T 145
574.88
49653

CENTRAL [illegible]
TEZPUR UNIV[illegible]
Accession No. 49653
Date 14/9/11
[illegible] LIBRARY T. U.
[illegible] T.145

REFERENCE BOOK
NOT TO [illegible]ISSUED
TEZPUR UNIVERSITY LIBRARY

PRODUCTION, ISOLATION AND CHARACTERIZATION OF MICROBIAL ALKALINE PROTEASE AND THEIR INDUSTRIAL APPLICATIONS

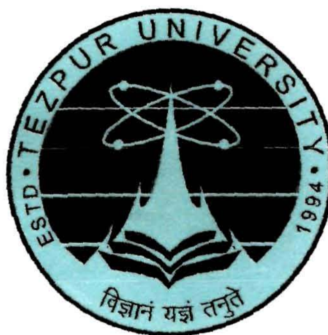
A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

BY

SUDHIR KUMAR RAI, M.Sc.

Registration no. 003 of 2007



School of Science and Technology

Department of Molecular Biology and Biotechnology

Tezpur University

September 2010

Dedicated to my beloved family members

Honr Lt Shri Nandu Prasad Rai

Smt. R. Rai

Er. Shri Shailesh Kumar Rai

Smt. Kiran Rai

Miss Hasita Rai

&

Prof. Ashis K. Mukherjee and his family members

Abstract

Microbial proteases constitute approximately 60% of the total worldwide production of enzymes. They have diverse applications in a wide variety of industries, such as in detergent, food, pharmaceutical, leather, silk and for recovery of silver from used X-ray films. Of these, alkaline proteases are particularly important because they are both stable and active at high pH solutions and in the presence of surfactants and oxidizing agents. Their major application is in detergent industry, because the pH of laundry detergents is generally in the range of 9.0-12.0. The industrial demand of highly active preparations of proteolytic enzymes with appropriate specificity and stability to pH, temperature, metal ions and surfactants continues to stimulate the search for new enzyme sources. Isolation and screening of microorganisms from naturally occurring alkaline habitats is expected to provide novel strains capable of producing high-titer of industrially important proteases which will retain its activity and stability in high alkaline conditions. Based on above mention requirements, present study was initiated with the objectives of isolation of high-titer alkaline protease producing bacteria by screening the soil / water samples of north-east India, taxonomic identification of above potential strains , optimization of culture conditions of potential microbes for maximizing the protease production using solid-state fermentation (SSF) and submerged fermentation (SmF) systems. Finally, effort has been given for the isolation, downstream processing and biochemical characterization of an alkaline protease produced by the promising microbial strain(s). Furthermore, the possible industrial applications of the isolated alkaline proteases were also investigated.

Thirty three alkaline protease producing bacterial cultures were obtained by initial screening of environmental samples collected from various parts of NE India, and amongst them only 12 bacteria showed zones of hydrolysis ≥ 25.0 mm in skim-milk agar plate. These strains were further characterized. On the basis of (i) bacterial growth kinetics, (b) alkaline protease yield, (c) alkaline pH and detergent stability, and (d) thermal stability of isolated protease five potential strains viz. **AS-S10-II, AS-S18 AS-S20-I, AS-S24-I and AS-S24-II** were selected for taxonomic identification and characterization of their protease enzyme(s). Pure culture of bacteria was obtained by following the standard protocol. On the basis of the data obtained from phenotypic, chemotaxonomic and phylogenetic analyses of pure cultures, the strain AS-S10-II, AS-S18, AS-S20-I, AS-S24-I and AS-S24-II were identified as *Brevibacillus* sp. AS- S10-II, *B.subtilis* AS-S18, *Bacillus* sp. AS-S20-I , *B. licheniformis* AS-S24-I, and *P. tezpurensis* sp. nov. AS-S24-II, respectively. Further, a protease producing promising strain isolated previously, in our laboratory viz. *B.subtilis* DM-04 was also included in the present study.

Screening of parameters for optimized protease production by *B. subtilis* RM-01, *B. subtilis* DM-04, *Brevibacillus* sp. AS-S10-II, *Bacillus* sp. AS-S20-I, *B.licheniformis* AS-S24-I, and *P.tezpurensis* sp. nov. AS-S24-II under SmF and SSF was performed using Plackett Burman design followed by Response surface methodology. Under statistically optimized conditions maximum alkaline protease production by all the strains was varied in a process controlled bioreactor. Bacterial strains such as *Paenibacillus*

tezpurensis sp.nov.strain AS-S24-II, *B.subtilis* strain RM-01, *Bacillus* sp.strain AS-S20-I, *Brevibacillus* sp. strain AS-S10-II demonstrated preference for inorganic nitrogen source whereas *B.subtilis* DM-04 and *Bacillus licheniformis* strain AS-S24-I showed preference for organic nitrogen source for maximum protease production under submerged fermentation. All strains except *B.subtilis* strain DM-04 demonstrated the choice of complex carbon source casein for optimum growth and protease production; *B.subtilis* strain DM-04 displayed maximum protease production in presence of maltose. However *Bacillus licheniformis* strain AS-S24-I preferred D-glucose for protease production under submerged fermentation.

In the present study, some alkaline proteases were purified their biochemical properties and biotechnological application were studied. For example, two alkaline proteases viz., Bsubap-I and Alzwiprase was purified from *B.subtilis* strain DM-04 with a molecular masses 33.1 kDa and 16.9 kDa respectively; detergent stable alkaline protease was purified from *P.tezpurensis* sp.nov. strain AS-S24-II with a molecular mass 43.0 kDa; Alkaline β-keratinase was purified from *B.subtilis* strain RM-01 with molecular mass 20.1 kDa. Another alkaline keratinase viz. Alkarnase having the molecular mass of 43.1 kDa was purified from *Bacillus licheniformis* strain AS-S24-I; Brevicarnase, an alkaline keratinase was purified from *Brevibacillus* sp. strain AS-10-II with molecular mass 83.2 kDa.

All purified alkaline proteases demonstrated suitable for industrial applications thus biochemical properties suggesting their applications in

laundry detergent formulations and other industries where alkaline proteases are employed. For examples, Bsubap-I and Alzwiprase isolated from *B.subtilis* strain DM-04, Alkarnase isolated from *Bacillus licheniformis* strain AS-S24-I, detergent stable alkaline protease from *P.tezpurensis* sp.nov. strain AS-S24-II and partially purified protease isolated from *Bacillus* sp. strain AS-S20-I demonstrated optimum pH in the range of 10.0-10.5 and optimum incubation temperature at 45.0ºC, whereas Brevicarnase isolated from *Brevibacillus* sp. strain AS-S10-II and alkaline β-keratinase isolated from *B.subtilis* strain RM-01 demonstrated optimum pH at 12.5 and 8.0, respectively. Substrate specificity study demonstrated maximum specificity for casein by Bsubap-I, Alzwiprase, and purified protease from *P.tezpurensis* sp.nov. strain AS-S24-II, whereas Brevicarnase, Alkarnase and β-keratinase showed maximum preference for hydrolyzing raw chicken-feather. In contrast, partially purified protease from *Bacillus* sp. strain AS-S20-I displayed maximum specificity for degrading fibrin thus showing its fibrinolytic activity and suggesting its future application as thrombolytic and anticancer agent. It was observed that Fe^{2+} enhanced the proteolytic activity of Bsubap-I, Alzwiprase and alkaline β-keratinase, whereas other metal ions decreased the protease activity of purified enzymes. All purified / partially purified proteases demonstrated appreciable stability in presence of various non-ionic and ionic surfactants, oxidizing and bleaching agents. Thermo-stability study demonstrated thermostable nature of the purified proteases by virtue of possessing intramolecular disulphide bond in protein molecule. Inhibition study demonstrated that all purified/ partially proteases / keratinase were inhibited by PMSF, 4-BPB and IAA suggesting that Ser-His-Cyst residues

are function as a catalytic triad during enzymatic reaction. All purified proteases demonstrated more or less organic solvent stability suggesting their application in peptide synthesis.

All purified proteases demonstrated very good stability in presence of various commercial laundry detergents at a very low dose of enzyme. For example, Bsubap-I (7.0 μg /ml), Alzwiprase (0.1 mg /ml), purified protease from *P.tezpurensis* sp.nov. AS-S24-II (3.0 μg /ml), Brevicarnase (6.0 μg /ml), Alkarnase (7.0 μg /ml), alkaline β-keratinase (0.5 mg / ml) , and partially purified protease from *Bacillus* sp. AS-S20-I (6.0 μg /ml) showed detergent stability at a very low dose, much below the dose recommended for inclusion of protease in detergent formulation. Pharmacological properties of all purified / partially purified proteases / keratinases demonstrated negligible toxicity at the tested concentration, suggesting eco-friendly applications of these proteases for laundry detergent formulations and other industries.

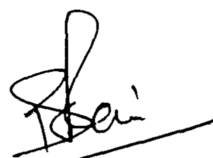
All the purified protease in the present study did not show collagenase activity suggesting their applications in leather industries as dehairing agents. Some of the alkaline protease such as Bsubap-I and Alzwiprase demonstrated excellent antibacterial activity advocating their application in food industry as a preserving and sterilizing agent, and in laundry detergent industries they may used as sanitizing agents. Partially purified protease from *Bacillus* sp. strain AS-S20-I degraded β-chain from fibrinogen and α-chain from fibrin clot in *invitro* condition implying their application in pharmaceutical industries as a thrombolytic agent.

Declaration

I hereby declare that due to the lack of proper facility at Tezpur University, following experiments / sample analyses were carried out at other institutes. Further, I declare that no part of this has been reproduced elsewhere for award of any other degree.

1. GC analyses of FAME of the bacterial strains were done by Dr. Shanmugam Mayilraj at Institute of Microbial technology, Chandigarh, India.

2. 16S-rRNA gene analysis was done by Prof. V.K.Chaudhary, at DBT sponsored National DNA Sequencing facility, Department of Biochemistry, University of Delhi South Campus, New Delhi.



Date: 10/09/10
Place:Tezpur University, Napaam

(Sudhir Kumar Rai)
Registration No.003 of 2007

TEZPUR UNIVERSITY

(A Central Govt. University)

DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY

Tezpur, 784028, Assam, India

Dr. Ashis K Mukherjee **Ph:** 03712-267007/8/9 Ext.5405 (O)

Professor and Head **Mobile:** +919957184351

E-mail: akm@tezu.ernet.in **Fax:** 03712-267005/6

CERTIFICATE OF THE PRINCIPAL SUPERVISOR

This is to certify that the thesis entitled "Production, isolation and characterization of microbial alkaline protease and their industrial applications" submitted to the Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of original research work carried out by Mr. Sudhir Kumar Rai under my personal supervision and guidance.

All helps received by him/her from various sources have been duly acknowledged. No part of this thesis has been reproduced elsewhere for award of any other degree.

AKMukherjee

Date: 10 September, 2010

Place: Tezpur University

(A. K. Mukherjee)
Signature of Principal Supervisor
Professor and Head
Department of Molecular Biology and Biotechnology
School of Science and Technology

TEZPUR UNIVERSITY

CERTIFICATE OF THE EXTERNAL EXAMINAR AND ODEC

This is to certify that the thesis entitled "Production, isolation and characterization of microbial alkaline protease and their industrial applications" submitted by Mr. Sudhir Kumar Rai to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us on 18/01/2011 and found to be satisfactory.

The Committee recommends for the award of the degree of Doctor of Philosophy.

Signature of:

Principal Supervisor

Date: 18/01/2011

External examiner

Date: 18/1/2011

CONTENTS | **Page No.**

Abstract	i-v
Declaration	
Certificate by the supervisor	
Certificate of the external examiner and ODEC	
Contents	
List of tables	vi-xix
List of figures	xx-Liv
List of abbreviation	
Acknowledgements	
CHAPTER I:	1
INTRODUCTION	1
1. Introduction	2-3
1.1 Historical aspect of Industrial enzymes	3
1.2 Industrial enzymes and Development of bio industrial sectors: An overview	4-5
1.2.1 Indian scenario of industrial enzyme's	5-6
1.2.1.1 Futuristic application of enzymes for the growth of Indian Bio-Industrial sector	6-9
1.3 Present scenario of microbial protease: a leader of industrial enzymes	10
1.4. Microbial protease: bread and butter for enzyme industries	11-12
1.4.1 Proteolytic enzymes	12-14
1.4.2 Classification of proteases	14-15
1.4.2.1 Exopeptidases	15-16
1.4.2.2 Endopeptidases	16
1.5 Serine proteases	16-17
1.5.1 Serine alkaline proteases	17
1.5.1.1 Subtilisins	17
1.6 Aspartic proteases	18
1.7 Cysteine/thiol proteases	18

1.8 Metalloproteases 19
1.9 Structure and Mechanism of action of proteases 19-20
1.9.1 Structure of proteases 20
1.9.2 Mechanism of action of proteases 20-21
1.9.2.1 Mechanism of action of serine protease 21-26
1.9.2.2 Mechanism of action of aspartic protease 27-28
1.9.2.3 Mechanism of action of metallo-protease 29
1.9.2.4 Mechanism of action of cysteine protease 30-31
1.10 Production of microbial alkaline proteases 32-35
1.11 Protease purification techniques 36-40
1.12 Physiological functions of proteases 41-43
1.13 Immobilization 43
1.13.1 Historical aspect of immobilized enzymes 43-44
1.13.2 Classification of immobilized methods and selection of carrier 44-45
1.13.3 Main Application Areas of Immobilization Technology 46
1.13.3.1 Bioprocess Technology 46
1.13.3. 2 Enzyme Technology 46
1.13.3.3. Waste Technology 46
1.13.3.4 Environmental Technology 46
1.14 Industrial application of microbial proteases 46-48
1.14.1 Food and feed industry 48-49
1.14.2 Peptide synthesis 49-50
1.14.3 Leather industry 50-51
1.14.4 Management of industrial and household waste 51
1.14.5 Proteases in the detergent industry 51-52
1.14.6 Selection and evaluation of detergent protease performance 52-53
1.15 Major obstacles and future prospect of microbial proteases 53-54
1.16 Aims and objectives of present study 54-55

CHAPTER II: 56
REVIEW OF LITERATURE 56
2. Screening and detection of protease producing potential micro-organisms 57-66

2.1 A brief review on alkaline protease production by submerged fermentation (SmF) and solid-state fermentation (SSF) system	66
2.1.1 Solid-state fermentation (SSF) systems	66-68
2.1.2 Solid-supports in SSF systems	68-69
2.2 Protease production under SSF system using agro-industrial wastes	69-74
2.3 Protease production under submerged fermentation system	75
2.4 Statistical optimization of protease production	76-81
2.5 Purification and biochemical characterization of alkaline protease	82-89
Immobilization of alkaline proteases	89
2.6.1 Cell-free immobilization	89-90
2.7 Industrial application of proteases	90
2.7.1 Detergent industries	90-99
2.7.2 Leather industry	100
2.7.3 Pharmaceutical industry (with especial reference with fibrinolytic enzymes)	100
2.7.4 Solid-waste management	101-103
CHAPTER III:	104
MATERIALS AND METHODS	105
3.1 Materials	105
3.1.1 Plasticware / Glassware /Columns	105
3.1.2 Chemicals	105
3.1.2.1 Analytical grade	105
3.1.2.2 Microbiological grade culture media/chemicals	105-106
3.1.2.3 Molecular biology grade chemicals / kits	106
3.1.2.4 Raw materials	106
3.1.2.5 Microbial strains	106
3.2 Methods	106
3.2.1 Collection of environmental samples	106-107
3.2.2 Sample collection from soil	107
3.2.3 Sample collection from water	107-108
3.3 Screening of alkaline protease producing thermophilic bacteria	108
3.3.1 Measurement of zone of hydrolysis	108

3.3.2 Growth kinetics and protease production	109
3.3.3 Thermo stability study	109
3.3.4 Detergent compatibility study	109
3.3.5 Storage stability of protease enzyme	109-110
3.4 Pure culture of alkaline protease secreting bacterial isolates	110
3.4.1 Spread plate technique	110
3.4.2 Streak-plate Technique	110
3.5 Routine maintenance and preservation of micro organism	110-111
3.6 Taxonomic identification of alkaline protease producing bacteria	111
3.6.1 Morphological tests	111
3.6.1.1 Simple staining	111
3.6.1.2 Negative staining	111
3.6.1.3 Gram's staining	111-112
3.6.1.4 Spore staining	112
3.6.2 Biochemical tests	112
3.6.2.1 Hydrolysis test for casein, starch, lipid and gelatin	112
3.6.2.2 Carbohydrate fermentation test	112
3.6.2.3 Triple sugar iron (TSI) agar test	113
3.6.2.4 IMViC test	113
3.6.2.4.1. Indole production test	113
3.6.2.4.2. Methyl red - Voges-Proskauer (MR-VP) test	113
3.6.2.4.3. Citrate Utilization test	113
3.6.2.4.4 Hydrogen sulfide test	113
3.6.2.4.5 Urease test	113
3.6.2.4.6 Litmus-milk reactions test	114
3.6.2.4.7 Nitrate reduction test	114
3.6.2.4.8 Catalase test	114
3.6.2.4.9 Oxidase test	114
3.6.3 Chemotaxonomic identification: analysis of bacterial cellular fatty acid methyl esters (FAME)	115
3.6.4 Ribotyping using 16S rRNA gene amplification	115
3.6.4.1 Isolation of chromosomal DNA	115
3.6.4.1.1 DNA extraction by alkaline lysis	115-116

3.6.4.1.2 Direct chromosomal DNA extraction 116
3.6.4.1.3 PCR amplification of 16S-rRNA gene 116-117
3.6.4.1.4 Phylogenetic analysis 117
3.6.5. PCR-RFLP analysis of selected strains 118
3.6.5.1 Synthesis of 16S-23S Inter specific region (ISR) primers for *Bacillus subtilis*, and *Bacillus licheniformis* strains 118
3.6.6 16S-rRNA gene species-specific amplification of AS-S20-I and AS-S24-II 118-119
3.7 Optimization of culture condition for optimum growth and maximum alkaline protease production by selected bacteria under SSF and SmF systems 119
3.7.1 Alkaline protease production under submerged fermentation (SmF) system 119
3.7.2 Effect of various carbon sources on alkaline protease production 119
3.7.3 Effect of various inorganic and organic nitrogen sources on alkaline protease production 120
3.7.4 Effect of pH on alkaline protease production 120
3.7.5 Effect of agitation rate on alkaline protease production 120
3.7.6 Effect of temperature on alkaline protease production 120
3.7.7 Effect of incubation time on alkaline protease production 120
3.8 Alkaline protease production under solid-state fermentation system 120
3.8.1 Process optimization of various parameters in SSF for alkaline protease production 121
3.8.2 Preparation of substrates for alkaline protease production 121
3.8.3 Preparation of substrates for alkaline keratinase production 122
3.8.4 Solid-state fermentation and optimization of process conditions for protease production 122-123
3.8.5 Solid-state fermentation and optimization of process conditions for alkaline keratinase production 123
3.8.6 Optimization of protease / keratinase extraction from fermented matter 123-124
3.9 Statistical optimization of alkaline protease production in SmF 124

and SSF
3.9.1 Screening of factors effecting protease production using Plackett-Burman design 124-125
3.9.2 Statistical optimization of alkaline protease production using Response surface method (RSM) 125-126
3.9.3 Validation of response surface: Batch fermentation under optimized conditions 126
3.9.3.1 Alkaline protease production under SSF systems 126-127
3.9.3.2 Alkaline keratinase production under SSF systems 127
3.9.3.3 Alkaline protease production under SmF systems 127
3.10. Isolation and downstream processing of alkaline proteases / keratinase 128
3.10.1 Isolation and purification of Bsubap-I, an alkaline protease from *B. subtilis* DM-04 128-129
3.10.2 Isolation and purification of Alzwiprase (an alkaline protease) from *B. subtilis* DM-04 129-130
3.10.3 Isolation and purification of alkaline β-keratinase protease from *B. subtilis* RM-01 130
3.10.4 Isolation and purification of detergent-stable alkaline protease from *Peanibacillus tezpurensis* sp.nov. AS-S24-II 130-131
3.10.5 Isolation and purification of Alkarnase (an oxidant and detergent-stable alkaline protease) from *Bacillus licheniformis* AS-S24-I 131
3.10.6 Isolation and purification of Brevicarnase (detergent-stable alkaline protease) from *Brevibacillus* sp. AS-S10-II 132
3.10.7 Partial purification of alkaline protease from *Bacillus* sp. AS-S20-I 132
3.10.8 Criteria of purity and determination of molecular weight 132-133
3.10.9 Determination of active alkaline protease/ fibrinolytic / keratinase activity using zymographic study 133
3.10.9.1 Casein zymographic study 133-134
3.10.9.2 Fibrin zymographic study 134
3.10.9.3 Keratin zymographic study 134-125
3.10.10 Estimation of protein content 125

