## List of abbreviations

<sup>0</sup>C Degree Celsius

AFLP Amplified fragment length polymorphism

ANOVA Analysis of Variance

ATP Adenosine Triose Phosphate

BLAST Basic Local Alignment Search Tool

C/N ratio Carbon/ Nitrogen ratio

CCD Central Composite Design

CVD Cardiovascular Disease

DNA Deoxy Ribose Nucleic Acid

dNTP Deoxyribo Nucleotide Triose phosphate

ECLT Euglobulin Clot Lysis Time

EDTA Ethylene Diammine Tetraacetatetae

F/C ratio Fibrinolytic activity/ Caseinolytic activity

ratio

FC reagent Folin-Ciocalteu's Reagent

FFD Fractional Factorial Design

g Gram

HCI Hydrochloric acid

ISR Inter Spacer Region

kDa Kilo Dalton

M Molar

MEGA Molecular Evolutionary Genetic

Algorithm

ml Milí Liter

mM Mili Molar

MR Methyl Red

NB Nutient Broth

NCBI National Center for Biotechnological

Information

NE, India North-East, India

NK Natto kinase

O.D Optical Density

PCR Polymerase Chain Reaction

PEG Poly Ethylene Glycol

RAPD Rapid Amplification of Polymorphic DNA

RFLP Restriction Fragment Length

Polymorphism

RNA Ribose Nucleic Acid

RPM Rotation Per Minute

rRNA Ribosomal Ribose Nucleic Acid

RSM Response Surface Methodology

S.D Standard Deviation

SDS Sodium Dodecyl Sulphate

SIM Sulfur Indole Motility

SMA Skimmed Milk Agar

SmF Submerged Fermntation

TAE Tris Acetate EDTA

TCA Tricholoacetic acid

t-PA Tissue plasminogen activator

TSI Triple Sugar Iron

U Unit (Enzyme activity unit)

VP Voges proskaure

w/v Weight/ Volume

w/w Weight/ Weight

WHO World Health Organization

μg Micro gram

μί Micro-liter

## Contents

Legends		Page
		No
	Chapter 1: Introduction	1-17
1.0	Introduction	2-6
1.2	Information on Cardiovascular diseases, available using	7-8
	thrombolytic drugs and its limitations	
1.3	Microbial protease (fibrinolytic enzymes) a boon for health sector	8-11
1.4	Classification of protease (fibrinolytic enzymes)	11-14
1.4.1	Type of serine proteases	11-12
1.4.2	Chymotrypsin-like proteases	12-13
1.4.3	Subtilisin-like protease or Subtilases	13
1.4.4	Wheat serine carboxypeptidase Il-like protease	13
1.4.5	Prolyloligopeptidae-like serine protease	13
1.4.6	Myxobacter ∝-lytic proteases	13
1.4.7	Staphylococcal protease	13-14
1.5	Protease (fibrinolytic) enzyme fermentation and yield	14
	improvement	
1.5.1	Fermentation methods	14-17
1.5.2	Aim and objectives	16-17
	Chapter 2: Review of literature	18-25
2.1	Non-food sources	19-20

2.2	Food sources	20-22
2.3	Comprehensive review on fibrinolytic enzyme production	23-24
	by submerged fermentation	
2.4	Application and perspectives	25
	Chapter 3: Materials and Methods	26-43
3.1	Materials	27
3.1.1	Plastic ware / Glass ware / Columns	27
3.1.2	Chemicals	27-28
3.1.2.1	Analytical grade	27
3.1.2,2	Microbiological grade culture media/chemicals	27
3.1.2.3	Molecular biology grade chemicals /kits	28
3.1.2.4	The bacterial strain	28
3.2	Methods	28
3.2.1	Pure culture preparation of fibrinolytic protease secreting	28
	bacterial isolates	
3.2.1.1	Spread plate technique	28-29
3.2.1.2	Streak-plate technique	29
3.2.2.3	Routine maintenance and preservation of Microorganism	29
3.2.3	Taxonomic identification of alkaline protease (Fibrinolytic	29-36
	enzyme) producing bacteria	
3.2.3.1	Morphological test	29-30
32311	Gram staining	29-30

3.2.3.2	Biochemical test	30-33
3.2.3.2,1	Hydrolysis test for casein, starch, lipid and gelatin	30
3.2.3.2.2	Carbohydrate fermentation test	30
3.2.3.2.3	Triple sugar iron (TSI) agar test	31
3.2.3.2.4	IMVIC test	31
3.2.3.2.5	Hydrogen sulphide test	32
3.2.3.2.6	Urease test	32
3.2.3.2.7	Litmus –milk test	32
3.2.3.2.8	Nitrate reduction test	32
3.2.3.2.9	Catalase test	32-33
3.2.3.2.10	Oxidase test	33
3.2.3.3	Ribotyping using 16S rRNA gene amplification	33-36
3.2.3.3.1	Isolation of DNA	33-34
3.2.3.3.2	PCR amplification of 16S rRNA gene	34-35
3.2,3.3,3	PCR amplification of the 16S-23S inter spacer region (ISR)	35
	region of the isolated DNA	
3.2.3.4	Phylogenetic analysis	36
3.2.4	Determination of protease activity	36-37
3.2.5	Optimization of culture condition for optimum growth and	37
	Maximum fibrinolytic protease production by by selected bacteria	
	under SmF systems	
3.2.5.1	Fibrinolytic protease production under submerged Fermentation	37

