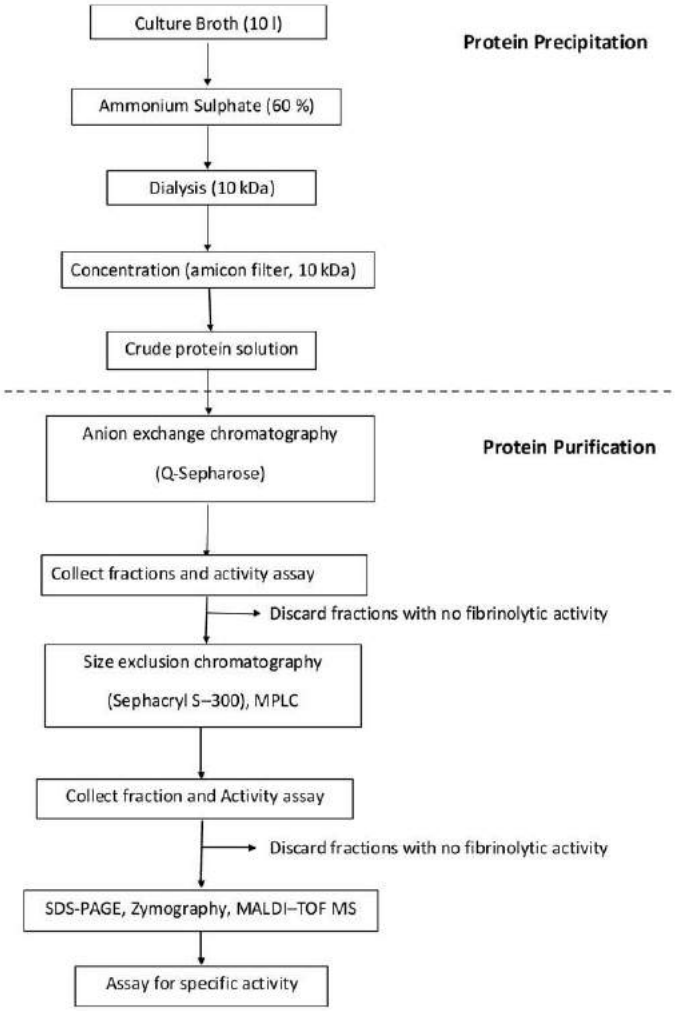


Fig 4.2: Flow chart displaying the scheme for purification of fibrinolytic enzyme from culture filtrate of #37 CRSTBRT

4.9.3 Size exclusion chromatography

The protein fractions were further purified by medium pressure liquid chromatography (MPLC) (Akta Prime, GE Healthcare Life Sciences) using Sephacryl S–300 column (72 cm column length, 1.5 cm internal diameter, 60 cm gel bed volume, Sigma Aldrich, USA) with a flow rate 1 ml/min, pressure 1 mPa and fraction size of 2 ml. The protein content of the eluted fractions was analysed at 280 nm. The fractions obtained were tested for their fibrinolytic activity using *in vitro* fibrin plate assay, those fractions having fibrinolytic activity were pooled, concentrated and used as pure enzyme. The homogeneity of the pure enzyme was confirmed by SDS–PAGE (Bi et al 2013a; Choi et al 2013a).

4.10 SDS-PAGE of eluted proteins

SDS–PAGE of the eluted protein fractions was performed following Laemmli's method (1970) with 5 % stacking and 10 % separating gel (refer appendix (A) for composition). The gel was 0.75 mm thick. The molecular mass of the denatured protein was estimated using a standard protein weight marker (20–205 kDa, Merck, Millipore, USA). The resolved proteins were detected in the gel by modified silver staining method (Blum et al 1987; Cha et al 2010).

Modified Silver Staining Method: The gel was soaked in destain I (40 % methanol + 7 % acetic acid) for 30 min at 37 °C. Destain I was removed and the gel was then gently agitated in destain II solution (5 % methanol + 7 % acetic acid) for 30 min. After removal of destain II solution, the gel was then placed in cross linking solution (10 % glutaraldehyde, Sigma Aldrich, USA) for 30 min. The cross linking solution was decanted and the gel was then washed with several changes of double distilled water for two hours with change after every 15 min. Further, the gel was placed in dithiothreitol (DTT, Sigma Aldrich, USA) solution (5 mg/ml) for 30 min. DTT solution was drained out and the gel was then transferred to silver nitrate solution (0.1 %, Sigma Aldrich, USA) for 30 min in dark. The traces of silver nitrate solution were removed by washing the gel with several changes of double distilled water. Finally, the gel was developed by adding developing solution (3 % sodium bicarbonate, 0.1 % sodium thiosulfate, 0.019 %

formaldehyde). As the resolved protein bands started to appear in the gel, the reaction was stopped by adding destain II solution.

In vitro fibrinolytic assay of the eluted proteins were carried out as per the fibrin plate assay (Section 4.4.2) based on which the fibrinolytic protein was further characterized. The purified enzyme was named 'Xylarinase' meaning fibrinolytic enzyme from *Xylaria.*

4.11 Molecular weight determination of xylarinase

The molecular weight of the purified fibrinolytic enzyme (xylarinase) was ascertained using Native–PAGE, SDS–PAGE, fibrin zymography and MALDI–ToF MS. Further the purity of enzyme was determined by HPLC.

4.11.1 Native–PAGE of xylarinase

Native PAGE was performed with 4 % stacking and 10 % separating gel in a Bio–Rad electrophoresis system at room temperature. A constant voltage of 50 mA was maintained in 1X Tris–glycine buffer until the tracking dye reached the bottom of the gel. The resolved protein was detected in the gel by using silver staining method as described earlier in section 4.10 (Laemmeli 1970; Blum et al 1987; Ju et al 2012; Shirasaka et al 2012).

4.11.2 SDS-PAGE of xylarinase

SDS–PAGE of xylarinase was performed as described in section 4.10 (Laemmli 1970; Blum et al 1987; Cha et al 2010).

4.11.3 Fibrin zymography of xylarinase

Fibrin zymography was performed according to the method of Kim et al (1998). Resolving gel solution (10 %) contained 0.12 % (w/v) fibrinogen prepared in a total volume of 10 ml and centrifuged to remove insoluble impurities which were induced when SDS stock solution was mixed. Thrombin solution (1 U/ml) and TEMED (N, N, N', N'–tetramethylethylenediamine) were added to the gel solution in final concentrations of 0.1 U/ml and 0.028 % (v/v), respectively. The purified enzyme was electrophoresed on

a fibrin gel. The gel was then washed in 2.5 % Triton X–100 solution, incubated in zymogram reaction buffer {30 mM Tris (pH 7.4) containing 200 mM NaCl, 10 mM $CaCl_2$, and 0.02 % NaN_3} at 37 °C overnight, stained with Coomassie brilliant blue R–250, and subsequently de–stained. The cleared white area against the blue gel represents fibrin degradation (Kim et al 2011; Ju et al 2012).

4.11.4 MALDI–ToF MS analysis of xylarinase

SDS–PAGE of the purified protein sample was carried out and resolved protein band was carefully sliced from the gel using a scalpel blade and minced into small sections. The gel pieces were dehydrated using 100 % acetonitrile (40 µl) for 5 min. Further, acetonitrile was removed and the gel pieces were destained thrice with 25 mM ammonium bicarbonate (30% acetonitrile) for 30 min. The gel pieces were again dehydrated in acetonitrile for 10 min and then vacuum dried. About, 10 µl of trypsin solution (0.02 µg/µl, MS grade, Merck Millipore, USA) was added and incubated at 37 °C for an hour. Further, 40 µl of the trypsin solution was added and incubated at 37 °C overnight. The trypsinized samples were then centrifuged 13,000 ×g (Hitachi RX II series, Japan) and supernatant collected. The proteolyzed fragments were then sonicated with 40 µl of 60 % acetonitrile in 0.1 % trifluro acetic acid for 30 min. The extracted sample was concentrated to about 10 µl by speed-vac. Further, zip–tip purification of the above sample was performed using C18/C4 resin. The molecular weight of the protein sample was then determined by Matrix–assisted Laser desorption/ionization–time of flight mass spectrometry (MALDI–ToF MS) using an AB SCIEX TOF/TOF™ 5800 system and molecular mass was calculated using MASCOT and ProteinPilot software (Kim and Kim 1999; Choi et al 2013b; Kang et al 2016).

4.11.5 Determination of purity of xylarinase by HPLC

Purity of xylarinase was analysed using High Performance liquid chromatography (HPLC) (Shimadzu LC system, Japan) equipped with C_{18} column (Merck Millipore, USA). The mobile phase was water (HPLC grade, Merck Millipore, USA) and acetonitrile (HPLC grade, Merck Millipore, USA) in the ratio 1:1. The purity was authenticated at 250 nm (Bi et al 2013b; Babu and Devi 2015).

4.12 N–terminal amino acid sequencing of xylarinase

To determine the N–terminal amino acid sequence, the purified enzyme was first subjected to SDS–PAGE. Further, the SDS gel and polyvinylidenedi fluoride membrane were sandwiched in a blotting cassette. The proteins were transferred at 350–400 mA for 35 min. The membrane was then rinsed 2–3 times in Milli Q water. The membrane was subsequently stained with 0.1 % amido black in 1 % Ponceau S for 2–3 min and then destained with 50 % methanol. The desired bands were excised from the membrane and amino acids of the N–terminal sequence were determined using Applied Biosystems PROCISE 491 cLC protein sequencer at Institute of Microbial Technology (IMTECH) Chandigarh, India. The homology of the purified enzyme with other proteinases was performed using NCBI "BLASTp" search program and the sequences so obtained were aligned in CLUSTALW (Bi et al 2013a; Choi et al 2013a).

4.13 Fibrinolytic and fibrinogenolytic activity of xylarinase

The fibrinolytic activity was analyzed according to the modified protocol of Datta et al (1995). 200 µl of 1 % fibrinogen in 20 mM Tris–HCl buffer was mixed with 20 µl of thrombin (10 NIH U/ml). The fibrin clot was allowed to form at room temperature for 1 h. Further, 50 µl (1 mg/ml) of xylarinase was added to the fibrin clot and incubated at 37 °C for different intervals ranging 10 to 180 min and overnight (10, 20, 30, 40, 50, 60, 90, 120, 150, 180 min and overnight). Plasmin served as positive control. At various time intervals, an aliquot of 20 µl was withdrawn and 10 µl of the denaturing solution (1 M urea, 4 % β–mercaptoethanol and 4 % SDS) was added and analysed by 10 % SDS–PAGE (Kim et al 2011; Choi et al 2013a)

Fibrinogenolytic activity of xylarinase was determined by modified method of Matsubara et al (2000). About 250 µl of 1% fibrinogen in 20 mM Tris–HCl buffer (pH 7.8) was incubated with 50 µl (1 mg/ml) of xylarinase at 37 °C. At various time intervals, 20 µl of the fraction was withdrawn and analysed by SDS–PAGE. Plasmin was used as a control (Ju et al 2012; Choi et al 2013a).

4.14 Fibrinogen clotting time assay

Fibrinogen clotting time (FCT) assay was performed according to the method described by Zingali et al (1993) with minor modifications. Four treatments were used to ascertain the proteolytic action of xylarinase on fibrinogen or thrombin. First, the normal clotting time was determined by using 50 µl of 1 % fibrinogen and 30 µl of 5 U/ml thrombin, this was referred to as control. Treatment A comprised of incubation of 20 µl xylarinase (0.5 mg/ml) with 50 µl of 1 % fibrinogen (20 mM Tris HCl, pH 7.8) for 5 min at 37 °C followed by addition of 30 µl of 5 U/ml of thrombin and determination of clotting time after addition of thrombin.

In treatment B1, xylarinase (20 µl) was incubated with 100 µl of 1 % fibrinogen while in treatment B2, xylarinase (20 µl) was incubated with 150 µl of 1 % fibrinogen (20 mM Tris–HCl, pH 7.8) for 5 min at 37 °C was used. Thereafter, 30 µl of 5 U/ml of thrombin was added in both treatment B1 and B2 and clotting time was determined. In treatment C1, xylarinase (20 µl) was incubated with 50 µl of 1 % fibrinogen for 5 min at 37 °C. Subsequently, 60 µl of 5 U/ml thrombin was added and clotting time determined. The C2 treatment was similar to C1 except that the volume of thrombin added was 90 µl after which the clotting time was determined (Wu et al 2009a). All the experiments were carried out in triplicates and their mean ± SD values were calculated.

4.15 Biochemical characterization of xylarinase

Herein different biochemical properties of xylarinase was established by varying pH, temperature, effect of metal ions and inhibitors which further helped in determination of kinetic constants and probable nature of enzyme.

4.15.1 Effect of pH on fibrinolytic activity of xylarinase

The optimal pH for fibrinolytic activity was determined within a pH range of 3.0–10.0. Xylarinase (10 µl, 1 mg/ml) was added to 90 µl of 20 mM glycine–HCl (pH 4), sodium citrate (pH 5), sodium phosphate (pH 6 and 7), Tris–HCl (pH 8), glycine–NaOH (pH 9 and 10) buffer system. The master mixtures were

incubated for 1 h and the enzyme activities were measured by *in vitro* fibrin plate assay as described earlier 4.4.2. Native enzyme at pH 7.8 served as control. All the tests were performed in triplicates. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values (Cui et al 2008).

4.15.2 Effect of temperature on fibrinolytic activity of xylarinase

The optimal temperature for fibrinolytic activity was determined by measuring the residual activity after incubating xylarinase (10 µl, 1 mg/ml) with 90 µl of 20 mM Tris–HCl buffer at different temperatures from 25–70 °C for 1 h. All the tests were performed in triplicates and the relative enzyme activities were measured by *in vitro* fibrin plate assay as described earlier. Native enzyme at 35 °C served as control in the study. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values (Cui et al 2008).

4.15.3 Effect of metal ions on fibrinolytic activity of xylarinase

Effect of metal ions on fibrinolytic activity was evaluated using $CaCl_2$, $MgCl_2$, KCl, $CoCl_2$, $ZnCl_2$, $MnCl_2$, $CuCl_2$, $FeCl_2$, $AlCl_3$, NaCl. Xylarinase (10 µl, 1 mg/ml) was incubated in presence and absence of cations such as Al^{3+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and Na^{+} with a final concentration of 2 mM in 20 mM Tris–HCl buffer for 1 h at 37 °C. Fibrinolytic enzyme without addition of cation served as control. The effect of metal ions on fibrinolytic activity was then analysed by *in vitro* fibrin plate assays as described earlier and expressed as relative mean fibrinolytic activity. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values (Bi et al 2013a).

4.15.4 Effect of protease inhibitors on fibrinolytic activity of xylarinase

The nature of the fibrinolytic enzyme was determined by assessing the effect of inhibitors like phenylmethyane sulfonyl fluoride (PMSF), toslyl pheneylalanine chloromethyl ketone (TPCK), ethylenediaminetetraacetic acid (EDTA), ethylene glycolbis–(2–aminoethyl)–*N',N',N',N'*tetraacetic acid

(EGTA), leupeptin, aprotinin and β–mercaptoethanol on xylarinase. Xylarinase (10 µl, 1 mg/ml) was incubated with 2 mM concentration of the protease inhibitors for 1 h at 37 °C. Xylarinase without any inhibitor served as control. The residual fibrinolytic activity was analysed by *in vitro* fibrin plate assay and expressed as relative mean fibrinolytic activity. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values (Bi et al 2013a).

4.16 Determination of kinetic constants of xylarinase

The kinetic constants K_m and V_{max} of xylarinase were determined by modified method of Mukherjee et al 2012. The initial reaction rate with increasing concentration of fibrin (2–10 mg/ml) and xylarinase (10 µl, 1mg/ml) per reaction well were recorded. The titre plate was then incubated at 37 °C for 3 h and the kinetic read was recorded every 10 min using BIOTEK® Powerwave 340 microplate reader at 630 nm. Non–linear regression fit tool was used in GraphPad Prism 5.0 to determine the K_m and V_{max} of xylarinase. One unit of enzyme activity was defined as the amount of enzyme causing conversion of 1 mM of substrate per minute per milligram of protein at 37 °C. Plasmin was used as standard (Mukherjee et al 2012; Kotb 2014b).

4.17 Spectrophotometric assay for *in vitro* fibrinolytic activity of xylarinase

For spectrophotometric analysis of the fibrinolytic activity, 90 µl of the 1 % bovine fibrinogen (20 mM Tris–HCl) was mixed with 10 µl of thrombin (10 NIH U/ml). The clot was allowed to form at 37 °C for 30 min. Subsequently, 10 µl xylarinase (1 mg/ml) was added in each well followed by incubation at 37 °C for 1 h. The reaction was terminated by addition of 100 µl of chilled trichloro acetic acid (TCA, 10 %) and incubated over ice bath for 10 min. This was followed by centrifugation at 8,000 ×g for 10 min. Subsequently, Folin's reagent (Sigma Aldrich, USA) was added and the absorbance measured at 660 nm using BIOTEK® Powerwave 340 microplate reader. Plasmin (10 U) was used as standard and 20 mM Tris–

HCl buffer act as control. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg of L-tyrosine/ml/min at 37 °C (Fig 4.3, Appendix)(Kotb 2014b).

4.18 *In vitro* thrombolytic activity assay

To assess the *in vitro* thrombolytic activity of xylarinase, fresh human blood was collected from a healthy volunteer. About 500 µl of the blood was dispensed in a microcentrifuge tube and allowed to coagulate at 37 °C for 1 h after which it was rinsed, weighed and then dipped in different concentration of enzyme (10–50 µg). Physiological saline was used as negative control whereas plasmin served as positive control. After 1 h, the residual thrombus was weighed and the dissolution ratio of the thrombus was calculated using the following equation;

$$\text{Dissolution ratio} = (\text{Weight}_0 - \text{Weight}_t / \text{Weight}_0) \times 100$$

Where, Weight_0 is the weight of the thrombus prior to treatment, Weight_t is the weight of the thrombus post treatment (Wu et al 2009a; Babu and Devi 2015).

4.19 *In vitro* anticoagulant activity assay

Blood was collected from the healthy volunteer in a test tube containing 3.2 % sodium citrate solution. Platelet poor plasma was obtained by centrifugation of the blood sample at 2,000 ×g for 10 min. For pro–thrombin (PT) time assay, 100 µl of the platelet poor plasma was mixed with different concentration (5 µg, 10 µg and 20 µg) of xylarinase (20 µl) and incubated at 37 °C for three minutes. Further, 100 µl of the PT solution was added and clotting time was observed using automated coagulometer (Chem 7, Erbamallhein, Germany). Human plasma without addition of fibrinolytic enzyme served as control (Choi et al 2013b; Kang et al 2016).

For activated partial thromboplastin time (APTT) assay, 100 µl of the platelet poor plasma was mixed with varying concentration (5 µg, 10 µg and 20 µg) of xylarinase (20 µl) and incubated at 37 °C for three minutes. Further 100 µl of the APTT solution (kaolin) was added and again incubated at 37 °C for 3 minutes. Subsequently, 100 µl of $CaCl_2$ was added and coagulation time was estimated using automated

coagulometer (Chem 7, ErbaMallhein, Germany). Plasma without addition of xylarinase was used as control. Similarly, Thrombin time (TT) was estimated. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values (Choi et al 2013b; Kotb 2014b; Kang et al 2016).

4.20 *In vitro* cytotoxicity assay

The *in vitro* cytotoxicity of xylarinase was determined using RAW 264.7 murine macrophage cell lines. Cells were seeded at a density of 1×10^5 cell/ ml and incubated for 24 h prior to experimental treatments. The cells were then treated with different concentrations of the fibrinolytic enzyme (5–50 µg). After 48 h of incubation, 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added to each well and incubated for 3 h at 37 °C followed by addition of 100 µl of DMSO to dissolve the formazan crystals. The absorbance was then measured at 540 nm using a BIOTEK® Powerwave 340 microplate reader. Cytotoxicity (percent cell death) after the addition of enzyme was assayed by an MTT–based method. For controls, cell cultures were treated without fibrinolytic enzyme served as negative control whereas medium without cells was used as blank (Mukherjee et al 2012; Choi et al 2013b).

4.21 Solid state fermentation (SSF) for production of fibrinolytic enzyme by #37 CRSTBRT

4.21.1 Selection of agro–residue for production of fibrinolytic enzyme

Substrates such as rice chaff, wheat bran, egg shell, orange peel and banana peel were collected for the local market and fruit vendors, dried for several days and powdered. 10 g of each of the substrate was taken is an Erlenmeyer flask and moistened with 50 % of salt solution (KH_2PO_4: 0.5 g, $MgSO_4$: 0.5 g, $MnSO_4$: 0.001 g, $ZnSO_4$: 0.002 g, $FeSO_4$: 0.0005 g, double distilled water: 10 ml). The contents were sterilized by autoclaving twice at 121 °C for 25 min and then cooled at room temperature and inoculated with 2 ml (0.91% physiological saline) of the finely ground mycelium of #37 CRSTBRT aseptically. Flasks were incubated at 28 ± 1°C for 15 days. Each flask was soaked with sterile 20 mM Tris–HCl buffer (pH

7.8) and incubated in orbital shaker at 130 rpm for 2 h at 28 °C. Further, the enzyme extract was obtained by filtration through Whatman filter paper. For 1 g dry substrate used, 3 ml of filtrate was obtained. The enzyme extract obtained from each substrate was checked for its fibrinolytic property using the fibrin plate method as described previously in section 4.4.2. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values (Tao et al 1997; Gopinath et al 2011; Hariharan et al 2014; Vijayaraghavan and Vincent 2014; Vijayaraghvan et al 2016).

4.21.2 Physio–chemical characterization of rice chaff

Determination of volatile matter, ash and moisture content was done as per the protocol described in, ASTM D3175-07, ASTM D 3174-04 and ASTM D3175-07 respectively. Fixed carbon was determined as the resultant of the summation of percentage moisture, ash, and volatile matter subtracted from 100. All percentages were on the same moisture reference base.

Fixed carbon (%) = 100 – (moisture % + ash % + volatile matter %).

Further, the calorific value was determined according to (Indian Standard) IS 1350 (part 2) protocol using static bomb calorimeter; BCM211059 and the basic elemental analysis (CHN) was performed at SAIF, IIT Mumbai, India (Akhtar et al 2016).

4.21.3 Optimization of culture conditions for *in vitro* production of fibrinolytic enzyme

Rice chaff was used as substrate for production of fibrinolytic enzyme in SSF. SSF was carried out in a 250 ml Erlenmeyer flask containing 10 g of the substrate moistened with salt. The contents were sterilized by autoclaving twice at 121 °C for 25 min, cooled at room temperature and then inoculated with 2 ml (1 g) of the finely ground mycelium of #37 CRSTBRT under aseptic conditions. Various parameters such as incubation period (2–20 days), moisture content (20–100 %), particle size (200–1000 µm) and incubation temperature (20–45 °C) were optimized following one factor at a time approach. Each experiment set contained three replicates and their mean and standard deviation were calculated.

Further, the data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values.

4.21.3.1 ***Optimization of incubation period for production of fibrinolytic enzyme by SSF***

The optimal incubation period for production of fibrinolytic enzyme was evaluated by subjecting the culture #37 CRSTBRT to grow over rice chaff for 20 days and regularly checking the enzyme production. Briefly, 10 g of rice chaff supplemented with 50 % salt solution was inoculated with 2 ml finely ground mycelium in and incubated at 28 ± 1°C. The production of fibrinolytic enzyme was regularly checked after every 24 h. The data was analysed using one way ANOVA followed by Tukey's analysis (GraphPad Prism 5.0) to determine significant difference between the mean values. The optimal incubation period obtained by this step was taken in account for subsequent SSF experiments (Tao et al 1997; Gopinath et al 2011**)**.

4.21.3.2 ***Determination of optimal moisture level for in vitro production of fibrinolytic enzyme by SSF***

To study the effect of moisture on production of fibrinolytic enzyme, the moisture content of the substrate was varied between 20 to 100 % (20, 30, 40, 50, 60, 80 and 100 %) and adjusted with salt solution. 2 ml of the inoculum was dispensed over the substrate under sterile conditions and incubated at 28 ± 1°C for 15 days under static position. The most favourable moisture content inducing maximum fibrinolytic activity by this step was further used in succeeding SSF experiments. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values (Tao et al 1997; Gopinath et al 2011**)**.

4.21.3.3 ***Effect of particle size of substrate on production of fibrinolytic enzyme by SSF***

To study the effect of particle size of the substrate on fibrinolytic enzyme production, SSF was carried out using various particle sizes (200 –800 μm) of rice chaff. Various particle sizes were obtained by using sieves of different sizes. 5 ml of the salt solution (50%) was added to the substrate (10 g) and autoclaved twice at 121 °C for 25 min and cooled at room temperature. Further, 2 ml of the inoculum was

aseptically inoculated to each flask and incubated at 28 ± 1 °C for 15 days. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values. The most effective particle size which induced maximum *in vitro* fibrinolytic activity was selected for further studies (Tao et al 1997; Gopinath et al 2011).

4.21.3.4 ***Effect of incubation temperature on in vitro production of fibrinolytic enzyme by SSF***

To study the effect of incubation temperature on production of fibrinolytic enzyme, pre–sterilized rice chaff (10 g) supplemented with 50 % salt solution was inoculated with 2 ml of the inoculum and incubated at varying temperature (20, 30, 35, 40 and 45° C) (Gopinath et al 2011; Hariharan et al 2014). The most favourable incubation temperature induced maximum *in vitro* fibrinolytic activity. The data was analysed using one way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism 5.0) to determine significant difference between the mean values.

4.21.4 ***In vitro*** **fibrinolytic enzyme production by SSF and extraction**

Briefly, 10 g of rice chaff substrate (particulate size 500 µm) in 250 ml Erlenmeyer flasks was supplemented with 50 % salt solution and autoclaved at 121 °C, 15 psi for 25 min. Subsequently, on cooling, the substrate was inoculated with 2 ml (1 g) of finely ground mycelium of #37 CRSTBRT aseptically. It was incubated at 28 ± 1°C for a period of 15 days. After 15 days, fresh moldy pith in each flask was soaked with 20 mM Tris-HCl buffer (3 ml buffer/g of dry substrate) and incubated in an orbital shaker at 28 °C, 130 rpm for 2 h to obtain the enzyme extract. Further, the enzyme extract was obtained by filtration through Whatman filter paper no. 4 followed by centrifugation at 10,000 ×g (Hitachi RX II series, Japan) for 10 min at 4 °C (Tao et al 1997).

4.21.5 Fibrinolytic activity of enzyme extract

The enzyme extract obtained after each step was checked for its *in vitro* fibrinolytic potential using the fibrin plate assay described in section 4.4.2.