3.11.1 Assay of proteolytic activity 135-136

3.12 Biochemical characterization of alkaline protease 136
3.12.1 Dose-dependent protease activity 136
3.12.2 Effect of pH on protease activity and stability 136
3.12.3 Determination of optimum temperature for protease activity 136
3.12.4 Heat-inactivation study 137
3.12.5 Influence of metal ions on protease activity 137
3.12.6 Chemical modification of protease activity 137
3.12.7 Effect of surfactants, chelators, denaturing, oxidizing and bleaching agents 137
3.12.8 Organic-solvent stability 137-138
3.12.9 Substrate specificity 138
3.12.10 Enzyme kinetics 138
3.13 Immobilization of purified alkaline keratinase onto iron-oxide magnetic nanoparticle for improvement of catalytic activity and biotechnological potential of enzyme 138
3.13.1 Preparation of iron-oxide magnetic nanoparticle (MNPs) 138
3.13.2 Optimization of enzyme coupling to magnetic nanoparticle 139
3.13.3 Preparation of aminofunctionalized MNP 139
3.13.4 Covalent immobilization of keratinase from *B.subtilis* RM-01 on Fe_3O_4 MNP 139
3.13.5 Attachment of purified enzyme onto MNP by carbodimide/cyanimide activation 139-140
3.13.6 Binding of MNP binding to purified keratinase enzyme: dose-dependent study 140
3.13.7 Statistical procedure for optimizing conditions for maximal coupling of keratinase on to iron-oxide MNP 140-141
3.14 Characterization of the enzyme-coupled iron-oxide magnetic nanoparticle 141
3.14.1 XRD analysis for determination of nanocrystalllite size 141
3.14.2 FTIR analysis 142
3.14.3 SEM analysis for determination of particle size 142

3.14.4 Comparison of stability storage of free and MNP-bound β-keratinase 142
3.15 Industrial application of purified / partially purified alkaline proteases / keratinase 142
3.15.1 Clot lysis (thrombolytic) activity 142
3.15.2 Detergent compatibility test 142
3.15.3 Wash performance test 143
3.15.4 Pharmacological characterization of proteases 143
3.15.4.1 Interference in blood coagulation 143
3.15.4.2 Assay of haemolytic activity 144
3.15.4.3 In-vitro tissue damaging activity 144
3.15.5 Dehairing activity 144-145
3.15.6 Antibacterial property of purified alkaline protease 145
3.15.7 Feather degradation using MNP-Bound β-keratinase 145-146
3.15.8 Release of free-amino acids from the chicken-feather 146
3.15.9 Analysis of liberated free fatty acids from chicken-feather by β-keratinase 146-147
CHAPTER IV :
SCREENING OF MICRO-ORGANISMS FROM DIFFERENT HABITATS OF NORTH-EAST INDIA (WITH SPECIAL EMPHASIS ON EXTREME HABITATS) FOR THE PRODUCTION OF PROTEASE WITH PARTICULAR REFERENCE TO THE ALKALINE, THERMOPHILIC ENZYMES
4.1 Results 148
CHAPTER V:
PROCESS OPTIMIZATION, ISOLATION, PURIFICATION, BIOCHEMICAL CHARACTERIZATION AND INDUSTRIAL APPLICATIONS OF ALKALINE PROTEASES ISOLATED FROM *Bacillus subtilis* DM-04
5.1 Results 230
CHAPTER VI:
PROCESS OPTIMIZATION, ISOLATION, PURIFICATION, BIOCHEMICAL CHARACTERIZATION AND INDUSTRIAL APPLICATIONS OF ALKALINE PROTEASE ISOLATED FROM

Paenibacillus tezpurensis sp.nov. strain AS-S24-II
6.1 Results 293
CHAPTER VII:
PROCESS OPTIMIZATION, ISOLATION, PURIFICATION, BIOCHEMICAL CHARACTERIZATION AND INDUSTRIAL APPLICATIONS OF AN ALKALINE Β-KERATINASE (PROTEASE) ISOLATED FROM *Bacillus subtilis* RM-01
7.1 Results 337
CHAPTER VIII:
PROCESS OPTIMIZATION, PARTIAL PURIFICATION, BIOCHEMICAL CHARACTERIZATION AND INDUSTRIAL APPLICATION OF AN ALKALINE PROTEASE ISOLATED FROM *Bacillus* sp. AS-S20-I
8.1 Results
CHAPTER IX: 401
PROCESS OPTIMIZATION, PURIFICATION, BIOCHEMICAL CHARACTERIZATION, AND INDUSTRIAL APPLICATION OF AN ALKALINE PROTEASE ISOLATED FROM *Bacillus licheniformis* AS-S24-I
9.1 Results 446
CHAPTER X:
PROCESS OPTIMIZATION, PURIFICATION, BIOCHEMICAL CHARACTERIZATION, AND INDUSTRIAL APPLICATION OF AN ALKALINE PROTEASE ISOLATED FROM *Brevibacillus* sp. strain AS-S10-II
10.1 Results 492
CHAPTER XI:
DISCUSSION 537
CONCLUSION 636
REFERENCES 638
Appendix
LIST OF PUBLICATIONS

List of Tables

Table No	Titles	Page No
	Chapter 1 : Introduction	
1.1	Bioindustrial companies by Revenue (2009-2010)	7
1.2	Potential enzyme distributors/suppliers in India	8-9
1.3	Specificity of proteases	13
1.4	Classification of proteases. Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and the triangles signify the blocked termini. Arrows show the sites of action of the enzyme	14-15
1.5	Statistical methods used to improve the protease production from microorganisms	34-35
1.6	Combination of chromatographic techniques applied for purification of microbial alkaline proteases	39-40
1.7	Comparison of Different Immobilization Methods	45
1.8	Commercial application of alkaline protease in various biotech industries	47-48
	Chapter 2: Review of literature	
2.1	Screening results of isolated alkaline proteases from different environmental samples reported from Abroad	59-63
2.2	Protease producing microbes reported from India	63-65
2.3	Advantages and disadvantages of the SSF system	67-68
2.4	Use of solid substrates as support for protease production in SSF systems	68-69
2.5	Alkaline protease production under solid-state fermentation system	71-74
2.6	Alkaline keratinase production under solid-state fermentation system	74
2.7	Statistical optimization of alkaline protease production	77-80
2.8	Statistical optimization of alkaline keratinase production	81

Table No	Titles	Page No
2.9	Properties of few selected proteases from different micro-organisms	84-86
2.10	Properties of few selected keratinase from different microorganims	88-89
2.11	Industrial application of different class of protease in various biotech sectors	93-100
2.12	Industrial application of alkaline keratinase	103
	Chapter 3 : Materials and Methods	
3.1	Optimal PCR reaction conditions for amplification of conserved region of 16S-rRNA gene of selected protease secreting bacterial strains	117
3.2	Taxonomic Description of the plant *Imperata cylindrica*	121-122
3.3	Independent variables for alkaline protease / keratinase production with respect to each strain under SSF and SmF systems using central composite design	126
3.4	Validation of response surface for alkaline protease/keratinase production	128
	Chapter 4 :Results	
4.1	Collection of soil samples from various regions of North-East India	149
4.2	Screening for alkaline protease producing alkalophilic bacteria from different samples of North- East India. The medium used was skim-milk agar, pH 8.0	150-152
4.3	Selection of alkaline protease producing 12 bacteria having zone of hydrolysis ≥ 25.0 mm (see Table 4.2)	153
4.4	Selection of alkaline protease producing potential bacteria on the basis of alkaline protease production in culture medium. Results represent mean ± S.D of three individual experiments.	154
4.5	Detergent compatibility study of crude protease pre-incubated at 45°C. Enzyme activity in absence of	155

Table No	Titles	Page No

	detergent was considered as 100% activity and other values were compared with that. Results represent mean ± S.D of three individual experiments.	
4.6	Thermo stability study of protease from selected bacterial isolates. The crude protease (2 mg/ml) was heated at 60°C for 15 to 60 mins followed by assay of protease activity. Results Represent mean ± S.D of three individual experiments.	156
4.7	Biochemical and morphological tests of strain AS-S10 II. Experiments were repeated thrice to assure the reproducibility.	158
4.8	Biochemical and morphological tests strain AS-S18. Experiments were repeated thrice to assure the reproducibility.	159
4.9	Biochemical and morphological tests of strain AS-S20-I. Experiments were repeated thrice to assure the reproducibility.	160
4.10	Biochemical and morphological tests of strain AS-S24-I. Experiments were repeated thrice to assure the reproducibility.	161
4.11	Biochemical and morphological tests AS-S24-II. Experiments were repeated thrice to assure the reproducibility.	162
4.12	Composition of cellular fatty acids content of selected bacterial strains	165
4.13	A similarity index of FAME analysis of protease producing selected bacteria	166
4.14	Partial DNA sequence of conserved region of 16S-rRNA gene of all bacterial isolates selected in present study	167-169
4.15	Homologous search results of 16S-rRNA gene partial sequence of strain AS-S10-II using Basic Local	171-175

Table No	Titles	Page No
	Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI). The 16S-rDNA sequences from microbes showing upto 99% identity are shown	
4.16	A Homologous search results of 16S-rRNA gene partial sequence of AS-S20-I using Basic Local Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI)	179-191
4.17	Homologous search results of 16S-rRNA partial sequence gene of strain AS-S24-I using BLAST from NCBI database	195-206
4.18	Homologous search results of 16S-rRNA partial gene of sequence of AS-S24-II using BLAST tool from NCBI	210-221
4.19	A taxonomic classification of alkaline protease producing potential bacteria	228
4.20	Taxonomic identification and nomenclature of selected bacterial strains	229
	Chapter 5 :Results	
5.1	Influence of different combinations of co-substrates on alkaline protease production by *B. subtilis* strain DM-04 under solid-state fermentation. Values are mean± S.D of three determinations	240
5.2	Plackett–Burman store design showing six variables with coded values along with the observed results for protease production by *B.subtilis* strain DM-04 in SmF. Values (protease yield) are mean of triplicate determinations	247-248
5.3	Statistical analysis of Plackett–Burman design showing coefficient values, t- and p-value for each variable for protease production (p-value <0.05)	248-249

Table No	Titles	Page No
5.4	Observed responses and predicted values of alkaline protease produced by *B.subtilis* strain DM-04. Boundaries of the experimental domain and spacing of levels are expressed in coded (within parenthesis) and natural units. C_1, IC: PP level (% w/v); C_2, beef-extract level (% w/w); C_3, incubation time (h)	250-251
5.5	Analysis of Variance (ANOVA) of alkaline protease produced by *B.subtilis* strain DM-04	252
5.6	Model coefficients estimated by multiple linear regressions (significance of regression coefficients) *Bacillus subtilis* strain DM-04 in SmF under shake-flask study	252
5.7	Summary of purification of Bsubap-I from *Bacillus subtilis* strain DM-04. Values are from a typical experiment	261
5.8	Biochemical characterization of Bsubap-I purified protease	261
5.9	Effect of surfactants, chelators, inhibitors, denaturing, oxidizing and bleaching agents on enzymatic activity of Bsubap-I . Values are mean ± S. D. of three determinations	266
5.10	Pharmacological properties of Bsubap-I from *B.subtilis* strain DM-04. Values are mean± S.D. of three experiments	268
5.11	Summary of purification of Alzwiprase from *Bacillus subtilis* strain DM-04. Values are from a typical experiments	274
5.12	Biochemical properties and substrate specificity of Alzwiprase from *Bacillus subtilis* strain DM-04. Substrate specificity values are mean ± S.D of three experiments	279

Table No	Titles	Page No
5.13	Effect of surfactants, inhibitors, EDTA, denaturing agents, oxidizing and bleaching agents on protease activity of Alzwiprase from *B. subtilis* strain DM-04. Assay was carried out at pH 10.0 and 45°C. Values are mean ± S.D. of three determinations	281-282
5.14	Pharmacological properties of Alzwiprase from *B.subtilis strain DM-04. Values are mean ± S. D. of three* determinations	287
	Chapter 6 : Results	
6.1	Influence of different combinations of co-substrates on alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II under solid-state fermentation. Values are mean± S.D of three determinations	302
6.2	Plackett–Burman store design showing six variables with coded values along with the observed results for alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF	309-310
6.3	Statistical analysis of Plackett–Burman design showing coefficient, t- and p-values for each variable (p-value <0.05)	310
6.4	Observed and predicted values of alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II post 48 h of incubation at 45°C and 200 rpm. The observed values are mean ± S.D of triplicate determinations. Boundaries of the experimental domain and spacing of levels are expressed in coded (within parenthesis) and natural units C_1, pH of the medium; C_2, casein level (% w/v); C_3, ammonium sulphate (%w/v)	312-313

Table No	Titles	Page No
6.5	Analysis of Variance (ANOVA) of alkaline protease produced by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II	313
6.6	Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF under shake-flask study	313-314
6.7	Summary of purification of a detergent stable alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Data represent a typical experiments	319-320
6.8	Biochemical properties and substrate specificity of alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Values are mean ± S.D. of triplicate determinations	324
6.9	Effect of different inhibitors, surfactant, denaturing agents, oxidizing and bleaching agents and metal chelators on purified protease. Values are mean ± S.D. of triplicate determinations	329-330
6.10	Pharmacological properties of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Values are mean ± S. D. of three determinations	331-332
	Chapter 7 : Results	
7.1	Plackett–Burman store design showing six variables with coded values along with the observed results for keratinase production by *B.subtilis* strain RM-01.	352
7.2	Statistical analysis of Plackett–Burman design showing coefficient values, t- and P-value for each variable for keratinase activity (p-value <0.05),	353
7.3	Observed and predicted values of alkaline β-keratinase	354-355

Table No	Titles	Page No
	incubation at 50°C. The observed values are average ± S.D of triplicate determination. Boundaries of the experimental domain and spacing of levels are expressed in coded (within parenthesis) and natural units. C_1, pH of the medium; C_2, maltose level (% w/v); C_3, sodium nitrate (% w/v).	
7.4	Model coefficients estimated by multiple regression (model adequacy determination)	356
7.5	Analysis of Variance (ANOVA) for alkaline β-keratinase produced by *B.subtilis* strain RM-01	356-357
7.6	Summary of purification of β-keratinase from *B.subtilis* strain RM-01. Results represent a typical experiment.	365
7.7	Effect of denaturing agent, oxidizing and bleaching agent, inhibitors, chelators, and surfactants on purified β-keratinase from *Bacillus subtilis* strain RM-01 at pH 8.0 and 45°C temperature. Values are mean± S.D. of three experiments.	372
7.8	Pharmacological properties of purified β-keratinase from *Bacillus subtilis* strain RM-01. Values are mean ± S. D. of three determinations.	375-376
7.9	A comparison of binding efficiency (under non-optimized condition) of β-keratinase onto iron-oxide MNP and increase in specific activity of the enzyme post binding to MNP by two different methods. Values are mean ± S.D. of three different experiments	376-377
7.10	Observed and predicted values of Y response by RSM. The observed values are mean of triplicate determinations. Data within bracket indicates the coded value.	377-378

Table No	Titles	Page No
7.11	Model co-efficients estimation by multiple regression analysis (model adequancy checking)	378
	Chapter 8 : Results	
8.1	Influence of different combinations of co-substrates on alkaline protease production by *Bacillus* sp. AS-S20-I in solid-state fermentation. Values are mean ± S.D. of three experiments	412
8.2	Plackett–Burman design showing six variables with coded values along with the observed results for protease production by *Bacillus* sp. strain AS-S20-I	419-420
8.3	Statistical analysis of Plackett–Burman design showing coefficient values, t- and p-value for each variable for protease activity (p-value <0.05)	420
8.4	Observed responses and predicted values of alkaline protease production by *Bacillus* sp. strains AS-S20-I in SmF. Values are mean ± S.D. of three experiments	422
8.5	Analysis of Variance (ANOVA) of alkaline protease produced by *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments	423
8.6	Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for alkaline protease production by *Bacillus* sp. strain AS-S20-I in SmF under shake-flask study. Values are mean ± S.D. of three experiments	423
8.7	Substrate specificity of partially purified alkaline protease from *Bacillus* sp.strain AS-S20-I. Values are mean ± S.D. of three experiments	432
8.8	Effect of chemical/chelators/ inhibitors/ denaturing/ oxidizing/ bleaching agents on catalytic activity of partially purified protease from *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D of triplicate	436-437

Table No	Titles	Page No
	determinations	
8.9	Pharmacological properties of partially purified protease from *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments	440-441
	Chapter 9 : Results	
9.1	Influence of different combinations of co-substrates on alkaline protease production by *B. licheniformis* strain AS-S24-I under solid-state fermentation Values are mean ± S.D. of three experiments.	457
9.2	Plackett–Burman store design showing six variables with coded values along with the observed results for protease (β-keratinase) production by *B.licheniformis* strain AS-S24-I.	464-465
9.3	Statistical analysis of Plackett–Burman design showing coefficient values, t- and P-value for each variable for protease (keratinase) activity (p-value <0.05).	465
9.4	Observed responses and predicted values of β-keratinase activity of culture supernatants from *B.licheniformis* strain AS-S24-I. The observed values are average of triplicate determinations	467
9.5	Analysis of variance (ANOVA) of alkaline β-keratinase activity produced by *B.licheniformis* strain AS-S24-I strain. Values are mean ± S.D. of three experiments.	468
9.6	Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for protease (β-keratinase) production by *B.licheniformis* strain AS-S24-I in SmF under shake-flask study (p<0.05).	469
9.7	Summary of purification of Alkarnase (an alkaline β-keratinase) from *B.licheniformis* strain AS-S24-I. Data represent a typical experiment.	477

Table No	Titles	Page No
9.8	Substrate specificity of Alkarnase from *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments.	479
9.9	Effect of protease inhibitors, chelator, surfactants, urea, oxidizing and bleaching agents on catalytic activity of Alkarnase from *B.licheniformis* AS-S24-I strain. *Experiment was done as described in the text.* Values represent mean ± S.D of three determinations.	482-483
9.10	Pharmacological properties of Alkarnase from *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments.	487-488
	Chapter 10 : Results	
10.1	Influence of different combinations of co-substrates on alkaline protease production by *Brevibacillus* sp.strain AS-S10-II under solid-state fermentation. Values are mean ± S.D of triplicate determinations.	503
10.2	Plackett–Burman store design showing six variables with coded values along with the observed results for alkaline protease (β-keratinase) production by *Brevibacillus* sp. AS-S10-II.	510-511
10.3	Statistical analysis of Plackett–Burman design showing coefficient values, t- and P-value for each variable for protease (β-keratinase) activity (p-value <0.05).	511
10.4	Observed responses and predicted values of alkaline β-keratinase production by *Brevibacillus sp.* strain AS-S10-II. The observed values are average of triplicate determinations	512-513
10.5	Analysis of Variance (ANOVA) of alkaline protease produced by *Brevibacillus* sp. AS-S10-II strain. Values are mean ± S.D. of three experiments.	513-514

Table No	Titles	Page No
10.6	Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for alkaline protease (β-keratinase) production by *Brevibacillus* sp. AS-S10-II in SmF under shake-flask study.($p<0.05$)	514-515
10.7	A summary of purification of an Brevicarnase (alkaline β-keratinase) from *Brevibacillus sp.* strain AS-S10-II. Data represent a typical experiment.	523
10.8	Biochemical characterization of Brevicarnase from *Brevibacillus* sp. strain AS-S10-II. Values are mean± S.D. of three experiments.	527-528
10.9	Effect of chemical inhibitors, chelator, metal ions, surfactants and urea on Brevicarnase. Values represent mean ± S.D of three determinations.	530-531
10.10	Pharmacological properties of Brevicarnase. Values are mean ± S.D. of three experiments.	533
	Chapter 11: Discussion	
11.1	Distinctive phenotypic properties of isolate AS-S10-II and related species of *Bacillus* sp. ND: Not determined	543-545
11.2	Percentage of cellular fatty acid composition of isolate AS-S10-II and related species of *Bacillus subtilis.*	548
11.3	Some phenotypic properties of isolate AS-S20-I and related species of *Kocuria* sp. ND: Not determined.	551-554
11.4	A comparison of percentage cellular fatty acid composition of isolate AS-S20-I with related species of *Kocuria*	556
11.5	Distinctive phenotypic properties of isolate AS-S24-I and related species of *Peanibacillus*	560-561
11.6	Percentage cellular fatty acid composition of isolate AS-S24-I and related species of *Paenibacillus* species	563

Table No	Titles	Page No
11.7	Distinctive phenotypic properties of isolate AS-S24-II and related *Bacillus licheniformis.* NT, not tested.	565-568
11.8	Percent cellular fatty acid composition of isolate AS-S24-II-and related species of *Paenibacillus* as determined by GC analysis	570
11.9	Phenotypic properties of isolate AS-S18 with related genus of *Bacillus*	572-574
11.10	Percentage of cellular fatty acid composition of isolate AS-S18 with other related genus of *Bacillus*	576
11.11	A comparison of statistically optimized alkaline protease yield by isolated strains and other reported bacteria	590-591
11.12	A comparison of molecular weight of isolated proteases (Bsubap-I and Alzwiprase (*B.subtilis* DM-04), Brevicarnase (*Brevibacillus* sp. AS-S10-II), Alkarnase (*B.lichenformis* AS-S24-I) and detergent stable alkaline protease (*P.tezpuresnsis* sp.nov. strain AS-S24-II) with molecular weight of reported protease purified from *Bacillus* species	592-594
11.13	A comparison of molecular weight of alkaline β-keratinase (in the present study) with the reported molecular weight of the same enzyme purified from *B. subtilis* and other species of *Bacillus*	596
11.14	A comparison of requirement of optimum temperature and pH for purified / partially purified proteases in the present study with reported alkaline proteases isolated from *Bacillus* species	598-599
11.15	A comparison of requirement of optimum temperature and pH of purified keratinase in the present study with reported alkaline keratinase purified from *Bacillus* species	599-600
11.16	A comparative study of effect of different surfactants,	604-607