	(SmF) systems	
3.2.5.2	Effect of various carbon sources on fibrinolytic protease	38
	production	
3.2.5.3	Effect of various inorganic and organic nitrogen sources on	38
	fibrinolytic protease production	
3.2.5.4	Effect of pH on fibrinolytic protease production	38
3.2.5.5	Effect of incubation time on fibrinolytic enzyme Production	38
3.2.6	Fibrinolytic protease production under submerged fermentation	38
	system	
3.2.6.1	Statistical optimization of protease production in SmF	38-43
3.2.6.1.1	Screening of factors effecting fibrinolytic protease Production	39-40
	using Plackett-Burman design	
3.2.6.1.2	Statistical optimization of fibrinolytic protease production using	41-42
	Response Surface Methodology(RSM)	
3.2.7	Validation of response surface; Batch fermentation Under	43
	optimized condition	
	Chapter 4: Results	44-83
	Results	
4.1	Pure culture preparation of the bacterial strain: FF01	45
4.2	Taxonomic identification of fibrinolytic enzyme producing FF01	46-56
	bacterial isolate	
4.2.1	Phenotypic study of FF01 bacterial isolate	46

4.2.1.1	Morphological identification: Gram staining of the bacterial strain	46
4.2.1.2	Biochemical identification	47-50
4.3.2	Genotyping profiling of FF01 bacterial isolate	51
4.3.2.1	PCR amplification of 16S rRNA gene of FF01 Bacterial isolate	51-52
4.3.2.2	Phylogenetic tree construction using 16S-rRNA gene	53
4.3.2.3	PCR amplification of 16S-23S rRNA gene of FF01 bacterial	54-55
	isolate	
4.3.2.4	Phylogenetic tree construction using 16S-23S rRNA gene	56
4.3.2.5	Bacterial naming and identification	58
4.4	Screening of initial process parameters for fibrinolytic enzyme	58-72
	production from FF01 bacterial isolate under submerged	
	fermentation	
4.5	Statistical optimization of influencing parameters for Plackett-	74
	Burman design	
4.5.1	Screening of influencing parameters by Plackett-Burman design	73-75
4.5.2	Statistical optimization of fibrinolytic enzyme production using	75-80
	Central composite design (CCD)	
4.5.3	Validation of the model	80-81
	Chapter 5: Discussion	82-88
5.1	Isolation and culture maintenance techniques	83
5.1.1	Pure culture techniques	83
5.1.2	Streak plate method	84

	Chapter 6: Conclusion	89-91
5.3.1.2	Response surface method	88
5.3.1.1	Plackett-Burman Design	87
	fibrinolytic protease production using statistical tools	
5.3.1	Screening of influencing process parameters for microbial	87-89
	fermentation system (SmF)	
5.3	Microbial fibrinolytic protease production under submerged	86-87
5.2.2	Genotypic approach	86
5.2.1	Phenotypic approach	85
5.2	Bacterial identification	84
5.1.3	Pour plate Method	84

Reference and Appendix

## **List of Tables**

Table	Table Legends	Pages
No.		
	Chapter 1: Introduction	1-17
1.1	Enzymes in various industrial segments and their	4-6
	applications	
1.2	Sources of microbial fibrinolytic enzymes	9
1.3	Fibrinolytic enzyme producing Bacilli, isolated from traditional	10
	fermented food	
1.4	Statistical method to improve protease production from	16
	microorganisms	
	Chapter 2: Review of Literature	18-25
2.1	Recent reports on isolation of fibrinolytic enzymes from	21
	various sources from India	
2.2	Some latest and selected reports on isolation of fibrinolytic	22
	enzymes from various sources from abroad	
2.3	Recent reports on statistically optimized process parameters	24
	for fibrinolytic enzyme prouction under submerged	
	fermentation	
	Chapter 3: Materials and Methods	26-43
3.1	Optimal PCR reaction conditions or amplification of	35
	conserved region of 16S-rRNA gene of selected fibrinolytic	

protease secreting	bacterial	strain
--------------------	-----------	--------

3.2	Independent variables for fibrinolytic protease production	40
	under SmF systems using Plackett-Burman design.	
3.3	Independent variables for fibrinolytic protease production	42
	under SmF system using Central Composite Design	
3.4	Validation of response surface for fibrinolytic protease	43
	production	
	Chapter 4: Results	44-81
4.1	Biochemical and morphological tests of bacteria FF01	50
	isolate. Experiments were repeated thrice to assure	
	reproducibility	
4.2	Homologous search results of 16S-rRNA gene sequence	52
	using BLAST tool from NCBI.	
4.3	Homologous search results of 16S-23S ISR gene sequence	55
	using BLAST tool from NCBI	
4.4	Representation of bacterial identification and designated	57
	name.	
4.5	Plackett-Burman store design showing three variables with	74
	coded values along with the observed results for fibrinolytic	
	enzyme production by FF-01 bacterial isolate	