4.21.6 Enzyme purification

Solid ammonium sulphate (HiMedia, India) was added to the enzyme extract to achieve 60% saturation. The mixture was stored at 4 °C for overnight. The next day, precipitate was collected by centrifugation at 13,000 ×g, 15 min at 4 °C. The pellet was then dissolved in 20 mM Tris–HCl buffer (pH 7.8), dialysed against the same buffer for 24 h before ultrafiltration using amicon filters (10 kDa, Millipore). The insoluble material was removed by centrifugation at 10,000 ×g for 10 min. Further, the protein was purified by medium pressure liquid chromatography (Akta Prime, GE Healthcare, USA) using Sephacryl S–300 column (72 cm column length, 1.5 cm internal diameter, 60 cm gel bed volume) with a flow rate of 1 ml/min, pressure 1 MPa and fraction size of 2 ml. Protein content was analysed at 280 nm. Protein fractions having fibrinolytic activity were pooled, concentrated and used as pure enzyme (Cui et al 2008; Cha et al 2010; Lu et al 2010).

4.21.7 Determination of molecular weight and purity of the purified enzyme

To determine the molecular weight of the purified fibrinolytic enzyme produced over rice chaff, SDS–PAGE was performed according to the method of Laemmli (1970) using 10 % resolving and 5 % stacking gels. The resolved protein was detected in the gel by staining with Coomassie Brilliant Blue R–250 for 2 h and then destained. The molecular mass of the denatured enzyme was estimated using a standard protein weight marker (Merck Millipore, USA) (Choi et al 2013a). The purity of the enzyme obtained from gel filtration chromatography was analysed using HPLC (Shimadzu LC system, Japan) equipped with C_{18} column as described earlier in section 4.11.5 (Bi et al 2013b; Babu and Devi 2015).

4.21.8 *In vitro* fibrinolytic activity of purified enzyme

In vitro fibrinolytic activity of the purified enzyme was determined using the fibrin plate assay described in section 4.4.2.

4.22 Comparative analysis of properties of enzyme "xylarinase" by submerged fermentation and SSF

The properties of the enzyme "xylarinase" in terms of fibrinolytic activity, molecular weight, N-terminal sequencing, protein content, enzyme yield and biochemical nature were compared to explore possible route for the mass production of the enzyme.

Chapter 5

Results

5.1 Isolation of endophytic fungi from plant samples

A total of 211 endophytic fungal isolates representing 35 fungal taxa were isolated from different parts of the host plants collected during the forays. These endophytic fungal isolates were assigned codes based on their host plant along and tentatively identified (Table 5.1, Appendix A). The tentative identification was based on their morphological and microscopic characters (Fig 5.1, Fig 5.2).

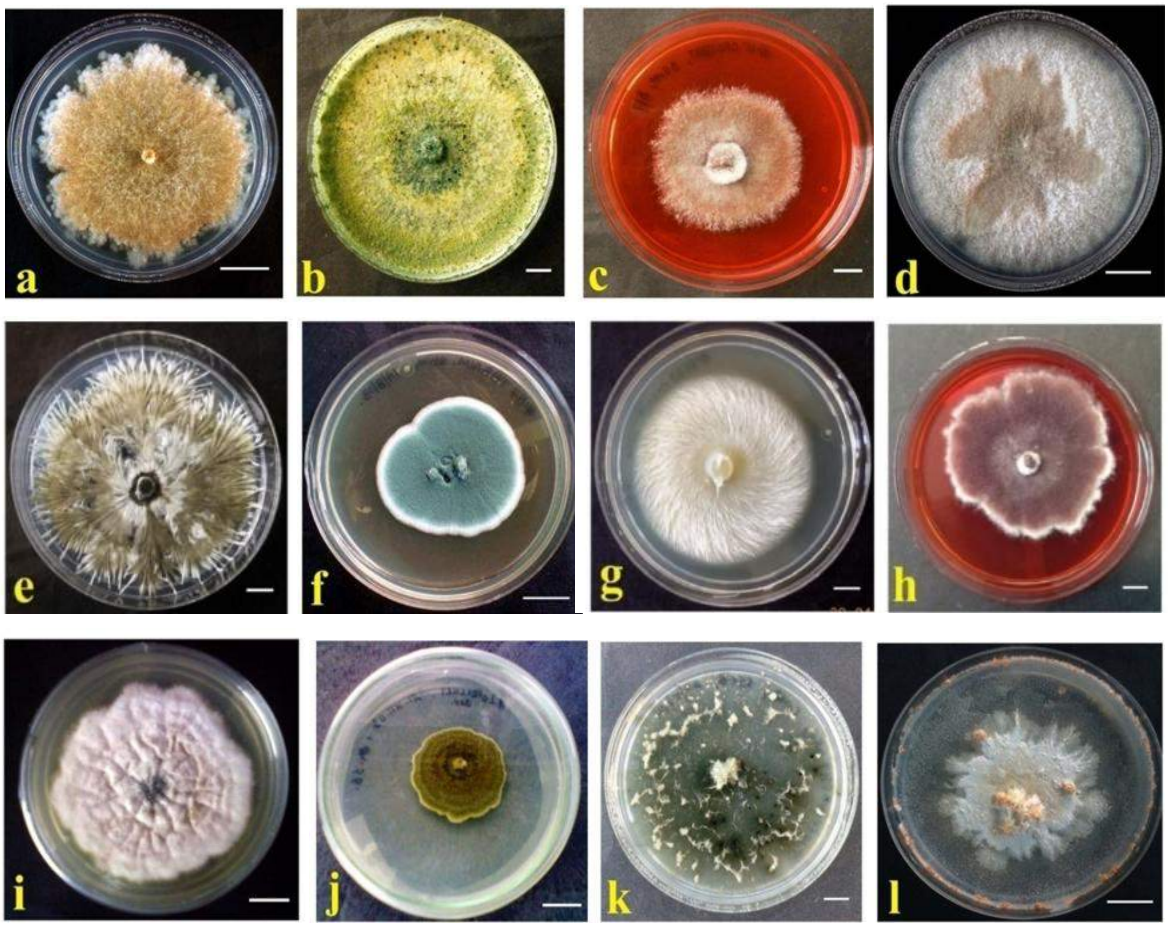


Fig 5.1: Endophytic fungal isolates from various medicinal plants used in the study. (a, d) *Fusarium equiseti*, (b) *Trichoderma viride*, (c) Unidentified, (e) *Xylaria Curta*, (f) *Penicillium* sp. (g) *Muscodor camphora*, (h) *Fusarium solani*, (i) *Pestalotiopsis microspora*, (j) *Alternaria alternata,* (k) *Lasiodiplodia pseudotheobromae*, (l) *Bionectria* sp. (Bar: 10 mm)

Endophytic fungal isolates were predominantly isolated from *Aegle marmelos* (33 %), followed by *Cinnamomum malabaricum* (27 %) and *Taxus baccata* (12 %). The host tissue of each plant sample exhibited a variation in colonization of the endophytic mycoflora (Table 5.2; Fig 5.3a). The maximum colonization of fungal endophytes were observed in stem (38.4 %) followed by bark (25.6 %), stem internal tissue (19.4 %) and leaves (19.6 %) (Table 5.2; Fig 5.3b). Further, the total

endophytic fungi isolated were grouped in hyphomycetes, coelomycetes, ascomycetes, basidiomycetes and unidentified. The maximum number of endophytic isolates belonged to hyphomycetes (48.3 %) followed by ascomycetes (19.9 %), coelomycetes (18.4 %), unidentified (12.3 %) and basidiomycetes (0.94 %). No member of zygomycetes was reported during the study (Fig 5.4).

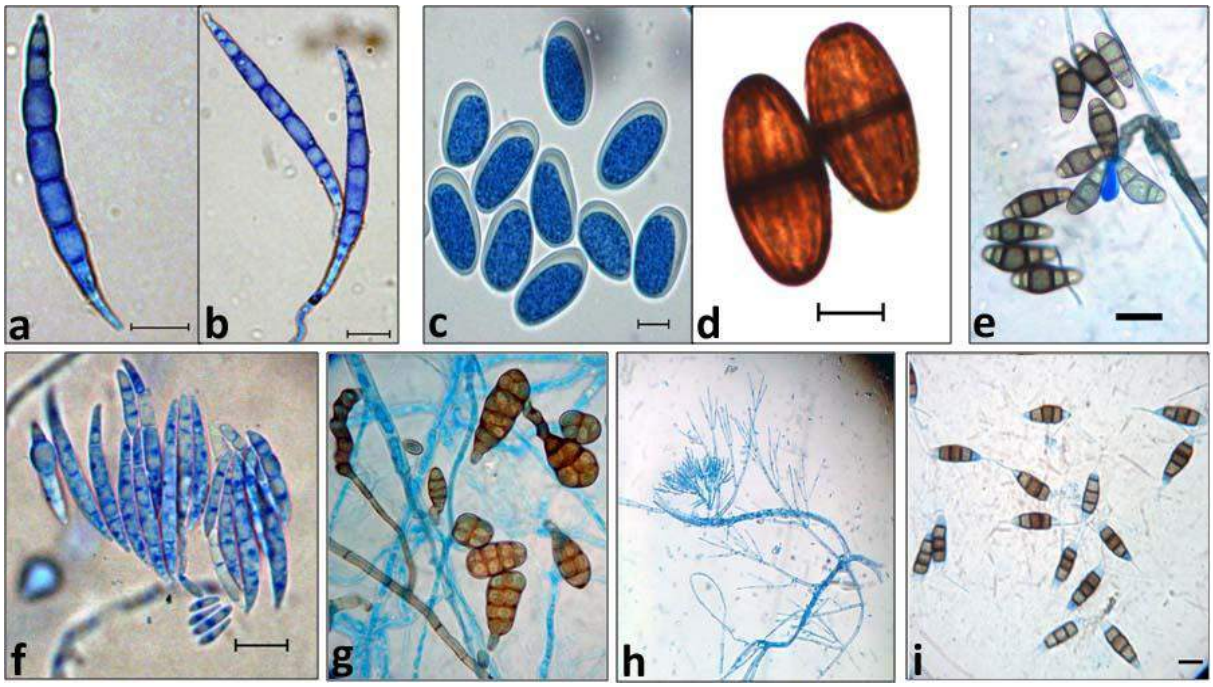


Fig 5.2: Microscopic features of endophytic fungi isolated during the study. (a–b) macroconidia of *Fusarium equiseti*, (c–d) immature and mature conidia of *Lasiodiplodia pseudotheobromae,* (e) Conidia of *Curvularia* sp. (f) macrconidia and microconidia of *Fusarium oxysporum,* (g) Conidia *Alternaria* sp. (h) penicillate and verticillate conidial arrangement of *Bionectria* sp. (i) Conidia of *Pestalatiopsis* sp. (Bar: 10 µm)

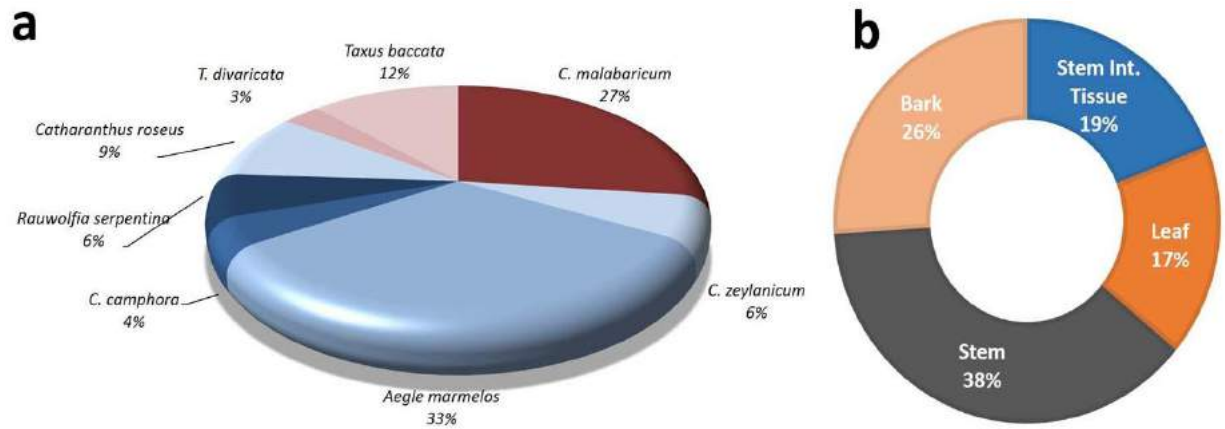


Fig 5.3: Distribution of endophytic fungi (%) in (a) host plants, (b) different tissues of host plants

Table 5.2: Summary of endophytic fungi isolated from different tissues of host plant

S. no	Host plant	Endophytic fungi				Total
		Leaf	Bark	Stem	Stem internal tissue	
1.	*Aegle marmelos*	16	5	30	19	70
2.	*Cinnamomum malabaricum*	12	16	26	4	58
3.	*Cinnamomum zeylanicum*	0	5	0	7	12
4.	*Cinnamomum camphora*	0	2	0	6	8
5.	*Cathranthus roseus*	0	0	18	0	18
6.	*Tabernaemontana divaricata*	0	0	7	0	7
7.	*Rauwolfia serpentina*	7	0	0	5	12
8.	*Taxus baccata*	0	26	0	0	26
	Total	35	54	81	41	**211**

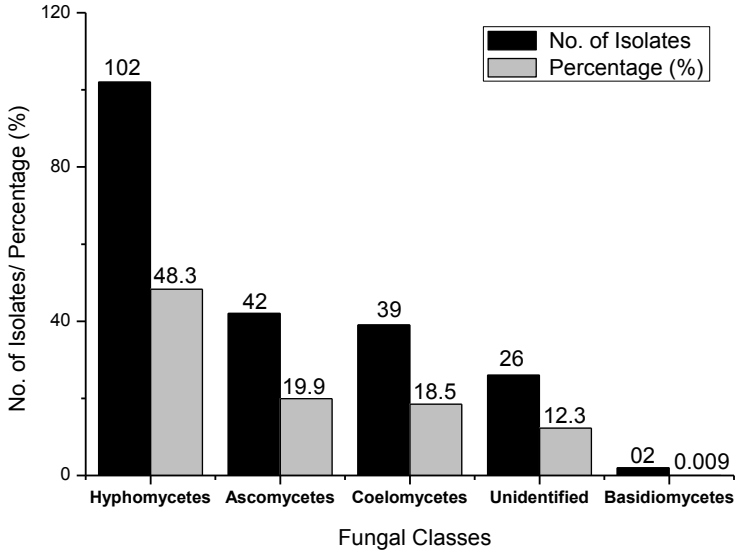


Fig 5.4: Distribution of endophytic fungal isolates in different classes of fungi

Among the taxa identified, *Alternaria*, *Botryosphaeria*, *Fusarium* and *Pestalotiopsis* sp. were the most frequently isolated whereas *Clonostachys*, *Drechslera*, *Sphaeropsis* and *Togninia* spp. were the least frequently isolated fungal isolates in the study (Table 5.3). The predominant endophytic colonizers of *Aegle marmelos* were *Fusarium* sp. followed by *Botryosphaeria* sp., *Pestalotiopsis* sp. and *Alternaria* sp. while dominant endophytic colonizers in *C. malabaricum* were *Pestalotiopsis* sp., *Alternaria* sp., *Curvularia* sp., *Colletotrichum* sp. and *Fusarium* sp. Volatile antimicrobial producing *Muscodor* species have also been reported from both *Aegle marmelos* and Cinnamon plants (Meshram et al 2013; Meshram et al 2014; Saxena et al 2014; Meshram et al 2015; Saxena et al 2015). In *Taxus baccata,* the prominent endophytic colonizers were *Fusarium* and *Pestalotiopsis* spp.

Table 5.3: Summary of endophytic fungal isolate obtained during the study from different medicinal plants

S. no	Endophytic fungi	*Rutaceae*	*Lauraceae*			*Apocynaceae*			*Taxaceae*	Total
		AM	CM	CZ	CC	CR	TMD	RS	TB	
1.	*Acremonium* sp.	–	2	–	–	–	–	–	–	**2**
2.	*Alternaria* sp.	4	4	–	1	2	–	1	1	**13**
3.	*Arthrinium* sp.	–	3	–	–	–	–	–	–	**3**
4.	*Ascobolus* sp.	–	–	–	–	–	–	3	–	**3**
5.	*Aspergillus* sp.	1	1	–	–	–	–	–	–	**2**
6.	*Aureobasidium* sp.	3	–	–	–	–	–	–	–	**3**
7.	*Barriopsis* sp.	3	–	–	–	–	–	–	–	**3**
8.	*Bionectria* sp	–	4	1	–	–	–	–	–	**5**
9.	*Botryosphaeria* sp.	10	1	–	–	–	–	–	–	**11**
10.	*Botrytis* sp.	–	–	–	–	–	–	–	2	**2**
11.	*Chaetomium* sp.	–	2	–	–	–	–	–	2	**4**
12.	*Cladosporium* sp.	–	–	–	–	2	–	–	–	**2**
13.	*Clonostachys* sp.	–	1	–	–	–	–	–	–	**1**
14.	*Colletotrichum* sp.	–	4	–	–	–	–	–	–	**4**
15.	*Curvularia* sp	2	4	–	–	–	–	–	–	**6**
16.	*Drechslera* sp.	–	–	–	–	–	–	–	1	**1**
17.	*Fusarium* sp.	24	4	1	1	**3**	4	3	5	**45**
18.	*Glomerella* sp.	–	–	2	–	–	–	–	–	**2**
19.	*Hypoxylan* sp.	–	–	–	–	**2**	–	–	–	**2**
20.	*Lasiodiplodia* sp.	6	1	1	–	–	–	–	–	**8**
21.	*Muscodor* sp.	1	–	1	5	–	–	–	–	**7**
22.	Mycelia sterilia	–	2	–	–	–	–	–	2	**4**
23.	*Neofussicoccum* sp.	2	–	–	–	–	–	–	–	**2**
24.	*Nectria* sp.	–	2	–	–	–	–	–	–	**2**
25.	*Nigrospora* sp.	–	3	–	–	–	–	–	–	**3**
26.	*Penicillium* sp.	2	–	–	1	–	–	–	1	**4**
27.	*Pestalotiopsis* sp.	4	7	5	–	2	–	–	5	**23**
28.	*Phaeoacremonium* sp.	2	2	–	–	–	–	–	–	**4**
29.	*Phoma* sp.	–	–	–	–	–	–	1	1	**2**
30.	*Phomopsis* sp.	–	–	–	–	–	–	–	2	**2**
31.	*Schizophyllum* sp.	–	–	–	–	–	1	1	–	**2**
32.	*Sphaeropsis* sp.	1	–	–	–	–	–	–	–	**1**
33.	*Togninia* sp.	1	–	–	–	–	–	–	–	**1**
34.	*Trichoderma* sp.	2	–	–	–	–	–	1	–	**3**
35.	*Xylaria* sp.	–	1	–	–	2	–	–	–	**3**
36.	Unidentified	2	10	1	–	5	2	2	4	**26**
	Total	**70**	**58**	**12**	**8**	**18**	**7**	**12**	**26**	
									Grand Total	**211**

AM: *A. marmelos*, CM: *C. malabaricum,* CZ*: C. zeylanicum,* CC*: C. camphora;* CR*: C. roseus,* TMD: *T. divaricata,* RS: *R. serpentina,* TB: *T. baccata*

5.2 Screening of fungal isolates for enzymatic properties

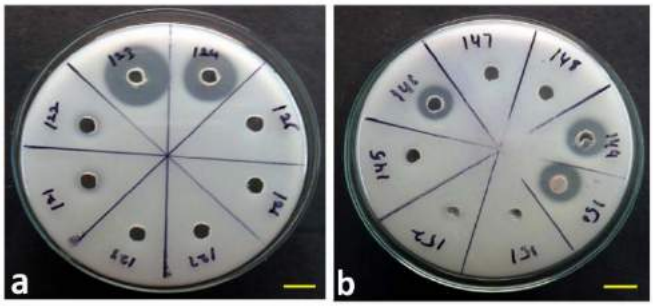
5.2.1 In vitro proteolytic assay

In this assay, culture broth of only 31.7 % endophytic fungi i.e. 67 isolates, exhibited a clear halo in the range of 6.7-13.7 mm diameter on 1 % skimmed milk agar plate indicating proteolytic activity (Table 5.4). The highest proteolytic activity was exhibited by the culture broth of #37 CRSTBRT and #22 AMSTYEL while the least activity was shown by #59 CMSTNEY (Fig 5.5).

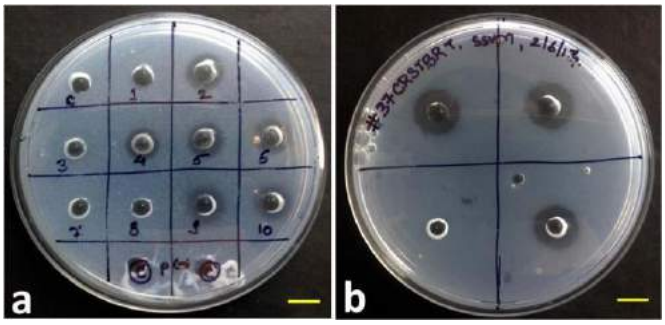
5.2.2 In vitro fibrinolytic activity

Screening of *in vitro* fibrinolytic activity was carried out in two modes- plasminogen dependent and plasminogen independent. Of the 211 isolates screened in both modes, 17.5 % i.e. 37 isolates exhibited plasminogen dependent fibrinolytic activity while 3.31 % i.e. six isolates exhibited plasminogen independent activity (Table 5.5).

The fibrinolytic potency of the culture broth was directly proportional to the size of the halo formed. The diameter of halo's formed by culture broth of endophytic fungi exhibiting plasminogen dependent activity ranged between 8.0–15.0 mm while those exhibiting plasminogen independent activity ranged between 10.0–13.0 mm. Maximum *in vitro* fibrinolytic activity (plasminogen dependent as well as plasminogen independent) was exhibited by culture broth of #37 CRSTBRT followed by #22 AMSTYEL and #4 RSLBRT (Fig 5.6).



← Fig 5.5: *In vitro* proteolytic activity of culture broth of endophytic isolates on1 % skimmed milk plate. {Well id a: (123): #37 CRSTBRT, (124): #45 CRSTBRT}, {b (146): #1048 AMSTITYEL, (149): #4 AMLBRT, (150): #17 AMLBRT}. (Bar: 10 mm)



← Fig 5.6: *In vitro* fibrinolytic activity of endophytic isolates on plasminogen free fibrin plate. {a: (1): 1003 AMSTITYEL (2): #1013 AMSTITYEL (3): #1010 AMSTITYEL, (4):#1013 AMSTITYEL (5): #1048 AMSTITYEL (6): #1079 AMSTITWLS (7): #1022 AMSTITYEL (8): #1058 AMSTITYEL (9):#1095 AMSTITWLS. (10): #1099 AMSTITWLS (c): Control}, {b: Fibrinolytic activity by #37 CRSTBRT, (T): Test, (C): Control Bar: 10 mm)

Other cultures which exhibited both plasminogen independent and plasminogen dependent fibrinolytic activity was based on the standard curve of plasmin (Fig 5.7, Appendix).

Table 5.4 *In vitro* proteolytic activity assay of culture broth endophytic fungi

S. No.	Culture Code	Proteolytic halo* (diameter in mm)	S. No.	Culture Code	Proteolytic halo* (diameter in mm)
1.	#37 CRSTBRT	$13.7^{a} \pm 0.58$	34.	#57 TBBALM	$10.7^{bcdefghi} \pm 1.12$
2.	#22 AMSTYEL	$13.7^{a} \pm 0.58$	35.	#28 AMSTWLS	$10.7^{bcdefghi} \pm 1.12$
3.	#13 TMDSTYEL	$13.5^{ab} \pm 0.50$	36.	#43 TBBALM	$10.7^{bcdefghi} \pm 1.12$
4.	#7 CRSTBRT	$13.5^{ab} \pm 0.50$	37.	#16 RSLBRT	$10.3^{cdefghij} \pm 0.58$
5.	#7(a) AMSTYEL	$13.5^{ab} \pm 0.50$	38.	#7 TBBALM	$10.3^{cdefghij} \pm 0.58$
6.	#11CRSTBRT	$13.3^{ab} \pm 0.58$	39.	#35 TBBALM	$10.3^{cdefghij} \pm 0.58$
7.	#18 CMBABRT	$13.3^{ab} \pm 1.12$	40.	#4 CMLBRT	$10.3^{cdefghij} \pm 0.58$
8.	#64 CZSTITG	$13.3^{ab} \pm 0.58$	41.	#4 CMBABRT	$10.3^{cdefghij} \pm 0.58$
9.	#6 AMLWLS	$13.3^{ab} \pm 0.58$	42.	#35 CMSTNEY	$10.3^{cdefghij} \pm 0.58$
10.	#2(a) TMDSTYEL	$13.3^{ab} \pm 0.58$	43.	#28 CZBAWLS	$10.0^{defghijk} \pm 0.00$
11.	#32 AMSTYEL	$13.3^{ab} \pm 0.58$	44.	#17 AMSTYEL	$9.80^{efghijk} \pm 0.76$
12.	#9(b) AMSTYEL	$13.3^{ab} \pm 1.12$	45.	#96 CMSTNEY	$9.50^{efghijk} \pm 1.32$
13.	#4 RSLBRT	$13.0^{abc} \pm 1.00$	46.	#73 TBBALM	$9.30^{fghjikl} \pm 0.58$
14.	#7 AMSTYEL	$12.7^{abcd} \pm 0.58$	47.	#14 RSBANEY	$9.30^{fghijkl} \pm 1.53$
15.	#2 CRSTBRT	$12.7^{abcd} \pm 0.58$	48.	#93 TBBALM	$9.20^{fghijkl} \pm 1.04$
16.	#4 TMDSTYEL	$12.3^{abcde} \pm 0.58$	49.	#33 TBBALM	$9.00^{ghijkl} \pm 0.00$
17.	#20 TBBALM	$12.3^{abcde} \pm 1.12$	50.	#37 AMSTWLS	$8.80^{hijkl} \pm 0.76$
18.	#1007 AMLBRT	$12.0^{abcdef} \pm 0.00$	51.	#1079 AMSTITWLS	$8.70^{hijkl} \pm 0.58$
19.	#45 CRSTBRT	$11.7^{abcdefg} \pm 1.53$	52.	#36 CCSTITD	$8.70^{hijkl} \pm 0.58$
20.	#1088 AMSTITYEL	$11.7^{abcdefg} \pm 0.76$	53.	#31CZSTITG	$8.70^{hijkl} \pm 0.58$
21.	#17 CRSTBRT	$11.5^{abcdefgh} \pm 0.50$	54.	#20 CMBANEY	$8.70^{hijkl} \pm 0.58$
22.	#25 AMSTWLS	$11.5^{abcdefgh} \pm 0.50$	55.	#1 RSLBRT	$8.70^{hijkl} \pm 1.12$
23.	#11 CMSTNEY	$11.3^{abcdefgh} \pm 0.58$	56.	#7 RSBANEY	$8.70^{hijkl} \pm 0.58$
24.	#26 CMBANEY	$11.3^{abcdefgh} \pm 0.58$	57.	#12 CMBANEY	$8.70^{hijkl} \pm 0.58$
25.	#17 CMSTNEY	$11.3^{abcdefgh} \pm 0.58$	58.	#1048 AMSTITYEL	$8.00^{ijkl} \pm 1.00$
26.	#2b TMDSTYEL	$11.2^{abcdefgh} \pm 0.31$	59.	#17 AMLSTWLS	$8.00^{ijkl} \pm 0.00$
27.	#12 CMBABRT	$11.2^{abcdefgh} \pm 0.76$	60.	#2 CMLNEY	$7.80^{jkl} \pm 1.44$
28.	#16 AMLWLS	$11.2^{abcdefgh} \pm 0.76$	61.	#40 CMLBRT	$7.70^{jkl} \pm 1.12$
29.	#23 CRSTBRT	$11.2^{abcdefgh} \pm 0.76$	62.	#3 AMSTYEL	$7.70^{jkl} \pm 0.58$
30.	#1013 AMSTYEL	$11.0^{abcdefgh} \pm 0.00$	63.	#43 CMSTITBRT	$7.70^{jkl} \pm 1.12$
31.	#43 CMSTNEY	$11.0^{abcdefgh} \pm 1.00$	64.	#1639 CCSTITID	$7.30^{kl} \pm 0.58$
32.	#1095 AMSTITIWLS	$10.8^{bcdefghi} \pm 1.04$	65.	#4 AMLBRT	$7.30^{kl} \pm 0.58$
33.	#9 AMLBRT	$10.7^{bcdefghi} \pm 0.58$	66.	#17 AMLBRT	$7.30^{kl} \pm 0.58$
			67.	#59 CMSTNEY	$6.70^{l} \pm 1.12$

*Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post hoc test ($p<0.05$)

Table 5.5: Screening for *in vitro* fibrinolytic activity of endophytic isolates

S. no.	Culture Code	Fibrinolytic halo (diameter in mm)*	
		Plasminogen rich plates	**Plasminogen devoid plates**
1.	#37 CRSTBRT	15.0^{a}± 1.00 (5.2)**	13.0^{a} ± 1.00 (3.9)**
2.	#22 AMSTYEL	13.5^{ab}± 0.50 (3.8)	12.0^{a} ± 1.00 (3.2)
3.	#9(b) AMSTYEL	12.5^{abc} ± 0.50(3.5)	12.5^{a} ± 1.32 (3.5)
4.	#4 RSLBRT	12.3^{abc}± 1.53 (3.4)	12.5^{a} ± 0.50 (3.5)
5.	#12 CMBANEY	12.3^{abc}± 0.58 (3.4)	ND
6.	#1088 AMSTITYEL	12.3^{ab}± 0.58 (3.4)	12.3^{a} ± 1.53 (3.4)
7.	#6 AMLWLS	12.2^{bc}± 1.04 (3.2)	ND
8.	#17 AMSTYEL	11.7^{bc} ± 0.58 (3.0)	ND
9.	#2a TMDSTYEL	11.5^{bc} ± 0.50 (2.9)	12.5^{a} ± 1.5 (3.5)
10.	#4 TMDSTYEL	11.5^{bc} ± 0.50 (2.9)	ND
11.	# 12 CMBABRT	11.3^{bc} ± 0.58 (2.7)	ND
12.	#2b TMDSTYEL	11.2^{bc} ± 0.50 (2.6)	ND
13.	#18 CMBABRT	11.0^{bc} ± 1.00 (2.5)	ND
14.	#28 AMSTWLS	10.7^{c} ± 1.53 (2.3)	ND
15.	#25 AMSTWLS	10.7^{c} ± 1.16 (2.3)	ND
16.	#13 TMDSTYEL	10.7^{c} ± 1.16 (2.3)	ND
17.	#1095 AMSTITWLS	10.7^{c} ± 1.16 (2.3)	ND
18.	#1007 AMLBRT	10.7^{c} ± 1.16 (2.3)	ND
19.	#28 CZBAWLS	10.7^{c} ± 1.16 (2.3)	ND

* Data presented are mean ± standard deviation of three replicates. Means within the same column with different superscript letters are different by Tukey's post hoc test ($p<0.05$). **Equivalent plasmin units (U/ml) are given in the brackets. ND refers to Not detected

5.3 Selection of protein precipitation method for crude enzyme preparation using fibrinolytic assay

Crude enzyme extract of six isolates exhibiting both plasminogen dependent and plasminogen independent activity were prepared using ammonium sulphate and acetone precipitation methods and their fibrinolytic potential assessed using fibrin plate assay. It was observed that ammonium sulphate precipitation method for preparation of the crude enzyme was a better method as compared to acetone precipitation since the *in vitro* fibrinolytic activity of the six isolates was significantly higher in the crude enzyme obtained through ammonium sulphate precipitation method. Further, Bland Altman analysis of the two precipitation methods to obtain crude enzyme for *in vitro* fibrinolytic activity exhibited a significant bias of 1.36 which corroborated our finding that

ammonium sulphate precipitation method was a better option to obtain crude enzyme which significantly exhibited higher fibrinolytic activity.

Table 5.6: Comparison of precipitation methods to obtain crude enzyme of the selected endophytic isolates by their *in vitro* fibrinolytic activity

S. No	Culture Code	Fibrinolytic activity (U/ml)* of crude enzyme	
		Ammonium sulphate precipitation	Acetone Precipitation
1.	#37 CRSTBRT	9.4^{a}± 0.38	7.2^{a}± 0.67
2.	#22 AMSTYEL	8.3^{ab} ± 0.38	5.6^{ab} ± 0.77
3.	#4 RSLBRT	7.8^{bc} ± 0.66	6.1^{ab} ± 0.38
4.	#2(a) TMDSTYEL	7.0^{c} ± 0.38	5.6^{ab} ± 0.76
5.	#9(b) AMSTYEL	5.6^{d} ± 0.38	5.0^{b} ± 0.77
6.	#1088 AMSTITYEL	3.9^{e} ± 0.66	2.9^{c} ± 0.38
7.	Control	0^{f}	0^{d}

*Data presented are mean ± standard deviation of three replicates. Means within the same column with different superscript letters are different by Tukey's post hoc test ($p<0.05$)

There was a statistically significant difference in the *in vitro* fibrinolytic activity expressed by crude enzyme of the different isolates obtained through ammonium sulphate precipitation method by one way ANOVA ($F(6, 14)=148.3$, $p=<0.0001$). Further, Tukey's post hoc analysis confirmed a significantly highest mean *in vitro* fibrinolytic activity by crude enzyme of #37 CRSTBRT as compared to other isolates. In case of *in vitro* fibrinolytic activity expressed by crude enzyme of isolates obtained by acetone precipitation, there was a significant statistical difference among the isolates by one way ANOVA ($F (6, 14) =48.94$, $p=<0.0001$). In case of crude enzyme obtained by acetone precipitation method the highest mean *in vitro* fibrinolytic activity was again exhibited by #37 CRSTBRT which was confirmed by Tukey's post-hoc analysis (Table 5.6). Hence based on above analysis, #37 CRSTBRT was selected for further enzyme production, purification and characterization.

5.4 Optimization of culture conditions for optimal *in vitro* fibrinolytic activity

Culture conditions of endophytic isolate, #37 CRSTBRT exhibiting maximum extracellular fibrinolytic activity during preliminary screening were further optimized

5.4.1 Selection of suitable medium for production of fibrinolytic enzyme

As evident from Fig 5.8, *in vitro* fibrinolytic activity of crude enzymes obtained by growing #37 CRSTBRT from different growth media were found to be significantly different by one way ANOVA ($F(6,14)=132.4$, $p=<0.0001$). Further, post hoc comparisons using Tukey's HSD indicated that the mean fibrinolytic activity of crude enzyme obtained from CDB was significantly highest, followed by MEB and RB which did not have significant differences among their mean fibrinolytic activity while it was quite low on PDB. No fibrinolytic activity was recorded by the crude enzyme obtained from YEB and TSB. Hence, CDB was selected as a medium for the production of fibrinolytic enzyme by #37 CRSTBRT.

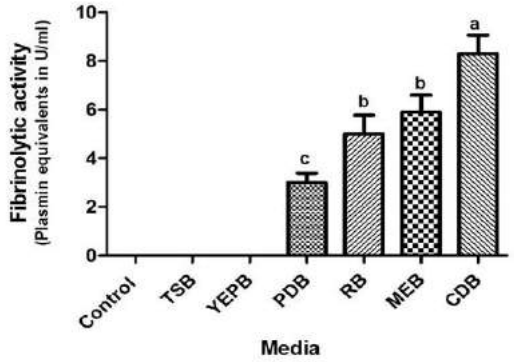


Fig 5.8: *In vitro* fibrinolytic activity of the crude enzyme produced on different media viz. PDB, RB, CDB, MEB, TSB and YEPB. Data represented are mean ± standard deviation of three replicates. Means with different letters are significantly different by Tukey's post hoc test at $p<0.05$

5.4.2 In vitro evaluation of intracellular fibrinolytic activity of #37 CRSTBRT

The intracellular extract of #37 CRSTBRT did not exhibit any *in vitro* fibrinolytic activity.

5.4.3 Evaluation of optimal ammonium sulphate concentration for protein precipitation

A significant difference in the *in vitro* fibrinolytic activity of protein precipitate (referred as crude enzyme) obtained at different concentrations of ammonium sulphate was observed by one way ANOVA analysis ($F\ (5,\ 12)\ =134.9$, $p\leq 0.0001$). Further Tukey's post hoc comparisons indicated that the mean *in vitro* fibrinolytic activity was highest in the protein precipitate obtained at 60 % ammonium

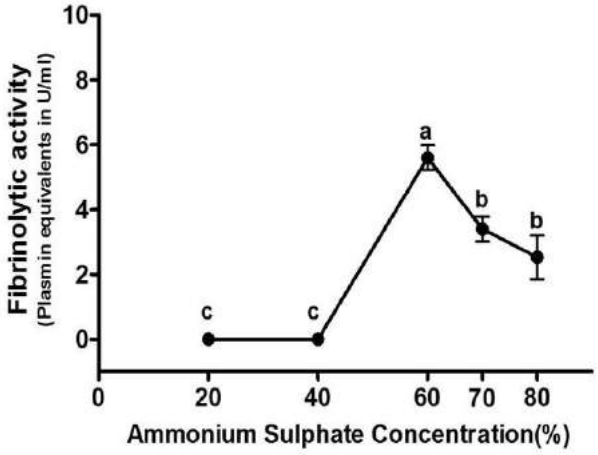


Fig 5.9: *In vitro* fibrinolytic activity of the crude enzyme at various ammonium sulphate concentrations. Data represented are mean ± standard deviation of three replicates. Means with different letters are significantly different by Tukey's post hoc test at $p<0.05$

sulphate concentration when compared to 70 % and 80 % ammonium sulphate concentration. Least fibrinolytic activity was observed by protein precipitate obtained by using 20 % and 40 % concentration of ammonium sulphate (Fig 5.9). Hence, 60 % ammonium sulphate was used for protein precipitation to obtain crude enzyme for purification and characterization.

5.4.4 Selection of optimal buffer concentration for in vitro fibrinolytic activity

A significant difference in the *in vitro* fibrinolytic activity of the protein precipitate was recorded when it was dissolved in Tris–HCl buffer of different strength using one way ANOVA ($F(6, 14) = 41.45$, $p \leq 0.0001$). Further based on Tukey's post hoc analysis the mean *in vitro* fibrinolytic activity was found significantly different and highest at 20 mM Tris–HCl buffer concentration (Mean =7.4 ± 1.01), while the least *in vitro* fibrinolytic activity was recorded at 60 mM Tris–HCl buffer concentration (M=1.70, SD=0.38). Tukey's analysis did not exhibit a significant difference in the *in vitro* fibrinolytic activity at 30 mM, 40 mM and 50 mM of Tris–HCl buffer concentration and grouped them together (Fig 5.10). Hence, 20 mM concentration of Tris–HCl buffer was considered best for dissolving the protein precipitate for use as a crude enzyme as it exhibited optimal *in vitro* fibrinolytic activity and was used for further purification and characterization of the enzyme.

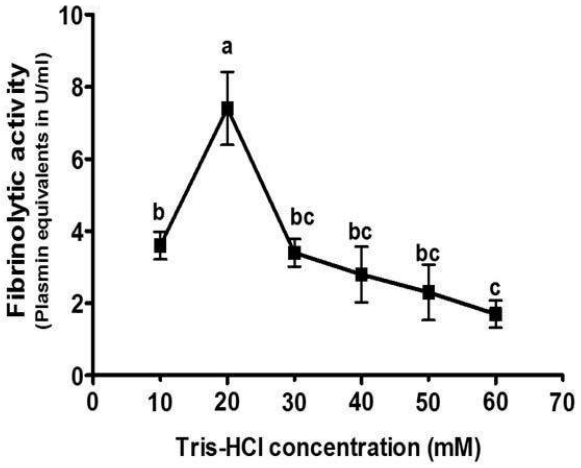


Fig 5.10: *In vitro* fibrinolytic activity of the crude enzyme at different Tris-HCl concentration. Data represented are mean ± standard deviation of three replicates. Means with different letters are significantly different by Tukey's post hoc test at $p<0.05$

5.5 Identification of the selected fibrinolytic enzyme producing endophytic fungus.

The selected fibrinolytic enzyme producing endophytic fungus #37 CRSTBRT was identified using morphotaxonomic and molecular taxonomic methods.

5.5.1 Morphotaxomony

The endophytic fungus #37 CRSTBRT produced white, moderately growing, floccose to downy colonies on different media after 15 days of incubation with 12 h photoperiod (Fig 5.11). The fungus produced volatile organic compounds (VOCs) on PDA, MEA, YEPDA, OMA, CMA and TSB. No VOC production was observed by the fungus on SNA, WA, CPA, PLA, and CLA.

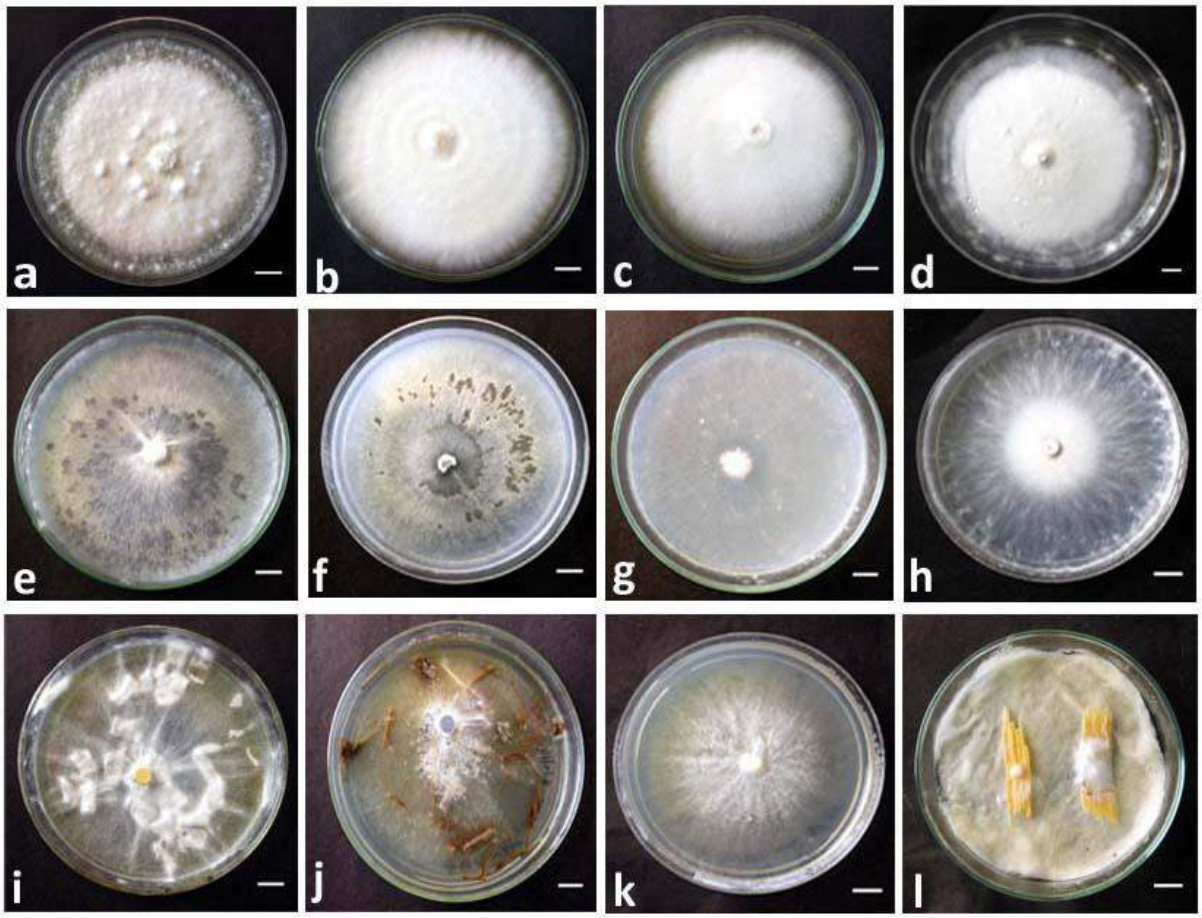


Fig 5.11: Growth pattern of #37 CRSTBRT on different media [a: PDA; b: YEPDA; c: TSB; d: RA; e: MEA; f: OMA; g: CMA; h: SNA; i: CLA; j: PLA; k: CPA; l: WP (wood pieces)]. (Bar: 10 mm)

The endophytic fungus #37 CRSTBRT did not develop any reproductive structure like stromata, conidia, conidiogenus cells or ascospores after 2–9 weeks of incubation. Further, the isolate did not sporulate even after providing stress conditions like incubation under complete darkness and ultraviolet radiation. The morphological characteristics on different growth media have been summarized in table 5.7 while the microscopic features have been displayed in the Fig 5.12.

Table 5.7: Morphological and microscopic features of #37 CRSTBRT produced on different medium after two weeks

Medium	Colony colour		Colony diameter (mm)*	Appearance	Elevation	Margin	VOC/pigment production
	Front	Back					
PDA	White	Cream	46.4 ± 2.12	Floccose	Flat	Smooth	VOCs, no pigment
MEA	White	Cream	70.5 ± 0.76	Floccose	Flat	Smooth	VOCs, no pigment
TSB	White	Cream	53.8 ± 0.71	Floccose	Flat	Smooth	VOCs, no pigment
CMA	White	White	58.5 ± 2.12	Floccose	Flat	Smooth	VOCs, no pigment
YEPDA	White	White	49.5 ± 3.54	Floccose	Flat	Smooth	VOCs, no pigment
OMA	White	White	90.0 ± 0	Floccose	Flat	Smooth	VOCs, no pigment
SNA	White	White	90.0 ± 0	Floccose	Flat	Smooth	No VOCs, no pigment
PLA	White	hyaline	33.0 ± 2.83	Downy	Flat	Smooth	No VOCs, no pigment
CLA	White	hyaline	65.0 ± 1.41	Downy	Flat	Smooth	No VOCs, no pigment
CPA	White	hyaline	90.0 ± 0	Downy	Flat	Smooth	No VOCs, no pigment
WA	Hyaline	hyaline	67.0 ± 2.83	Downy	Flat	Smooth	No VOCs, no pigment
WP	White	White		Slow	Flat	Smooth	No VOCs, no pigment

* Data presented are mean ± standard deviation of triplicate readings

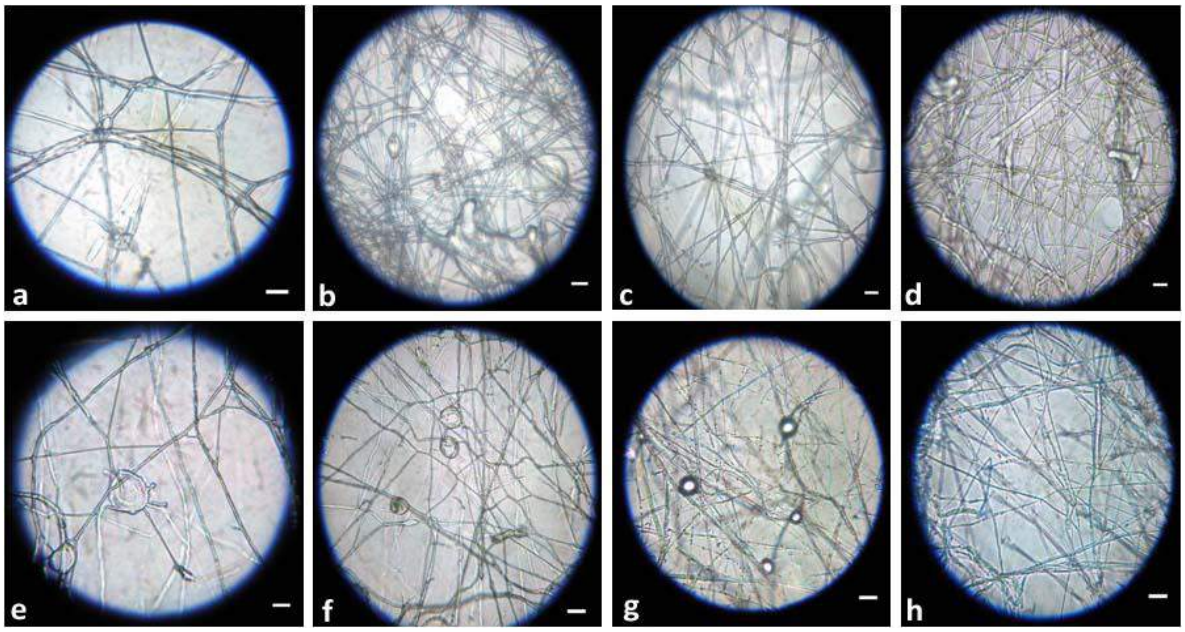


Fig 5.12: Microscopic features of #37 CRSTBRT on different media. (a): PDA, (b): CMA, (c): CPA (d):OMA, (e):WA, (f): SNA, (g): CLA, (h): CSA. (Bar: 10 µm)

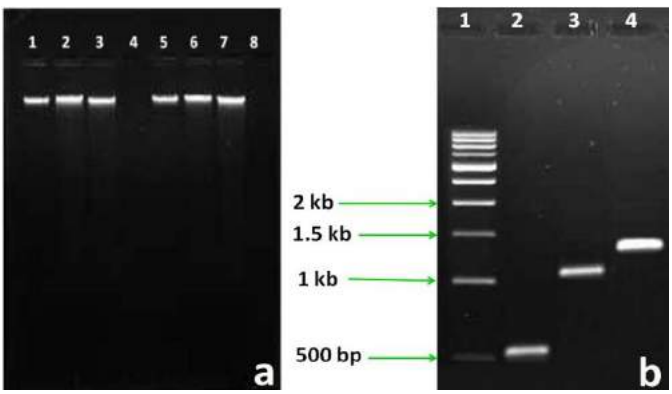
5.5.2 Molecular Taxonomy

Several attempts were made to induce sporulation in #37 CRSTBRT but were unsuccessful. Further, the morphology of the culture was uninformative and therefore molecular phylogeny was employed in which ITS-rDNA sequence was amplified from the genomic DNA (concentration 80 ng/µl). The purity of the DNA (A_{260}/ A_{280}) exhibited a ratio of 1.76. The amplicon of ITS1-5.8S-ITS2 region of

rDNA was found to be pure (Fig 5.13a) and the size was ~500 bp (Fig 5.13b). The sequence data was submitted in GenBank under accession number KM880132. BLAST analysis of the ITS sequence revealed its homology with *Xylaria* species with 98% identity suggesting that the isolate was a member of *Xylariaceae* and probably was *Xylaria curta* (Table 5.8).

Many times for accurate identification of the fungus, sequence analysis of highly conserved region apart from ITS-rDNA becomes a necessity (Atkins and Clark 2004; Rakeman et al 2005). Large subunit (LSU) rRNA genes have been extensively used for fungal phylogeny and taxonomic placement (Arnold et al 2009). Although, ITS region provides a useful "barcode" for environmental biodiversity studies, the extent of sequence variability does not allow a robust sequence alignment. The LSU-rRNA region of #37 CRSTBRT was also amplified using a specific primer and an ~1100bp amplicon was obtained (Fig. 5.13b). The sequenced LSU amplicon of #37 CRSTBRT was submitted in GenBank with accession number KM880131. The BLAST analysis of this sequence gave the first two hits with *Xylaria curta* suggesting #37 CRSTBRT to be a member of *Xylariaceae* and similar to *Xylaria curta* (Table 5.9).

Another viable molecular marker for identification of fungi in ribosomal polymerase II (RPBII), which was amplified from the genomic DNA of #37 CRSTBRT. A ~1500 bp amplicon of RPBII (Fig 5.13b) was obtained which was sequenced and submitted in GenBank with accession number KM880133. Further, in BLAST analysis of this sequence the first two hits again exhibited 100 % similarity to *Xylaria curta* (Table 5.10).



← Fig 5.13: (a) Genomic DNA of #37 CRSTBRT (b) Amplification of #37 CRSTBRT; Lane 1: 500 bp ladder, Lane 2: ITS1–5.8S–ITS2 (~500 bp), Lane 3: LSU amplicon (~1100 bp), Lane 4: RPB region (~1500 bp) amplification

Table 5.8. BLAST search summary of homology analysis of ITS1–5.8S–ITS2 sequence of #37 CRSTBRT

GenBank Accession No.	Organism	Query Coverage (%)	Sequence Similarity (%)	e-value
KM880132	#37 CRSTBRT	Present study		
KM066560	*Xylaria* sp. BAB-3299	99	99	0.00
GU322444	*Xylaria curta* 494	98	99	0.00
HQ435666	*Xylaria sp*. XF10	98	99	0.00
GU322443	*Xylaria curta* 92092022	98	98	0.00
KJ883611	*Xylaria curta* MST7-25	97	99	0.00
KJ883609	*Xylaria curta* MST7-6	97	99	0.0
JQ341078	*Xylaria* sp.D12b4a	97	99	0.00
KP133353	*Xylaria curta* Isolate R1	97	97	0.00
KP133354	*Xylaria curta* 1118	97	97	0.00
KP133310	*Xylaria curta* RV-2015_1022	97	96	0.00

Table 5.9 BLAST search summary of homology analysis of LSU(28S) sequence of #37 CRSTBRT

GenBank Accession No.	Organism	Sequence Similarity (%)	Query Coverage (%)	e-value
KM880131	#37 CRSTBRT	Present study		
JQ862604	*Xylaria curta* *	100	99	0.0
JQ862630	*Xylaria curta**	100	99	0.0
JQ862632	*Xylaria* sp.5195	100	99	0.0
DQ674817	*Xylariaceae* sp.DIS 360g	100	99	0.0
DQ674827	*Xylariaceae* sp.DIS 216i	100	99	0.0
DQ674826	*Xylariaceae* sp.DIS 327b	100	99	0.0
DQ327623	*Xylaria* sp.DIS 125b	100	99	0.0
AY544676	*Xylaria acuta* AFTOL ID-63	100	98	0.0
JQ862611	*Xylaria acuta* 5092	100	98	0.0
KT281899	*Xylaria polymorpha* MUCL 49884	100	98	0.0

Chen et al. (2013), PLoS ONE 8(3): e58268. doi:10.1371/journal.pone.0058268

Table 5.10 BLAST search summary of homology analysis of RPBII sequence of #37 CRSTBRT

GenBank Accession No.	Organism	Sequence Similarity (%)	Query Coverage (%)	e-value
KM880133	Present study			
GQ844831	*Xylaria curta* 494	100	99	0.00
GQ844830	*Xylaria curta* 92092022	100	99	0.00
GQ844824	*Xylaria sp*. 8 HMH	100	86	0.00
GQ844825	*Xylaria juruensis* 92042501	99	85	0.00
GQ848338	*Xylaria digitata* 919 (HAST)	98	86	0.00
GQ844823	*Xylaria apoda* 90080804	98	86	0.00
GQ848356	*Xylaria allantoidea* 94042903	98	86	0.00
GQ 848357	*Xylaria regalis* 920720001	98	84	0.00
GQ844829	*Xylaria culleniae* 189 (JDR)	95	86	0.00
GQ848362	*Xylaria berteri* 90112623	93	86	0.00

For proper delineation of endophytic isolate, three individual phylogenetic tree were made using ITS, LSU and RPBII regions. The phylogenetic tree of ITS1–5.8S–ITS2 region sequences of *Xylaria* species resolved in two major clades – Clade I and Clade II (Fig 5.14). In clade I, seven strains of *Xylaria curta* were clustered including the isolate under study (#37 CRSTBRT). #37 CRSTBRT showed closed relatedness with *X. curta* ST2382 strain with 98 % bootstrap support value thereby confirming its identity as a *X. curta* species. In clade II, three strains of *X. laevis* were clustered with 98 % bootstrap support value whereas *X. montagnei*, *X. apiculata* and *X. polymorpha* emerged basal to clade II with 95 %, 100 % and 79 % bootstrap support values, respectively. *Fusarium incarnatum* was used as outgroup to root the tree (Meshram et al 2016a).