Table No	Titles	Page No
	oxidizing and bleaching agent's on purified alkaline proteases	
11.17	A comparison of influence of metal ions on purified / partially purified proteases in the present study with proteases purified by other workers	609-610
11.18	A comparison of kinetic properties of purified/ partially purified protease in the present study with the reported proteases purified from *Bacillus*	613
11.19	Comparison of fibrinolytic / casinolytic (F/C) ratio of *Bacillus* sp strain AS-S20-I with other reported strains	619
11.20	Comparison of K_m and V_{max} of partially purified protease *Bacillus* sp strain AS-S20-I with other reported strains	619-620
11.21	General composition of detergents for laundry and dishwashing	622-623
11.22	A comparison of detergent compatibility properties of purified / partially purified protease with detergent stability of previously reported proteases	626-628

List of Figures

Figure No	Figure legends	Page No
	Chapter No 1: Introduction	
1.1	Global enzyme markets by application sectors, through 2012 (In $ Millions), according to Business Communications Company (BCC 2009).	5
1.2	Consumption of industrial enzymes in India: Pharmaceuticals; Food and feed; Detergent manufacturing; Leather and paper and; Textile processing (Source: Biospectrum, June,2010. (http://biospectrumindia.ciol.com/content/BSTOP20/110061423.asp)	6
1.3	Distribution of enzyme sales. The contribution of different enzymes to the total sales of enzymes is indicated. The shaded portion indicates the total sales of proteases.	10
1.4	Three-dimensional structure of proteases: (a) The crystal structure of trypsin, a serine protease resolution(http://www.proteases.org/structure.php?start=1200)	20
1.5	Active sites of proteases. The catalytic site of proteases is indicated by * and the scissile bond is indicated by —S1; through Sn and S1' through Sn' are the specificity subsites on the enzyme, while P1 through Pn and P1' through Pn' are the residues on the substrate accommodated by the subsites on the enzyme	21
1.6	Catalytic mechanism of the serine proteases	24-26
1.7	Catalytic mechanism of aspartic proteases	28
1.8	Schematic illustration of catalytic mechanism of metalloprotease (thermolysin)	29
1.9	Catalytic mechanism of cysteine protease	31

Figure No	Figure legends	Page No
	Chapter 2: Review of literature	56-103
	No figures	
	Chapter 3 : Materials and Methods	104-147
	No figures	
	Chapter 4 :Results	
4.1	Storage stability of crude protease from selected bacteria at zero day (■) and post 30 days of storage at 4°C(□). Results represent mean ± S.D of three individual experiments.	157
4.2(a)	Phylogenetic relationships of strain AS-S10-II and *other closely related Brevibacillus species based on* 16S rDNA sequencing. The tree was generated using the neighbour-joining method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.	176
4.2(b)	Phylogenetic relationships of strain AS-S10-II and other closely related *Brevibacillus* species based on 16S rDNA sequencing. The tree was generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.	177
4.3(a)	Phylogenetic relationships of strain AS-S20-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the	192

Figure No	*Figure legends*	Page No
	neighbour-joining method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.	
4.3(b)	Phylogenetic relationships of strain AS-S20-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.	193
4.4(a)	Phylogenetic relationships of strain AS-S24-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the neighbour-joining method and the sequence from *Escherichia coli strain KesE6 (accession no* EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.	207
4.4(b)	Phylogenetic relationships of strain AS-S24-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data	208

Figure No	Figure legends	Page No
	set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.	
4.5(a)	Phylogenetic relationships of strain AS-S24-II and other closely related *Paenibacillus* species based on 16S rDNA sequencing. The tree was generated using the neighbour-joining method and the sequence from *Bacillus* sp. HSCC 1649 T (accession no. AB045097) was considered as out-group. The data set was resampled 1,000 times by using the bootstrap option, and percentage values are given at the nodes. Bar, 0.01 substitutions per site.	222
4.5(b)	Phylogenetic relationships of strain AS-S24-II and other closely related *Paenibacillus* species based on 16S rDNA sequencing. The tree was generated using the unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Bacillus* sp. HSCC 1649 T (accession no. AB045097) was considered as out-group. The data set was resampled 1,000 times by using the bootstrap option, and percentage values are given at the nodes. Bar, 0.01	223
4.6	PCR amplification of ISR of 16S-23S r RNA gene of strain AS-S10-IILane 1 molecular weight marker in kbp [(a) 1.0 kb and (b) 0.5 kbp)], Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-S10-II with primers (p1/p2) in Fig. (a) and in Fig. (b) nested PCR of 0.7kb fragment with primer p3/p4.	224
4.7	PCR amplification of ISR of 16S-23S r RNA gene of strain AS-S20-I: Lane 1 molecular weight marker in kbp ((a) 1.0 kb and (b) 0.5 kbp), Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-	225

Figure No	Figure legends	Page No
	S20-I with primers (p1/p2) in fig. (a) and in fig. (b) nested PCR of 0.7kb fragment with primer p3/p4.	
4.8	PCR amplification of ISR of 16S-23S r RNA gene of strain AS-S18: Lane 1 molecular weight marker in kbp ((a) 1.0 kb and (b) 0.5 kbp), Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-S18 with primers (p1/p2) in fig. (a) and in fig. (b) *nested PCR of 0.7kb fragment with primer p3/p4.*	226
4.9	PCR amplification of ISR of 16S-23S r RNA genes of strain AS-S24-I: Lane 1 molecular weight marker in kbp ((a) 1.0 kb and (b) 0.5 kbp), Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-S24-I with primers (p1/p2) in fig. (a) and in fig. (b) nested PCR of 0.7kb fragment with primer p3/p4.	227
	Chapter 5 :Results	230
5.1	Screening of different waste residues such as MOC (◆), WB (◘), RB (▲), IC(X), PP (☒), BL (○), and TL (■) for the production of alkaline protease by *B.subtilis* strain DM-04 strain at different time intervals. Values are mean ± S.D. of three experiments.	232
5.2	Time course of alkaline protease production by *Bacillus subtilis* strain DM-04. Legends show bacterial dry biomass (◆) and protein concentration of cell free extract (◘).Values are mean ± S.D. of three experiments.	233
5.3	Influence of initial moisture content of the substrates [PP (■) or IC (◆)] on alkaline protease production by *B.subtilis* strain DM-04. Values are mean ± S.D. of three determinations post 24h incubation at 50°C. Moistening agent was distilled water, pH adjusted to 8.0.	234

Figure No	Figure legends	Page No
5.4	Influence of moistening agents on alkaline protease production by *B.subtilis* strain DM-04 using either IC (■) or PP (▣) as a substrate. Values represent mean ± S.D of three experiments post 24h of incubation of *B.subtilis* strain DM-04 at 50°C.	235
5.5	Influence of inoculum size on alkaline protease production by *B.subtilis* strain DM-04 using PP (Δ) or IC (■) *as substrate under SSF. Values are mean ±* S.D. of three determinations.	236
5.6	Effect of supplementation of co-carbon sources (10 % w/w) to IC (substrate) on alkaline protease production by *B.subtilis* strain DM-04.Values are means ± S.D. of three determinations.	237
5.7	Effect of supplementation of co-nitrogen sources (1.0% w/w) to IC (99 % w/w) on alkaline protease production by *B.subtilis* DM-04.Values are means ± S.D. of three determinations.	238
5.8	Screening of best extraction medium for alkaline protease extraction from the fermented matter. Values are means ± S.D. of three determinations.	239
5.9	Effect of different carbon sources on alkaline protease production from *B.subtilis* strain DM-04 under SmF. Values are mean ± S.D of three determinations.	241
5.10	Effect of various nitrogen sources on alkaline protease production from *B.subtilis* strain DM-04 under SmF. Values are mean ± S.D of three determinations.	242
5.11	Effect of pH on alkaline protease production from *B.subtilis* strain DM-04. Values are mean ± S.D of three determinations.	243
5.12	Effect of incubation temperature on alkaline protease	244

Figure No	Figure legends	Page No
	production from *B.subtilis* strain DM-04. Values are mean ± S.D of three determinations.	
5.13	Effect of incubation time on alkaline protease production (◆), bacterial dry biomass (▣) and protein content (▲) of culture supernatant from *B.subtilis* strain DM-04 under submerged fermentation condition. The pH and temperature of the medium were adjusted to 8.0 and 45°C, respectively. Values are mean ± S.D of three determinations	245
5.14	Effect of agitation rate on alkaline protease *production from B.subtilis strain* DM-04 under submerged fermentation. Values are mean± S.D of three determinations.	246
5.15	Response surface plots for alkaline protease production by *B. subtilis* strain DM-04. The interaction between (a) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and concentration (%, w/v) of beef-extract level (C_2), hold value incubation time (C_3) = 0; (b) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and incubation time (C_3),; hold value beef extract level (C_2) =0; (c) concentration (%, w/v) of beef extract (C_2) and incubation time (C_3), hold value IC:PP level (C_1)= 0.	253
5.16	Countor surface plots for alkaline protease production by *B. subtilis* strain DM-04. The interaction between (a) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and concentration (%, w/v) of beef-extract level (C_2), hold value incubation time (C_3) = 0; (b) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and incubation time (C_3),; hold value beef extract level (C_2) =0; (c) concentration (%, w/v) of beef extract (C_2) and incubation time (C_3), hold value	254

Figure No	Figure legends	Page No
	IC:PP level (C_1)= 0.	
5.17	Fractionation of anionic proteases secreted from *B.subtilis* strain DM-04 by DEAE Sephadex A-50 column. Elution was performed stepwise with phosphate buffers of the following molarities and pH values: (a) 20 mM K-phosphate, pH 7.0); (b) 20 mM K-phosphate (pH 6.5); (c) 20 mM K-phosphate (pH *6.0); (d) 20 mM K-phosphate (pH 5.5); (e) 100 mM K-*phosphate (pH 5.0); (f) 120 mM K-phosphate (pH 5.0). Data represent a typical experiment.	257
5.18	Sephacryl S-200 gel filtration profile of DE-I fraction. Data represent a typical experiment.	258
5.19	12.5 % SDS-PAGE of crude protease and Bsubap-I from *B. subtilis* DM-04. Molecular weight markers are phosphorylase b (97,400 Da), BSA (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), soybean trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da); Lane 1: crude protease (50 µg); Lane 2: DEAE-Sephadex A-50 flow through fraction (50 µg); Lane 3: DE-01 fraction (50 µg); Lane 4: Bsubap-I *under reduced condition* (10 µg); Lane 5: Bsubap-I under non-reduced condition (10 µg), Lane 6: Casein zymograph of Bsubap-I (20 µg) post incubation at 45 °C for 4 h.	259
5.20	RP-HPLC of GF-II fraction. HP-I represents solvent peak.	260
5.21	Lineweaver-Burk plot to determine the K_m and V_{max} values for Bsubap-I using casein as a substrate. Values are mean of triplicate determinations.	262
5.22	Thermo *stability* study of Bsubap-*I* from *B.subtilis* strain DM-04. Values are mean ± S. D. of three determinations	263

Figure No	Figure legends	Page No
	Effect of polyols on Bsubap-I stability at 60ºC for 120 min. Values are mean ± S. D. of three determinations	
5.24	Effect of divalent metal ions on catalytic activity of Bsubap-I. Values are mean ± S. D. of three determinations	265
5.25	Organic solvent stability of Bsubap-I. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.	267
5.26	Detergent stability and compatibility of Bsubap-I (7.0 µg / ml) pre-incubated at 25 (■), 37(□), and 45°C (■). Enzyme activity in the absence of detergent was considered as 100% activity and other values were compared with that. The values represent mean ± S.D. of three determinations	270
5.27	Wash performance test of Bsubap-I at 37°C. The values represent mean ± S.D. of three determinations	271
5.28	Antibacterial activity of purified Bsubap-I (0.25mg /ml) from *B.subtilis* DM-04. Values represent mean ± S.D of three experiments	272
5.29	Dehairing activity of Bsubap-I (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with Bsubap-I (50 U / ml) for 12 h at 37°C.	273
5.30	Gel filtration profile of zwitterionic proteins from *B.subtilis* strain DM-04.	275
5.31	Reverse-phase HPLC of GF-I fraction. HP-S stand for solvent peak	276
5.32	12.5% SDS-polyacrylamide gel electrophoresis of	277

Figure No	Figure legends	Page No
	purified protease. Molecular weight markers were phosphorylase b (97,400 Da), BSA (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), soybean trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da); lane 1, crude protease (40 µg); lane 2, CM-Cellulose flow through (CMFT) (30 µg); lane 3, DEAE-Sephadex A-50 Flow through (DEFT) (30 µg); lane 4, ethanol precipitated fraction (8 µg); lane 5, HP-I fraction under reduced condition (15.0 µg); lane 6, HP-I fraction under non-reduced condition (15.0 µg).	
5.33	*Effect of incubation temperature on* Alzwiprase activity. Each value represents mean ± S.D. of three experiments	278
5.34	Effect of pH on Alzwiprase activity. Each value represents mean ± S.D. of three experiments.	278
5.35	Thermo-stability study of Alzwiprase *from B.subtilis* strain DM-04 at 60°C. Each value represents mean ± S.D. of three experiments	280
5.36	Effect of divalent ions on Alzwiprase from *B.subtilis strain DM-04. Each value represents mean* ± S.D. of three experiments.	283
5.37	Effect of polyols against heat-denaturation of catalytic activity of Alzwiprase. The activity of enzyme in absence of polyols was considered as 100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.	284
5.38	Lineweaver-Burk plot to determine the K_m and V_{max} values of Alzwiprase using casein as a substrate. Each value represents mean of three experiments.	285
5.39	Organic solvent stability of Alzwiprase. Enzyme activity in the absence of solvents was considered as	286

Figure No	Figure legends	Page No
	100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.	
5.40	Detergent stability and compatibility of Alzwiprase at pre-incubation temperatures of 25(□), 37 (▨) and 45 (■)°C for 60 min. Enzyme activity in the absence of detergent was considered as 100% activity and other values were compared with that. The values represent mean ± S.D. of three determinations.	288
5.41	Wash performance study of Alzwiprase at 37°C.The values represents mean ± S.D. of three determinations	289
5.42	Antibacterial study of Alzwiprase (0.30 mg /ml) from *B.subtilis* strain DM-04 post 12h of incubation at 37°C. Values represent mean ± S.D of three experiments.	290
5.43	Dehairing activity of Alzwiprase (a) goat skin incubated in 100mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with Alzwiprase (50U / ml) for 12 h at 37°C.	291
	Chapter 6 : Results	293
6.1	Screening of different waste residues such as MOC (◇), WB (□), RB (▲), IC(X), PP(●), BL(●), and TL(X) for the production of alkaline protease by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II strain at different time intervals. Values are mean ± S.D. of three experiments.	294
6.2	Kinetics of alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Legends show bacterial dry biomass (◇) and protein	295

Figure No	Figure legends	Page No
	concentration of cell free extract (□).Values are mean ± S.D. of three experiments.	
6.3	Influence of initial moisture content of the substrates [PP (■) or IC (♦)] on protease production. Values are mean ± S.D. of triplicate determinations post 24h incubation at 45°C. Moistening agent was distilled water, adjusted to pH 8.0 with 0.01N NaOH.	296
6.4	Influence of moistening agents on alkaline protease production by *Paenibacillus tezpurensis* sp.nov.strain AS-S24-II in SSF using either IC (■) or PP (□) as a solid substrate. Values represent mean ± S.D of three experiments post 24h at 45°C.	297
6.5	Influence of inoculum size on protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II using PP (◆) or IC (□) under SSF. Values are mean ± S.D. of three determinations	298
6.6	Effect of supplementation of co-carbon sources (10 % w/w) to IC (99 % w/w) on alkaline protease production by *Paenibacillus tezpurensis* sp.nov. *strain AS-S24-II. Values are means ± S.D. of three* determinations.	299
6.7	Effect of supplementation of co-nitrogen sources (1.0% w/w) to IC (99.0%) (substrate) on protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Values are means ± S.D. of three determinations	300
6.8	Screening of extraction mediums for maximum protease extraction from fermented substrate (IC). Values are means ± S.D. of three determinations.	301
6.9	Effect of different carbon sources on alkaline protease production from *Paenibacillus tezpurensis*	303

Figure No	Figure legends	Page No
	sp.nov. strain AS-S24-II under SmF. Values are means ± S.D. of three determinations.	
6.10	Effect of various nitrogen sources on alkaline protease production from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. Values are means ± S.D. of three determinations	304
6.11	Effect of pH on alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. Values are means ± S.D. of three determinations.	305
6.12	Effect of incubation temperature on alkaline protease production from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SSF. The pH of the medium was adjusted to 8.0, respectively. Values are means ± S.D. of three determinations.	306
6.13	Effect of incubation time on alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. Legends show alkaline protease production (◆) bacterial dry biomass (■) and protein content (▲). Values are means ± S.D. of three determinations.	307
6.14	Effect of agitation rate on alkaline protease production from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II under SmF. Values are means ± S.D. of three determinations.	308
6.15	Response surface plots for alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SSF. The interaction between (a) pH of the medium (C_1) and concentration (%, w/v) of casein level (C_2), hold value C_3= 0 (b) pH of the medium (C_1) vs concentration (%, w/v) of ammonium	315

Figure No	Figure legends	Page No
	sulphate level (C_3), hold value C_2= 0 and (c) concentration (%, w/v) of casein (C_2) vs concentration of ammonium sulphate level (C_3) (% w/v) hold value C_1 = 0	
6.16	2D Countor surface plots for alkaline protease production by *P.tezpurensis* sp.nov. strain AS-S24-II in SSF. The interaction between (a) pH of the medium (C_1) and concentration (%, w/v) of casein level (C_2), hold value C_3= 0 (b) pH of the medium (C_1) vs concentration (%, w/v) of ammonium sulphate level (C_3), hold value C_2= 0 and (c) concentration (%, w/v) of casein (C_2) vs concentration of ammonium sulphate level (C_3) (% w/v) hold value C_1 = 0	316
6.17	Gel filtration of 80% acetone fraction of alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II on Sephacyrl S-200 column. Data represent a typical experiment	321
6.18	RP-HPLC of GF-II protease fraction of alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Initial peak with a retention time of 2.0 min represents the solvent peak.	322
6.19	SDS-polyacrylamide gel (15%) electrophoresis of purified protease (HP-III). Lane 1, Protein molecular weight standards: ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (20.1 kDa); lane 2, reduced crude protease (30 µg); lane 3, 60% acetone precipitated fraction (30 µg), lane 4, 80% acetone precipitated fraction (30 µg), lane 5, reduced alkaline protease (8 µg), lane 6, non-reduced alkaline protease (8 µg)	323
6.20	Thermo-stability study of detergent-stable protease from *Paenibacillus tezpurensis* sp.nov. strain AS-	325

Figure No	Figure legends	Page No
	S24-II in presence of $CaCl_2$ (◆) and in absence of $CaCl_2$ (▨). Values are mean ± S.D. of triplicate determinations	
6.21	Lineweaver-Burk plot to determine the K_m and V_{max} values of detergent stable alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. The substrate used was casein, at pH 9.5 and incubation temperature was 45°C.	326
6.22	Effect of polyols on thermostability of protease under heated condition from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. The enzyme without polyols served as control (100% residual activity). Values are mean ± S.D. of three experiments.	327
6.23	Effect of metal ions on protease activity of detergent stable alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II Values are mean ± S.D. of three experiments.	328
6.24	Organic solvent stability of alkaline protease from *Paenibacillus tezpurensis* sp.nov.strain AS-S24-II. Enzyme activity in the absence of solvent was considered as 100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.	330
6.25	Detergent compatibility pattern of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II at the tested temperature ranges 25 (□), 37 (■) and 45°C (▨). Values are mean ± SD of triplicate determinations.	333
6.26	Dose-dependent and wash performance study of purified protease in presence of sunlight® detergent(7.0 mg /ml)	334

Figure No	Figure legends	
6.27	Wash performance study of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II at 45°C. Values are mean ± SD of triplicate determinations.	[illegible]
6.28	Dehairing activity of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II (50 U / ml) for 12 h at 37°C.	336
	Chapter 7 : Results	
7.1	Alkaline keratinase production from *Bacillus subtilis* strain RM-01 under solid state fermentation. Values are mean ± S.D. of three experiments.	339
7.2	Influence of initial moisture content on alkaline keratinase production from *B.subtilis* strain RM01. Values are mean ± S.D. of three determinations post *96 incubation at 50°C temperature. Moistening agent* was distilled water, pH adjusted to 8.0.	340
7.3	Influence of moistening agent on alkaline β-keratinase production by *B.subtilis* strain RM-01 using chicken feather as a substrate. Values represent mean ± S.D. of three experiments post 96 h of incubation at 50°C.	341
7.4	Influence of inoculum size on alkaline β-keratinase production by *B.subtilis* strain RM-01 on raw chicken-feather under SSF. Values are mean ± S.D. of three experiments.	342
7.5	Effect of supplementation of co-carbon sources (10%, w/w) to chicken feather (90%, w/w) on β-keratinase production by *B. subtilis* strain RM-01.	343