4.6	Statistical analysis of Plackett–Burman design showing	75
	coefficient values, t- and p-value for each variable for	
	fibrinolytic enzymes from FF-01 bacterial isolate (p-value	
	<0.05).	
4.7	Observed responses and predicted values of fibrinolytic	76
	enzyme production by FF01 bacterial isolates.	
4.8	Analysis of Variance (ANOVA) of fibrinolytic enzyme	77
	produced by FF01 bacterial isolate	
4.9	Model coefficients estimated by multiple linear regressions	78
	(significance of regression coefficients) for fibrinolytic enzyme	
	production by FF01 bacterial isolate in SmF under shake-	
	flask study (p<0.05).	
	Chapter 5: Discussion	82-88
	Chapter 6: Conclusion	89-91

## List of Figure legends

Figure	Figure Legends	Page
No.		No.
	Chapter 1: Introduction	
1.1	The steps involved in classical versus state-of the- art	3
	development of enzymes	
1.2	The proportion of deaths by causes in WHO regions, estimates for	7
	2000 (WHO, 2001)	
	Chapter 2: Review of Literature	
	Chapter 3: Materials and Methods	
	Chapter 4: Results	
4.1	Preparation of pure culture	45
4.2	Gram staining of FF01 cells at a magnification of 1000x under	46
	compound microscope	
4.3	Biochemical fingerprints of FF01 bacterial isolate	47
4.4	Biochemical profile of FF01 bacterial isolate	48
4.5	Biochemical property of FF01 bacterial isolate	49
4.6	PCR amplification of 16S rRNA gene from FF01 bacterial isolates	51
4.7	Phylogenetic tree construction of 16S-rRNA gene using Neighbor-	53
	Joining method.	
4.8	PCR amplification of 16s-23s ISR region amplification	54
4.9	Phylogenetic tree construction off 16S-23S ISR gene using	56
	Neighbor-Joining method.	
4.10	Effect of pH on bacterial growth (◆ ) and dry biomass (■) post	58
	incubated for 24h at 37°C. Values are mean ± S.D are of triplicate	
	experiments	

4.11	Effect of pH on protein content (♦), caseinolytic activity (■), and	59
	fibrinolytic activity (▲) of FF01 bacterial isolate. Values are means	
	± S.D are of triplicate experiments.	
4.12	Effect of pH on F/C ratio of culture supernatant FF01 bacterial	60
	isolates. Value are means ± S.D are of triplicate experiments.	
4.13	Effect of carbon sources of bacterial growth (■) and bacterial dry	62
	biomass ( ◆) from FF01 bacterial isolate. Values are mean ± S.D	
	are of triplicate experiments.	
4.14	Effect of carbon sources on protein content (◆ ), caseinolytic	63
	activity (■ ) and fibrinolytic activity (▲) fibrinolytic enzyme	
	production from FF01 bacterial isolate. Values are mean $\pm$ S.D are	
	of triplicate experiments.	
4.15	Effect on Carbon source on F/C ratio on Fibrinolytic enzyme	64
	production by FF01 bacterial isolates. Values are mean $\pm$ S.D of	
	triplicate values.	
4.16	Effect of nitrogen sources on bacterial growth (◆) and bacterial dry	66
	biomass (■) of FF-01 bacterial isolate. Values are mean ± S.D are	
	of triplicate experiments.	
4.17	Effect of nitrogen sources on protein yield (■), caseinolytic activity	67
	(◆) and fibrinolytic activity (▲) of FF01 bacterial isolate. Values	
	are mean ± S.D are of triplicate experiments.	
4.18	Effect of nitrogen sources on F/C ratio of fibrinolytic enzymes from	68
	FF01 bacterial isolates. Values are mean ± S.D are of triplicate	
	experiments	
4.19	Effect of incubation time on bacterial growth (+) and bacterial dry	70
	biomass (閨) of FF01 bacterial isolate Values are mean ± S.D	
	are of triplicate experiments.	

4.20	Effect of incubation time on protein content (♦), caseinolytic	71
	activity (■), and fibrinolytic activity (▲) of FF-01 bacterial isolate.	
	Values are mean ± S.D are of triplicate experiments.	
4.21	Effect of incubation time on F/C ratio of fibrinolytic enzymes	72
	produced by FF-01 bacterial isolate. Values are mean ± S.D are	
	of triplicate experiments	
4.22	Response surface methodology plots for F/C ratio for fibrinolytic	79
	enzymes from FF-01 bacterial isolate (a) response surface plot,	
	(b) Countor plot.	
4.23	Validation of model for fibrinolytic enzyme production from FF01	81
	bacterial isolate.	
	Chapter 5: Discussion	82-88
	Chapter 6: Conclusion	89-91
	Reference and Appendix	