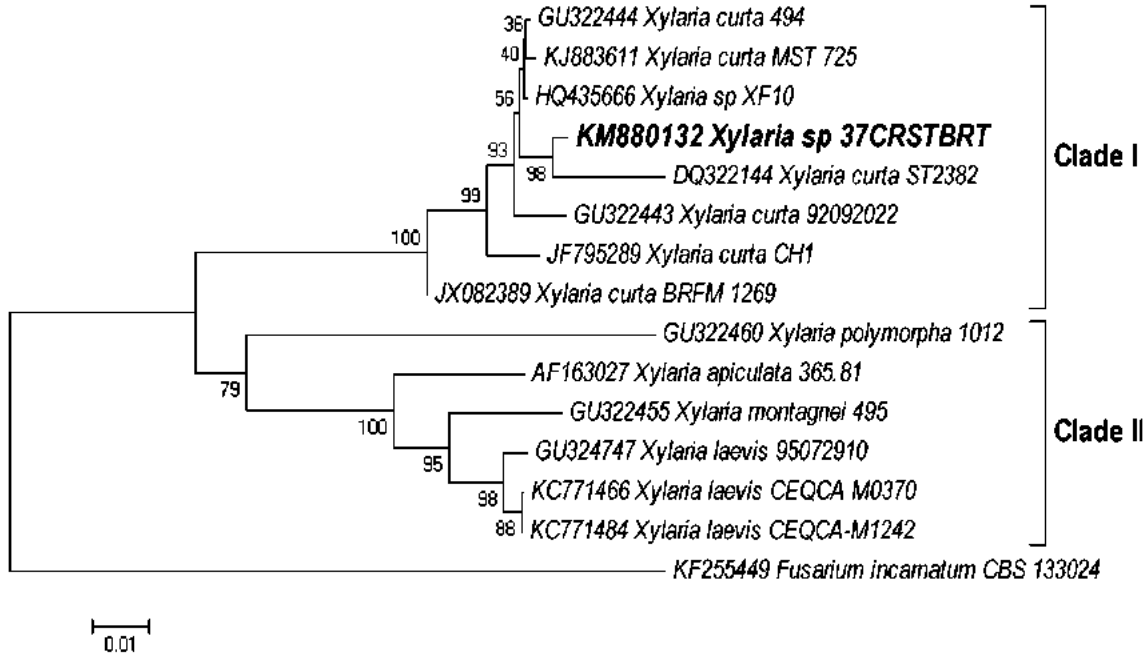


Fig 5.14: Neighbor-joining tree based on the ITS1–5.8S–ITS2 region. The optimal tree with the sum of branch length= 0.4305 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates).

The Neighbor joining phylogenetic tree based upon LSU region resolved into four clades- Clade I clustered 3 strains of *X. curta* along with #37 CRSTBRT with significant bootstrap support value. Clade II clustered *X. acuta* and *X. apoda. Xylaria acuta* AFTOL emerged basal from both of these clades. Three strains of *X. grammica* were grouped in clade III and two strains of *X. papules* were clustered in clade IV. *F. solani* was used to root the tree (Fig 5.15).

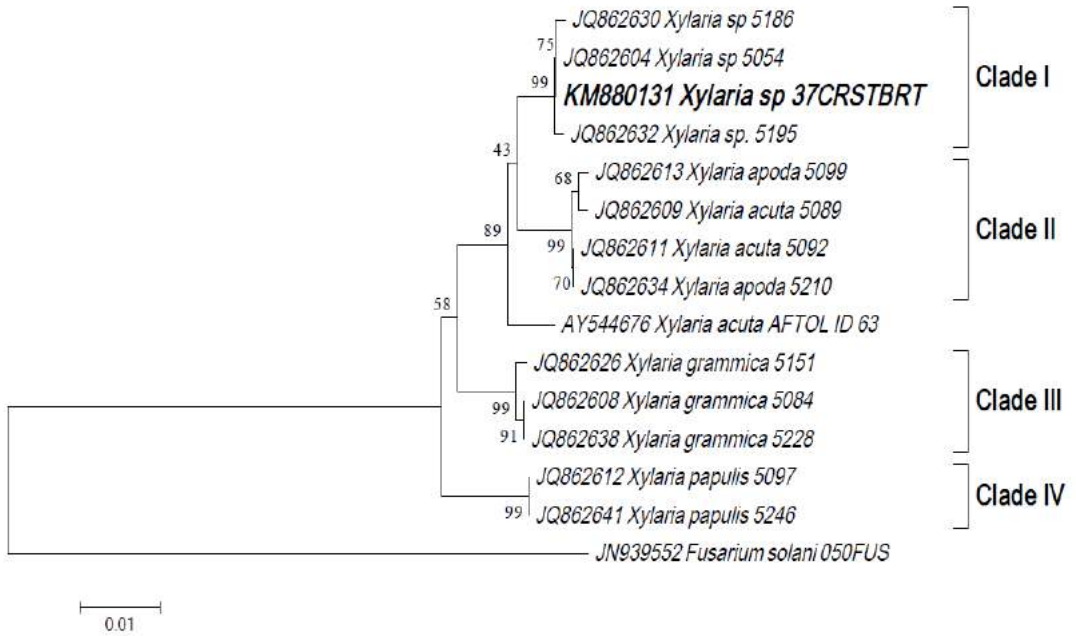


Fig 5.15: Neighbor–joining tree based on the 28S–LSU region. The optimal tree with the sum of branch length= 0.178 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates)

The phylogenetic tree based upon RPBII region divided into four major clades. Clade I was divided into three subclades clustering three strains of *X. cubensis*, two strains of *X. laevis* and *X. berteri* in subclade I, II and III respectively. Clade II was further sectioned into two subclades of *X. telfairii* and *X. regalis* with bootstrap support value of 95. Clade III grouped *X. frustulosa* with bootstrap value of 100. Clade IV clustered selected isolate, #37 CRSTBRT, along with *X. curta* species

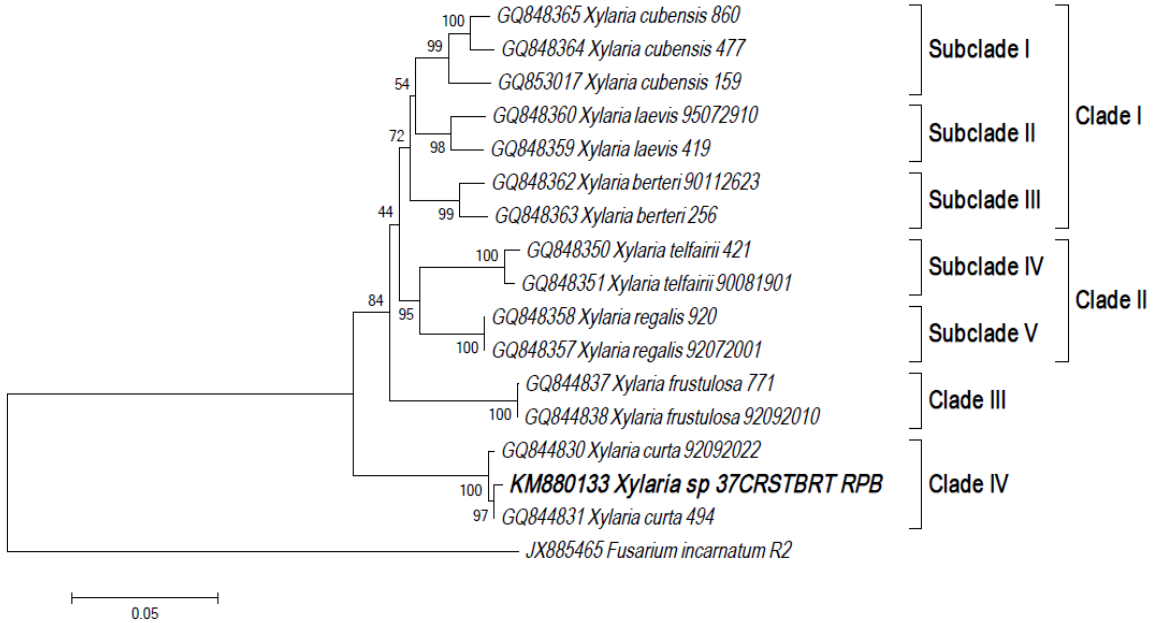


Fig 5.16: Neighbor–joining tree based on the RPBII region. The optimal tree with the sum of branch length= 0.596 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates)

with bootstrap value of 97, thereby confirming the placement of #37 CRSTBRT as *Xylaria curta. F. incarnatum* was chosen as out-group to root the tree (Fig 5.16).

5.6 Enzyme purification

Bioactivity guided purification of enzyme from the culture broth of #37 CRSTBRT (*X. curta*) was carried out using ion exchange chromatography followed by gel filtration chromatography. 96.34 mg of crude protein was obtained from 10 l of culture broth by using ammonium sulphate precipitation. The *in vitro* fibrinolytic activity of the culture broth and crude protein was 3.6 U/ml and 9.83 U/ml using plasmin equivalence (Fig 5.17). However, the specific *in vitro* fibrinolytic activity of the crude protein was found to be 10.2 U/mg of protein (Table 5.11). Subsequently, the crude protein was partially purified and concentrated using Sepharose- Mono-Q Column. Five fractions exhibiting *in vitro* fibrinolytic activity were pooled and further purified through Sephacryl 300 columns yielded two Peaks. Peak1, which eluted between fraction number 29 to 35 and Peak 2, which eluted between fraction number 36 to 52 (Fig 5.18). SDS-PAGE of both Peak 1 and Peak 2 exhibited homogeneity and their molecular mass was ~60 kDa and ~33 kDa respectively (Fig 5.19). Further, the *in vitro* fibrinolytic activity was only exhibited by Peak 2 which was having a molecular mass of ~33kDa (Fig 5.20). The pure enzyme (~33kDa) exhibited a specific activity of 38 U/mg after 9.5 fold purification.

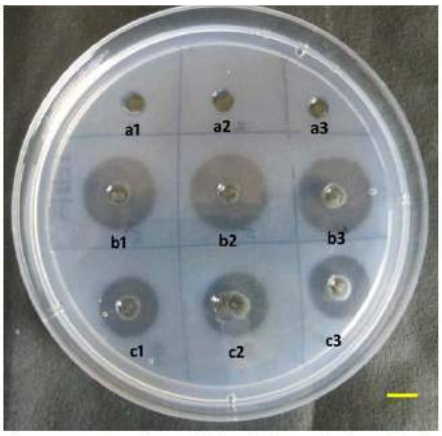


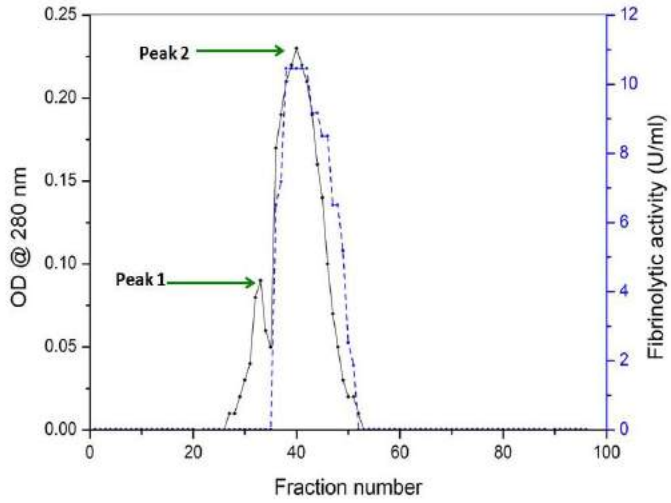
Fig 5.17: *In vitro* fibrinolytic activity of the culture broth and crude protein (ammonium sulphate precipitate). Well id a1-a3: Control; b1-b3: Crude protein, c1-c3: culture broth. Bar 10 mm

Table 5.11: Purification summary of xylarinase from culture broth of *Xylaria curta*

Purification step	Volume (ml)	Protein (mg)	Activity (U/ml)*	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Culture broth	10000	9004.7	3.6	36,000	4.00	1	100
Ammonium sulphate	100	96.3	9.8	980	10.2	2.6	2.7
Sepharose-Mono-Q-Column	12	8.5	10.5	126	14.8	3.7	0.4
Sephacryl S-300	3	0.9	11.4	34.2	38.0	9.5	0.09

The activity was determined by the fibrin plate assay as described in materials and methods. The units of activity are calculated on the basis of the equivalent standard plasmin units.

The purified enzyme was named as "xylarinase" after the name of its producing organism *Xylaria curta.* This was further taken up for further characterization.



← Fig 5.18: Gel filtration chromatography (Sephacryl 300) of the six pooled fibrinolytic fractions obtained from Sepharose-Mono-Q-Column chromatography. The optical density as well as *in vitro* fibrinolytic activity of the fractions contributing in formation of the two peaks have been depicted.

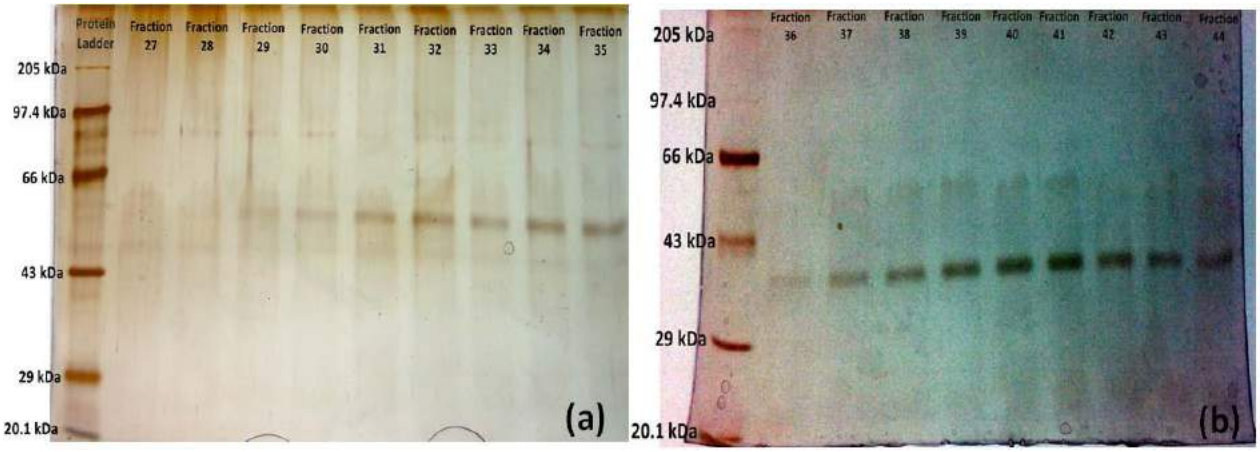
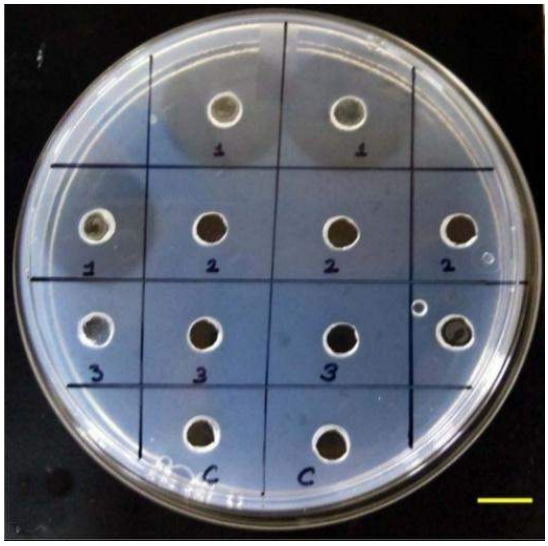


Fig 5.19: SDS–PAGE of fractions obtained by gel filtration chromatography (a) Fraction no. 27–35 (Peak 1, ~ 60 kDa) (b) Fraction no. 36–43 (Peak 2, ~ 33kDa)



← Fig 5.20: *In vitro* fibrinolytic activity of Peak 1(~60 kDa) and Peak 2 (~33 kDa) obtained after gel filtration chromatography. Well id 1: Peak 1 (~33 kDa); Well id 2: Peak 2 (~60 kDa); Well id 3: 20 mM Tris–HCl; Well id C: Blank. (Bar 10 mm)

5.7 Confirmation of the molecular weight and fibrinolytic activity of xylarinase

The molecular weight of xylarinase was reconfirmed by Native–PAGE, SDS–PAGE, fibrin zymography and MALDI–ToF MS analysis while purity was reconfirmed by HPLC. The purified enzyme (xylarinase) was monomeric in nature and migrated as a single band of ~33 kDa under native and SDS- PAGE (Fig 5.21 a, b). To ascertain the *in vitro* fibrinolytic activity of xylarinase, fibrin zymography was carried out which reaffirmed that the enzyme possessed *in vitro* fibrinolytic activity by presence of the clear area on fibrin gel at the same molecular weight as depicted by the enzyme (~33kDa) on SDS-PAGE (Fig 5.21c). Further, on HPLC analysis, xylarinase exhibited a single peak at retention time of 2.3 min. (Fig 5.22). Based on MALDI–ToF MS analysis, the molecular weight of xylarinase was found to be 33.8 kDa which corroborates our electrophoretic findings (Fig 5.23).

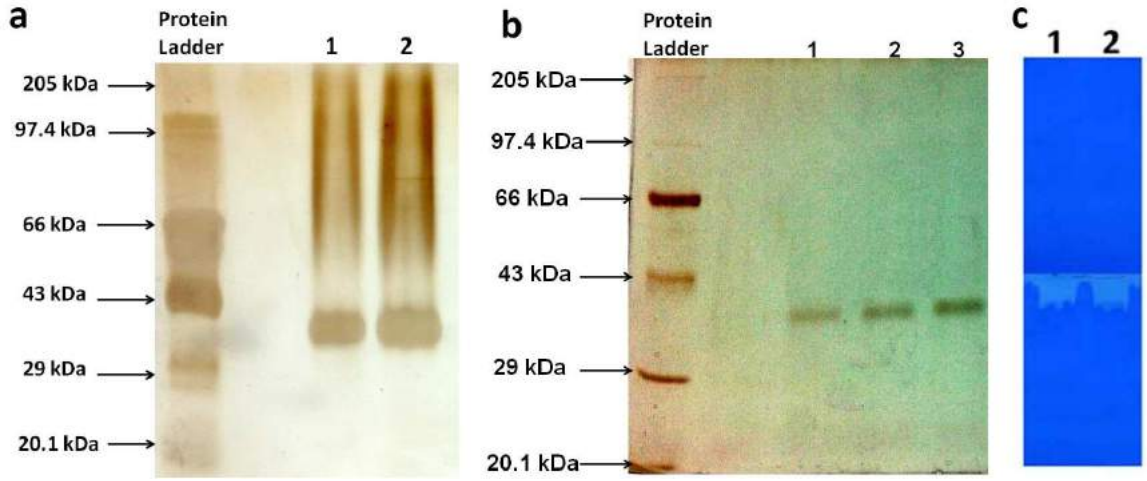


Fig 5.21: Molecular weight determination of xylarinase by (a) Native–PAGE (b) SDS–PAGE (c) Zymography for fibrinolytic activity of xylarinase

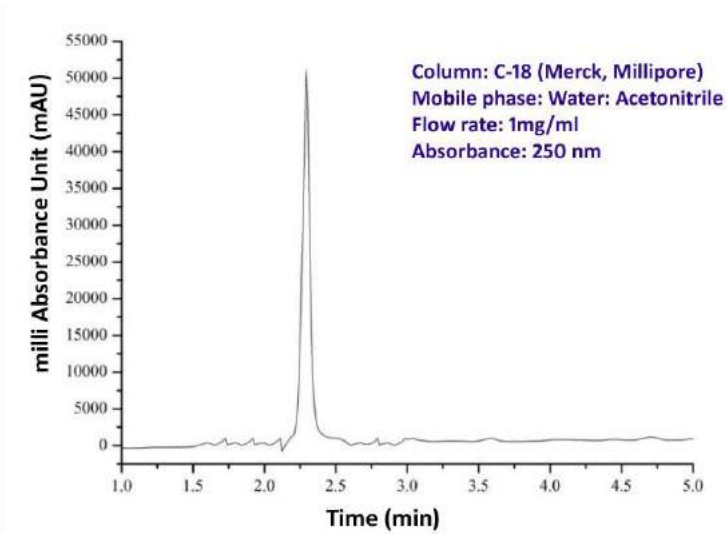


Fig 5.22 Purity of xylarinase by HPLC

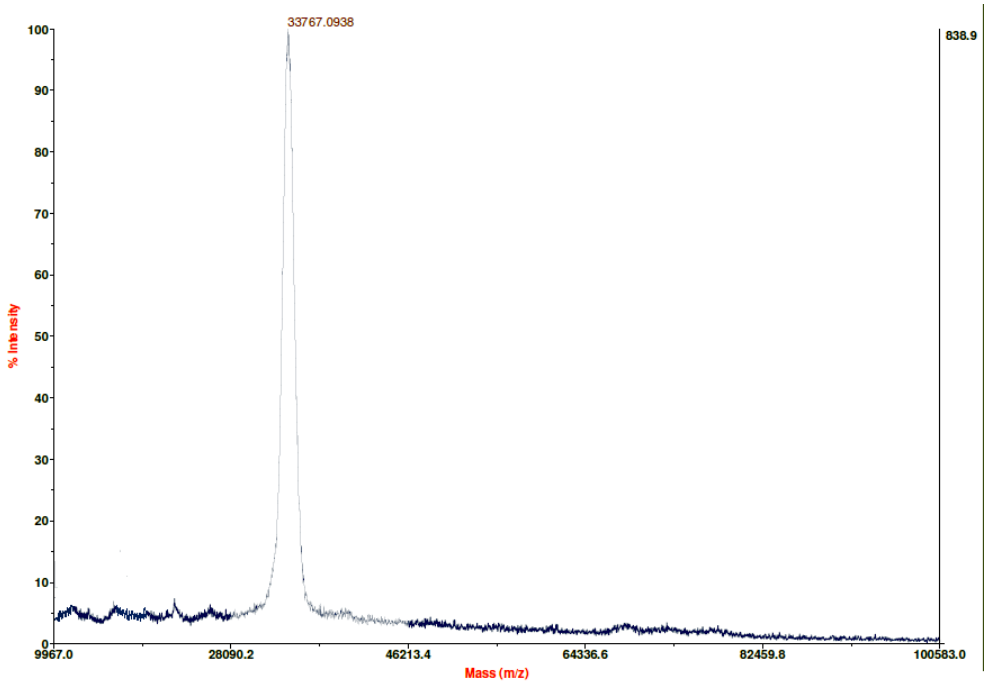


Fig 5.23: Determination of molecular weight of xylarinase by MALDI–ToF MS

5.8 N-Terminal amino acid sequence of xylarinase

First 13 amino acids residues of xylarinase obtained by automated Edman degradation method were Ser–Asp–Gly–Pro–Leu–Pro–Gly–Gly–Val–Val–Try–Ala–Gly (SNGPLPGGVVWAG). No putative conserved domains were detected in the NCBI database. However, this N-terminal sequence showed 100 % identity with proteases and peptidases from various *Streptomyces* species (Table 5.12). The N-terminal sequence of xylarinase was found to be different from those previously reported fibrinolytic enzymes from fungi thereby suggesting its novelty.

Table 5.12: Alignment of N-terminal acid sequence of xylarinase and other enzymes

Organism	Accession number	Alignment												
Xylaria curta(xylarinase)	Present study	S	N	G	P	L	P	G	G	V	V	W	A	G
Streptomyces globisporus *	WP030576741	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces baarnensis#	WP030087295	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces sp. CNB091#	WP018956326	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces griseus#	WP012379135	S	P	S	P	L	P	G	G	V	V	W	E	C
*Streptomyces roseosporus**	EFE77436	S	P	S	P	L	P	G	G	V	V	W	E	C
*Streptomyces globisporus**	WP010061589	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces sp. MNU77 #	WP047178678	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces sp. CNS654 #	WP032763306	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces sp. JS01	WP032783415	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces griseus#	WP030716777	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces albus #	WP030631310	S	P	S	P	L	P	G	G	V	V	W	E	C
Streptomyces anulatus#	WP030580451	S	P	S	P	L	P	G	G	V	V	W	E	C

* refers to proteases; # refers to peptidases

5.9 Fibrinolytic and fibrinogenoyltic activity of xylarinase

The fibrin degradation pattern obtained in the present study revealed that there is a significant difference in the hydrolysis pattern of different chains of fibrinogen by xylarinase. Xylarinase rapidly hydrolyzed Aα chain of fibrin in about 5 min followed by Bβ chain in appx. 10 min, however no activity was observed against the γ chain of fibrinogen over a period of 180 min (Fig. 5.24a). On the other hand, plasmin also hydrolyzed Aα chain and Bβ chain in 5 min. However, the γ-chain was hydrolyzed after 60 min by plasmin (Fig 5.24b). Hence, the fibrinolysis pattern exhibited by xylarinase was different from plasmin.