Figure No	Figure legends	Page No
	Values are *mean* ± S.D. of three experiments	
7.6	Effect of supplementation of co-nitrogen sources (1.0%, w/w) to chicken feather (substrate) for β-keratinase production by *B. subtilis* strain RM-01. Values are mean ± S.D. of three experiments	344
7.7	Screening of best extraction medium for maximum alkaline β-keratinase extraction from fermented material. Values are mean ± S.D. of three experiments.	345
7.8	*Effect of different carbon sources on alkaline* β-keratinase production from *B.subtilis* strain RM-01 under SSF. Values are mean ± S.D. of three experiments.	346
7.9	Effect of various nitrogen sources on alkaline β-keratinase production from *B.subtilis* strain RM-01 under SSF. Values are mean ± S.D. of three experiments.	347
7.10	Effect of pH on alkaline β-keratinase production from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.	348
7.11	Effect of incubation temperature on alkaline β-keratinase production from *B.subtilis* strain RM-01 in SmF. Values are *mean* ± S.D. of three experiments.	349
7.12	Effect of incubation time on alkaline β-keratinase production in SmF (▪), bacterial dry biomass (●), and protein content (▫) from *B.subtilis* strain RM-01 under SSF. Values are mean ± S.D. of three experiments.	350
7.13	Effect of agitation rate on alkaline β-keratinase production from *B.subtilis* strain RM-01 under SmF. Values are mean ± S.D. of three experiments.	351
7.14	Response surface plots for alkaline β-keratinase production by *B.subtilis* strain RM-01. The interaction	358

Figure No	Figure legends	Page No

Figure No	Figure legends	Page No
	between (a) pH of the medium and concentration (%, w/v) of maltose, hold value C_3=0 (b) pH of the medium vs concentration (%, w/v) of sodium nitrate, hold value C_2=0 and (c) concentration (%, w/v) of maltose vs concentration of sodium nitrate (% w/v) hold value C_1=0. Values are mean ± S.D. of three experiments.	
7.15	Countor plots for alkaline β-keratinase production by *B.subtilis* strain RM-01. The interaction between (a) pH of the medium and concentration (%, w/v) of maltose, hold value C_3=0 (b) pH of the medium vs concentration (%, w/v) of sodium nitrate, hold value C_2=0 and (c) concentration (%, w/v) of maltose vs concentration of sodium nitrate (% w/v) hold value C_1=0. Values are mean ± S.D. of three experiments	359
7.16	Batch fermentation for alkaline β-keratinase production from *B.subtilis* strain RM-01 under statistically optimized conditions in SSF. Values are mean ± S.D. of three experiments.	361
7.17	CM-Cellulose profile of cationic keratinase secreted from *B.subtilis* strain RM-01. Buffers used for step elution were : (a) 100 mM K-phosphate buffer, pH 7.5 ; (b) 100 mM K-phosphate buffer, pH8.0 ; (c) 150 mM K-phosphate buffer,pH8.0 ; (c) 200 mM K-phosphate buffer, pH 8.0; (d) 200 mM K-phosphate buffer, pH 8.0 ; (e) 300 mM K-phosphate buffer, pH 8.0 ; (f) 300 mM K-phosphate buffer, pH 8.5. Data represent a typical experiment.	362
7.18	Gel-filtration profile of CM-1 (non-retained by CM-cellulose) fraction from *B.subtilis* RM-01 bacterial strain on Sephacryl S-200 column. Data represent a	363

Figure No	Figure legends	Page No

	typical experiment.	
7.19	RP-HPLC profile of fraction GF-III. The HPLC experiment condition is described in the text. Data represent a typical experiment.	364
7.20	SDS-polyacrylamide gel (12.5%) electrophoresis of purified β-keratinase. Lane 1, Protein molecular weight standards: phosphorylase b (97.4 kDa), BSA *(66.0 kDa), ovalbumin (43 kDa), carbonic anhydrase* (29 kDa), and soybean trypsin inhibitor (20.1 kDa); lane 2 reduced crude β-keratinase (30 μg); lane 3 non-reduced crude β-keratinase (30 μg); lane 4 reduced β-keratinase (15 μg); lane 5 non-reduced β-keratinase (15 μg).	365
7.21	pH optimization of alkaline β-keratinase from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.	366
7.22	Effect of incubation temperature on alkaline β-keratinase activity from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.	367
7.23	Substrate specificity study of purified alkaline β-*keratinase from B.subtilis strain RM-01. Values are* mean ± S.D. of three experiments.	368
7.24	Thermostability of alkaline β-keratinase from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments	369
7.25	Lineweaver-Burk plot to determine the K_m and V_{max} values of purified alkaline β-keratinase from *Bacillus subtilis* strain RM-01.	370
7.26	Effect of metal ions on alkaline β-keratinase activity from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments	371
7.27	Effect of polyols on alkaline β-keratinase stability at	373

Figure No	Figure legends	Page No
	60°C for 30 min. The enzyme activity without polyols served as control (100%). Values are mean ± S.D. of three experiments.	
7.28	Organic solvent stability of alkaline β-keratinase from *B.subtilis* strain RM-01. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean± S.D. of three experiments.	374
7.29	Legend shows response surface plot (a) and contour plot (b) of specific activity of iron-oxide MNP bound β-keratinase vs pH of buffer (Tris-HCl) system used. C_1, *amount of MNP (in coded value)*, C_2, *pH of Tris-*HCl (in coded value), and C_3 (specific activity in U /mg).	379
7.30	Scanning electron microscopic images of (a) bare bare Fe_3O_4 MNP, and (b) Fe_3O_4 MNP bound β-keratinase	382
7.31(a)	FTIR spectra of iron oxide magnetic nanoparticle	383
7.31(b)	FTIR spectra of MNP-coupled β-keratinase.	384
7.32	Magnetization curve of iron oxide magnetic *nanoparticle (a) Before (b) after enzyme* immobilization.	385
7.33	X-ray diffraction pattem of (A) bare Fe_3O_4 MNP (B) Fe_3O_4 MNP bound β-keratinase.	386
7.34	Effect of pH on free β-keratinase (◆) and MNP coupled β-keratinase (□).Values are mean ± S.D. of three experiments.	388
7.35	Thermostability study of free β-keratinase and MNP – coupled β-keratinase at 60°C for 120 min. Values are mean ± S.D. of three experiments	390
7.36	Storage stability study of free β-keratinase and MNP –coupled β-keratinase for 30 days at 4°C. Values are	391

Figure No	Figure legends	Page No
	mean ± S.D. of three experiments.	
7.37	Detergent stability and compatibility of alkaline β-keratinase from *B.subtilis* strain RM-01 at 25 (□), 37 (▨) and 45 (□)°C. Enzyme activity in absence of detergent was considered as 100% activity and other values were compared with that. Values represent mean ± S.D of three experiments.	392
7.38	Wash performance study of alkaline β-keratinase from *B.subtilis* strain RM-01 at 37°C. From left (a) control (tap water with blood stain cloth), (b) alkaline β-keratinase (0.5 mg / ml) with blood stain cloth, (c) unheated detergent with blood stain cloth, (d) heated detergent with blood stain cloth, (e) unheated detergent with purified alkaline β-keratinase (0.5 mg / ml) with blood stain cloth, (f) heated detergent with purified alkaline β-keratinase (0.5 mg / ml) with blood stain cloth. Values represent mean ± S.D of three experiments.	393
7.39	Dehairing activity of alkaline β-keratinase (a)goat skin incubated in 100 mM Tris- buffer, pH 8.0 for 6 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with alkaline β-keratinase (50U / ml) for 6 h at 37°C.	394
7.40	Kinetics of chicken-feather hydrolysis by free β-keratinase (◆) and MNP-coupled β-keratinase (■). Values represent mean ± S.D of three experiments	395
7.41	Scanning electron microscopic images of (a) native chicken-feather (X 270) (b) native chicken-feather barb (X 1000) (c) rachis of chicken-feather post 24 h treatment with Fe_3O_4 MNP bound β-keratinase (X 10,000) and (d) chicken-feather post 40 h treatment with Fe_3O_4MNP bound β-keratinase (X 500)	396

Figure No	Figure legends	Page No
7.42	Gas chromatographic profile of free β-keratinase treated chicken feather fermented products	398
7.43	Gas chromatographic profile of MNP coupled β-keratinase treated chicken feather fermented products.	399
7.44	Mass spectrum (MS) of volatile product released from hydrolyzed chicken feather post treated with MNP-coupled β-keratinase. Legends show ion chromatogram of each peak: (a) retention time *19.430; (b) retention time 25.936; (c) retention time* 28.363, 31.405, and 32.615 have similar products; (d) retention time 30.078.	400
	Chapter 8 : Results	401
8.1	Screening of different agro-industrial waste residues such as MOC (◆), WB (■), RB (▲), IC(X), PP(⊠), BL(●), and TL(■) for the production of alkaline protease by *Bacillus* sp. strain AS-S20-I at different time intervals. Values are mean ± S.D. of three experiments.	403
8.2	Kinetics of alkaline protease production by *Bacillus* sp. strain AS-S20-I in SSF. Legends show bacterial dry biomass (◆) and protein concentration (□).Values are mean ± S.D. of three experiments	404
8.3	Influence of initial moisture content of the substrates [PP (◆) or IC (□)] on protease production. Values are mean ± S.D. of three experiments post 24h incubation at 50°C. Moistening agent was distilled water, pH adjusted to 11.0.	405
8.4	Influence of moistening agent on protease production using either IC (□) or PP (□) *as a substrate.* Values are mean ± S.D. of three experiments post 24h of incubation of *Bacillus* sp. strain AS-S20-I at 50°C.	406

Figure No	Figure legends	Page No
8.5	Influence of inoculum size on protease production by *Bacillus* sp. strain AS-S20-I strain using PP (◇) or IC (■) as substrate under SSF. Values are mean ± S.D. of three experiments.	407
8.6	Effect of supplementation of co-carbon sources (10 % w/w) to IC (substrate) on protease production by *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments	409
8.7	*Effect of supplementation of co-nitrogen sources* (1.0% w/w) to IC (substrate) on protease production by *Bacillus* sp.strain AS-S20-I. Values are mean± S.D. of three experiments.	410
8.8	Screening of extraction medium for optimum protease recovery from fermented matter. Values are mean ± S.D. of three experiments.	411
8.9	Effect of different carbon sources on alkaline protease production by *Bacillus* sp.strain AS-S20-I *under submerged fermentation. Values are mean ±* S.D. of three experiments.	413
8.10	Effect of various nitrogen sources on alkaline protease production from *Bacillus* sp. strain AS-S20-I under SmF. Values are mean ± S.D. of three experiments.	414
8.11	Effect of medium pH on protease production by *Bacillus* sp. strain AS-S20-I. Values are mean± S.D. of three experiments.	415
8.12	Influence of incubation temperature on alkaline protease production by *Bacillus* sp.strain AS-S20-I in SSF. The pH of the medium was adjusted to 9.5. Values are mean ± S.D. of three experiments.	416
8.13	*Effect of incubation time on alkaline protease*	417

Figure No	Figure legends	Page No
	production by *Bacillus* sp. strain AS-S20-I in SmF. Values are mean ± S.D. of three experiments.	
8.14	Effect of agitation rate on alkaline protease production from *Bacillus* sp. strain AS-S20-I under SmF. Values are mean ± S.D. of three experiments.	418
8.15	Response surface plots for alkaline protease production by *Bacillus* sp. strain AS-S20-I. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0 (coded value) (b) pH of the medium vs concentration (%, w/v) of ammonium sulphate, hold value C_2 = 0(coded value), and (c) concentration (%, w/v) of casein vs concentration of ammonium sulphate (% w/v) hold value C_1 = 0(coded value). Values are mean ± S.D. of three experiments	425
8.16	Countor plots for alkaline protease production by *Bacillus* sp. strain AS-S20-I. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0 (coded value) (b) pH of the medium vs concentration (%, w/v) of ammonium sulphate, hold value C_2 = 0 (coded value) and (c) concentration (%, w/v) of casein vs concentration of ammonium sulphate (% w/v) hold value C_1 = 0 (coded value). Values are mean ± S.D. of three experiments.	427
8.17	12.5 % SDS-polyacrylamide gel electrophoresis of crude and acetone precipitated proteases. Molecular weight markers are phosphorylase b (97,400 Da), BSA (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da); lane 1, crude protease in reduced condition (40.0 µg); lane 2, crude protease in non-reduced condition (40.0 µg); lane 3, acetone	429

Figure No	Figure legends	Page No
	precipitate fraction under reduced condition (15.0 μg); lane 4, acetone precipitate fraction under non-reduced condition (15.0 μg).	
8.18	Influence of pH on alkaline protease activity from *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments.	430
8.19	Thermo-stability study of partially purified protease *from Bacillus sp. strain* AS-S20-I *heated at* 60°C *for* various time interval. Values are mean ± S.D. of three *experiments.*	431
8.20	Effect of polyols against heat-denaturation of partially purified alkaline protease from *Bacillus* sp. strain AS-S20-I. The protease was heated at 60°C for 120 min either in presence or absence of polyols. The enzyme activity without polyols was considered as control (100%) and values were compared with that. Values are mean ± S.D. of three experiments.	433
8.21	Lineweaver-Burk plot to determine the K_m and V_{max} values of partially purified protease using casein as a substrate. Values are mean ± S.D. of three experiments.	434
8.22	Effect of metal ion on partially purified protease from *Bacillus sp. strain* AS-S20-I. *Values are mean* ± S.D. of three experiments.	435
8.23	Organic-solvent stability of partially purified protease from *Bacillus* sp. strain AS-S20-I. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean ± S.D. of three experiments.	438
8.24	Degradation of fibrin by partially purified alkaline protease from *B.* sp. strain AS-S20-I. The fibrin *degradation products were separated by 12.5% SDS-*	439

Figure No	Figure legends	Page No
	PAGE. Lanes 1: control; Lane 2-7: fibrin degradation pattern by 5.0 µg of partially purified alkaline protease post 30, 60, 120, 240, 360, 480, and 720 min of incubation, respectively at 37°C.	
8.25	Degradation pattern of fibrinogen by partially purified alkaline protease from *B.* sp. strain AS-S20-I. The fibrinogen degradation products were separated by 12.5% SDS-PAGE. Lanes 1-7: fibrin degradation pattern by 5.0 µg of partially purified alkaline protease post 30, 60, 120, 240, 360, 480, and 720 min of incubation, respectively at 37°C; Lane 8: control.	439
8.26	Thrombolytic activity of partially purified protease from *Bacillus* sp. strain AS-S20-I. Values represent mean ± S.D of three experiments.	441
8.27	Detergent stability and compatibility of partially purified protease (6.0 µg / ml) from *Bacillus* sp. strain AS-S20-I at the tested temperature ranges 25 (□), 37 (■) and 45°C (▩). Values are mean ± SD of triplicate determinations.	443
8.28	Wash performance test of partially purified protease (6.0 µg /ml) from *Bacillus* sp. strain AS-S20-I at 37°C. The values represent mean ± S.D. of three determinations	444
8.29	*Dehairing activity of partially purified protease from Bacillus* sp. AS-S20-I (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with partially purified protease (50 U / ml) for 12 h at 37°C.	445
	Chapter 9 : Results	446

Figure No	Figure legends	Page No
9.1	Screening of different waste residues such as MOC (◇), WB (■), RB (▲), IC(○), PP(⊠), BL(○), and TL(+) for the production of alkaline protease by *B. licheniformis* strain AS-S24-I at different time intervals. Values are mean ± S.D. of three experiments.	448
9.2	Kinetics of alkaline protease production from *B. licheniformis* strain AS-S24-I. Legends show bacterial dry biomass (◇) and protein concentration (■).Values are mean ± S.D. of three experiments.	449
9.3	Influence of initial moisture content of the substrates [PP (▣) or IC (◆)] on protease production. Values are mean± S.D. of three experiments post 60h incubation at 45°C. Moistening agent was distilled water, adjusted to pH 10.0 with 0.01N NaOH.	450
9.4	Influence of moistening agents on alkaline protease production by *B.licheniformis* strain AS-S24-I in SSF using either IC (■) or PP (□) as a solid substrate. Values are mean ± S.D. of three experiments post 60h of incubation 45°C.	451
9.5	Influence of inoculum size on protease production by *B. licheniformis* strain AS-S24-I using PP (◆) or IC (▣) as substrate under SSF. Values are mean ± S.D. of three experiments.	452
9.6	Effect of supplemented co-carbon sources (10 % w/w) to IC (substrate) on alkaline protease production by *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments	454
9.7	Effect of supplemented co-nitrogen sources (1.0% w/w) to IC (substrate) for protease production from *B.*	455

Figure No	Figure legends	Page No
	licheniformis strain AS-S24-I. *Values are mean±* S.D. of three experiments	
9.8	Screening of best extraction mediums for alkaline protease extraction from the fermentation matter. Values are mean ± S.D. of three experiments	456
9.9	Effect of different carbon sources on alkaline protease production from *Bacillus licheniformis* strain AS-S24-I under SmF. Values are mean ± S.D. of three experiments	458
9.10	Effect of *various nitrogen* sources on alkaline protease production from *B. licheniformis* strain AS-S24-I in SmF. Values are mean ± S.D. of three experiments.	459
9.11	Effect of pH on alkaline protease production by *B. licheniformis* strain AS-S24-I in SmF. Values are mean ± S.D. of three experiments	460
9.12	Effect of incubation temperature on alkaline protease production by *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments.	461
9.13	Effect of incubation time on alkaline protease production (◆), bacterial dry biomass (□), and protein content (Δ) from *B. licheniformis* strain AS-S24-I under SmF condition. Values are mean ± S.D. of three experiments.	462
9.14	Effect of agitation rate on alkaline protease production from *B. licheniformis* strain AS-S24-I under SmF. Values are mean± S.D. of three experiments.	463
9.15	Response surface plots for alkaline protease production by *B.licheniformis* strain AS-S24-I. The interaction between (a) pH of the medium and	470

Figure No	Figure legends	Page No
	concentration (%, w/v) of glucose, hold value C_3 = 0 (b) pH of the medium vs concentration (%, w/v) of yeast extract, hold value C_2 = 0 and (c) concentration (%, w/v) of glucose vs concentration of yeast extract (% w/v), hold value C_1=0. Values are mean ± S.D. of three experiments.	
9.16	Countor plots for alkaline protease production by *B.licheniformis* strain AS-S24-I. The interaction between (a) pH of the medium and concentration (%, w/v) of glucose, hold value C_3 = 0 (b) pH of the medium vs concentration (%, w/v) of yeast extract, hold value C_2 = 0 and (c) concentration (%, w/v) of glucose vs concentration of yeast extract (% w/v), hold value C_1=0. Values are mean ± S.D. of three experiments	471
9.17	Sephacryl S-200 gel filtration profile of 80 % (v/v) acetone precipitated fraction. Data represent a typical experiment.	474
9.18	RP-HPLC profile of fraction GF-VI from *B.licheniformis* strain AS-S24-I.	475
9.19	SDS-PAGE (12.5%) of HP-III fraction (Alkarnase) from *B. licheniformis* strain AS-S24-I. Lane 1 –crude keratinase (50 µg); Lane 2- acetone precipitated keratinase (50 µg), Lane 3- reduced Alkarnase (10.0 µg); Lane 4- non-reduced Alkarnase (10.0 µg); Lane 5- Protein molecular weight marker (kDa) :phosphorylase b (97.4), BSA (66.0), ovalbumin (43.0), carbonic anhydrase (29.0).	476
9.20	Effect of pH on protease activity. Values are mean ± S.D. of three experiments	478
9.21	Effect of incubation temperature on protease activity of Alkarnase. Values are mean ± S.D. of three	478

Figure No	Figure legends	Page No
	experiments	
9.22	Lineweaver–Burk plot for determination of K_m and V_{max} values of Alkarnase. Values are mean ± S.D. of three experiments.	480
9.23	Effect of metal ions on activity of Alkarnase. Values are mean ± S.D. of three experiments	481
9.24	Thermo-stability study of Alkarnase from *Bacillus licheniformis* AS-S24-I heat at 60ºC for 120 min. Values are mean ± S.D. of three experiments.	484
9.25	Effect of polyols on thermostability of Alkarnase from *B.licheniformis* strain AS-S24-I post heated at 60ºC for 120 min. The enzyme without polyols served as control (100%). Values are mean ± S.D. of three experiments.	485
9.26	Organic solvent stability of Alkarnase from *B. licheniformis* strain AS-S24-I. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean ± S.D. of three experiments.	486
9.27	Detergent stability and compatibility of Alkarnase (7.0 µg / ml) from *B. licheniformis* strain AS-S24-I at the tested temperature ranges 25(■), 37 (□) and 45 °C (▩). Values are mean ± SD of triplicate determinations.	489
9.28	Wash performance test of Alkarnase from *B. licheniformis* strain AS-S24-I at 45°C. The values represent mean ± S.D. of three determinations.	490
9.29	Dehairing activity of purified alkaline protease (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with purified alkaline protease (50U / ml) for 12 h at 37°C.	491

Figure No	Figure legends	Page No
	Chapter 10 : Results	492
10.1	Screening of different agro-industrial waste residues such as MOC (◇), WB (□), RB (Δ), IC(X), PP (◆), BL (●), and TL (■) for the production of alkaline protease by *Brevibacillus* sp. strain AS-S10-II strain at different time intervals. Values are mean ± S.D. of three experiments.	494
10.2	Kinetics of alkaline protease production by *Brevibacillus* sp. strain AS-S10-II. Legends show bacterial dry biomass (▩) and protein concentration (◆).Values are mean± S.D. of three experiments	495
10.3	Influence of initial moisture content of the substrates [PP (■) or IC (◆)] on alkaline protease production. Values are mean ± S.D. of triplicate determinations post 48h incubation at 50°C. Moistening agent was distilled water, adjusted to pH 12.5 with 0.1N NaOH.	496
10.4	Influence of moistening agent on alkaline protease production using either IC (▣) or PP (□) as a substrate. Values represent mean ± S.D of three experiments post 48h of incubation of *Brevibacillus* sp. strain AS-S10-II at 50°C.	497
10.5	Influence of inoculum size on protease production by *Brevibacillus* sp. strain AS-S10-II using PP (◆) or IC (□) under SSF. Values are mean ± S.D. of triplicate determinations	498
10.6	Effect of supplementation of co-carbon sources (10 % w/w) to IC (substrate) on protease production by *Brevibacillus* sp. strain AS-S10-II.Values are means ± S.D. of three determinations	500
10.7	Effect of supplementation of co-nitrogen sources (1.0% w/w) to IC (substrate) on protease production by *Brevibacillus sp.* strain AS-S10-II.Values are	501

Figure No	Figure legends	Page No
	means ± S.D. of three determinations	
10.8	Screening of best extraction medium for extraction of alkaline protease from fermented matter. Values are means ± S.D. of three determinations.	502
10.9	Effect of different carbon sources on alkaline protease production by *Brevibacillus* sp. strain AS-S10-II under SmF. Values are mean ± S.D. of three experiments	504
10.10	Effect of various nitrogen sources on alkaline protease production from *Brevibacillus* sp. strain AS-S10-II under SmF. Values are mean± S.D. of three experiments.	505
10.11	Effect of medium pH on alkaline protease production by *Brevibacillus* sp. strain AS-S10-II in SSF. Values are mean ± S.D. of three experiments	506
10.12	Effect of incubation temperature on alkaline protease production from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.	507
10.13	Effect of incubation time on alkaline protease production (◆), bacterial dry biomass (▫), and protein content (▲) from *Brevibacillus* sp. strain AS-S10-II under SmF condition. Values are mean ± S.D. of three experiments.	508
10.14	Effect of agitation rate on alkaline protease production from *Brevibacillus* sp. strain AS-S10-II under SmF. Values are mean ± S.D. of three experiments.	508
10.15	Response surface plots for alkaline protease production by *Brevibacillus* sp. strain AS-S10-II. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0(b) pH of the medium vs concentration (%, w/v) of	516

Figure No	Figure legends	Page No
	potassium nitrate, hold value C_2 = 0 and (c) concentration (%, w/v) of casein vs concentration of potassium nitrate (% w/v) hold value C_1 = 0. Values are mean ± S.D. of three experiments	
10.16	Countor plots for alkaline protease production by *Brevibacillus* sp. strain AS-S10-II. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0 (b) pH of the medium vs concentration (%, w/v) of potassium nitrate, hold value C_2 = 0 and (c) concentration (%, w/v) of casein vs concentration of potassium nitrate (% w/v) hold value C_1 = 0. Values are mean ± S.D. of three experiments	517
10.17	Gel–filtration pattern of 80% acetone precipitate protease (β-keratinase) fraction from *Brevibacillus* sp. strain AS-S10-II on Sephacryl S-200 column.	520
10.18	SDS-PAGE (12.5%) of GF-I fraction from *Brevibacillus* sp. strain AS-S10-II. Lane 1 - protein molecular weight markers (kDa) :phosphorylase b (97.4), BSA (66.0), ovalbumin (43.0), carbonic anhydrase (29.0); Lane 2- crude protease under reduced condition (45.0µg); Lane3- crude protease non-reduced condition (45.0µg); Lane 4 – 80% acetone precipitate fraction (10.0µg); Lane 5- GF-I fraction under reduced condition (8.0 µg); Lane 6 – keratin zymographic study of GF-I fraction (8.0 µg).	521
10.19	RP-HPLC of GF-I fraction, HP-I represents solvent peak	522
10.20	Effect of incubation temperature on catalytic activity of Brevicarnase from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments	524

Figure No	Figure legends	Page No
10.21	Influence of pH on Brevicarnase activity from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.	525
10.22	Heat-inactivation study of Brevicarnase from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.	526
10.23	Effect of stabilizers on thermostability of Brevicarnase post heating at 60°C for 120 min. The enzyme with out stabilizers (polyols) served as control (100%). Values are mean ± S.D. of three experiments.	527
10.24	Lineweaver-Burk plot to determine the K_m and V_{max} values of Brevicarnase. Values are mean ± S.D. of three experiments.	528
10.25	Effect of metal ions on Brevicarnase activity . Values are mean ± S.D. of three experiments.	529
10.26	Organic - solvent stability of Brevicarnase. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean ± S.D. of three experiments.	532
10.27	*Detergent stability and compatibility of Brevicarnase* (6.0 µg / ml) from *Brevibacillus* sp. strain AS-S10-II at the tested temperature ranges 25 (■), 37 (□) and 45(□)°C. Values are mean ± SD of triplicate determinations.	534
10.28	Wash performance study using blood stain containing cotton fabrics with Brevicarnase (6.0 µg / ml) at 45°C. From left (a) control (tap water with blood stain cloth), *(b) Brevicarnase (6.0 µg / ml) with blood stain cloth,* (c) unheated detergent with blood stain cloth, (d) heated detergent with blood stain cloth, (e) unheated detergent with Brevicarnase (6.0 µg / ml) with blood	535

Figure No	Figure legends	Page No
	stain cloth, (f) heated detergent with Brevicamase (6.0 µg / ml) with blood stain cloth. Values represent mean ± S.D of three experiments	
10.29	Dehairing activity of Brevicamase (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with Brevicamase (50 U / ml) for 6 h at 37°C.	536
	Chapter 11: Discussion	537
11.1	Diagrammatic representation of techniques and markers used in modern polyphasic approach for resolving the bacterial hierarchy	541
11.2	Inflow and outflow diagram for leather processing. Temporarily preserved (salted) hides and skins are treated with variety of chemicals in a water medium, through a series of unit processes and operations, to produce leathers. This leads to a variety of solid (red text) and air (purple text) pollutants at various stages of processing	631

Acknowledgements

It gives me immense pleasure and joy to express my heartfelt gratitude to my supervisor, Prof. Ashis K. Mukherjee, Head and Co-crdinator, Department of Molecular Biology and Biotechnology, Tezpur University, who has guided me throughout the entire work. Without his constant inspiration, encouragement and constructive criticism, the present investigation would not have been possible.

I would like to express my sincere thanks to Prof. M.K Chaudhari, Vice chancellor, Tezpur University, Napaam for their kind support and constant encouragement during the tenure of my research period.

I express my deep sense of gratitude to Dr. G.maitri Scientist –I DBT nodel centre, Miss Rupamoni thakur, ,Jetender, Rocktotpal, Satish, Hemant adhikari, saurov mazumdar and Miss Debashree Saikia for their technical support and careful thesis editing.

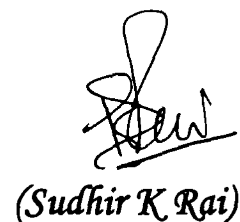
It is a great pleasure to offer my sincere thanks to all faculties and staff members of the Department, specifically Mr. Kalyan Hazarika, Mr. Pranab Mudoi, Mr. Samar Kakoti, Mr. Depak and Mr.D.D. Bania for their support during the entire course of investigation. Special thanks are to my colleague Mr. Deepak, Research scholar, Dept. of Biological science, Birla institute of

Technology (BITS), Pilani for his friendly advices and constant encouragement. I am also grateful to my senior collegaues Dr. Kishore das, Dr. N.K.Bordoloi, Dr. (Mrs) Pallabi Kalita , Dr. R.Doley and all other friends of the department and also all AT hostel boarders of TU for their help, encouragement and co-operation.

I would like to express my deep sense of gratitude to all of my family members –for their enthusiastic inspiration all throughout my academic career. Thanks are also to my close associates Khem, Ambi, and Rakesh for their constant help and support.

Last but not the least, I also offer thanks to master students of MBBT departmet and various other departments for their support throughout the work.

The financial assistance in the form of a Junior and Senior Research Fellowship received from the DBT and CSIR , New Delhi is duly acknowledged.



Date 10/09/10 *(Sudhir K Rai)*

List of abbreviation

Abbreviations	Full form
CFU	Colony forming unit
DF	Degree of freedom
f-value	Fishure value
FTIR	Fourier transform infrared spectroscopy
gm	Gram
g	gravity
GC-FAME	Gas chromatography of fatty acid methyl ester
KBr	potassium bromide
kbp	Kilobase pair
kDa	Kilo dalton
MTCC	Microbial type culture collection
M	Molar
µg	Microgram
mM	Millimolar
mg /ml	Milligram / milliliter
ml	Milliliter
µl	Microlitre
Na-EDTA	Disodium ethylene diamine tetra acetate
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium nitrate

Abbreviations	Full form
$(NH_4)_2SO_4$	Ammonium sulphate
$NaNO_3$	Sodium nitrate
N	Normality
OD	Optical density
p-value	Probability value
rpm	Revolution per minute
RBC	Red blood cells
S.D	Standard deviation
TCA	Tri carboxylic acid
U	Units
v/v	Volume / volume
w/v	Weight / volume
w/w	Weight / Weight

CHAPTER I

INTRODUCTION

Chapter 1

Introduction

1.0 Introduction

Biotechnology which can simply be defined as the application of living organisms and their components to industrial products and processes is important technology that has a large impact on many different industrial sectors. Biotechnology offers the potential for new industrial processes that require less energy and are based on renewable raw materials[1,2]. Biotechnology is currently considered as a useful alternative to conventional process technology in industrial and analytical fields. Unlike the chemical catalysts, the biological systems have the advantages of accomplishing complex chemical conversions under mild environmental conditions with high specificity and efficiency. Biological systems help in ingredient substitution, processing aid substitution, more efficient processing, less undesirable products, increased plant capacity, increased products yields, and improved or unique products. The variety of chemical transformations catalyzed by enzymes has made these catalysts a prime target of exploitation by the emerging biotech industries[2,3].

Industrial biotechnology is the application of biological based systems and their components in the manufacture of industrial products or in the support of industrial processes. Industrial biotechnology (also referred to as "White Biotechnology") is one of the three areas of the biotechnology sector, the others being Healthcare biotechnology ("Red biotechnology") and Agricultural biotechnology ("Green biotechnology") as described by Soetaert and Vandamme[4].

Industrial biotechnology is mainly based on fermentation technology and bio-catalysis. Fermentation is a biological process in which an organism or its components an aerobically convert organic compounds into alternative organic compounds[5]. In industrial biotechnology this

typically involves the conversion of complex organic material into simpler products (e.g. the conversion of carbohydrates into alcohol). In Bio-catalysis processes mostly the microbial enzymes, are applied to accelerate (catalyze) the conversion of substrate into a product[5]. Industrial biotechnology covers a broad range of industrial applications and sectors, including polymer manufacturing, biofuels production, fine and specialty chemical manufacturing and bio-remediation[5].

1.1 Historical aspect of Industrial enzymes

Most of the reactions in living organisms are catalysed by protein molecules called enzymes. Enzymes can rightly be called the catalytic machinery of living systems. Man has indirectly used enzymes since the beginning of human history. Enzymes are responsible for the bio catalytic fermentation of sugar to ethanol by yeasts, a reaction that forms the basis of beer and wine manufacturing. Enzyme oxidises ethanol to acetic acids. This reaction has been used in vinegar production for thousands of years. Similar microbial enzymatic reactions of acid forming bacteria and yeasts are responsible for aroma forming activities in bread making and in preserving activities in sauerkraut preparation[6,7].

Probably the first application of cell free enzymes was the use of rennin isolated from calf or lamb stomach. Rennin is an aspartic protease which coagulates milk protein and has been used for hundreds of years by cheese manufacturing industry. Rohm in Germany prepared the first commercial enzyme preparation in 1914[8,9]. This trypsin enzyme isolated from animals degraded proteins and was used as a components of detergent. It proved to be so powerful compared to traditional washing powders that the original small package size made the German housewives suspicious so that the product had to be reformulated and sold in larger packages[10,11]. The real breakthrough of enzymes occurred with the introduction of microbial proteases into washing powders. The first commercial bacterial (*Bacillus*) protease Bio-40 was marketed in 1959 and it revolutionized the enzyme market when Novozymes a Denmark based bio-industry started to manufacture it and major detergent manufactures started to use it around 1965.

1.2 Industrial enzymes and development of bio industrial sectors: An overview

The field of industrial enzymes is now undergoing major R&D initiatives, resulting in both the development of a number of new products and in improvement in the process and performance of several existing products. According to a report from Business Communications Company, Inc the global market for industrial enzymes was estimated to touch $2.7 billion in 2012[12] .Volume growth of industrial enzymes is between 4% and 5% AAGR (average annual growth rate), which is accompanied by decreasing prices, due to the increase in the number of minor players competing in the market. As a result, the market is expected to rise at an AAGR (average annual growth rate) of a little over 3% over the next 4 years, and the total industrial enzyme market in 2012 is expected to reach nearly $2.4 billion[13]. The industrial enzyme market is divided into three application segments: technical enzymes, food enzymes, and animal feed enzymes (Fig. 1.1). The growth of animal feed enzymes is somewhat higher, expected to be close to 4% AAGR, which in large part is boosted by increased use of phytase enzyme as an animal feed additive in diets for monogastric animals, for high-temperature feed pelleting processes[14,15].

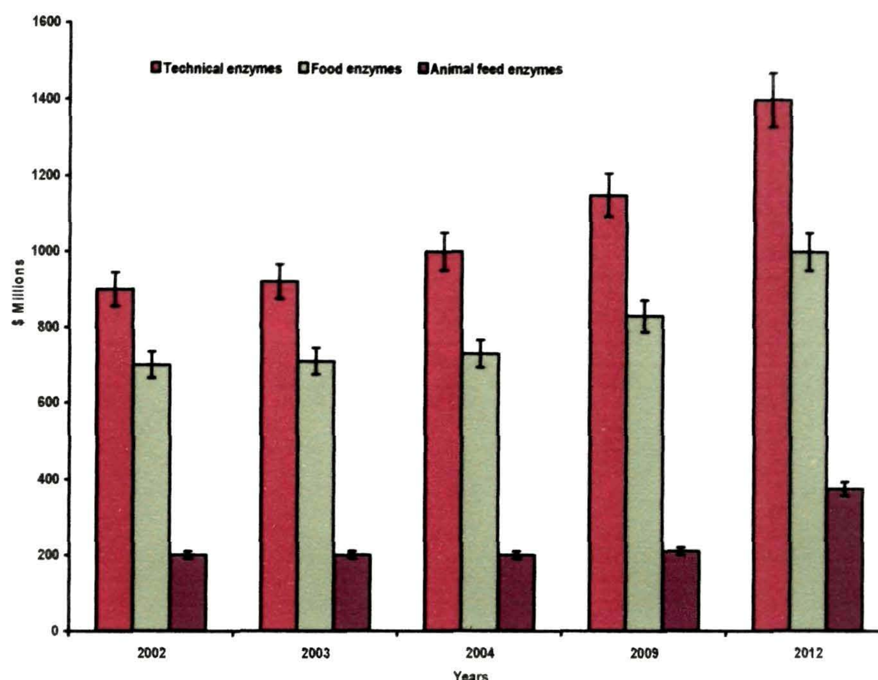


Fig.1.1 Global enzyme markets by application sectors, through 2012 (In $ Millions), according to Business Communications Company (BCC 2009).

1.2.1 Indian scenario of industrial enzymes

Indian biotech sector has attained critical mass in manufacturing and research services. Indian biotechnology is now poised to leverage its scientific skills and technical experiences to make a global impact on a strong innovation-led platform. The technological capability of a firm to produce some products is only a part of the requirement for the commercial success of the business. Apart from technological capabilities, the firm must be able to position its product in pharmaceutical industries, followed by food / feed and textiles market. The market, thus, serves as the link between consumer's needs and the pattern of industrial response [16].

In India, the industrial enzyme consumption is predominantly in the pharmaceutical market (50 percent), followed by the detergent market (20 percent) (Fig.1.2). The other important segments are food and feed, textiles, leather, pulp and paper[17] (Fig.1.2). In recent years, enzymes

have found numerous applications in the food, pharmaceutical, diagnostic and chemical processing industries. The trend for their application in almost every sector is ever increasing.

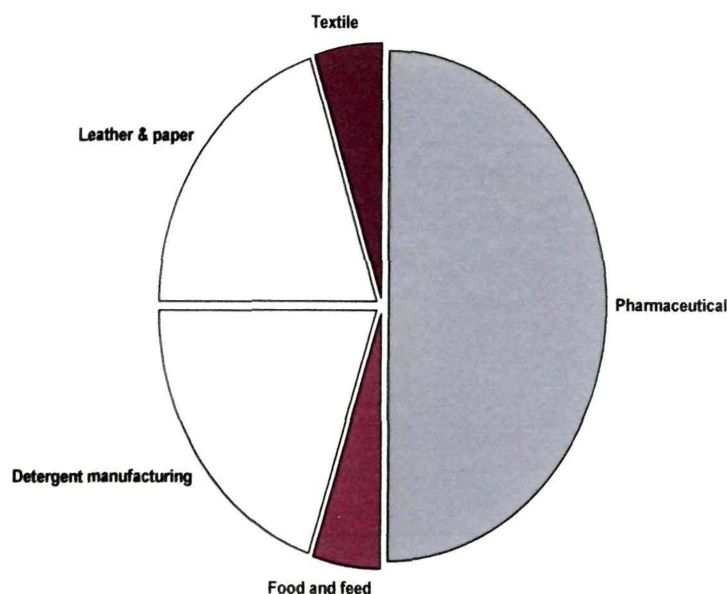


Fig.1.2 Consumption of industrial enzymes in India: Pharmaceuticals; Food and feed; Detergent manufacturing; Leather and paper and; Textile processing[17]

1.2.1.1 Futuristic application of enzymes for the growth of Indian Bio-Industrial sector

The Bio-Industrial market mainly comprising industrial enzymes is estimated to be Rs 564 crore in the year 2009-10 as against Rs 478 crore in 2008-09. The segment grew 16 percent in FY 2009-10[17] .Though the overall enzyme consumption figures of India are comparatively low with respect to other countries and 60-70 percent of domestic demand is imported, the segment on an average has been growing over 15 percent in the last five years. The leading players in this segment include Novozymes which has over 50 percent market share followed by Advanced Enzyme Technologies holding 25-30 percent market share (Table 1.1). Other important players are Maps, Genecor, Lumis and Kerry Biosciences. Besides, a new entrant Anthem Cellutions, a part of Anthem Biosciences group has gained significant market share over a short period of time[17].

Table 1.1 Bioindustrial companies by Revenue (2009-2010)

Rank 2010	Company	2009-10 (Revenue in Rs Cr)	2008-09 (Revenue in Rs Cr)	% Change Over 2008-09
1	Novozymes South Asia*	268	250.00	7.2
2	Advanced Enzymes	121	88.60	36.57

* Biospectrum estimates   All the figures in Rs. Crore unless indicated otherwise

Apart from focusing on the local market, companies like Advanced Enzyme Technologies are looking at foreign markets such as China. Considering the potential opportunity in the other markets Advanced Enzyme has registered appreciable growth in its export sales[17]. The Indian enzyme companies are reporting a good growth in terms of activity; a lot of R&D and exploration of new applications of microbial enzymes. However, with respect to global enzyme industry India still needs to do a lot for expanding its market including production of enzyme. The leading distributor / suppliers of enzymes in India are shown in Table 1.2.