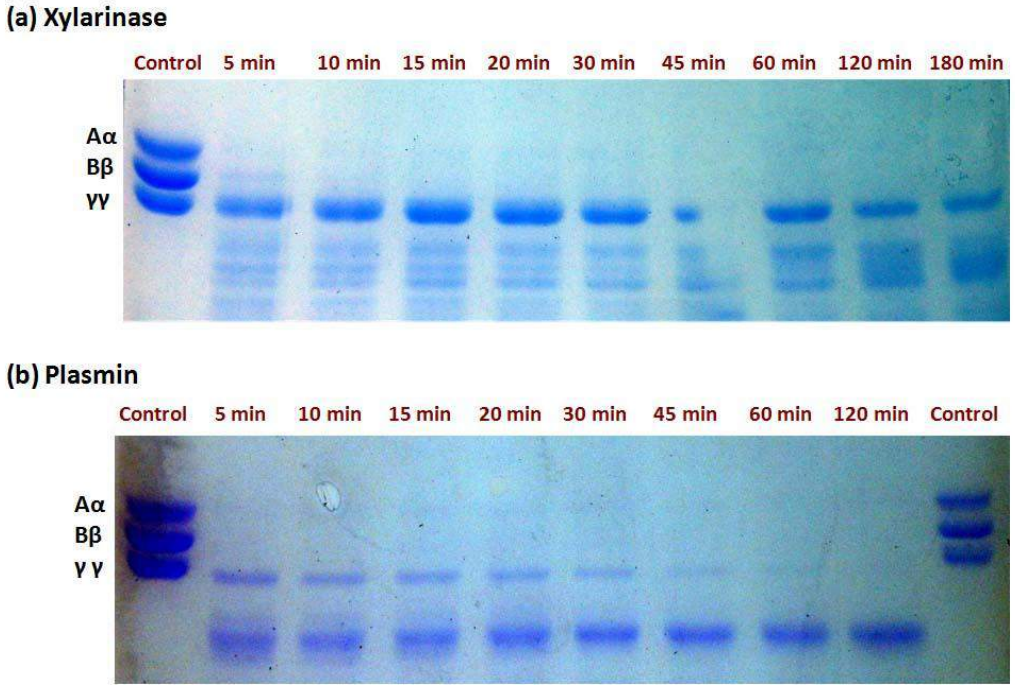


Fig 5.24: Hydrolysis pattern of the constituent proteins of fibrin at different incubation time by (a) xylarinase (b) plasmin

Similarly, the fibrinogen hydrolysis pattern by xylarinase exhibited a preference for Aα chain followed by Bβ chain, however it could not degrade the γ chain till three hours (180 min) incubation. Further, after 24 h of incubation with xylarinase, the γ chain remained intact. This implies that xylarinase exhibited α and β fibrinogenase activity (Fig 5.25a). However, it was found that plasmin was able to degrade Aα chain after 10 min of incubation, however both Bβ and γ chains were degraded at 90 min of incubation (Fig 5.25b).

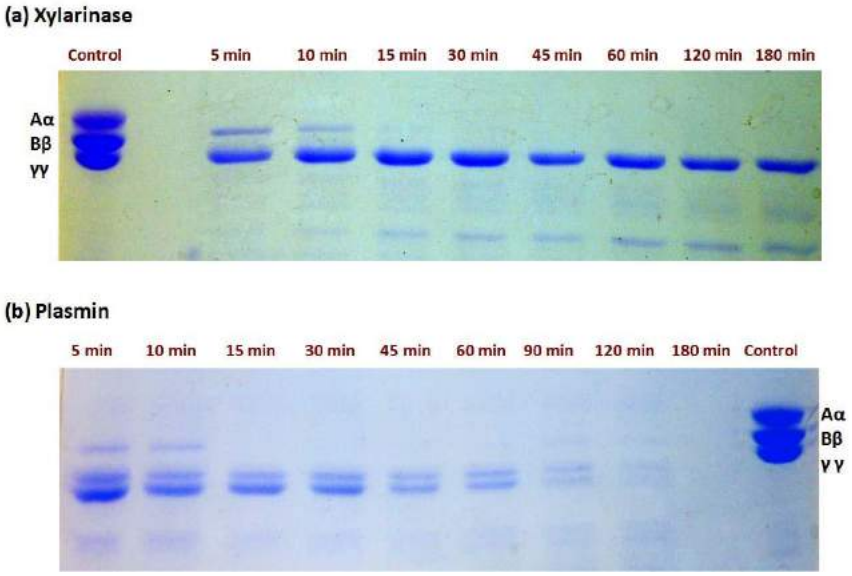


Fig 5.25: Hydrolysis pattern of constituent proteins of fibrinogen at different incubation time by (a) xylarinase (b) plasmin

5.10 Fibrinogen clotting time assay

The fibrinogen clotting time assay was used to assess the action of xylarinase on conversion of fibrinogen into fibrin. Prolongation of FCT suggests two possible sites of action for xylarinase which could either be fibrinogen or thrombin. As evident from Table 5.13, xylarinase was found to significantly extend the clotting time when compared to control (Control vs. Treatment A). Further, addition of fibrinogen (100 µl and 150 µl) enhanced the clotting time significantly till 430 s i.e. appx. 7 min. However, no significant difference in clotting time was observed between treatment A and C1 (60 µl thrombin) and C2 (90 µl thrombin). This suggests that the probable site of action of xylarinase is fibrinogen and not thrombin.

Table 5.13: Effect of xylarinase on fibrinogen clotting time

Treatment	**Fibrinogen clotting time (in seconds)**
Control (fibrinogen + thrombin)	297.3^{d}±3.1
A (fibrinogen+ thrombin+ xylarinase)	323.3^{c}± 4.5
B1 (fibrinogen*+ thrombin + xylarinase)	339.7^{b}± 8.0
B2 (fibrinogen**+ thrombin+ xylarinase)	430.3^{a} ± 11.0
C 1 (fibrinogen+ thrombin¶ +xylarinase)	321.7^{c}± 5.5
C2 (fibrinogen+ thrombin¶¶+xylarinase)	323.3^{c}± 6.8

The data presented are mean ± SD of three replicates. Means represented by different alphabets are significantly different from by Tukey's post hoc analysis ($p \leq 0.05$). * represents 100 µl of fibrinogen, ** represent 150 µl of fibrinogen; ¶ represents 60 µl of thrombin, ¶¶ represents 90 µl of thrombin

5.11 Biochemical characterization of xylarinase

5.11.1 Effect of pH on in vitro fibrinolytic activity of xylarinase

The *in vitro* fibrinolytic activity of xylarinase ranged between a pH of 6-9. A significant difference in the *in vitro* activity of xylarinase was observed at different pH levels in the range of 4-9 by one way ANOVA ($F(6, 14) = 84.38$, $p \leq 0.0001$). The highest *in vitro* fibrinolytic activity by xylarinase was observed at a pH 8 which was significantly different from *in vitro* fibrinolytic activity exhibited by xylarinase at other pH tested by Tukey's post hoc analysis (Fig 5.26). The pH stability for *in vitro* fibrinolytic activity of xylarinase resided between pH 7 to 8. As per Tukey's analysis, a non-significant *in vitro* activity of xylarinase was found at pH 6 and 9. Least *in vitro* fibrinolytic activity of xylarinase was present at pH 4 and 5 which did not exhibited significant difference among them.

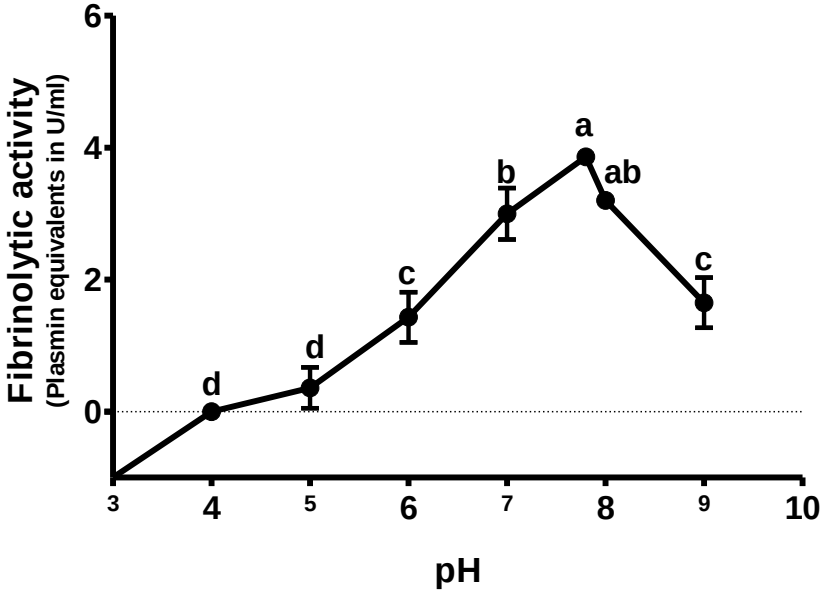


Fig 5.26: *In vitro* fibrinolytic activity of xylarinase at different pH. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letter are not significantly different by Tukey's post hoc test at $p<0.05$

5.11.2 Effect of temperature on in vitro fibrinolytic activity of xylarinase

One way ANOVA exhibited a statistically significant effect of different temperatures on the *in vitro* fibrinolytic activity of xylarinase at $p<0.05$: $F(9, 20) = 108.4$, $p \leq 0.0001$). Highest *in vitro* fibrinolytic activity of xylarinase was observed at 35 °C which was significantly different from the fibrinolytic activity at other incubation temperatures as evident by Tukey's post hoc analysis (Fig 5.27). There

was no significant effect of temperature observed at 25 °C, 30 °C and 40 °C as per Tukey's post hoc analysis. A significant drop in the fibrinolytic activity of xylarinase was observed at 45°C and 50 °C. The fibrinolytic activity was completely lost at 60 °C and above.

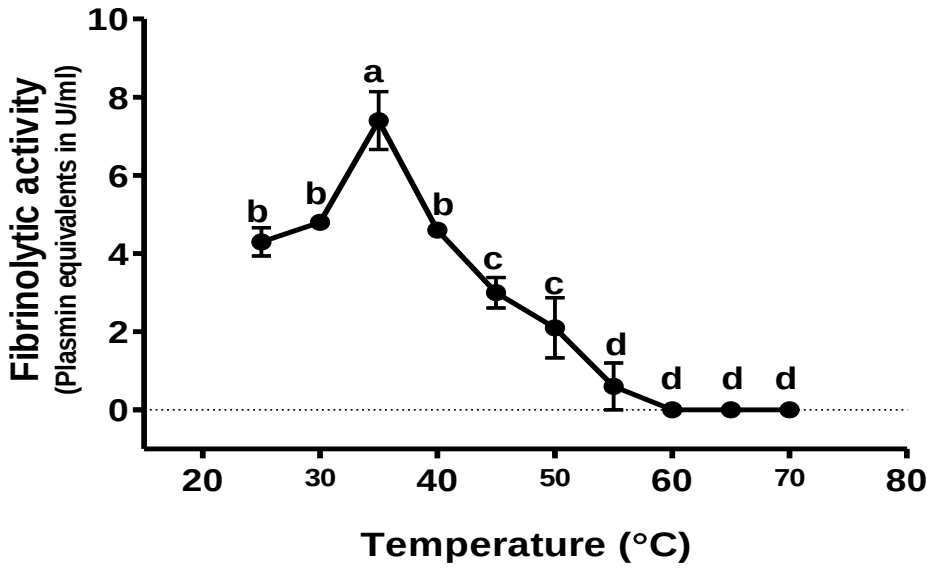


Fig 5.27: *In vitro* fibrinolytic activity of xylarinase at different incubation temperature. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letter are not significantly different by Tukey's post hoc test at $p<0.05$

5.11.3 Effect of metal ions on in vitro fibrinolytic activity of xylarinase

Metal ions significantly affected the *in vitro* fibrinolytic activity of xylarinase at 2 mM concentration as evident from one way ANOVA at $p \leq 0.05$: $F(10, 22) = 20.07$, $p \leq 0.0001$). The major finding of this experiment was the inhibition or complete loss of *in vitro* fibrinolytic activity of xylarinase in the presence of zinc and ferric ions (Fig 5.28). No significant difference in the *in vitro* fibrinolytic activity was observed in the presence of cobalt and calcium ions when compared to control using Tukey's post hoc analysis. Na^+, K^+, Al^{3+}, Mg^{2+}, Mn^{2+} and Cu^{2+} ions significantly reduced the *in vitro* fibrinolytic activity when compared to the control. However, the *in vitro* fibrinolytic activity of xylarinase in presence of K^+, Na^+ and Al^{3+} did not differ significantly as per Tukey's post hoc analysis.

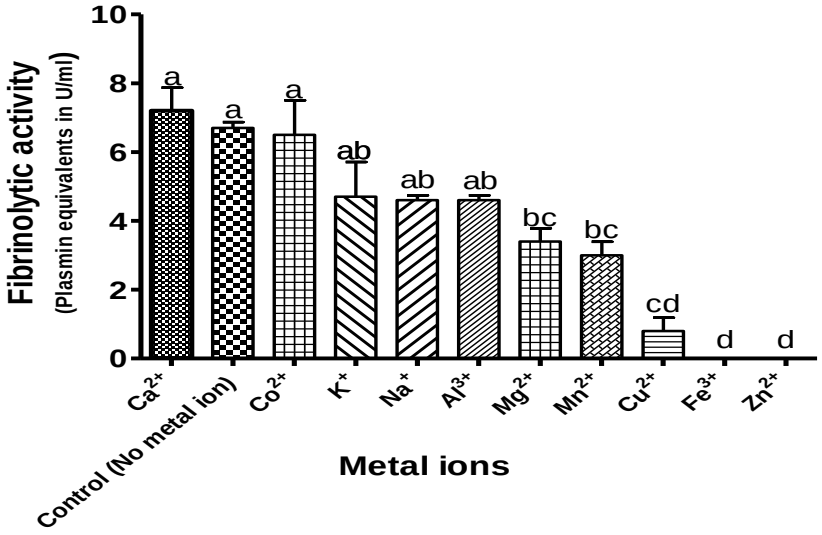


Fig 5.28: *In vitro* fibrinolytic activity of xylarinase in presence of different metal ions at 2 mM concentration. Data represented are mean ± standard deviation of three replicates. Mean values represented by different letters are significantly different by Tukey's post hoc test at p<0.05

5.11.4 Effect of protease inhibitors on in vitro fibrinolytic activity of xylarinase

EDTA and EGTA were found to be the strongest inhibitors of xylarinase as they completely inhibited the *in vitro* fibrinolysis (Fig 5.29). There was a significant difference in the *in vitro* fibrinolytic activity of xylarinase in the presence of protease inhibitors by one way ANOVA at p <0.05: F (7, 16) =136.6, p ≤ 0.0001.

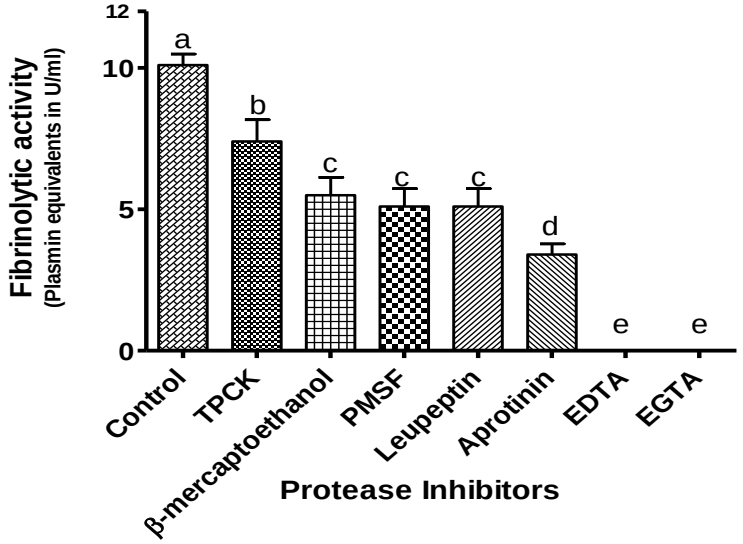


Fig 5.29: *In vitro* fibrinolytic activity of xylarinase in presence of different protease inhibitors at 2 mM concentration. Data represented are mean ± standard deviation of three replicates. Mean values represented by different letters are significantly different by Tukey's post hoc test at <0.05

Inhibition of *in vitro* fibrinolytic activity by Tukey's analysis was found to be similar in the case of β-mercaptoethanol, PMSF and leupeptin which exhibited ~ 50 % inhibition in the *in vitro* fibrinolytic activity of xylarinase.

5.12 Kinetic constants of xylarinase

The K_m value of xylarinase for fibrin was 246 µM while the V_{max} was 1.22 µM/min. However the K_m value of plasmin for fibrin was found to be higher i.e. 282 µM while the V_{max} was 0.13 µM/min (Fig. 5.30)

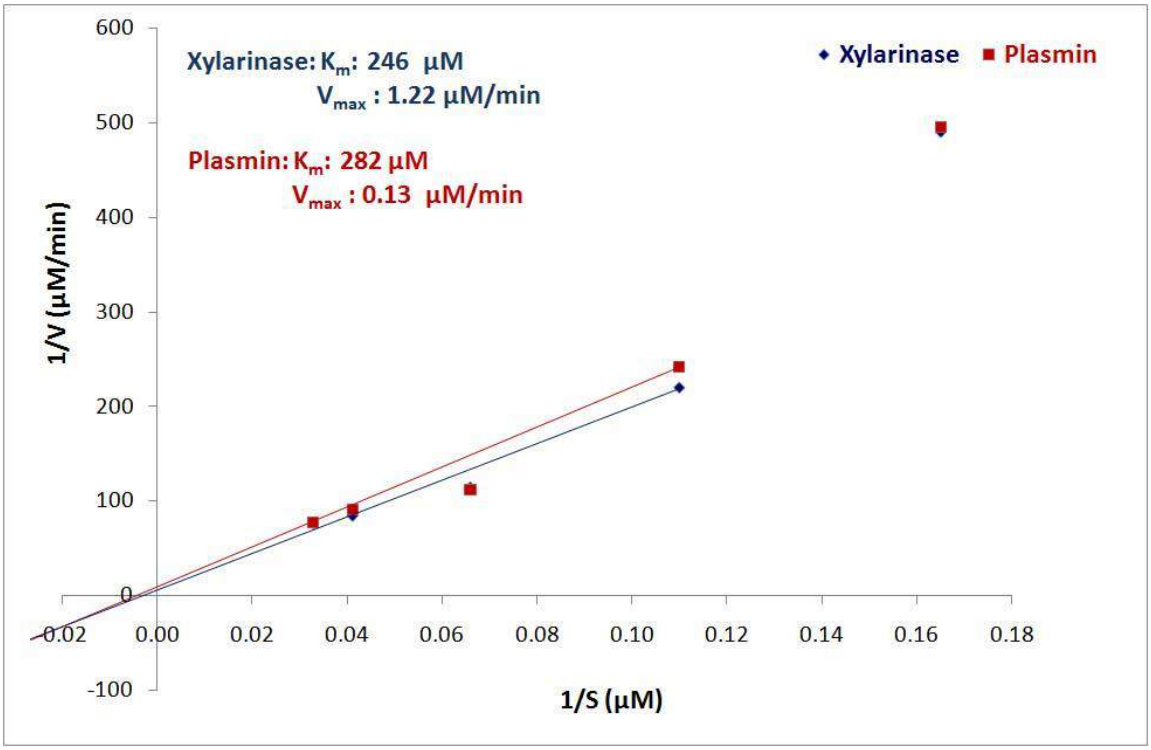


Fig 5.30: Lineweaver–Burk plot for the hydrolysis of fibrin by xylarinase and plasmin

5.13 Spectrophotometric assay for *in vitro* fibrinolytic activity of xylarinase

In spectrophotometric assay, xylarinase exhibited a fibrinolytic activity of 12.5 ± 0.9 U/ml where as plasmin displayed fibrinolytic activity of 11.8 ± 0.6 U/ml. From this assay, it becomes quite clear that xylarinase possessed a better fibrinolytic activity than plasmin under *in vitro* conditions.

5.14 *In vitro* thrombolytic activity of xylarinase

Addition of xylarinase resulted in dose dependent dissolution of the thrombus. 50 µg of xylarinase completely dissolved the thrombus whereas similar concentration of plasmin resulted in 84 % hydrolysis of the thrombus. The results clearly depict that xylarinase possess a better thrombolytic efficacy than plasmin under tested conditions (Fig 5.31, 5.32) (Meshram et al 2016a).

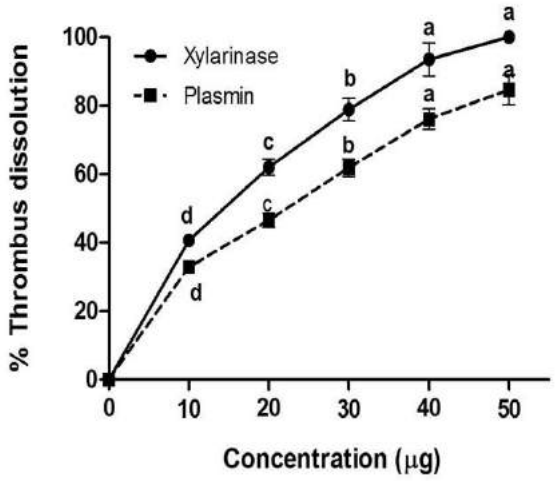


Fig 5.31. *In vitro* thrombolytic activity of xylarinase and plasmin. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letters are not significantly different by Tukey's post hoc test at $p<0.05$

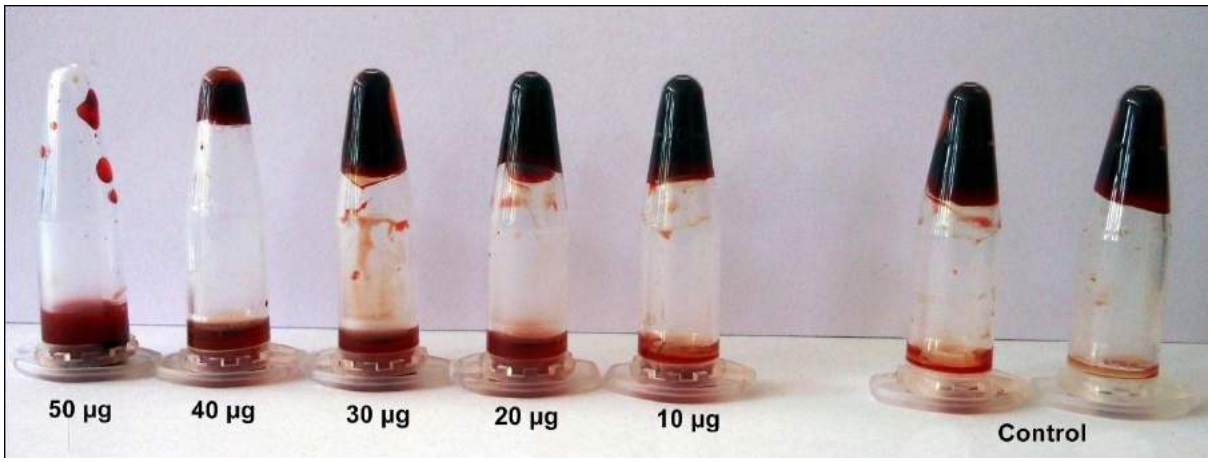


Fig 5.32: *In vitro* thrombolytic activity of xylarinase after 1 h of incubation

5.15 *In vitro* anticoagulant activity of xylarinase

Xylarinase at different concentrations was found to significantly enhance the APTT as per one way ANOVA analysis at $p<0.05$: $F(3, 8) = 1552$, $p \leq 0.0001$. Further, Tukey's post hoc analysis also indicated that there was a significant difference in prolongation of APTT by different concentrations of xylarinase (Table 5.14). Xylarinase also exhibited a significant prolongation in the PT as compared

to the control only at a concentration of 20 µg as per Tukey's post hoc analysis. A similar trend was observed in the case of TT (Meshram et al 2016a).

Table 5.14: Effect of xylarinase on blood coagulation

Enzyme concentration	Clotting time (s)¥		
	PT*	APTT**	TT***
Control (No enzyme)	**7.9^{c} ± 1.5**	**21.6^{d} ± 2.9**	**6.8^{b} ± 0.3**
Xylarinase (5µg)	10.4^{c} ± 1.7	116.8^{c}± 3.1	7.4^{b} ± 0.3
Xylarinase (10 µg)	19.2^{b} ± 2.3	156.0^{b} ± 5.5	8.0^{ab} ± 0.7
Xylarinase (20 µg)	**46.5^{a} ± 4.4**	**> 180.0^{a} ± 0.0**	**9.3^{a} ± 1.1**

¥Data presented are mean ± standard deviation of three replicates. Means within the same column with different superscript letters are different by Tukey's post hoc test ($p<0.05$). * refers to Prothrombin Time; ** refers to Activated partial thromboplastin time, *** refers to Thrombin time

5.16 *In vitro* assessment of cytotoxicity of xylarinase

Xylarinase did not have a significant cytotoxic effect on RAW murine macrophage cells (Fig 5.33a). The RAW murine macrophage cells did not exhibit a significant difference in viability when treated with 50 µg of xylarinase after 48 h based on the OD at 540 nm using MTT assay (Fig 5.33b).

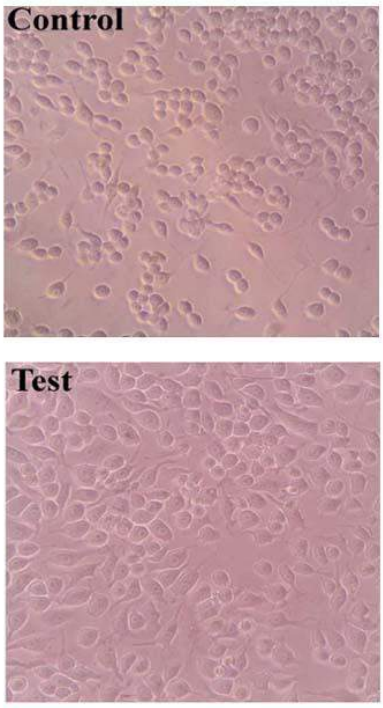


Fig 5.33 (a): Morphology of RAW murine macrophage cells after incubation with 50 µg of xylarinase

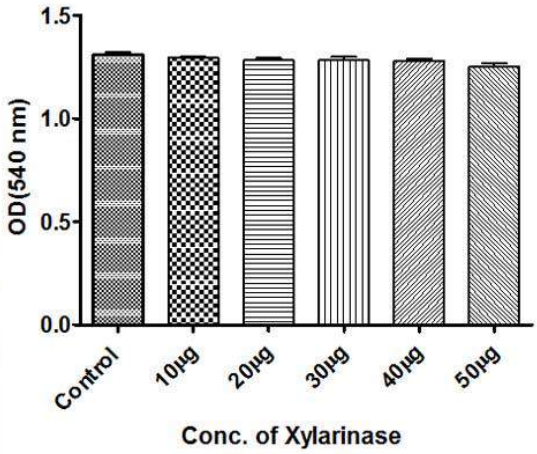


Fig 5.33 (b): *In vitro* cytotoxicity of xylarinase on RAW murine macrophage cell lines. Data represented are mean ± standard deviation of three replicates

5.17 SSF for production of fibrinolytic enzyme

5.17.1 Utilization of agro–industrial residues for production of fibrinolytic enzyme

Out of the five agro industrial residues tested for solid state production of the fibrinolytic enzyme by *Xylaria curta* (#37 CRSTBRT), maximum *in vitro* fibrinolytic activity was expressed by rice chaff followed by wheat bran and egg shell. Banana peel and orange peel as a solid substrate failed to produce fibrinolytic enzyme (Fig 5.34). A significant difference in the *in vitro* fibrinolytic activity induced on the agro-industrial residues was recorded by one way ANOVA analysis at $p<0.05$: $F(4, 10) = 94.17$. Tukey's post hoc analysis also confirmed that the highest significantly different *in vitro* fibrinolytic activity was shown by rice chaff. However, the analysis also indicated that the *in vitro* fibrinolytic activity of enzyme extract obtained from wheat bran and egg shell did not differ significantly. The fungus was unable to grow on banana and orange peel. Thus, based on the above results rice chaff was selected as a solid substrate fermentation medium for production of fibrinolytic enzyme by *Xylaria curta* (#37 CRSTBRT).