Table 1.2 Potential enzyme distributors/suppliers in India

Distributors/suppliers	Business type	Products offered
Texnzymes India, Mumbai, Maharashtra	Manufacturers	Textile auxiliaries and enzymes
Americos Industries Inc., Ahmedabad, Gujarat	Manufacturers And exporters	Textile auxiliaries and enzymes
United Biochemicals Private Limited, Mumbai, Maharashtra	Manufacturers and exporters	Enzymes and biochemicals for textiles
Ruchi Biochemicals, Mumbai, Maharastra	Manufacturers and suppliers	Biofertilizers and industrial enzymes
Trishul Chemicals, Chennai, Tamil Nadu	Manufacturers and exporters	Leather chemicals including bates and enzymes
Indian Textile Auxiliary Co., Bangalore, Karnataka	Manufacturers and exporters	Textile auxiliaries, enzymes, leather auxiliaries, leather enzymes, etc.
Zytex India Private Limited Mumbai, Maharashtra	Manufacturers and exporters	Industrial enzymes
Naruveli Ventures Chennai, Tamil Nadu	Dealers	Industrial enzymes, microbial products and biofertilizers

Distributors/suppliers	Business type	Products offered
Enzyme India Private Limited, Chennai, Tamil Nadu	Manufacturers and exporters	Enzymes for agro, bio-feed, food, detergent and textiles
Monozyme India Limited, Secunderabad, Andhra Pradesh	Manufacturers and exporters	Enzymes and diagnostic reagents
Prasanthi Leather Chem Private Limited, Kolkata, West Bengal	Wholesale suppliers/Distributor sellers	Enzymes and fat liquors, etc.
Biomax, Thane, Maharashtra	Manufacturers	Biofertilzers, soil modifi ers and enzymes
Genotex International Private Limited, Hyderabad, Andhra Pradesh	Manufacturers and exporters	Industrial enzymes, Aloe vera juice and patented peptides
Protos Trading Private Limited, New Delhi	Importers/buyers	Textile enzymes

1.3 Present scenario of microbial protease: a leader of industrial enzymes

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. Among them, alkaline proteases represent one of the three largest groups of industrial enzymes and account for about 60% of total worldwide enzyme sales (Fig.1.3). This dominance of proteases in the industrial market is expected to increase 15% for next five years is by 2015[17]. In recent years, there has been a phenomenal increase in the use of alkaline protease as industrial catalysts. These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons; for example, they exhibit higher catalytic activity and, a higher degree of substrate specificity. Besides they can be produced in large amounts in a relatively short period of time and are economically viable thus further expanding their industrial acceptability.

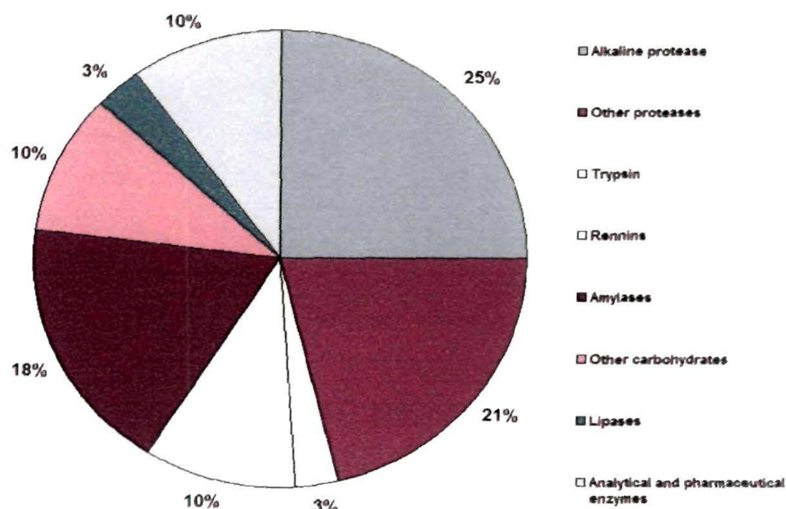


Fig.1.3 Distribution of enzyme sales. The contribution of different enzymes to the total sales of enzymes is indicated. The shaded portion indicates the total sales of proteases.

1.4. Microbial protease: bread and butter for enzyme industries

Microorganisms represent an excellent source of enzymes due to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 65% of the total worldwide enzyme sales[9, 18] and are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Most commercial proteases mainly neutral and alkaline are produced by the genus *Bacillus*. Fungi elaborate a wider variety of enzymes than bacteria, e.g., *Aspergillus oryzae* produces acid, neutral and alkaline proteases. Fungal acid proteases are useful in cheese-making industry, neutral proteases in reducing the bitterness of food protein hydrolysates and alkaline proteases in food protein modification. Most of the viral encoded peptidases are endopeptidases of serine, cysteine or aspartic type.

Among the broad sources of enzymes i.e., animals, plants and microbes, enzymes from micro organisms have become the choice of industrial production. The current trend is to use microbial enzymes since they provide a greater diversity of catalytic activities and can be produced more economically. Micro-organism have qualified as an average source of industrial enzyme owing to their consistency, ease of process optimization and modification and their potent industrial application. Micro-organism elaborates a large array of intracellular and / or extracellular processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and maintenance of the cellular protein pool. Extra cellular proteins are important for the hydrolysis of proteins in cell free environments and enable the cell to absorb and utilize hydrolytic products[19].

The technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzymes unrecommendable. Thus, the search for new microbial sources is a

continual exercise, but one must respect biodiversity. The microorganism habour diverse and exotic environments. Microbial diversity is a major resource for biotechnological products and processes. Extremophiles are considered an important source of enzymes, and their specific properties are expected to result in novel process applications. Infact only 2% of the world's micro-organism have been tested as enzymes sources[20,21]. Looking into the depth of microbial diversity , there is always a chance of discovery a promising micro-organism producing novel enzymes with better properties that are suitable for commercial exploitation[22]. The multitude of physicochemically diverse habitats has

challenged nature to develop equally numerous molecular adaptations in the microbial world. About 80% of the commercial enzymes are produced using micro organisms of different genera and species, isolated from different geographical regions. Although, northeastern part of India is considered as one of the mega biodiversity zones of the world, however, persusal of literature shows that limited attempt has been made to explore the industrially important microorganisms from this region.

1.4.1 Proteolytic enzymes

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields [23,24]. Proteases catalyze only a single reaction, i.e the hydrolysis of peptide bonds. Yet, the various ways they achieve this, their ubiquitous distribution among all life forms, their multiplicity of locations inside, outside and at the surface of cells and, above all, their enormous diversity of function makes them one of the most fascinating groups of enzymes[23,24]. The specificity of some of these proteases has been listed in Table 1.3.

CENTRAL LIBRARY, T. U.
ACC. NO. 49653

Table 1.3 Specificity of proteases

Enzyme	Peptide bond cleaved[a]
Trypsin	↓ --Lys(or Arg)------
Chymptrypsin, subtilisin	↓ ----Trp (or Tyr, Phe, Leu)--------
Staphylococcus V8 protease	↓ -----Asp(or Glu)-------
Papain	↓ -----Phe (or Val, Leu)- Xaa-------
Thermolysin	↓ --------Leu (or Phe)------
Pepsin	↓ ----Phe (or Tyr, Leu)-------Trp (or Phe, Tyr)

[a]The arrow indicates the site of action of the protease. Xaa, any amino acid residue.

They are presumed to have arisen in the earliest phases of biological evolution, some billion years ago since the present digestive proteases can be shown to have a common ancestry with those of the microbial origin. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications[23-25]. The involvement of proteases in the life cycle of disease causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. The major producers and distributors of proteases worldwide are Novo Industries (Denmark), Gist-Brocades (Netherlands), Genencor International and Miles Laboratories (United States).

CENTRAL LIBRARY, T. U.
ACC. NO. T 145

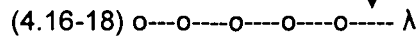
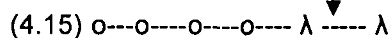
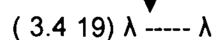
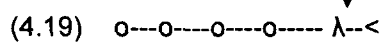
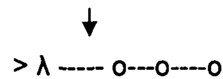
1.4.2 Classification of proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (International Union of Biochemistry. (1992). However proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (I) type of reaction catalyzed, (II) chemical nature of catalytic site, and (III) evolutionary relationship with reference to structure[26]. Depending on their site of action, proteases are grossly subdivided into two major groups, i.e. exopeptidases and endopeptidases (Table 1.4). Based on the functional group at the active site, proteases are further classified into serine, aspartic, cysteine and metalloproteases or of unknown type, e.g., ATP dependent proteases which require ATP for their activity[24, 27]. Based on their amino acid sequences, proteases are classified into different families[28] and further classified into clans to accommodate sets of peptidases that have diverged from a common ancestor [29].

Table 1.4 Classification of proteases. Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and the triangles signify the blocked termini. Arrows show the sites of action of the enzyme.

PROTEASES	
EXOPEPTIDASE ↓ λ ---o---o----o----o----o	**ENDOPEPTIDASE** ↓ o---o---o---o---o----o
Based on actions	Based on active sites
Aminopeptidases ↓ (3.4.11) λ---o---o----o----o----o	Serine endopeptiases (3.4.21)
Dipeptidyl peptidase ↓ (3.4.14) λ--- λ ---o----o----o----o	Cysteine endopeptidases (3.4.22)

PROTEASES

EXOPEPTIDASE	ENDOPEPTIDASE
Tripeptidyl peptidase	Aspartic endopeptidases (3.4.23)
Carboxylpeptidases	Metallo endopeptidases (3.4.24)
	
Carboxylpeptidase (serine) (3.4.16)	
Carboxylpeptidase (metallo) (3.4.17)	
Carboxylpeptidase (cysteine) (3.4.18)	
Peptidyl dipeptidases	Endopeptidase of unknow catalytic mechanism (3.4.99)
	
Dipeptidases	
	
Omegapeptidases	
	
	

1.4.2.1 Exopeptidases

Exopeptidases act only near the ends of polypeptide chains, further classified as amino or carboxypeptidases based on their site of action at the N or C terminus respectively[30]. Aminopeptidases liberate a single amino acid residue, a dipeptide (dipeptidyl peptidase) or a tripeptide (tripeptidyl peptidase)[30,31]. The substrate specificities of the enzymes from bacteria and fungi are distinctly different in that the organisms can be differentiated based on the profiles of the products of hydrolysis[30,32]. Aminopeptidases can also be classified as aminopeptidase N or aminopeptidase A, depending on their preference for neutral (uncharged) or acidic side chains, respectively[33]. Most of the aminopeptidases are metalloenzymes. Carboxypeptidases can be

divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes[34]. The enzymes can also hydrolyze the peptides in which the peptidyl group is replaced by a pteroyl moiety or by acyl groups. Other exopeptidases include dipeptidases, which cleave a dipeptide and omega peptidases which release modified residues from N- or C-termini.

1.4.2.2 Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N or C terminus. They are divided into five subgroups based on their catalytic mechanism, (i) serine proteases, (ii) cysteine proteases, (iii) aspartic proteases, (iv) metalloproteases and (v) unknown type denoted by the letters S, C, A, M, and U, respectively.

1.5 Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. Based on their structural similarities, they have been grouped into families, which have been further subdivided into about six clans with common ancestors[30,35,36]. The classification of peptidases based on the evolutionary relationships has been given in MEROPS – the peptidase database; url: http://www.bi.bbsrc.ac.uk/Merops/merops.htm. Trypsin, chymotrypsin are the well studied proteases of this subgroup[34,36]. Another interesting feature of the serine proteases is the conservation of glycine residues near the catalytic serine residue to form the motif Gly-Xaa-Ser-Yaa-Gly[37,38]. They are recognized by their irreversible inhibition by 3,4- DCI, E.64, DFP, PMSF and TLCK. Some are inhibited by thiol reagents such as PCMB due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities

including esterolytic and amidase activity. Their molecular masses range between 18 and 35 kDa. Their isoelectric points are generally between pH 4 and 6. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases.

1.5.1 Serine alkaline proteases

These are produced by several bacteria, molds, yeasts and fungi. They are inhibited by DFP but not by TLCK or TPCK. They hydrolyze a peptide bond, which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimal pH of the alkaline proteases is around pH 10, and their isoelectric point is around pH 9. Their molecular masses are in the range of 15 to 60 kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces*, and *Flavobacterium* spp[39], subtilisins produced by *Bacillus* spp. are the best known. Alkaline proteases are also produced by *S. cerevisae*[40] and filamentous fungi such as *Conidiobolus* spp.[41] and *Aspergillus* and *Neurospora* spp[42].

1.5.1.1 Subtilisins

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, Subtilisin Carlsberg produced by *Bacillus licheniformis* and Subtilisin Novo or BPN' produced by *B. amyloliquefaciens* have been identified[43-46]. Subtilisin Carlsberg was the first detergent protease formulated for detergent industries since 1960s and was isolated from *Bacillus licheniformis* strain. The active-site conformation of subtilisins is similar to that of trypsin and chymotrypsin despite the dissimilarity in their overall molecular arrangements. The serine alkaline protease from the fungus *Conidiobolus coronatus* was shown to possess a distinctly different structure from Subtilisin Carlsberg in spite of their functional similarities[47].

1.6 Aspartic proteases

Commonly known as acid proteases, these proteases depend on aspartic acid residues for their catalytic activity. They have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3)[36] and have been placed in the clan AA. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. The members of the pepsin family have a bilobal structure with the active-site cleft located between the lobes[48]. The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly in which Xaa can be Ser or Thr. The aspartic acid proteases are inhibited by pepstatin and diazocompounds such as DAN and EPNP in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin. They can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus, Penicillium, Rhizopus* and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* sp.

1.7 Cysteine/thiol proteases

The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His residues differs among the 20 families[26]. Generally cysteine proteases are active in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups, (i) papain-like, (ii) trypsin-like with preference for cleavage at the arginine residue, (iii) specific for glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, like lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal chelating agents.

1.8 Metalloproteases

These are the most diverse of the catalytic types of proteases[49] characterized by the requirement of a divalent metal ion for their activity. Out of 30 families of metalloproteases, 17 contain only endopeptidases, 12 contain only exopeptidases and 1 (M3) contains both endo- and exopeptidases. Families of metalloproteases have been grouped into different clans based on the nature of the amino acid that completes the metal-binding site; e.g., clan MA has the sequence HEXXH-E and clan MB corresponds to the motif HEXXH-H. Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I, and (iv) Myxobacter II[50,51]. The neutral proteases show specificity for hydrophobic amino acids, while alkaline proteases possess a very broad specificity. Myxobacter I is specific for small amino acid residues on either side of the cleavage bond, whereas protease II is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP. Thermolysin, collagenase and elastase are the well studied metalloproteases. Matrix metalloproteases play a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing, and may be useful in the treatment of diseases such as cancer and arthritis[52].

1.9 Structure and Mechanism of action of proteases

1.9.1 Structure of proteases

The three dimensional structure of proteases can provide valuable insights into their function and specificity providing a framework to focus previous biochemical data and a sound basis for the design of new experiments and therefore, can be a rich source of information for the modern biochemist. Structural data on proteases can have a profound influence on the search and design of novel specific inhibitors ("rational drug design"). The x-ray crystallographic structures of many proteases have been determined[53-56]. In 1994, Yamagata and his colleague's[57]

reported the structure of cucumisin, a thermostable alkaline serine protease that is found in the juice of melon fruits *(Cucumis melo* L.). Their studies on the primary structure of cucumisin revealed that it is synthesized as a precursor, consisting of four functional domains: a possible signal peptide (22 amino acid residues), an NH,-terminal pro-sequence*(8 8* residues), a 54-kDa protease domain (505 residues), which is the active enzyme domain of the 67-kDa native cucumisin, and a 14-kDa COOH-terminal polypeptide (116 residues), which arises by limited autolysis of the 67-kDa native cucumisin. Sequence comparisons revealed that cucumisin has several features in common with the microbial proteases of the subtilisin family (Fig. 1.4).

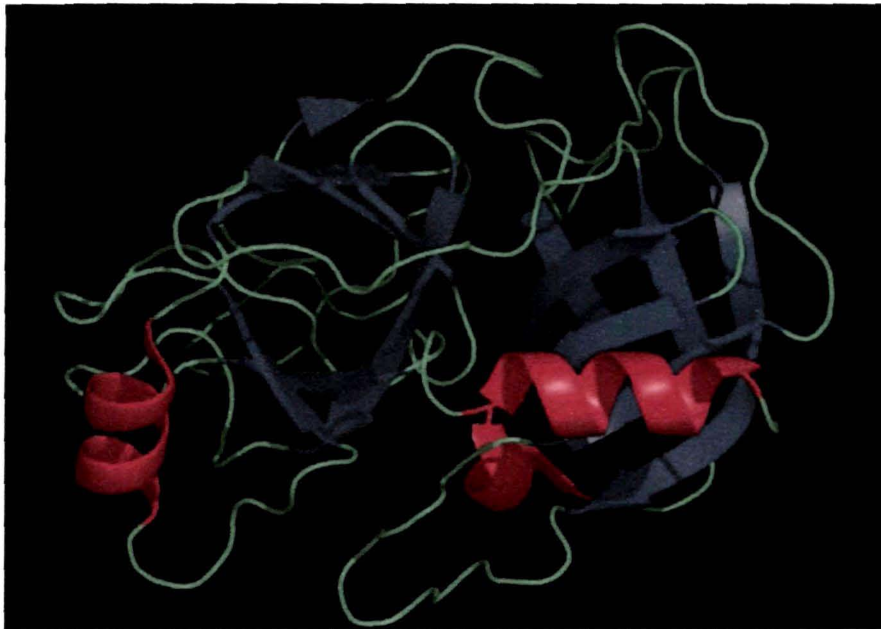


Fig.1.4 Three-dimensional structure of proteases: (a) The crystal structure of trypsin, a serine protease[58].

1.9.2 Mechanism of action of proteases

The mechanism of action of proteases has been a subject of great interest to researchers as it forms a basis for exploring various ways of

modifying its activity to make it suitable for its biotechnological application. Studies of the mechanism of proteases have revealed that they exhibit different types of mechanism based on their active-site configuration. The catalytic site of proteases is flanked on one or both sides by specificity sub-sites, each able to accommodate the side chain of a single amino acid residue from the substrate. These sites are numbered from the catalytic site S1 through Sn toward the N terminus of the structure and S1' through Sn' toward the C terminus. The residues which they accommodate from the substrate are numbered P1 through Pn and P1' through Pn', respectively (Fig.2.2). Proteolytic enzymes , in general, have three essential catalytic components[50,51]:

1. A nucleophilie to attack the carbonyl C atom of the scissile peptide to form a tetrahedral intermediate (Ser 195 serves this function in trypsin)
2. An elelctrophile to stabilize the negative charge that develops on the carbonyl O atom of the tetrahedral intermediate (the H-bonding donors lining the oxyanion hole, Gly 193 and Ser 195, do so in trypsin).
3. A proton donor so as to make the amide N atom of the scissile peptide a good leaving group (the imidazolium group of His 57 in trypsin)

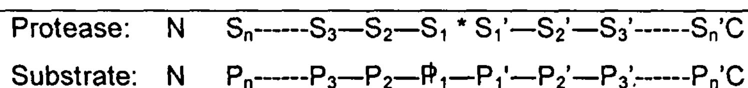


Fig.1.5. Active sites of proteases. The catalytic site of proteases is indicated by * and the scissile bond is indicated by —S1; through Sn and S1' through Sn' are the specificity subsites on the enzyme, while P1 through Pn and P1' through Pn' are the residues on the substrate accommodated by the subsites on the enzyme.

1.9.2.1 Mechanism of action of serine protease

Serine proteases usually follow a two-step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with

the loss of the amino acid or peptide fragment[59]. This acylation step is followed by a deacylation process which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide. The key steps in the hydrolysis have been illustrated in Fig.1.6. The serine endopeptidases can be classified into three groups based mainly on their primary substrate preference:

(I) Trypsin-like, which cleave after positively charged residues

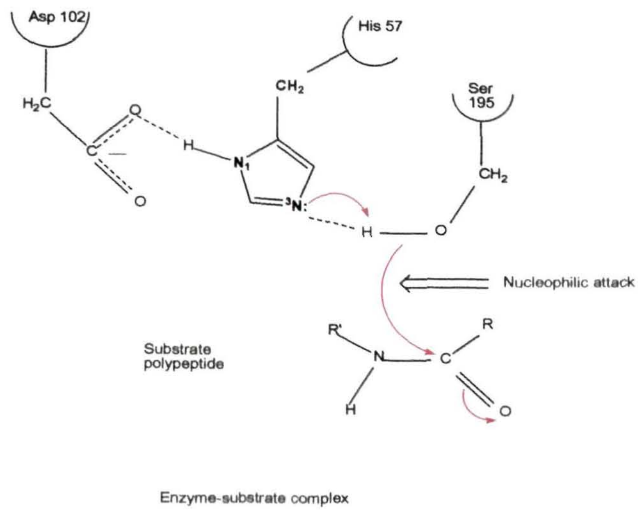
(II) Chymotrypsin-like, which cleave after large hydrophobic residues; and

(III) Elastase-like, which cleave after small hydrophobic residues.