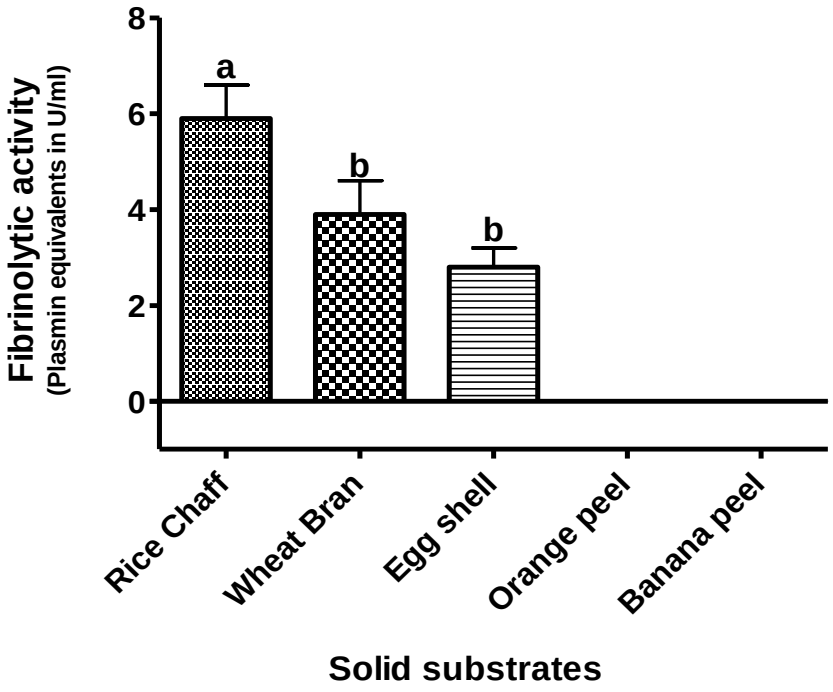


Fig 5.34: Screening of agro-industrial residues for *in vitro* fibrinolytic activity. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letters are not significantly different by Tukey's post hoc test at $p<0.05$

5.17.2. Physico–chemical characterization of rice chaff

As *Xylaria curta* exhibited maximum growth and fibrinolytic enzyme production over rice chaff, we proceeded with the characterization of rice chaff. The volatile matter, ash, moisture and fixed carbon content of rice chaff were found to be 60 %, 16.5 %, 10.5 % and 12.9 % respectively. Rice chaff exhibited a calorific value of 3.8 MJ/Kg and CHN content of rice chaff was estimated to be 40.9 %, 5.1 % and 0.3 % respectively.

5.17.3 Optimization of culture conditions for fibrinolytic enzyme production by SSF

One variable at a time approach was used for the optimization of culture conditions i.e (a) incubation period (b) moisture content (c) particle size and (d) incubation temperature.

5.17.3.1 Optimization of incubation period for fibrinolytic enzyme production by SSF

The production of fibrinolytic enzyme by *Xylaria curta* (#37 CRSTBRT) began after 6th day of incubation and increased till 15th day, it gets stabilized between 15–17 days of incubation. Fibrinolytic enzyme production by *Xylaria curta* under different incubation period was found to be significantly different by one way ANOVA at $p<0.05$: $F(14, 30) = 93.08$, $p \leq 0.0001$.

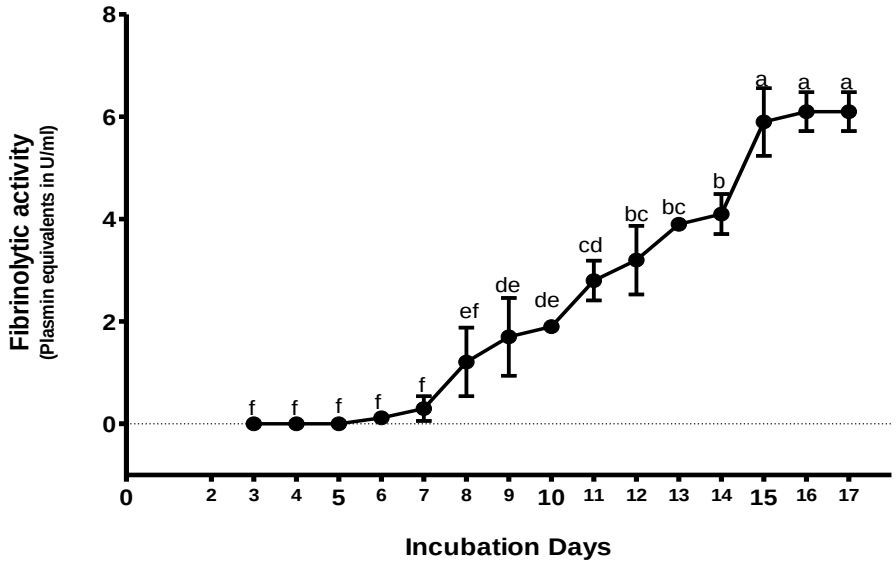


Fig 5.35: Determination of optimal time for production of fibrinolytic enzyme by *X. curta* by SSF. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letters are not significantly different by Tukey's post hoc test at $p<0.05$

The optimal fibrinolytic enzyme production was observed at 15th day of incubation by *in vitro* fibrinolytic assay. Tukey's post hoc analysis has revealed that there was no significant difference in

the production of fibrinolytic enzyme on 15th, 16th and 17th day of incubation (Fig 5.35). Therefore, 15 days was selected as an optimum incubation time for production of fibrinolytic enzyme by solid substrate fermentation using rice chaff as the solid substrate.

5.17.3.2 Optimization of moisture content for fibrinolytic enzyme production by SSF

Water has a profound effect on the fibrinolytic enzyme production by SSF. A significant difference in the fibrinolytic enzyme production at different moisture level of the solid substrate was observed by *in vitro* fibrinolytic activity using one way ANOVA, p≤0.05. Highest fibrinolytic enzyme production was observed at 50 % moisture level which was significantly different from the fibrinolytic enzyme production at other moisture level by Tukey's post hoc analysis. 100 % moisture level induced least fibrinolytic enzyme production (Fig 5.36). Hence, 50 % moisture level was selected for further production and purification of the fibrinolytic enzyme by solid substrate fermentation

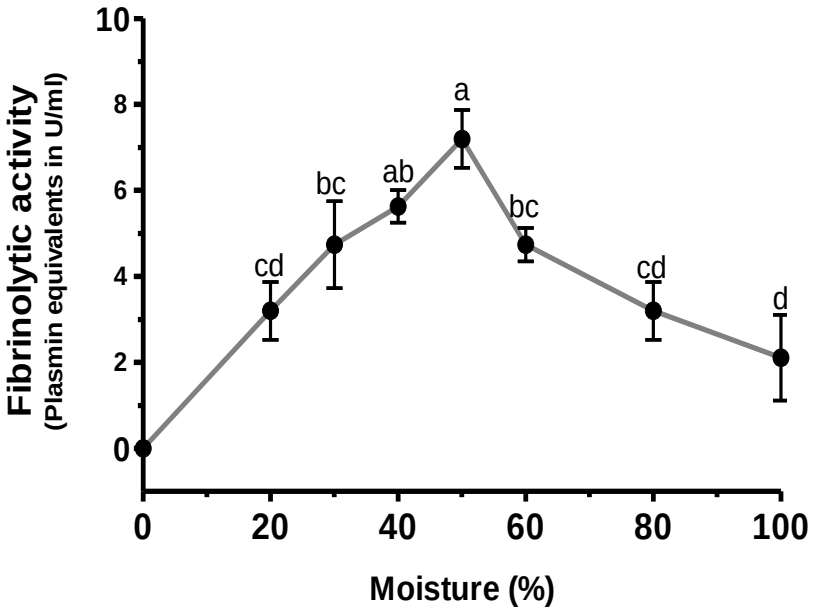


Fig 5.36: *In vitro* fibrinolytic activity at different moisture level of solid substrate to assess fibrinolytic enzyme production by *X. curta*. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letters are not significantly different by Tukey's post hoc test at p<0.05

5.17.3.3 Optimization of particle size of substrate for fibrinolytic enzyme production by SSF

In SSF, particle size plays an important role in the production of enzymes. The highest fibrinolytic enzyme production as assessed by *in vitro* fibrinolytic assay was observed at a particle size of 500

µm. Fibrinolytic enzyme production was significantly different on different particle size of rice chaff by one way ANOVA at p≤0.05: F (5,12)-24.8, p≤0.0001. Tukey's post hoc analysis further confirmed that the significant difference existed in the fibrinolytic enzyme production at different particle size with highest being at 500 µm (Fig 5.37). Lowest fibrinolytic production was observed at particle size of 200 and 800 µm. Hence, for the production of fibrinolytic enzyme by *Xylaria curta*, 500 µm was selected as the optimal particle size of the solid substrate i.e. rice chaff.

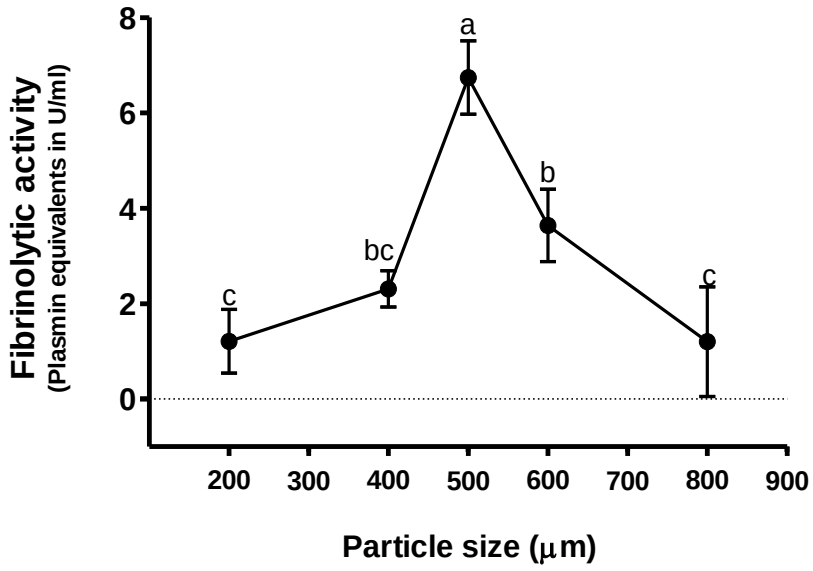


Fig 5.37: Determination of optimal particle size of rice chaff for the production of fibrinolytic enzyme by *X. curta* by SSF. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letters are not significantly different by Tukey's post hoc test at p<0.05

5.17.3.4 Optimization of incubation temperature for fibrinolytic enzyme production by SSF

Increase in production of fibrinolytic enzyme by *Xylaria curta* was observed till 28 °C after which there was a sharp decline (Fig 5.38). One way ANOVA at p≤ 0.05 suggested a significant difference in the fibrinolytic enzyme production by *in vitro* fibrinolytic assay F (5, 12) =112.2, p≤0.0001. Further, Tukey's post hoc analysis confirmed the significantly highest, fibrinolytic enzyme production at 28 °C. The enzyme production completely stopped at 37 °C. Thus, 28 °C was selected as the best incubation temperature for the SSF production of fibrinolytic enzyme by *Xylaria curta*.

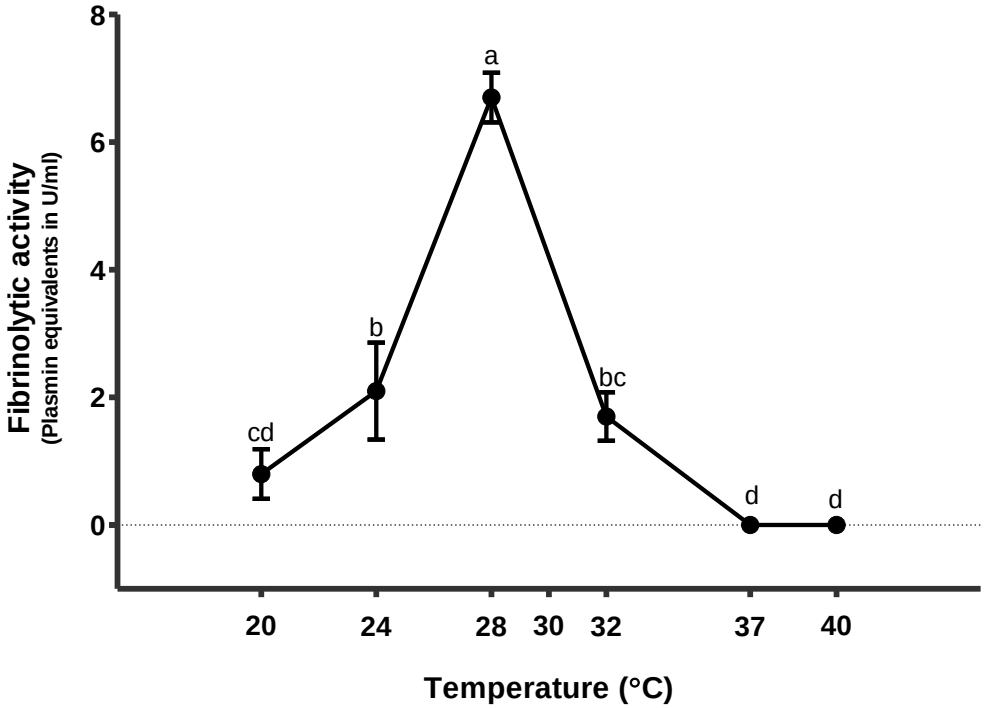


Fig 5.38: Determination of optimal temperature for the production of fibrinolytic enzyme by *X. curta* by SSF. Data represented are mean ± standard deviation of three replicates. Mean values represented by same letters are not significantly different by Tukey's post hoc test at $p<0.05$

5.17.4 Enzyme purification from SSF

The fibrinolytic enzyme produced by endophytic fungus, *Xylaria curta* (#37 CRSTBRT) over rice chaff was purified by employing single step purification technique using gel filtration chromatography with Sephacryl S–300 column. Around 32 pure fractions were obtained during the gel filtration chromatography out of which 13 fractions were found to exhibit significant *in vitro* fibrinolytic activity (≥10 mm zone) were isolated and pooled based on their SDS–PAGE profile (Fig 5.39). As summarized in Table 5.15, the crude rice chaff moldy pith contained 1090.5 mg of protein exhibiting a specific activity of 1.1 U/mg. After final purification, 11.2 mg of the purified protein was recovered from the crude rice chaff moldy pith with a recovery of 8.5 %. The purified protein exhibited a specific activity of 9.2 U/mg representing an 8.4 fold increase in the fibrinolytic activity over the native rice chaff moldy pith (Table 5.15).

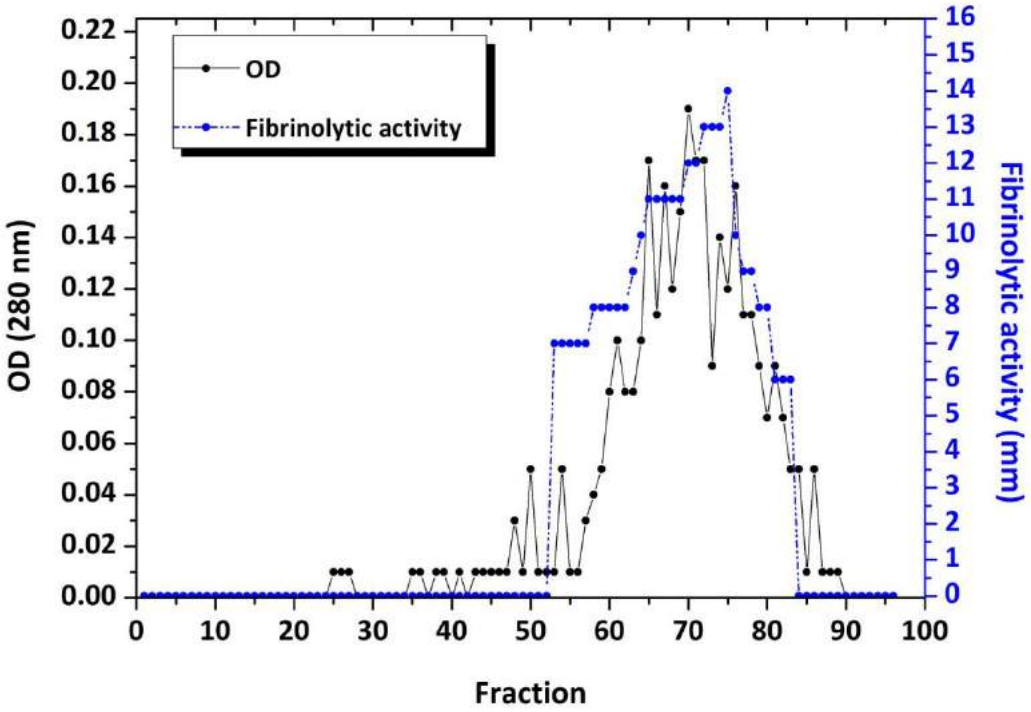


Fig 5.39: Gel filtration chromatogram and fibrinolytic activity profile of xylarinase produced by *Xylaria curta* over rice chaff

Table 5.15: Purification summary of xylarinase produced over rice chaff

Purification step	Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Fold	Yield (%)
Crude moldy pith	1090.5	1212	1.1	1	100
Ammonium sulphate	170.4	440	2.5	2.7	36.3
Sephacryl S–300	11.2	103.5	9.2	8.4	8.5

The activity was determined by the fibrin plate assay as described in materials and methods. The units of activity are calculated on the basis of the equivalent standard plasmin units.

5.17.5 Molecular weight determination by SDS–PAGE and fibrin zymography

The purified enzyme was monomeric in nature and exhibited a single band in the ~33 kDa region as observed by SDS–PAGE analysis. The purified enzyme also exhibited a strong fibrinolytic activity in similar region over fibrin zymography (Fig 5.40a). HPLC also exhibited single peak, determining its homogeneity (Fig 5.40b).

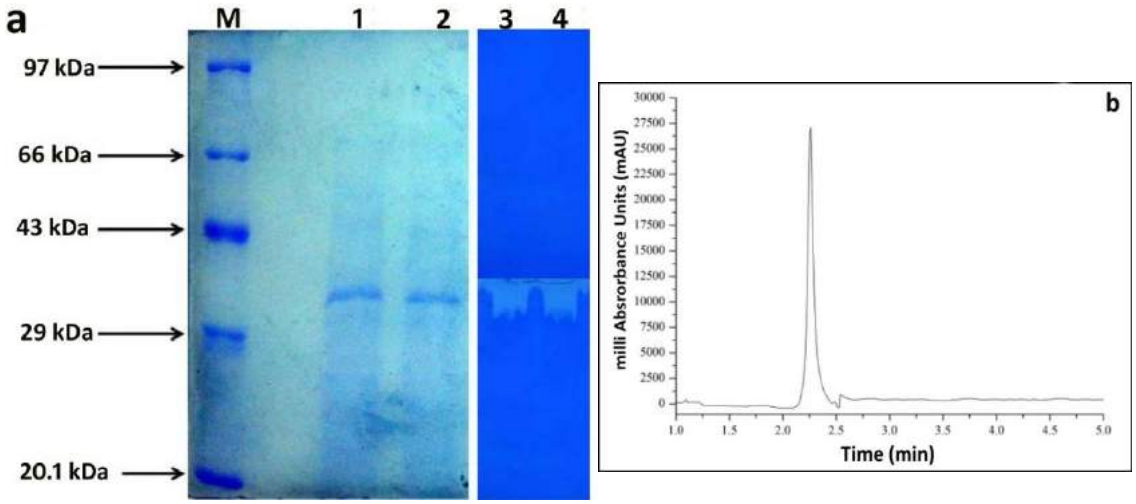


Fig 5.40: (a) Molecular weight determination of xylarinase produced over rice chaff by SDS-PAGE. M represents the protein ladder; Lane 1–2: fractions from Sephacryl S–300; Lane 3–4: Fibrin zymography of xylarinase (b) HPLC spectra of xylarinase depicting purity of the purified enzyme

5.18 Comparative analysis of the fibrinolytic enzyme “xylarinase" by SMF and SSF

Based on the comparative analysis of the two methods adopted for purification and characterization of xylarinase, SSF emerged as the most suitable, cost effective method for purification of xylarinase as the enzyme yield is quit high when compared to submerged fermentation (Table 5.16).

Table 5.16: Comparative analysis of the fibrinolytic enzyme “xylarinase" by SMF and SSF

S.no	Properties	Submerged fermentation	Solid state fermentation
1	Fibrinolytic activity	Bi–functional	Bi–functional
2	Molecular weight	33.76 kDa	~33 kDa
3	N-terminal sequence	SNGPLPGGVVWAG	SNGPLPGGVVWAG
4	Protein content	0.93 mg	11.2 mg
5	Enzyme yield	0.095 %	8.5 %
6	Biochemical nature		
a	*Maximum activity*	pH 8, 35 °C	pH 8, 35 °C
b	*Metal ions*	Ca^{2+} stimulates fibrinolytic activity Fe^{2+} and Zn^{2+} suppress fibrinolytic activity	Ca^{2+} stimulates fibrinolytic activity Fe^{2+} and Zn^{2+} suppress fibrinolytic activity
c	*Protease inhibitors*	EDTA and EGTA suppress fibrinolytic activity	EDTA and EGTA suppress fibrinolytic activity
7	Fibrinogenolytic activity	αβ fibrinogenase activity	αβ fibrinogenase activity

Chapter 6

Discussion

CVDs today are a major cause of mortality responsible for over 18 million deaths annually (WHO 2015; Choi et al 2014). The underlying pathology of CVDs (predominantly acute coronary syndrome, stroke and venous thromboembolism) and related thromboembolic events such as pulmonary embolism is thrombosis (Raskob 2014; Kang et al 2016; Meshram and Saxena 2016). Thrombosis generally refers to the formation of a blood clot or thrombus in the blood vessel which blocks or obstructs the blood flow in the affected area and leads to serious complications when it moves to different part of the circulatory systems such as heart, lungs or brain. Thus, dissolution of the thrombus is an integral part of the thrombolytic treatment to restore the blood flow in the affected area. Hence, thrombolytic therapy largely comprises of use of anti–coagulants, anti–platelets and thrombolytic agents. Anti–coagulants are generally administered to prevent the formation of blood clots, while anti–platelet agents generally interfere in the aggregation of platelets thereby inhibiting the thrombus formation in thrombotic cerebrovascular and CVDs. While thrombolytic agents generally involve the dissolution of the thrombus directly or indirectly i.e. they initiate the process of fibrinolysis (clot dissolution) either by activating plasmin or they can mimic the action of plasmin to dissolve the fibrin clots. Despite being widely used in treatment of CVDs, plasminogen activators such as t–PA, u–PA, streptokinase and APSAC have been found to have significant limitations such as short half-life, haemorrhage effect apart from cost implications in CVD therapy (Ju et al 2012; Choi et al 2013). Therefore, thrombolytic strategies which are safe as well as site specific are being explored (Cui et al 2008; Wu et al 2009b; Choi et al 2013).

The viable alternatives at present in the thrombolytic armamentarium are agents which directly act on fibrinogen and dissolve it into fibrin degradation product i.e. carry out fibrinolysis. These agents have gained much attention in the recent times due to their impressive thrombolytic efficacy and safety from bleeding (Mander et al 2011). However, they are rapidly inactivated by α_2–anti–plasmin which limits their use (Choi et al 2013b). Recently, several fibrinolytic agents possessing dual activity have been identified (Choi et al 2013a, b; Kang et al 2016; Meshram et al 2016a). These bi–functional fibrinolytic agents can provoke plasminogen activation and can simultaneously dissolve

the fibrin clot directly. Thus, fibrinolytic agents with dual activity could be a potential candidate for thrombolytic therapy (Choi et al 2013b). However, there exists an immense need of an ideal thrombolytic agent; hence, newer sources of thrombolytic agents are being explored from nature with least shortcomings for use as an ideal thrombolytic agent.

Exploration of microorganisms from different niches has been the mainstay in pharmaceutical industry for exploration of new chemical structures having novel functions for pharmaceutical drug development (Demain 1999; Demain and Sanchez 2009; Aly et al 2011; Amedei and D'Elios 2012; Katz and Baltz 2016). However, microbial interactions in unexposed biological niche opened gates for discovery of novel chemistries for pharmaceutical as well as industrial applications. Endophytic fungi since last two decades have been fountainheads for the discovery of new pharmaceutically active compounds apart from being a source of putative phytochemicals of their host (Strobel and Daisy 2003; Tejesvi et al 2007; Verma et al 2009; Aly et al 2011; Zhang et al 2015; Stadler 2015; Zheng et al 2016). In the recent past, endophytic fungi have also been found to be prolific producers of extracellular enzymes which they produce as a strategy to develop as well as maintain a healthy interaction with their host plant which in turn provides fitness to the plant to combat with biotic as well as abiotic stresses (Reddy et al 1996). Fungal endophytes have also been screened for their potential to degrade wood components and hence emphasis has been on xylanases, laccases, pectinases and ligninolytic enzymes (Suto et al 2002; Urairuj et al 2003; Wang et al 2006; Oses et al 2006). However, there are limited studies on exploration of the fibrinolytic potential of the extracellular proteases which are expressed by the endophytic fungi (Ueda et al 2007; Li et al 2007; Wu et al 2009; Rovati et al 2010). Thus, the present study was oriented towards exploring the fibrinolytic potential of extracellular proteases elaborated by endophytic fungi for their possible use as clot busting agents directly or indirectly.

In the present study, over two hundred endophytic fungal isolates belonging to 35 different taxa were isolated from different medicinal plants. The endophytic mycoflora in present study were diverse in their existence in the medicinal plants. *Aegle marmelos* exhibited maximum colonization

followed by *C. malabaricum* and *T. baccata*. Similarly, maximum colonization of endophytes was observed in stem followed by bark and stem internal tissue (vascular system) which exhibited geographical specificity. The dominant fungal species obtained during the present study were *Fusarium* sp., *Alternaria* sp. and *Botryosphaeria* sp*ecies. Fusarium* species dominantly colonised in stem and stem internal tissue of *A. marmelos* collected from Yelandur, Karnataka, whereas it was not isolated from *A. marmelos* collected from Wayanad, Kerala. The endophytic fungal isolates recovered in the present study were dominantly hyphomycetes followed by ascomycetes and coelomycetes. The results corroborate with the previous study carried out by Gond et al (2007) and Kharwar et al (2012) where hyphomycetous fungi were dominantly colonizing as compared to ascomycetes and coelomycetes.