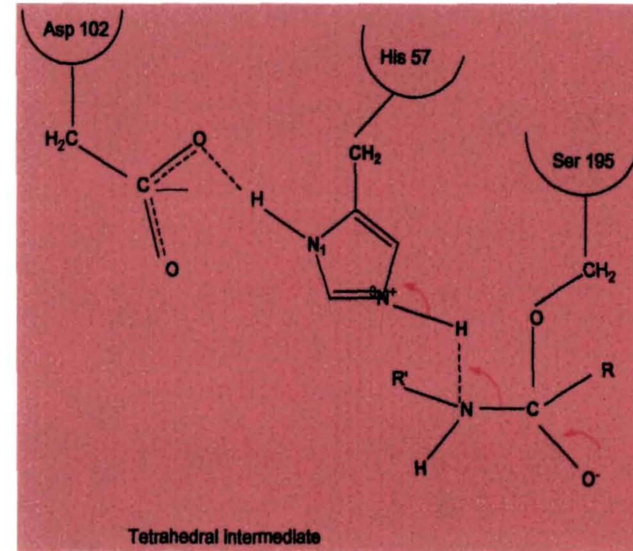
The recent studies based on the three dimensional structures of proteases and comparisons of amino acid sequences near the primary substrate-binding site in trypsin-like proteases of viral and bacterial origin suggest a putative general substrate binding scheme for proteases with specificity towards glutamic acid involving a histidine residue and hydroxyl function. However, a few other serine proteases such as peptidase A from *E. coli* and the repressor LexA show distinctly different mechanism of action without the classic Ser- His- Asp triad[26]. Some of the glycine residues are conserved near the catalytic serine residue, but their exact positions are variable[38].

A few of the serine proteases belonging to the subtilisin family show a catalytic triad composed of the same residues as in the chymotrypsin family; however, the residues occur in a different order (Asp-His-Ser). Some members of the subtilisin family from the yeasts *Tritirachium* and *Metarhizium* sp. require thiol for their activity. The thiol dependance is attributable to Cys 173 near the active-site histidine[60]. The carboxypeptidases are unusual among the serine-dependent enzymes in that they are maximally active at acidic pH. These enzymes are known to possesss a Glu residue preceding the catalytic Ser, which is believed to be responsible for their acidic pH optimum. Although the majority of the serine proteases contain the catalytic triad of Ser- His-Asp, a few use the Ser-base catalytic dyad. The Glu-specific proteases

display a pronounced preference for Glu-Xaa bonds over Asp-Xaa bonds[61].

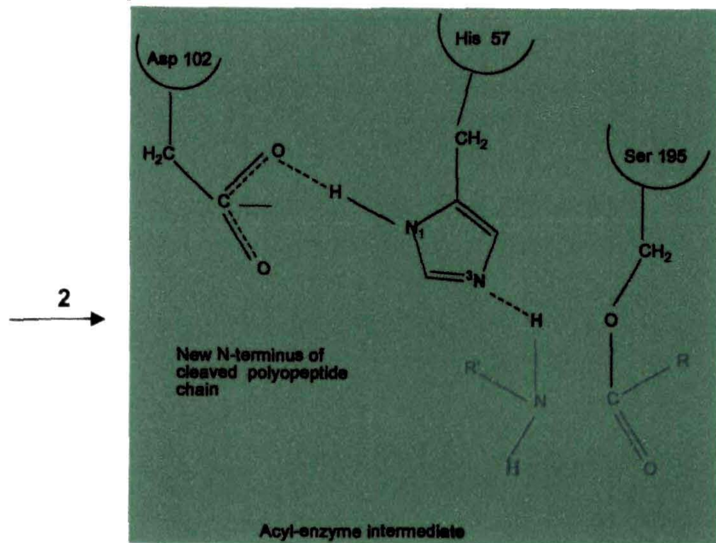


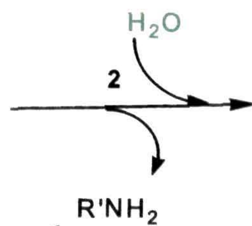
1 →

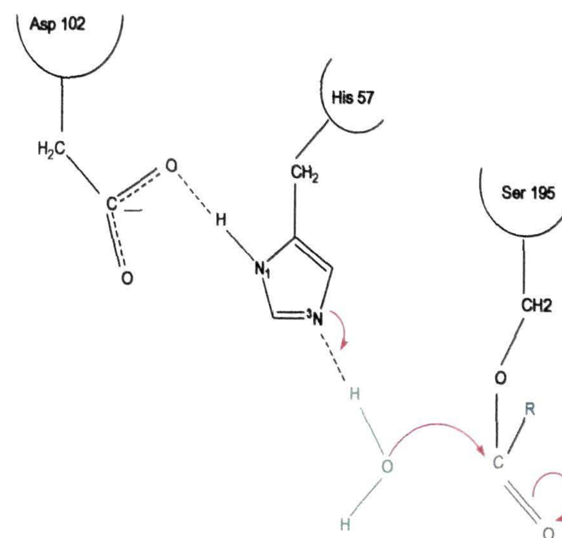


STEP 1

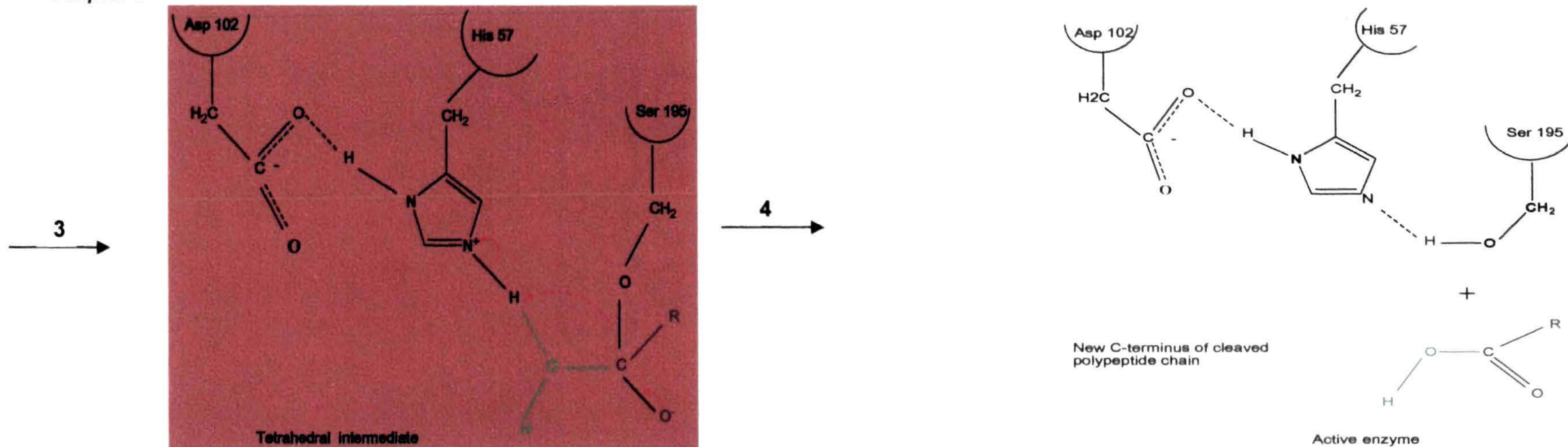
2







Step 2



Step 3

Fig.1.6 Catalytic mechanism of the serine proteases. The reaction involves (1) the nucleophilic attack of the active site Ser on the carbonyl carbon atom of the scissile peptide bond to form the teterhedral intermediate; (2) the decomposition of the tetrahedral intermediate to the acyl- enzyme intermediate through general acid catalysis by the active site Asp-polarized His, followed by loss of the amine product and its replacement by a water molecule; (3) the reversal of Step 2 to form a second tetrahedral intermediate; and (4) the reversal of Step1 to yield the reaction's carboxyl product and the active enzyme.

1.9.2.2 Mechanism of action of aspartic protease

Crystallographic studies have shown that the enzymes of the pepsin family are bilobed molecules with the active-site cleft located between the lobes and each lobe contributing one of the pair of aspartic acid residues that is essential for the catalytic activity[48,62]. In most of the enzymes from the pepsin family, the catalytic Asp residues are contained in an Asp-Thr-Gly-Xaa motif in both the N- and C-terminal lobes of the enzyme, where Xaa is Ser or Thr, whose side chains can hydrogen bond to Asp. However Xaa is Ala in most of the retropepsins. The pepsins and majority of the members of the family show specificity for the cleavage of bonds in peptides of at least six residues with hydrophobic amino acids in both the P1 and P1' positions[63]. The steps involve in catalytic mechanism

An active site Asp carboxylate group, acting as a general base, activates the bound water molecule, the so called lytic water, to nucleophilically attack the scissile peptide carbonyl C as an OH^- ion. Proton donation (general acid catalysis) by the second, previously uncharged active site Asp stabilizes the oxyanion that would otherwise form in the resulting tetrahedral intermediate. The N atom of the scissile peptide is protonated by the first Asp (general acid catalysis) resulting, through charge rearrangement and proton transfer to the second Asp (general acid catalysis), in amide bond scission.

Asparatic proteases are inhibited by compounds with tetrahedral carbon atoms at a position mimicking a scissile peptide bond (Fig.1.7). This strongly suggests that these enzymes preferentially bind their transition states (transition state stabilization), thereby enhancing catalysis. The structural and kinetic studies have suggested that the mechanism involves general acid-base catalysis with lytic water molecule directly participating in the reaction.

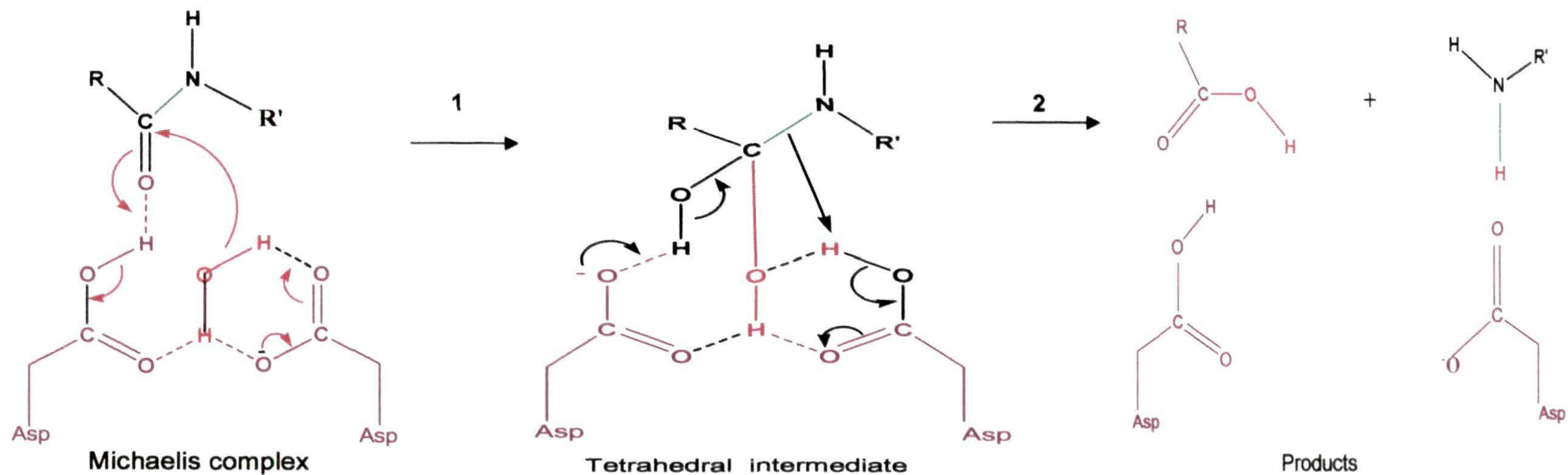


Fig.1.7 Catalytic mechanism of asparatic proteases. The nulceophilic attack of the enzyme –activated water molecule (red) on the carbonyl carbon atom of the scissile peptide bond (green) to form the tetrahedral intermediate.This reaction step is promoted by general base catalysis by the Asp on the right and general acid catalysis by the Asp on the left (Blue). (2) The decomposition of the tetrahedral intermediate to form products via general acid catalysis by the Asp on the right and general base catalysis by the Asp on the left.

1.9.2.3 Mechanism of action of metallo-protease

Metalloproteases depend on the presence of bound divalent cations and can be inactivated by dialysis or by the addition of chelating agents. Most of these are enzymes containing His-Glu-Xaa-Xaa-His (HEXXH) motif, which has been shown by X-ray crystallography to form a part of the site for binding of the metal, usually zinc. For thermolysin, based on the X-ray studies of the complex with a hydroxamic acid inhibitor, it has been proposed that Glu 143 assists the necleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond, which is polarized by the Zn^{2+} ion as shown in Fig.1.8[64].

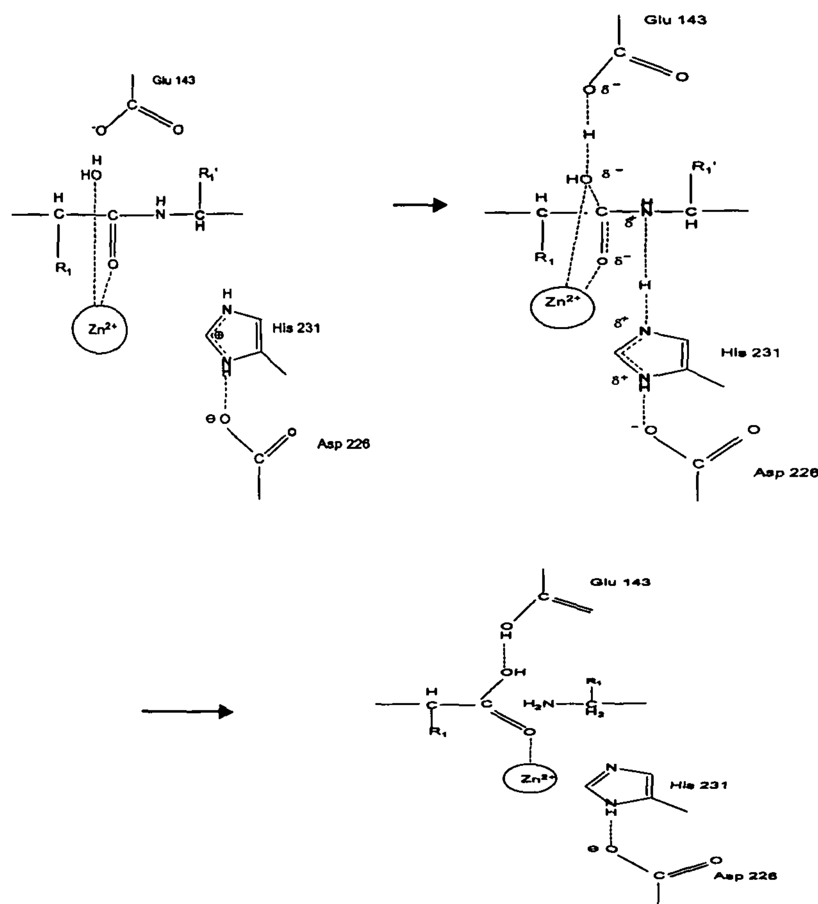


Fig.1.8 Schematic illustration of catalytic mechanism of metalloprotease (thermolysin).

1.9.2.4 Mechanism of action of cysteine protease

Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of the serine proteases. The initial step in the catalytic process involves the non-covalent binding of the free enzyme and the substrate to form the complex followed by the acylation of the enzyme with the formation and release of the first product, the amine R'-NH2 (Fig.1.9). In the next deacylation step, the acyl-enzyme reacts with a water molecule to release the second product, with the regeneration of free enzyme (Fig.1.9). The plant peptidase papain can be considered the archetype of cysteine peptidases and constitutes a good model for this family of enzymes, which catalyze the hydolysis of peptide, amide, ester, thiol ester and thiono ester bonds[65].

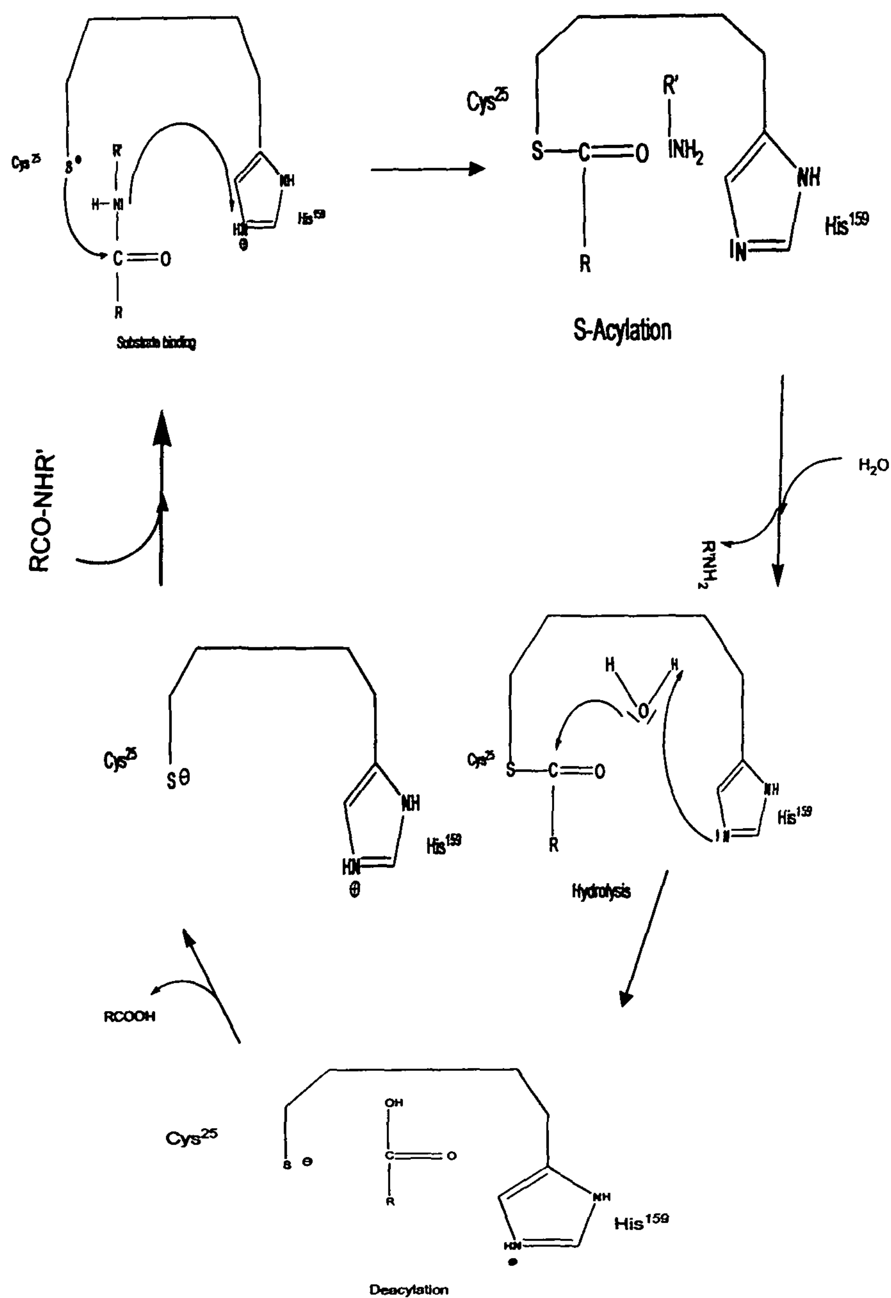


Fig.1.9 Catalytic mechanism of cysteine protease

1.10 Production of microbial alkaline proteases

Most alkalophilic microorganisms produce alkaline proteases, though interest is limited only to those that yield substantial amounts. It is essential that these organisms be provided with optimal growth conditions to increase enzyme production. The culture conditions that promote protease production were found to be significantly different from the culture conditions promoting cell growth[66]. In the industrial production of alkaline proteases, technical media are usually employed that contained very high concentrations (100–150 g dry weight/litre) of complex carbohydrates, proteins, and other media components[67]. With a view to develop an economically feasible technology, research efforts are mainly focused on (a) optimization of the fermentation medium and (b) production conditions to enhance the protease yield.

Alkaline proteases are generally produced by submerged fermentation. In addition, solid state fermentation processes has also been exploited though, to a lesser extent for the production of these enzymes[68-70]. Generally, proteases produced from microorganisms are constitutive or partially inducible in nature and, under most culture conditions, *Bacillus* species produce extracellular proteases during post-exponential and stationary phases. Extracellular protease production in microorganisms is also strongly influenced by media components, e.g. variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose[71,72], and metal ions[73]. Protease synthesis is also affected by rapidly metabolizable nitrogen sources, such as supplementation of amino acids in the medium. Besides these, several other physical factors, such as aeration, inoculum density, pH, temperature and incubation, also effect the level of protease production[74-78].

In order to scale up protease production from microorganisms at the industrial level, biochemical and process engineers have adopted several strategies to obtain high yields of protease in a fermentor. Controlled batch and fed-batch fermentations using simultaneous

control of glucose, ammonium ion concentration, oxygen tension, pH and salt availability[74,79-81] and chemostat cultures[82,83] have been successfully used for improving protease production for long-term incubations, using a variety of microorganisms.

In recent years, attempts have been made to utilize statistical methods, using different statistical software packages (Table 1.5) for process optimization studies, with the aim of obtaining high yields of alkaline protease in the fermentation medium[73, 75, 84]. The application of properly designed approaches with multi-factor models allows process and biochemical engineers to design scale-up strategies for increasing enzyme production. As such no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production and that must be fulfilled for obtaining a superior yield.