The culture filtrates were subjected to primary screening in which *in vitro* proteolytic activity and *in vitro* fibrinolytic activity were screened using agar plate based assays. In preliminary screening, culture filtrates of 27 isolates exhibited more than 80 % of the *in vitro* proteolytic activity while only eight isolates displayed more than 80 % of the *in vitro* fibrinolytic activity (Meshram et al 2016b; Meshram and Saxena 2016). Based on the preliminary screening, it was concluded that the fibrinolytic enzymes are proteolytic in nature, however all proteolytic enzymes are not fibrinolytic in nature. Further, we inferred that there was a higher propensity of *in vitro* proteolytic activity as compared to *in vitro* fibrinolytic activity in culture broths of endophytic fungi. Rovati et al (2010) screened 230 isolates using similar approach in which 62 % isolates exhibited proteolytic activity while only 1.3 % of those isolates displayed fibrinolytic activity. Similar results were obtained in the study by Shirasaka et al (2012) in which 544 fungal isolates were screened, of which only 2.4 % of the isolates exhibited fibrinolytic property. In the present study, ~47 % of the isolates (19) exhibiting *in vitro* fibrinolytic activity were isolated from *Aegle marmelos*. Similarly, ~39% of the isolates (23) from *Trachelospermum jasminoides* exhibited *in vitro* fibrinolytic activity (Li et al 2007). However, in the present study, maximum *in vitro* fibrinolytic activity was exhibited by an isolate recovered from the stem of *C. roseus*. It is generally difficult to quantify the fibrinolytic activity using *in vitro* agar plate

assays; hence it becomes imperative to use a calibration curve which indicates the fibrinolytic potential in terms of equivalent units of a known fibrinolytic agent. In most of the cases, fibrinolytic activity is expressed in terms of tyrosine released from the clot or the halo formation is directly converted to urokinase or streptokinase equivalent units (Rovati et al 2010). However, several workers have referred fibrinolytic activity in terms of plasmin equivalent units (Ueda et al 2007; Rovati et al 2010; Kim et al 2011)

Thus, 19 isolates were selected, which were exhibiting plasminogen dependent fibrinolytic activity. However, these isolates were further subjected to evaluate their plasminogen independent fibrinolytic activity; since direct fibrinolytic activity is preferred over plasminogen activation based fibrinolytic agents as discussed above. Only six isolates exhibited both plasminogen dependent and plasminogen independent fibrinolytic activity. These six isolates were then subjected for production of crude enzyme extract. Further, it was found that ammonium sulphate precipitation was a better method of obtaining the protein precipitate for crude enzyme preparation as compared to acetone precipitation method as evident by *in vitro* fibrinolytic activity. Further, Bland Altman analysis supported the obtained results and suggested that ammonium sulphate method is more suitable approach for protein precipitation. Similarly, isolation of crude fibrinolytic enzyme from the culture filtrate of *Fusarium* sp. BLB, *Fusarium* sp. CPCC 480097 and *Aspergillus oryzae* KSK–3 was carried out using ammonium sulphate precipitation method (Ueda et al 2007; Wu et al 2009b; Shirasaka et al 2012). Cold acetone precipitation method have been adopted for recovering crude enzyme from *Bacillus* sp. strain AS–S20–I and *Bacillus amyloliquefaciens* FCF–11 (Mukherjee et al 2012 and Kotb et al 2014b). However, the major advantage of using ammonium sulphate precipitate is that the enzyme is not denatured as in case of acetone precipitation. Thus, ammonium sulphate precipitation was adopted for isolation of crude enzyme from the culture filtrate of #37 CRSTBRT which was exhibiting the maximum plasminogen free as well as plasminogen dependent fibrinolytic activity when compared to #4 RSLBRT and #22 AMSTYEL by one way ANOVA followed by Tukey's post hoc

analysis. Thus, #37 CRSTBRT was selected for identification, mass production, purification and characterization.

Another pre–requisite for obtaining maximum fibrinolytic activity was ascertaining the optimal culture conditions. A significant impact on the fibrinolytic activity of the fungus has been observed when a variation was carried out in its fermentation medium. In the present study, CDB exhibited maximum fibrinolytic activity when compared to other media like PDB, RB, MEB, YEPB and TSB which was analysed statistically. CDB had been used as a fermentation medium to evaluate the fibrinolytic activity of *Rhizomucor miehei* and *Aspergillus japonicum* (Ali and Ibrahim 2008; Yadav and Siddalingeshwara 2015).

The present study exhibited that the optimal saturation level of ammonium sulphate for obtaining the crude enzyme exhibiting maximal *in vitro* fibrinolytic assay was 60 %. On increasing or decreasing the saturation level, the fibrinolytic activity tends to decline or was lost. Usually a range of 20 % to 80 % saturation level is generally tested to evaluate the precipitation of the proteins as well as to evaluate their *in vitro* enzymatic activity (Cha et al 2010). Wu et al (2009b) precipitated culture supernatant of *Fusarium* sp. CPCC 480097 with 40 % and 60 % of ammonium sulphate to obtain fibrinolytic enzyme of which 60 % saturated solution was used for further purification. Similarly, the culture filtrate of *Streptomyces* sp. CS684 was saturated with 0–75 % ammonium sulphate to obtain crude enzyme extract (Simkhada et al 2010). Further, the *in vitro* fibrinolytic activity was only exhibited by the culture broth of #37 CRSTBRT and no activity was observed with intracellular fraction of this culture. Thus, from the present study, it was established that the best medium for production of fibrinolytic enzyme by #37 CRSTBRT was CDB which was saturated with 60 % ammonium sulphate and the precipitate so obtained was dissolved in 20 mM Tris–HCl buffer of pH 7.8 to obtain the crude enzyme (Choi et al 2013a; Kang et al 2016, Meshram et al 2016a).

Morphotaxonomy is the most convenient and reliable way of identifying *xylariaceous* fungi. However, sometimes it becomes quite difficult to delineate endophytes exclusively on the basis of morphological features since most endophytic fungi failed to produce reproductive structures when

cultured under laboratory conditions (Petrini and Petrini 1985; Chen et al 2013). In the present study also, the isolate #37 CRSTBRT was found in its anamorphic form i.e. producing only hyphal structures and not producing any reproductive structures like stromata, conidia and ascospores even when subjected to spore inducing media and physical stress conditions like incubation in dark and UV light. Further, the isolate produced a fruity odour which is attributable to its VOCs.

Endophytic *Xylaria* sp. strain PB3f3 isolated from *Haematoxylon brasiletto* has also been reported of non–sporulation even after exposure to UV radiations and temperature differences including freezing. The isolate also produced VOCs which possessed anti–microbial properties (Sanchez–Ortiz et al 2016). Several sterile *Xylariaceous* endophytic fungi belonging to the genus *Muscodor* produces VOCs which are lethal to various plant and human pathogens (Meshram et al 2013; Saxena et al 2015; Meshram et al 2015).

As the endophytic fungus #37 CRSTBRT did not produce any reproductive structures, it was not possible to identify it solely on the basis morphological characters. For fungal diagnostics and phylogenetics the use of nuclear rRNA cistron has been effectively used over a period of two decades. The eukaryotic rRNA cistron comprises of the 18S, 5.8S and 28S rRNA genes transcribed as a unit by RNA polymerase I. Post transcriptional processes split this cistron, thereby removing two internal transcribed spacers. These two spacers, including the 5.8S gene, are referred to as the ITS region. Across the fungal kingdom, ITS was superior to LSU in discrimination of the species and has a clearly more defined barcode gap (Schoch et al 2012). Hence, for proper delineation of endophytic fungus #37 CRSTBRT, 18S ITS region was amplified and sequenced. On the basis of ITS region, the endophytic fungus was identified as *Xylaria curta.* Other fungi exhibiting fibrinolytic properties such as *Fusarium* sp. BLB, *Fusarium* sp. CPCC480097 were also identified using ITS sequence (Ueda et al 2007; Wu et al 2009a). Although ITS–rDNA region provided sufficient information regarding the phylogenetic placement of #37 CRSTBRT, speciation cannot be exclusively based on ITS sequences (Atkins and Clark 2004; Rakeman et al 2005). Hence, highly conserved housekeeping genes like LSU and RPBII were taken into account (O'Donnell 2009; Chen et al 2013). The evolutionary tree based

on LSU and RPBII region confirmed the identity of the endophytic isolate #37 CRSTBRT as *Xylaria curta* species. Molecular phylogenetics needs much more access to highly conserved, low copy number, protein encoding nuclear genes for representation of evolutionary history scenarios of the endophytic fungi. Previously, fibrinolytic enzymes producing fungi like *Bionectria ochroleuca, Cladosporium cladosporioides* and *Aspergillus oryzae KSK-3* were identified using LSU D1 and D2 region (Rovati et al 2010; Shirasaka et al 2012).

The endophyte *Xylaria curta* (#37 CRSTBRT) exhibiting maximum fibrinolytic activity was isolated from *Catharanthus roseus* or periwinkle which is an evergreen shrub possessing medicinal properties and has been used to cure diabetes and hypertension (Kharwar et al 2008; Aruna et al 2015). It is also the source of two very important compounds, vincristine and vinblastine which were isolated from leaves of plant and have been developed into anti–cancer drugs/chemotherapeutic agents. *C. roseus* is known to host plethora of endophytes, some of which viz *Fusarium oxysporum, Talaromyces radicus* are able to produce vincristine and vinblastin (Kumar et al 2013; Palem et al 2015). Endophytic fungi of *C. roseus* have largely been exploited for their potential to produce the anti–cancer compound vincristine, vinblastin and related vinca alkaloids such as vindoline (Pandey et al 2016). Recently, endophytic fungi like *Colletotrichum* sp. and *Fusarium solani* isolated from leaf of *C. roseus* have been reported to produce hydrolytic enzymes like cellulase, amylase and protease (Ayob and Simarani 2016). To best of our knowledge, the present study is the very first report an endophytic fungus *Xylaria curta* isolated from stem of *C. roseus* displaying fibrinolytic activity (Meshram et al 2016a).

Several endophytic fungi including *Bionectria ochroleuca, Fusarium* sp. BLB, *Fusarium* sp. CPCC 480097 and *Trachelospermum jasminoides* have been reported to display either plasminogen free or plasminogen dependent fibrinolytic activity (Li et al 2007; Ueda et al 2007; Wu et al 2009b; Rovati et al 2010). Bi–functional fibrinolytic enzymes have gained much attention in the recent past owing to their superiority to plasmin and t–PA (Choi et al 2014). They have been previously reported from algae (Choi et al 2013b, Choi et al 2014; Kang et al; 2016) and mushrooms (Choi et al 2013a).

However, to best of our knowledge, the current study is the very first report of an endophytic fungus exhibiting bi–functional fibrinolytic property (Meshram et al 2016a).

Endophytic *Xylaria* species has been reported to be a putative source of biologically active secondary metabolites possessing anti–fungal, anti–oxidant and enzyme inhibitors properties (Pongcharoen et al 2013). *Xylaria* sp. YX–28 existing as an endophyte in *Ginkgo biloba* has been found to produce 7-amino-4-methylcoumarin which exhibited antimicrobial activity against dreadful pathogens like *Staphyloccous aureus, Escherichia coli, Salmonella typhimurium, Vibrio anguillarum, Candida albicans* etc. Further, the same isolate is also known to display antioxidant property (Liu et al 2007). Endophytic *Xylaria* sp. isolated from *Glochidion ferdinandi* has been found to produce xanthones which possesses anti–staphylococal and anti–candidal activity (Healy et al 2004). Similarly, glucoside derivatives xylarosides 1 and 2 and sordaricin was isolated from spent broth of *Xylaria* sp. PSU–D14. Amongst them, sordaricin displayed anti–candidal activity (Pongcharoen et al 2008). Another endophytic *Xylaria* sp. produced 3-chloro-4-hydroxyphenylacetamide in its fermentation broth which confers anti–fungal activity (Davis et al 2005). Two new cytochalasans, cytochalasin Z_{27}, 1, and cytochalasin Z_{28}, 2 have been isolated from endophytic *Xylaria* sp. XC–16 which exhibited fungicidal activity (Zhang et al 2014). Xyloketal is being produced by endophytic *Xylaria* sp. which exhibit significant acetylcholine esterase inhibitory activity (Krohn and Riaz 2004). Cyclotripeptide X–13 isolated from *Xylaria* sp. (no. 2508) induces angiogenesis in zebrafish embryos and in human endothelial cells via PI3K/Akt/eNOS signaling pathways (Lu et al 2012). Recently, an endophytic *Xylaria* sp. strain PB3f3 isolated from *Haematoxylon brasiletto* exhibited anti–fungal and anti–mycotic effect by the virtue of its VOCs (Sanchez–Ortiz et al 2016).

Apart from being a potential source of bioactive metabolites, endophytic *Xylaria* species have also been reported to produce several important enzymes like cellulose, amylase, proteases, xylanase etc (Correa et al 2014). Several tropical endophytic *Xylariaceae* strains isolated from Thailand were producing ligninolytic enzyme (Urairuj et al 2003). Endophytic *Xylaria* species isolated from the forests of Western Ghats elaborated cellulase, laccase, lipase, pectinase and

pectatetranseliminase enzymes (Rajulu et al 2013). Similarly, endophytic *Xylaria* sp. isolated from *Opuntiaficus indica* produces cellulase, protease and xylanase (Bezerra et al 2012).

Fibrinolytic enzymes have been reported from mushrooms like *Cordyceps militaris, Pleurotus eryngii* and *Hericium erinaceum* (Cha et al 2010; Choi et al 2011; Choi et al 2013a). However, fibrinolytic enzymes has been purified and characterized from few endophytic fungi including *Fusarium* sp. BLB, *Fusrium oxysporum* and *Bionectria ochroleuca* (Ueda et al 2007; Wu et al 2009b; Rovati et al 2010). However, to best of our knowledge, the present study is the very first report of an endophytic *Xylaria curta* species (#37 CRSTBRT) producing a fibrinolytic enzyme (Meshram et al 2016a).

Chromatography is the most widely used analytical technique for the purification of enzymes. Combinations of two or more types of column chromatographic techniques are generally used to purify the enzyme for further characterization. The fibrinolytic enzyme was isolated and purified from *Xylaria curta* (#37 CRSTBRT) by using two chromatographic techniques viz. ion exchange chromatography and gel filtration chromatography (Meshram et al 2016a). The yield of xylarinase was better than fibrinolytic enzymes obtained from *Fusarium* sp. BLB and *Fusarium* sp. CPCC 480097 and *Paecilomyces* sp., (Ueda et al 2007; Wu et al 2009b; Kim et al 2011) whereas, *Cordyceps militaris* and *Hericium erinaceum* gave higher yield of purified enzyme (Choi et al 2011; Choi et al. 2013a). Further, the fold increase in the enzyme activity during purification was found to be better than *Streptomyces* sp. XZNUM 00004 (Ju et al 2012), comparable with *Streptomyces* sp. CS684 (Simkhada et al 2010) and less than *C. militaris* and *H. erinaceum* (Choi et al 2011; Choi et al 2013).

The molecular mass of the fibrinolytic enzyme from fungi have been reported to be in the range from 14 to 52 kDa. The molecular mass of xylarinase (~33 kDa) was found to be closer to fibrinolytic enzymes from *C. militaris*; *Fusarium* sp. BLB, *Fusarium* sp. CPCC 480097 (27 kDa), *Trachelospermum jasminoides* (31 kDa), *Bacillus* sp. strain AS–S20–I (32.3 kDa) and *Streptomyces* sp. CS684 (35 kDa) (Li et al 2007; Ueda et al 2007; Wu et al 2009b; Simkhada et al 2010, Choi et al 2011;

Mukherjee et al 2012). On the other hand, the fibrinolytic enzyme in the present study showed lower molecular mass as compared to fibrinolytic enzymes produced by *H. erinaceum* (51 kDa) and *C. militaris* (52 kDa) (Choi et al 2013a; Kim et al 2006) and much higher than the fibrinolytic enzymes isolated from *Armillariella mellea* (18.53 kDa) and *P. tenuipes* (14 kDa). From the above results, it becomes evident that the molecular masses of fibrinolytic enzyme from various microbial species are quite different (Kim and Kim 1999; Kim et al 2011).

Xylarinase can independently hydrolyse fibrin, imitating plasmin activity. Plasmin has high affinity towards fibrin and fibrin zymography is considered as one of the most sensitive and reliable technique to identify plasmin like enzyme (Kim et al 1998). Therefore, we subjected xylarinase to fibrin zymography and it produced a clear halo in the fibrin polymerised gel at ~33 kDa indicating that xylarinase possesses plasmin like activity. MALDI–ToF MS is considered as one of the most reliable technique to determine the apparent molecular mass of enzyme. The actual molecular mass of xylarinase analysed by MALDI-ToF MS was calculated to be 33.76 kDa (Meshram et al 2016a). Similarly, the molecular mass of *Armillariella mellea* was also estimated to be 18.53 kDa using MALDI–ToF MS (Kim and Kim 1999). The apparent molecular weight of microbial fibrinolytic enzymes like bafrinase (32.3 kDa), codiase (48.9 kDa), ulvease (51.48 kDa) has also been determined using MALDI–ToF MS technique (Choi et al 2011; Mukherjee et al 2012; Kang et al 2016).

The mechanism of fibrinolysis was investigated by examining the fibrinolytic activity on plasminogen free and rich plates. Xylarinase showed strong fibrinolytic activity on both plasminogen rich and plasminogen free plate indicating that the enzyme can directly hydrolyse fibrin clots or can activate plasminogen like streptokinase, urokinase and t–PA (Meshram et al 2016a). Fibrinolytic agents like herinase, codiase, starase, and UFEII have been reported to possess such bi–functionality (Bi et al 2013b; Choi et al 2013a,b; Choi et al 2014). Bi–functionality is the distinctive characteristic of xylarinase which makes it a better fibrinolytic agent as compared to others (Choi et al 2013a, b; Choi et al 2014; Kang et al 2016).

Another striking feature of xylarinase was the hydrolysis of both, fibrin and its precursor, fibrinogen. The obtained cleavage profile suggested that the mode of action of the enzyme was similar to that of α and β fibrinogenase in selectively cleaving the Aα and Bβ chain of fibrin/ fibrin(ogen) rather than γ chains (Mukherjee et al 2012). The degradation pattern differs from the other fibrinolytic enzymes as it only hydrolyses the Aα and Bβ chain of fibrin(ogen). Fibrinolytic enzymes from *Bacillus* sp. strain AS-S20-I, *A. mellea* and *Plueurotous eryngii*, exhibited similar fibrin(ogeno)lytic property (Kim and Kim 1999; Cha et al. 2010; Mukherjee et al 2012). In addition, this degradation pattern was dissimilar to those fibrinolytic enzymes produced by *H. erinaceum*, *P. tenuipes* and *C. militaris* which selectively hydrolysed either α or β chains of fibrin(ogen) respectively (Choi et al 2011; Kim et al 2011; Choi et al 2013a). On the other hand, fibrinolytic enzymes from *Fusarium* sp. CPCC 480097 and *C. militaris* digested Aα, Bβ and γ chain of fibrinogen (Kim et al 2006; Wu et al 2009b). αβ fibrinogenase is a special property exhibited by bi–functional fibrinolytic enzyme (Mukherjee et al 2012; Kang et al 2016).

The N–terminal sequence of xylarinase revealed that it's a novel protease exhibiting bi–functional fibrinolytic activity. Interestingly, the enzyme did not exhibit any sequence homology with the N–terminal amino acid sequence of other known fibrinolytic enzymes. The enzyme was found to differ significantly from fibrinolytic enzymes produced by other endophytic fungi or mushrooms including *C. militaris*, *Fusarium* sp. CPCC 480097, *Fusarium* sp. BLB and *H. erinaceum* (Choi et al 2011; Ueda et al 2007; Wu et al 2009a Choi et al 2013a). It showed a high degree of homology with proteases and peptidases from various *Streptomyces* species but has no or less similarities in the N-terminal sequence of the previously reported fibrinolytic enzymes.

The probable concept of studying the effect of different protease inhibitors is to have knowledge of the type of molecules which can interfere or inhibit the *in vitro* fibrinolysis and based on that knowledge we can decipher which molecules probable can interfere during clinical application of the enzyme. The use of a specific inhibitor also helps in understanding the nature of the proteolytic enzyme. As the fibrinolytic activity of xylarinase was completely inhibited by EDTA it

appears to be a metalloprotease, since the metal ion gets chelated and the enzyme loses its activity. Xylarinase was also inhibited by EGTA and hence it was a metalloprotease with dependence on calcium ion. Similar inhibition of fibrinolytic activity by EDTA has been observed in the enzymes produced by *R. chinensis* and *Armillaria mellea* (Xiao–lan et al 2005; Lee et al 2005). Xylarinase appears to be a serine/cysteine protease based on the inhibition profiles of PMSF, leupeptin, TPCK and PMSF. Further, $Fe^{2+}$and Zn^{2+} ion completely inhibited the fibrinolytic action of xylarinase. Cu^{2+} also exhibited inhibition of the fibrinolytic action over 95 %. The fibrinolytic activity of protease from *M. subtilissimus* also exhibited a similar pattern of inhibition with $Cu^{2+,}$ Fe^{3+} and Zn^{2+} ions. The Co^{2+} ion was also having an inhibitory effect on the fibrinolytic activity of *M. subtilissimus* protease, however in our case xylarinase was not affected by Co^{2+} ions (Nascimento et al 2016). The N-terminal sequence comparison and inhibition of enzyme activity by these chelating agents established it as a metalloprotease. The metal–dependence of the fibrinolytic enzyme could shield it from serpins attack which usually targets serine proteases involved in blood coagulation (Kim and Kim 1999; Lee et al 2005; Choi et al 2013). The biochemical nature of xylarinase was somewhat similar to herinase and ulvease (Choi et al 2013a; Kang et al 2016).

Xylarinase showed high specificity towards fibrinogen which is evident in fibrinogen clotting time assay where the variation in fibrinogen concentration prolong the clotting time whereas no such effect was observed when the concentration of thrombin was changed. Similar prolongation in clotting time was observed with endophytic *Fusarium* CPCC 480097. This helps in selection of target moiety for probable action of fibrinolytic agent (Wu et al 2009a). The K_m and V_{max} of xylarinase was found to be 246 µM and 1.22 µM/min for fibrin which is lower than that of plasmin found in the present study. These results were in concurrence with the fibrinogen clotting time assay where it was clearly evident that xylarinase is a fibrinogen dependent enzyme (Meshram et al 2016a). Xylarinase showed dose dependence in terms of its *in vitro* thrombolytic activity. 50 µg of xylarinase is required for complete dissolution of the thrombus. These results showed that the fibrinolytic enzyme possesses better thrombolytic efficacy than plasmin under *in vitro* conditions. The results

obtained were also found to be significantly better than those of *Streptococcus equinus* VIT_VB2 and *Fusarium* sp. CPCC 480097 (Wu et al 2009a; Babu and Devi 2015).

The blood clotting cascade involved three pathways: intrinsic, extrinsic and a common pathway. The present study demonstrated that xylarinase possesses anticoagulation properties as shown by elongation of PT and APTT. The prolongation of APTT might be due to decrease in coagulation factors such as VIII, IX and XI which results into inhibition of intrinsic and common pathways whereas the prolongation of PT suggests inhibition of extrinsic pathway due to decrease in clotting factors such as V, VII and X. Thus, xylarinase might block the activation of clotting cascade by suppressing the thrombin pathway (Meshram et al 2016a). Similar prolongation in APTT and PT was observed in case of bi–functional fibrinolytic enzymes like ulvease and codiase (Choi et al 2013b; Kang et al 2016).

In the *in vitro* cytotoxic assay, RAW 264.7 murine macrophage cells remained viable and no change in morphology was observed even after 48 h of treatment. Thus, xylarinase could be considered as a potentially non-toxic fibrinolytic agent. Similarly, fibrinolytic enzyme from *Bacillus amyloliquefaciens* FCF–11 and bafrinase did not display a significant cytotoxicity towards H29 cells (Mukherjee et al 2012; Kotb et al 2014b). Similarly, codiase was non–cytotoxic to NIH–3T3 cells (Choi et al 2013b).

To meet the market demand, it is very important to produce cost effective fibrinolytic enzyme using cheaper substrate. High activity, stability and low cost are the key requirement of an enzyme subjected for large scale production. Agro–industrial wastes can serve as novel and useful matrices for enzyme production, development and improvement. Usually, solid state fermentation is preferred when fungi are used for enzyme production. SSF offer greatest of opportunity where fungi are used because of their inherent property to grow over solid substrates like wood pieces, animal bones and skin which are low in moisture content (Bhargav et al 2008; Singhania et al 2009). Hence, for enhanced production of the desired enzyme using SSF, selection of a suitable substrate and its optimization is a crucial step (Vijayaraghavan and Vincent 2014).

From tested agro–industrial substrate, rice chaff emerged as the most productive substrate for fibrinolytic enzyme production followed by wheat bran. The fungus showed inability to grow over banana and orange peel. This might be due to the inherent anti–microbial properties of the respective substrates. The selection of suitable agro–residue for enzyme production largely depends on the availability and cost of the substrate. In earlier studies, *Fusarium oxysporum* and *Penicillium chrysogenum* SGAD12 efficiently produced fibrinolytic enzyme over rice chaff (Tao et al 1997; Gopinath et al 2011) whereas, a potential fibrinolytic enzyme has been purified from *Pleurotus eryngii* cultivated using corn cob as substrate under solid–state conditions (Cha et al 2011). Vijayaraghavan and Vincent (2014, 2015) found that wheat bran and cow dung is the most suitable substrate for production of fibrinolytic enzyme by marine bacteria *Bacillus cereus* IND1 and *Shewanella* sp. IND20 respectively. Corn husk was also used as a cheap substrate to produce a chymotrypsin–like serine fibrinolytic enzyme from *Bacillus amyloliquefaciens* FCF–11 (Kotb et al 2014). Recently, cow dung has been used as a novel feedstock for fibrinolytic enzyme production by *Bacillus* sp. IND7 (Vijayaraghavan et al 2016). Further, various parameters were optimized for production of fibrinolytic enzyme over rice chaff. Maximum fibrinolytic activity was observed on 15th day of incubation at 28 °C over rice chaff of particle size 500 µm containing 50 % moisture. Tao et al (1997) had optimized various parameters including moisture content, incubation time and particle size for production of fibrinolytic enzyme over rice chaff medium. They found that 5–10 % of the inoculum, 30–50 % moisture and substrate particle size of 400 µm exhibited maximum enzyme production. Gopinath et al (2011) found that rice chaff of particle size 500 µm supported maximum enzyme production. The enzyme was purified using gel filtration chromatography. The purified enzyme exhibited a similar band in SDS–PAGE as that observed in xylarinase. The biochemical properties of purified enzyme were similar at that of xylarinase including N–terminal sequencing. Thus, it can be concluded that the purified enzyme produced by #37 CRSTBRT (*Xylaria curta*) over rice chaff was xylarinase. However, a significant increase in enzyme yield was observed in comparison to that obtained in case of SmF. From 10 l of culture filtrate, 0.93 mg of purified enzyme

was obtained with recovery of 0.095 %. However in case of SSF, from 250 g of rice chaff, 11.2 mg of enzyme was purified with a yield of 8.5 %. Thus, for production and purification of xylarinase, SSF is a recommended approach.

Thus, the present study establishes that endophytic fungi are novel bio–resource for production of fibrinolytic enzyme and xylarinase is a novel metalloprotease showing dual property including direct hydrolysis of fibrin(ogen) or by stimulating the tissue plasminogen. Preliminary studies suggested that the enzyme does not possess any cytotoxic activity under *in vitro* conditions. Thus, xylarinase stands out as a potential candidate amongst the therapeutic agents in thrombolytic therapy. Further studies on animal model and molecular characterization of protein–protein interactions needs to be carried out.

Chapter 7

Conclusion

Conclusion

The present study establishes that endophytic fungi is a novel source of enzymes possessing fibrinolytic activity and provide immense possibilities for their development of novel thrombolytic agents.

1. This is the first report wherein a bi–functional fibrinolytic enzyme Xylarinase has been isolated from endophytic *Xylaria curta* residing in the stem of *Catharanthus roseus* in a rain forest in south India.
2. Xylarinase is a bi–functional, Ca^{2+} dependent metalloprotease exhibiting optimal *in vitro* fibrinolytic activity at pH8, 35 °C. The K_m and V_{max} of xylarinase are 246 µm and 1.22 µmol/min which is comparable to plasmin. Xylarinase exhibited a better activity than plasmin in spectrophotometric assay of for fibrinolytic activity.
3. This study also establishes that solid substrate fermentation using rice chaff is a better method for xylarinase as compared to submerged fermentation.

Chapter 8

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Chapter 9

Appendix

Appendix

Table 5.1: Endophytic fungi isolated from various tissues of medicinal plants collected from biodiversity hotspots of India

S.no	Culture code	Plant name	Plant part	Sampling location	Tentative identification
Family : *Rutaceae*					
1.	#1 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
2.	#2 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
3.	#3 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Pestalotiopsis* sp.
4.	#4 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
5.	#5 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
6.	#6 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Lasiodiplodia theobromae*
7.	#7 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium equiseti*
8.	#7(a) AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
9.	#8 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
10.	#9 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
11.	#9(b) AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium equiseti*
12.	#11 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Neofusicoccum parvum*
13.	#15 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Pestalotiopsis* sp.
14.	# 17 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
15.	#18 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Neofusicoccum* sp
16.	# 22 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
17.	# 23 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Aureobasidium* sp.
18.	# 23(b) AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Aureobasidium* sp.
19.	# 32 AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Botryosphaeria* sp.
20.	#1003 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Botryosphaeria* sp.
21.	#1010 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium* sp.
22.	#1011 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium* sp.
23.	#1013 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Botryosphaeria* sp.
24.	#1022 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium* sp.
25.	#1032 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Botryosphaeria* sp.

26.	#1048 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Lasiodiplodia pseudotheobromae*
27.	#1058 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium* sp.
28.	#1069 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium* sp.
29.	#1070 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium* sp.
30.	#1088 AMSTITYEL	*A. marmelos*	Stem internal tissue	Yelandur, Karnataka	*Lasiodiplodia pseudotheobromae*
31.	#6 AMLWLS	*A. marmelos*	leaves	Wayanad, Kerala	*Fusarium incarnatum*
32.	#16 AMLWLS	*A. marmelos*	leaves	Wayanad, Kerala	*Muscodor kashayum* sp. *nov*
33.	#61 AMLWLS	*A. marmelos*	leaves	Wayanad, Kerala	*Trichoderma* sp.
34.	#11 AMBAWLS	*A. marmelos*	Bark	Wayanad, Kerala	*Aureobasidium sp.*
35.	#22 AMBAWLS	*A. marmelos*	Bark	Wayanad, Kerala	*Aspergillus niger*
36.	#23 AMBAWLS	*A. marmelos*	Bark	Wayanad, Kerala	*Penicillium* sp.
37.	#24 AMBAWLS	*A. marmelos*	Bark	Wayanad, Kerala	*Penicillium* sp.
38.	#29 AMBAWLS	*A. marmelos*	Bark	Wayanad, Kerala	*Trichoderma* sp.
39.	#20 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria* sp.
40.	#25 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria* sp.
41.	#28 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria* sp.
42.	#33 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Alternaria* sp.
43.	#37 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Phaeoacremonium* sp.
44.	#42 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Phaeoacremonium* sp.
45.	#43 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Togninia* sp.
46.	#46 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Pestalotiopsis* sp.
47.	#47 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Pestalotiopsis* sp.
48.	#53 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria* sp.
49.	#59 AMSTWLS	*A. marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria stevensii*
50.	#1079 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Lasiodiplodia theobromae*
51.	#1082 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Lasiodiplodia pseudotheobromae*
52.	#1095 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Lasiodiplodia theobromae*
53.	#1099 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Botryosphaeria* sp.
54.	#1103 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Sphaeropsis sapinea*
55.	#1104 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Barriopsis iraniana*

56.	#1111 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Barriopsis iraniana*
57.	#1118 AMSTITWLS	*A. marmelos*	Stem internal tissue	Wayanad, Kerala	*Barriopsis iraniana*
58.	#3 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Fusarium* sp.
59.	#4 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Fusarium* sp.
60.	# 9 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Fusarium* sp.
61.	#12 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Alternaria* sp.
62.	#17 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Alternaria* sp.
63.	#20 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	Unidentified
64.	#24 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Curvularia* sp.
65.	#27 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Curvularia* sp.
66.	#1004 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	Unidentified
67.	#1005 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Alternaria marmelos* sp. *nov*
68.	#1006 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Fusarium* sp.
69.	#1007 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Fusarium* sp.
70.	#1016 AMLBRT	*A. marmelos*	leaves	BRT Wildlife sanctuary, Karnataka	*Fusarium* sp.
Family: Lauraceae					
71.	#1 CMSTITBRT	*C. malabaricum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
72.	#10 CMSTITBRT	*C. malabaricum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
73.	#43 CMSTITBRT	*C. malabaricum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
74.	#50 CMSTITBRT	*C. malabaricum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	Unidentified
75.	#1CMBABRT	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
76.	#4 CMBABRT	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	*Bionectria* sp.
77.	#12 CMBABRT	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	*Bionectria* sp.
78.	#16 CMBABRT	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	*Clonostachys* sp.
79.	#18 CMBABRT	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	*Bionectria* sp.
80.	#49 CMBABRT	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	Unidentified
81.	#4 CMLBRT	*C. malabaricum*	Leaf	BRT Wildlife sanctuary, Karnataka	*Xylaria* sp.
82.	#27 CMLBRT	*C. malabaricum*	leaves	BRT Wildlife sanctuary, Karnataka	*Aspergillus* sp.
83.	#29 CMLBRT	*C. malabaricum*	leaves	BRT Wildlife sanctuary, Karnataka	Unidentified
84.	#33 CMLBRT	*C. malabaricum*	leaves	BRT Wildlife sanctuary, Karnataka	Unidentified

85.	#40 CMLBRT	*C. malabaricum*	leaves	BRT Wildlife sanctuary, Karnataka	*Alternaria* sp.
86.	#1 CMLNEY	*C. malabaricum*	leaves	Neyyar, Kerala	*Nigrospora* sp.
87.	#2 CMLNEY	*C. malabaricum*	leaves	Neyyar, Kerala	*Nigrospora* sp.
88.	#17 CMLNEY	*C. malabaricum*	leaves	Neyyar, Kerala	*Chaetomium* sp.
89.	#18 CMLNEY	*C. malabaricum*	leaves	Neyyar, Kerala	*Fusarium* sp.
90.	#23 CMLNEY	*C. malabaricum*	leaves	Neyyar, Kerala	*Fusarium* sp.
91.	#29 CMLNEY	*C. malabaricum*	leaves	Neyyar, Kerala	Unidentified
92.	# 37 CMLNEY	*C. malabaricum*	leaves	Neyyar, Kerala	*Chaetomium* sp.
93.	# 2 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Alternaria* sp.
94.	#4 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Botryosphaeria* sp.
95.	#11 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	Unidentified
96.	#12 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Alternaria* sp.
97.	#14 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Fusarium* sp.
98.	#16 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Bionectria* sp.
99.	#18 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Alternaria* sp.
100.	#20 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Lasiodiplodia* sp.
101.	#21 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	Mycelia sterilia
102.	#26 CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	Unidentified
103.	#1 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Arthrinium phaeospermum*
104.	#4 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Arthrinium phaeospermum*
105.	#5 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Arthrinium phaeospermum*
106.	#11 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Pestalotiopsis sp.*
107.	#13 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Pestalotiopsis sp.*
108.	#17 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
109.	#18 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Nigrospora* sp.
110.	#21 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
111.	#26 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Curvularia* sp.
112.	#28 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Curvularia* sp.
113.	#31 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Curvularia* sp.
114.	#35 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Phaeoacremonium* sp.

115.	#36 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Mycelia sterilia
116.	#43 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Acremonium* sp.
117.	#44 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Acremonium* sp.
118.	#45 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Fusarium sp.*
119.	#49 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
120.	#52 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
121.	# 54 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Pestalotiopsis* sp
122.	#55 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
123.	#56 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
124.	#59 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Curvularia* sp.
125.	#60 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
126.	#64 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Phaeoacremonium* sp
127.	#68 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
128.	#79 CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified
129.	#5 CZBAWLS	*C. zeylanicum*	Bark	Wayand, Kerala	*Fusarium oxysporum*
130.	#20 CZBAWLS	*C. zeylanicum*	Bark	Wayand, Kerala	Unidentified
131.	#28 CZBAWLS	*C. zeylanicum*	Bark	Wayand, Kerala	*Glomerella* sp.
132.	#30 CZBAWLS	*C. zeylanicum*	Bark	Wayand, Kerala	*Glomerella sp*
133.	#31 CZBAWLS	*C. zeylanicum*	Bark	Wayand, Kerala	*Lasiodiplodia sp.*
134.	#2 CZSTITBRT	*C. zeylanicum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
135.	#5 CZSTITBRT	*C. zeylanicum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
136.	#6 CZSTITBRT	*C. zeylanicum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
137.	#7 CZSTITBRT	*C. zeylanicum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
138.	#10 CZSTITBRT	*C. zeylanicum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.
139.	#12 CZSTITBRT	*C. zeylanicum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Bionectria* sp.
140.	#6610 CZSTITBRT	*C. zeylanicum*	Stem internal tissue	BRT Wildlife sanctuary, Karnataka	*Muscodor strobelii* sp.*nov*
141.	#1CCSTITD	*C. camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor darjeelingensis* sp.*nov.*
142.	#2 CCSTITD	*C. camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor tigerii* sp.*nov*
143.	#6 CCSTITD	*C. camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor ghoomensis* sp.*nov.*
144.	#6(b) CCSTITD	*C. camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor indica* sp.*nov.*

145.	#16 CCSTITD	*C. camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor camphora* sp.*nov.*
146.	#36 CCSTITD	*C. camphora*	Stem internal tissue	Darjeeling, West Bengal	*Alternaria* sp.
147.	#1 CCBD	*C. camphora*	Bark	Darjeeling, West Bengal	*Fusarium* sp.
148.	# 2 CCBD	*C. camphora*	Bark	Darjeeling, West Bengal	*Penicillium* sp.
Family: Apocynaceae					
149.	#2 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Pestalotiopsis* sp.
150.	#6 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Hypoxylan* sp.
151.	#7 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Hypoxylan* sp.
152.	#11CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	Unidentified
153.	#13 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	Unidentified
154.	#15 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Fusarium* sp.
155.	#16 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Cladosporium* sp
156.	#17 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Fusarium oxysporum*
157.	#20 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Xylaria* sp.
158.	#22 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	Unidentified
159.	#23 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	Unidentified
160.	#26 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Pestalotiopsis* sp.
161.	#29 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Cladosporium* sp.
162.	#30 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	Unidentified
163.	#37 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Xylaria* sp.
164.	#42 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Fusarium* sp.
165.	#43 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Alternaria* sp.
166.	#45 CRSTBRT	*C. roseus*	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	*Alternaria* sp.
167.	#2 TMDSTYEL	*T. divaricata*	stem	Yelandur, Karnataka	*Fusarium* sp.
168.	#2(a) TMDSTYEL	*T. divaricata*	stem	Yelandur, Karnataka	*Fusarium oxysporum*
169.	#2(b) TMDSTYEL	*T. divaricata*	stem	Yelandur, Karnataka	*Fusarium oxysporum*
170.	#4 TMDSTYEL	*T. divaricata*	stem	Yelandur, Karnataka	*Fusarium solani*
171.	#13 TMDSTYEL	*T. divaricata*	stem	Yelandur, Karnataka	*Schizophyllum* sp.
172.	#15 TMDSTYEL	*T. divaricata*	stem	Yelandur, Karnataka	Unidentified

173.	# 24 TMDSTYEL	*T. divaricata*	stem	Yelandur, Karnataka	Unidentified
174.	# 2 RSSTBRT	*R. serpentina*	Stem	BRT Wildlife sanctuary, Karnataka	*Alternaria* sp.
175.	#3 RSSTBRT	*R. serpentina*	Stem	BRT Wildlife sanctuary, Karnataka	*Phoma* sp.
176.	#4 RSSTBRT	*R. serpentina*	Stem	BRT Wildlife sanctuary, Karnataka	*Schizophyllum* sp.
177.	#15 RSSTBRT	*R. serpentina*	Stem	BRT Wildlife sanctuary, Karnataka	*Ascobolus* sp.
178.	#23 RSSTBRT	*R. serpentina*	Stem	BRT Wildlife sanctuary, Karnataka	*Trichoderma* sp.
179.	#1 RSLBRT	*R. serpentina*	Stem	BRT Wildlife sanctuary, Karnataka	*Fusarium oxysporum*
180.	#4 RSLBRT	*R. serpentina*	Leaf	BRT Wildlife sanctuary, Karnataka	*Fusarium solani*
181.	#7 RSLBRT	*R. serpentina*	Leaf	BRT Wildlife sanctuary, Karnataka	Unidentified
182.	#10 RSLBRT	*R. serpentina*	Leaf	BRT Wildlife sanctuary, Karnataka	*Fusarium* sp.
183.	#11 RSLBRT	*R. serpentina*	Leaf	BRT Wildlife sanctuary, Karnataka	Unidentified
184.	#15 RSLBRT	*R. serpentina*	Leaf	BRT Wildlife sanctuary, Karnataka	*Ascobolus* sp.
185.	#16 RSLBRT	*R. serpentina*	Leaf	BRT Wildlife sanctuary, Karnataka	*Ascobolus* sp.
Family: *Taxaceae*					
186.	#7 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Pestalotiopsis* sp.
187.	#8 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Pestalotiopsis* sp.
188.	#11 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Pestalotiopsis* sp.
189.	#13 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Pestalotiopsis* sp.
190.	#17 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	Unidentified
191.	#20 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Fusarium* sp.
192.	#21 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	Unidentified
193.	#22 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Phoma* sp.
194.	#25 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	Unidentified
195.	#26 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Chaetomium* sp.
196.	#27 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Chaetomium* sp.
197.	#28 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	Mycelia sterilia
198.	#30 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Fusarium* sp.
199.	#31 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Fusarium* sp.
200.	#33 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Fusarium* sp.
201.	#34 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Fusarium* sp.

202.	#43 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Phomopsis* sp.
203.	#44 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Alternaria* sp.
204.	#46 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Phomopsis* sp.
205.	#48 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Pestalotiopsis* sp.
206.	#50 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	Mycelia sterilia
207.	#53 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Drechslera* sp.
208.	#57 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Penicillium* sp.
209.	#65 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	Unidentified
210.	#67 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Botrytis* sp.
211.	#69 TBBALM	*T. baccata*	Bark	Almora, Uttarakhand	*Botrytis* sp.

Figure 4.3: Standard Curve of tyrosine

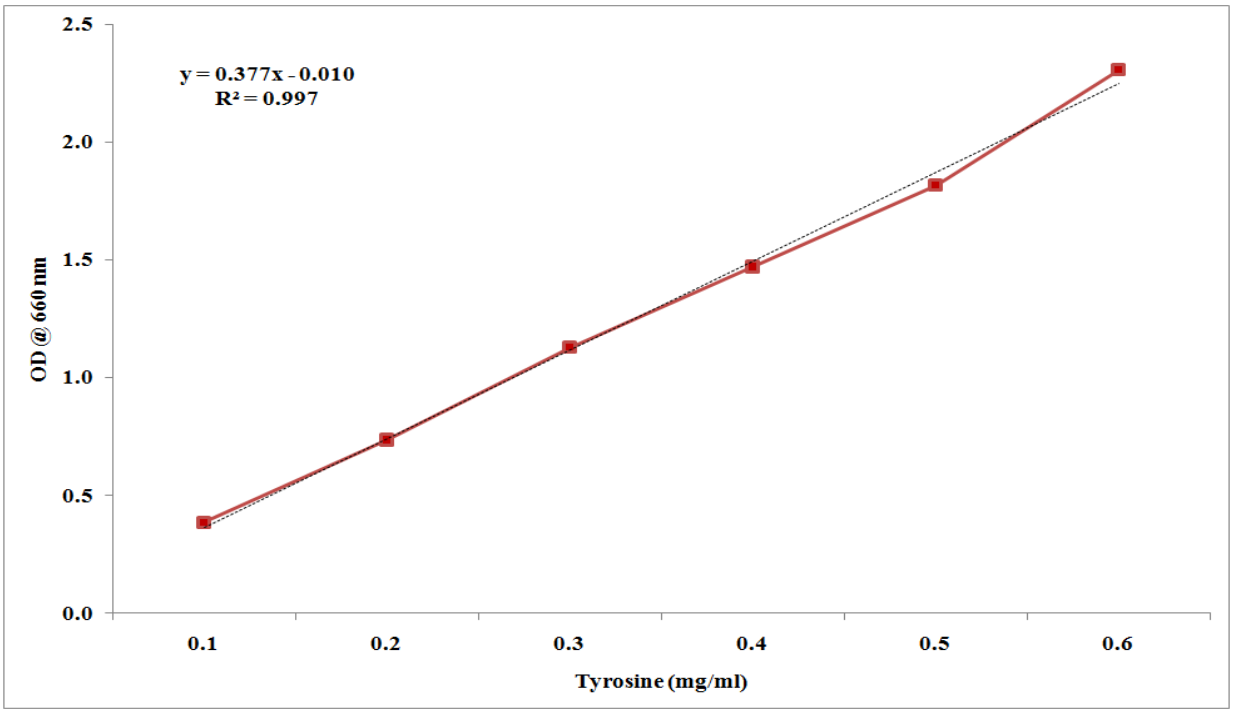
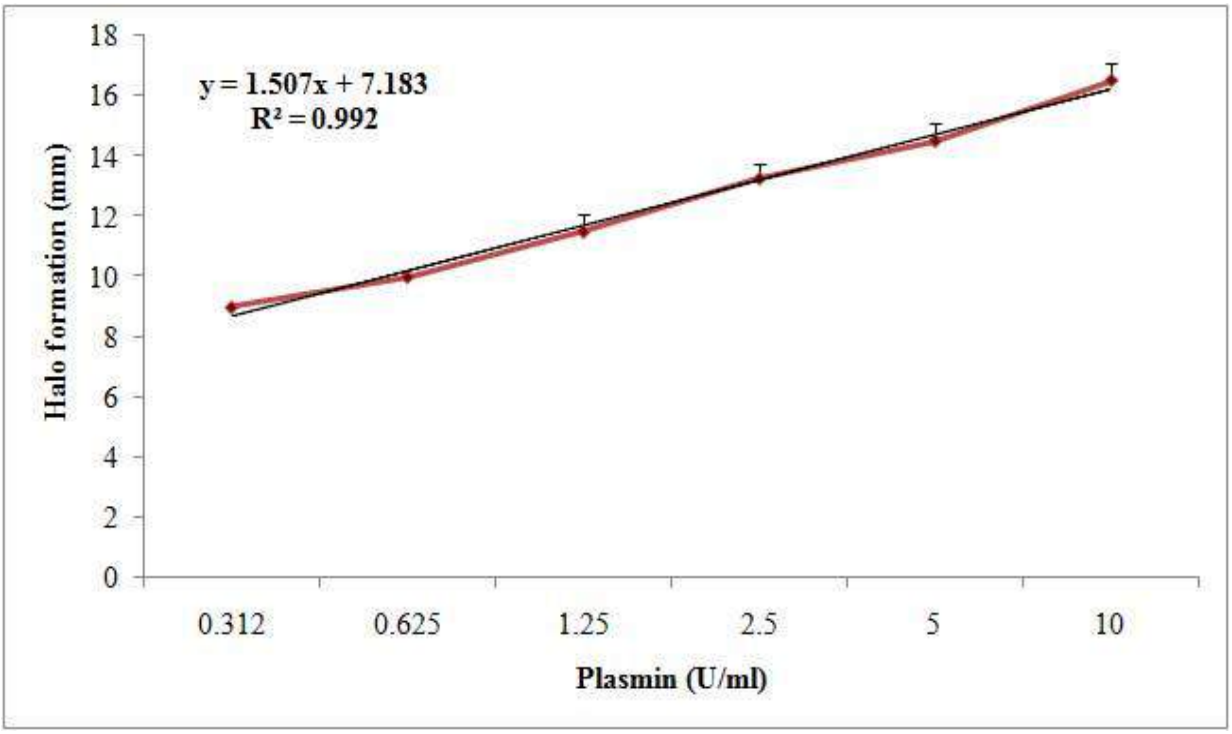


Fig 5.8: Standard curve of plasmin



SDS–PAGE gel composition

Resolving gel (10 %, 6ml)

1. Double distilled water - 2.4 ml
2. Acrylamide: Bis-acrylamide (29.2:0.8) - 2.04 ml
3. 1.5 M Tris HCl buffer (pH–8.8) - 1.5 ml
4. 10 % SDS - 60 µl
5. 10 % Ammonium Per-sulfate (APS) - 60 µl
6. TEMED - 6 µl

Stacking gel (4 %, 5 ml)

1. Double distilled water - 3.0 ml
2. Acrylamide: Bis-acrylamide (29.2: 0.8) - 880 µl
3. 0.5 M Tris HCl buffer (pH–6.8) - 1.25 ml
4. 10 % SDS - 25 µl
5. 10 % Ammonium Per-sulfate (APS) - 70 µl
6. TEMED - 7 µl

Loading Buffer (10 ml)

1. 0.5M Tris HCl Buffer (pH 6.8) - 2.5 ml
2. 10 % SDS - 4 ml
3. Glycerol (100 %) - 2 ml
4. β-mercaptoethanol - 0.8 ml
5. Bromophenol blue (0.1 %) - 300 µl

Electrophoretic buffer (10X/l)

1. Tris Base - 30.3 g
2. Glycine - 144 g
3. SDS - 10 g

(A) Native–PAGE gel composition

Resolving Gel (10 %, 6ml)

1. Double distilled water - 2.46 ml
2. Acrylamide: Bis-acrylamide (29.2:0.8) - 2.04 ml
3. 1.5 M Tris HCl buffer (pH- 8.8) - 1.5 ml
4. 10 % Ammonium Per-sulfate (APS) - 60 µl
5. TEMED - 6 µl

Stacking gel (4 %, 5 ml)

1. Double distilled water - 3.02 ml
2. Acrylamide: Bis-acrylamide (29.2:0.8) - 880 µl
3. 0.5 M Tris HCl buffer (pH- 6.8) - 1.25 ml
4. 10 % Ammonium Per-sulfate (APS) - 70 µl
5. TEMED - 7 µl

Loading buffer (10 ml)

1. 0.5M Tris–HCl Buffer (pH 6.8) - 2.5 ml
2. Glycerol (100 %) - 2 ml
3. Bromophenol blue (0.1 %) - 300 µl

Electrophoretic buffer (10X/l)

1. Tris Base - 30.3 g
2. Glycine - 144 g
3. SDS - 10 g

(B) Coomassie staining solutions

Staining Solution

1. Methanol - 40 %
2. Glacial acetic Acid - 10 %
3. Coomassie Blue R-250 - 0.15 %

De-staining Solution

1. Methanol - 30 %
2. Glacial Acetic Acid - 5 %

(C) Zymography

Native zymo gel composition

Resolving gel (10 %, 6ml)

1. Distilled water - 2.1 ml
2. Acrylamide: Bis-acrylamide - 2.04 ml
3. 1.5M Tris HCl (pH 8.8) - 1.5 ml
4. 10 % APS - 60 µl
5. TEMED - 6 µl
6. Fibrinogen - 300 µl (12 mg/300 µl of 50mM Tris HCl (pH7.8))
7. Thrombin - 15µl

Stacking gel (4 %, 5 ml)

1. Double distilled water - 3.02 ml
2. Acrylamide: Bis-acrylamide (29.2:0.8) - 880 µl
3. 0.5 M Tris HCl buffer (pH- 6.8) - 1.25 ml
4. 10% Ammonium Per-sulfate (APS) - 70 µl
5. TEMED - 7 µl

Loading Dye composition (10ml)

1. 0.5 M Tris HCl (pH-6.8) - 2.5 ml
2. 10% SDS - 4 ml
3. 10% Glycerol - 3.5 ml
4. Bromophenol Blue - 10 mg

Comb Used – 10 well

Spacer – 0.75 mm

Volt – 80V to 120V

Appendix

Buffers

1. **Glycine–HCl (pH 3.0)**

   Stocks solutions

   A: 0.2 M solution of glycine (15.01 g in 1 L)

   B: 0.2 M HCl

   50 ml of A + 11.4 ml of B + 39.6 ml of distilled water

2. **Acetate buffer (pH 4)**

   Stocks solutions

   A: 0.2 M solution of acetic acid (11.55 g in 1 L)

   B: 0.2 M solution of sodium acetate (16.4 g $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na.3H_2O$ in 1 L).

   41 ml of A + 9 ml of B, diluted to a total of 100 ml

3. **Phosphate buffer (pH 6.0)**

   Stocks solutions

   A: 0.2 M solution of monobasic sodium phospate (27.8 g in 1 L)

   B: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1 L).

   87.7 ml of A + 12.3 ml of B, diluted to a total of 200 ml

4. **Tris–HCl (pH 7.4)**

   Stocks solutions

   A: 0.2 M solution of tris(hydroxymethyl) aminomethane (24.2 g in 1 L)

   B: 0.2 M HCl

   50 ml of A + 41.4 ml of B, diluted to a total of 200 ml

5. **Tris–HCl (pH 7.8)**

   Stocks solutions

   A: 0.2 M solution of tris(hydroxymethyl) aminomethane (24.2 g in 1 L)

   B: 0.2 M HCl

   50 ml of A + 32.5 ml of B, diluted to a total of 200 ml

6. Glycine–NaOH

Stocks solutions

A: 0.2 M solution of glycine (15.01 g in 1 L)

B: 0.2 M NaOH

50 ml of A + 8.8 ml of B, diluted to a total of 200 ml