Table 1.5 Statistical methods used to improve the protease production from microorganisms.

Micro-organisms	Design	Software	References
Bacillus sp.	Central composite design	Statistica	Adinarayana, and Ellaiah [85]
Bacillus sp. L21	response surface methodology (RSM)	Minitab (Minitab Inc., State College, PA., USA)	Tari *et al*[86]
Bacillus clausii	L16 (45) orthogonal array of Taguchi design	Design Expert software (version6.0.10, Stat-Ease Inc., USA)	Oskouie *et al*[87]
Bacillus sp.	RSM: FCCCD as Experimental Design	Design-Expert® 6.0 (Stat-Ease, Min- neapolis, MN) was	Saran *et al*[88]
Bacillus sp. HS08	24 full- factorial central composite design (CCD) was	Statistica, Version 7.0 (Statsoft Inc., Tulsa, OK)	Guangrong *et al* [89]
Bacillus sp. RKY3	Plackett–Burman design & Central composite design	Design-Expert 7.1 (Stat- Ease, Inc., Minneapolis, MN, USA)	Reddy *et al*[90]
Colwellia sp. NJ341	Response surface method	Design Expert software (Version 7.0, USA)	Wang *et al*[91]

Micro-organisms	Design	Software	References
Bacillus pseudofirmus SVB1	Plackett-Burman experimental design. Central composite design	Minitab (Minitab Inc., State College, PA., USA)	Sen *et al*[92]
Bacillus pseudofirmus Mn6	Box-Behnken Design	Microsoft Excel 97	Abdel -Fattah *et al* [93]
Mangifera Indica Cv. Chokanan	central composite design (CCD)	Minitab v.14 statistical package (Minitab Inc., PA, USA).	Amid *et al*[94]

1.11 Protease purification techniques

Crude preparations of alkaline proteases are generally employed for commercial use[6,95,96]. Nevertheless, the purification of alkaline proteases is important from the perspective of developing a better understanding of the functioning of the enzyme[97,98].

After successful fermentation, when the fermented medium leaves the controlled environment of the fermenter, it is exposed to a drastic change in environmental conditions. The rapid lowering of the temperature of the fermented medium (to below 5°C) becomes indispensable to prevent microbial contamination as well as to maintain enzyme activity and stability. The removal of the cells, solids, and colloids from the fermentation broth is the primary step in enzyme downstream processing, for which vacuum rotary drum filters and continuous disc centrifuges are commonly used. To prevent the loss in enzyme activity caused by imperfect clarification or to prevent the clogging of filters, it is necessary to perform some chemical pretreatment of the fermentation broth before commencing separation[67,99]. Changes in pH may also be suitable for better separation of solids[98]. Furthermore, the fermentation broth solids are often colloidal in nature and are difficult to remove directly. In this case, addition of coagulating or flocculating agents becomes vital[100].

When isolating enzymes on industrial scale for commercial purposes, the prime consideration has been the cost of production in relation to the value of the end product. Because the amount of enzyme present in the cell-free filtrate is usually low, the removal of water is a primary objective[101-104]. Various techniques have been employed for removal of water molecule such as membrane based ultrafiltration, and temperature-sensitive hydrogel ultrafiltration for concentrating an alkaline protease[105] and precipitation (salt based and organic-solvent based) which is the most commonly used method for the isolation and

recovery of proteins from crude biological mixtures[106]. It also performs both purification and concentration steps (Table 1.6).

Alkaline proteases are generally positively charged and are not bound to anion exchangers[98,107,108]. Therefore, cation exchangers can be a rational choice and the bound molecules can be eluted from the column by an increasing salt or pH gradient[109]. Reports on the purification of alkaline proteases by different affinity chromatographic methods showed that an affinity adsorbent hydroxyapatite was used to separate the neutral protease[110] as well as purify the alkaline protease from a *Bacillus* sp.[111]. Other affinity matrices used were Sephadex-4-phenylbutylamine[112], casein agarose[113,114], or N-benzoyloxycarbonyl phenylalanine immobilized on agarose adsorbents[115]. However, the major limitations of affinity chromatography are the high cost of enzyme supports and the labile nature of some affinity ligands, which make them unrecommendable for use at industrial scale. This technique has been applied for purification of alkaline proteases using mixtures of polyethylene glycol (PEG) and dextran or PEG with salts such as H_3PO_4, $MgSO_4$[116-118].

In addition, other methods, such as the use of reversed micelles for liquid–liquid extraction[119], affinity precipitation, and foam fractionation[120] have also been employed for the recovery of alkaline proteases. The enzyme preparations used commercially are impure and are standardized to specified levels of activity by the addition of diluents and carriers. Further, the conditions for maximum stability of crude preparations may be quite different than for purified enzymes. Because loss of activity is encountered during storage in the factory, shipment to client(s) and/ or storage in client's facilities, storage stability is of prime concern to enzyme manufacturers. Protease solutions are subject to proteolytic and autolytic degradation that results in rapid inactivation of enzymatic activity. To maintain the enzyme activity and provide stability, addition of stabilizers like calcium salts, sodium formate, borate,

propylene glycol, glycerine or betaine, polyhydric alcohols, protein preparations, and related compounds have proved successful[121,122]. Also, to prevent contamination of the final commercial crude preparation during storage, addition of sodium chloride at 18–20% concentration has been suggested[67,123]. In certain cases, for the purpose of convenience in handling and storage, the liquid enzyme preparations are often converted to powder form. However, the handling of dry enzymes poses potential health hazards and therefore, it is customary to maintain the enzyme preparations in stabilized liquid form[11,124]

Table 1.6. Combination of chromatographic techniques applied for purification of microbial alkaline proteases

Micro-organisms	Concentration method	Column matrices	References
Clostridium sp.SPA3	Ammonium sulfate fractionation ,Dialysed and lyophilisation	Sephadex G-100 gel filtration column	Alam *et al*[125]
B. mojavensis	Ammonium sulfate saturation (0–85%),	anion-exchanger Q-sepharose column	Beg and Gupta,[126]
Engyodontium album BTMFS10	(NH4)2SO4 precipitated fraction (40–90%)	DEAE-cellulose column	Chellappan *et al* [127]
Aspergillus clavatus ES1	acetone precipitation	Sephadex G-100 gel filtration and CM-Sepharose ion exchange chromatography	Hajji *et al*[128]
Bacillus mojavensis A21	acetone precipitation	Sephadex G-75 gel filtration and CM-Sepharose ion exchange chromatography	Haddar *et al*[96]
Periserrula leucophryna	ammonium sulphate fractionation	gel filtration, ion exchange and Benzamidine-Sepharose chromatography	Joo *et al*[129]
Bacillus pseudofirmus	Not specified	Phenyl Sepharose 6 Fast Flow column.	Gupta *et al*[130]
Bacillus cereus	ammonium sulfate precipitation	ion exchange chromatography	Doddapaneni *et al*[131]
Haloalkaliphilic bacterium sp. AH-6	Ammonium sulphate precipitation	Phenyl sepharose 6FF HIC	Dodia *et al*[132]

Micro-organisms	Concentration method	Column matrices	References
Bacillus pumilus MS-1	Not specified	DEAE-cellulose (DE-52), CM-Toyopearl 650	Miyali *et al*[133]
Bacillus subtilis PE-11	Ammonium sulphate , dialyzed	Sephadex G-200	Adinarayana *et al* [134]
Botrylis cinerea	Ammonium sulphate precipitation	Sephacryl S-200 HR, DEAE sepahrose chromatography, HLPC with DEAE-Progel TSK, Gel filtration on TSK SW-200 HLPC	Abidi *et al*[135]
Bacillus licheniformis AP-1	Ammonium sulphate precipitation	DEAE Sephadex A50, CM- Sephadex C50, Sephadex G75	Tang *et al*[136]

1.12 Physiological functions of proteases

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. In general, extra-cellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell, whereas intracellular proteases play a critical role in the regulation of metabolism. Some of the major activities in which the proteases participate are described below.

Catabolism of proteins provides a ready pool of amino acids as precursors for the synthesis of proteins. Intracellular proteases such as ATP-dependent proteases in *E. coli*[137] and eukaryotes[138] are known to participate in executing the proper protein turnover for the cell. Formation of spores in bacteria[139], ascospores in yeast[140], fruiting bodies in slime moulds[141] and conidial discharge in fungi[142] involve intensive protein turnover. The alkaline serine protease of *Conidiobolus coronatus* was shown to be involved in forcible conidial discharge using a less conidia forming mutant. Formation of the less active protease by auto proteolysis represents a novel means of physiological regulation of protease activity in *Conidiobolus coronatus*[143]. Degradation of proteins in dormant spores by serine endoproteinases makes amino acids and nitrogen available for the biosynthesis of new proteins and nucleotides. Their activity is rapidly lost on germination of the spores[144]. Microconidal germination and hyphal fusion[145], also involves participation of a specific alkaline serine proteinase.

Extracellular acid proteases are used in the breakage of cell wall polypeptide linkages during germination of *Dictyostelium discoideum* spores[146] and *Polysphondylium pallidum* microcysts[147]. Activation of zymogenic forms of chitin synthase by limited proteolysis has been observed in *Candida albicans*, *Mucor rouxii* and *Aspergillus nidulans*. Kex-2 protease catalyses the hydrolysis of prohormones, integral

membrane proteins of the secretory pathway by specific cleavage at the carboxyl side of pairs of basic residues such as Lys-Arg or Arg-Arg[26]. Pepsin, trypsin and chymotrypsin occur as their inactive zymogenic forms, which are activated by the action of proteases. Proteinases A and B from yeast inactivate several enzymes in a two-step process involving covalent modification of proteins as a marking mechanism for proteolysis. Proteolytic modification of enzymes is known to result in a protein with altered physiological function e.g. leucyl-L-RNA synthetase from *E. coli* is converted into an enzyme which catalyzes leucine-dependent pyrophosphate exchange by removal of a small peptide from the native enzyme.

The extracellular enzymes play a major role in nutrition due to their depolymerizing activity. The microbial enzymes and the mammalian extracellular enzymes such as those secreted by pancreas are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition.

Modulation of gene expression mediated by protease has been demonstrated[148]. Proteolysis of a repressor by an ATP-requiring protease resulted in a de-repression of the gene. Change in the transcriptional specificity of the B-subunit of RNA polymerase in *Bacillus thuringiensis* was correlated to its proteolytic modification[149]. Modification of ribosomal proteins by proteases has been suggested to be responsible for the regulation of translation. Besides the general functions that are described so far, the proteases also mediate the degradation of a variety of regulatory proteins that control the heat shock response, the SOS response to DNA damage, the life cycle of bacteriophage[150] and programmed bacterial cell death[151].

Recently, a new physiological function has been attributed to the ATP-dependent proteases conserved between bacteria and eukaryotes. It is implied that they act as chaperones and mediate not only proteolysis

but also the insertion of proteins into membranes and disassembly or oligomerization of protein complexes [152].

1.13 Immobilization

Microorganisms in nature are irregularly distributed and often exist in Biofilms. Biofilms are surface-attached microbial communities consisting of multiple layers of cells embedded in hydrated matrices. In all known habitats, bacteria preferentially reproduce on a surface rather than in suspension in the liquid phase[153]. Biofilms were first extensively studied during the 1940s when it was not until the 1970s that it was appreciated that their formation occurs in almost all natural environments. A rock immersed in a stream, an implant in the human body, a tooth, a water pipe or conduit, etc. are all sites where Biofilms develop. This natural phenomenon encouraged human to utilize it for similar services. Since that time many studies have been undertaken on immobilized cells and its industrialization.

1.13.1 Historical aspect of immobilized enzymes

In 1916 it was reported that an enzyme in water-insoluble form inhibited catalytic activity. They illustrated that invertase extracted from yeast was adsorbed on charcoal, and the adsorbed enzyme showed the same activity as native enzyme[154]. In 1948 it was found that urease from jack bean became water- insoluble on standing in 30% alcohol and sodium chloride for 1-2 days at room temperature, and the water-insoluble urease was active . Thus, it has been known for a long time that enzymes in water-insoluble form show the catalytic activity[155]. However the first attempt to immobilize an enzyme to improve its properties for a particular application was not made until 1953, when enzymes such as carboxypeptidase, diastase, pepsin and ribonuclease was immobilized by using diazotized polyaminopolystyrene resin. On the other hand, prior to this (1949), immobilization of physiologically active protein was carried out[156,157]. In 1973 scientists succeeded in the

first industrialization of continuous process for production of L-aspartic acid using immobilization.

1.13.2 Classification of immobilized methods and selection of carrier

The bio-immobilization methods include adsorption, covalent bond combination, crosslink and embedment. Their principles and characteristics are shown in Table 1.7.

Table 1.7 Comparisons of Different Immobilization Methods

Methods	Principles	Carriers	Characteristics
Carrrier combination (covalent, electrovalent bond, physical adsorption, etc.)	Covalent bond, electrovalent bond or molecule gravitation formed between cells and carriers	Lacunaris glass bead, alumina,active carbon, kaoline, cellulose, collagen etc.,	Easy preparation; low fastness; cell's quantity, exhibited activities and life are greatly affected by carriers.
Crosslink	Crosslink between cell and reagent with two or more function	Glutaraldehyde, bis-diazo biphenylamine	Difficulty in execution, intense
Embedment	Embedded cells in gelatin grid or capsule composed of semipermeable membrane	Agar, polyurea, alginate polyvinyl alcohol (PVA) etc.	Easy operation ; high intensity of immobilized particles; suitable for the reaction between small molecule substrate and production

1.13.3 Main Application Areas of Immobilization Technology

Advances in biotechnology using immobilization techniques have shown that conditions can be modified to enhance the activity of specific desirable reactions or chemical process in extreme environments[157]. This advantage of immobilization opened the gate for immobilization technology to be applied in every field in our life

1.13.3.1 Bioprocess Technology

Historically, the most important area of immobilization is namely brewing, antibiotics, mammalian cell culture, etc; extensive development is in progress with new products envisaged, namely *polysaccharides, medically important drugs, solvents & protein-*enhanced foods. Novel fermenter designs are being used to optimize productivity.

1.13.3. 2 Enzyme Technology

Used for the catalysis of extremely specific chemical reactions; immobilization of enzymes; to create specific molecular converters (bioreactors).Products formed include-amino acids, high fructose syrup, semi-synthetic penicillin, starch and cellulose hydrolysis, etc.,

1.13.3.3 Waste Technology

Greater emphasis is now being made to couple the process of waste management & bio-immobilization with the conservation and recycling of resources-foods and fertilizers & biological fuels.

1.13.3.4 Environmental Technology

Great scope exists for the application of immobilization concepts for solving many environmental problems-pollution controls,

1.14 Industrial application of microbial proteases

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology.

There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community. The extra cellular proteases particularly of microbial origin have been commercially exploited to catalyze protein / peptide degradation in various industrial processes[158-160].Alkaline protease used in various industrial segments and their applications are shown in Table1.8[9]

Table 1.8 Commercial application of alkaline protease in various biotech industries.

Industry	Type of protease	Applications
Detergent industries	Alkaline protease, subtilisin	Laundry detergent for protein stain removal
Dairy industries	Fungal protein chymosin other protease	Replacement of calf rennet, whey protein processing. Production of enzyme modified cheese (EMC)
Beverage industries	Papain	Chill profile removal of haze in beverages
Baking industries	Neutral protease	Dough conditioner.
Food processing	Several protease	Modification of protein rich nature
Leather industries	Trypsin , other proteases	Bating of leather, dehairing of skins
Meat and fish	Papain, other proteases	Meat tenderization, recovery of protein -

Industry	Type of protease	Applications
		-from bones and fish waste
Medicine	Trypsin	Dead tissue removal, blood clot dissolution
Photography	Several proteases	Recovery of silver from X-ray and photographic films
Sweetner	Thermolysin	Reverse hydrolysis in aspartame synthesis

Alkaline proteases account for a major share of the enzyme market all over the world[18,19]. Alkaline proteases from bacteria find numerous applications in various industrial sectors and different companies worldwide have successfully launched several products based on alkaline proteases (Table 1.8). The bulk uses of alkaline proteases in industrial sectors are described in the following section.

1.14.1 Food and feed industry

Traditionally, microbial proteases have been exploited in the food industries in many ways. Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks[161, 162]. The basic function of proteases is to hydrolyze proteins; and this property has been exploited for the preparation of protein hydrolysates of high nutritional value. The alkaline proteases are used in hydrolysate production from various natural protein substrates. Keratinolytic activity of alkaline protease has also been exploited in the production of proteinaceous fodder from waste feathers or keratin-containing materials. Dalev[163,164] and Cheng *et al*[165] reported the use of alkaline proteases (B72 from *B. subtilis* and *B. licheniformis* PWD-1) for the

hydrolysis of feather keratin, to obtain a protein concentrate for fodder production.

1.14.2 Peptide synthesis

Proteases in organic synthesis have attracted a great deal of attention in recent years[166,167]. Under normal aqueous conditions, proteases catalyze the hydrolysis reaction, but the reverse actions of proteases have been found in water-restricted media such as synthesis of peptides and esters[167,168]. There are a great many advantages associated with the application of enzymes to organic synthesis including enantioselectivity, specificity, nonhazardous reaction conditions and so on[167,169]. These approaches require enzymes that are stable in the presence of organic solvents, but enzymes are generally very labile catalysts and easily lose their activities in non-aqueous media. Several current techniques such as medium engineering, substrate engineering and protein engineering have been employed to improve enzymes for synthesis in organic solvent[166,167]. However, if enzymes were naturally stable and active in hostile environments, they would be very useful for organic synthesis. Recently, solvent-tolerant bacteria as a relatively novel group of extremophilic microorganisms with unique ability to live in presence of organic solvents have attracted the great attention of many researchers. Some of these microbes have been found to be sources of solvent-stable enzymes, but only a few are reports available in the literature concerning the screening of organic solvent-stable protease producers[170-173]. At the same time, solvent-tolerant bacteria usually secrete protease in low yield. However, if the solvent-stable proteases could be used for high-yield production, they would be more suitable for industrial applications.

Since the first report of Bergman and Frankel-Conrat[174], on protease-catalyzed peptide synthesis using the reverse-enzymatic reaction of hydrolysis, the proteases have frequently been used for peptide synthesis[175-178]. Enzymatic peptide synthesis offers several advantages

over chemical methods, e.g. reactions can be performed stereo-specifically and reactants do not require side-chain protection, increased solubility of non-polar substrates, or shifting thermodynamic equilibria to favor synthesis over hydrolysis. There is less need for expensive protecting-groups, organic solvents, or hazardous chemicals, resulting in production costs competitive with those of chemical methods[178]. However, the major limitation for the use of protease in synthetic chemistry is the strongly reduced activity of the enzyme under anhydrous conditions.

1.14.3 Leather industry

Leather processing involves several steps such as soaking, dehairing, bating, and tanning. The major building blocks of skin and hair are proteinaceous. The conventional methods of leather processing involve hazardous chemicals such as sodium sulfide, which create problems of pollution and effluent disposal[179-181]. The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution[181]. Proteases are used for selective hydrolysis of noncollagenous constituents of the skin and for removal of nonfibrillar proteins such as albumins and globulins. The purpose of soaking is to swell the hide. Traditionally, this step was performed with alkali. Currently, microbial alkaline proteases are used to ensure faster absorption of water and to reduce the time required for soaking. The conventional method of dehairing and dewooling consists of development of an extremely alkaline condition followed by treatment with sulfide to solubilize the proteins of the hair root. At present, alkaline proteases with hydrated lime and sodium chloride are used for dehairing, resulting in a significant reduction in the amount of wastewater generated[180] .Currently, trypsin is used in combination with other *Bacillus* and *Aspergillus* proteases for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin, and the amount of enzyme needed depends on the type of leather (soft or hard) to be produced. Increased usage of enzymes for

dehairing and bating not only prevents pollution problems but also is effective in saving energy. Novo Nordisk manufactures three different proteases, Aquaderm, NUE, and Pyrase, for use in soaking, dehairing, and bating, respectively.

1.14.4 Management of industrial and household waste

Proteases solubilize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era in the use of proteases in waste management. Recently, poultry wastes (chicken feather) generation rate is increasing everyday and reaching a million ton per years, this creating serious environmental problems[182,183]. Waste feathers make up approximately 5% of the body weight of poultry and are considered to be a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. The use of keratinolytic protease in food and feed industry waste, for degrading waste keratinous material from poultry refuse[184] and as depilatory agent to remove hair from the drains[185] has been reported.

1.14.5 Proteases in the detergent industry

Enzymes have long been of interest to the detergent industry for their ability to aid in the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies[11,160,186]. Applications of detergent proteases have grown substantially and the largest application is in household laundry detergent formulations[160,186]. The increased reliance of detergent manufacturers on enzyme technology is because of consumer-recognizable cleaning benefits, the addition of completely new performance benefits, fabric restoration and an increased performance/cost ratio, because of the availability of more efficient enzymes and the industry trend toward reduced pricing. Current market

trends and consumer needs are influencing the development of enzymes for detergent applications, with the emphasis on enzymes that have improved performance/cost ratios, lower washing temperature, alteration in the detergent compositions with regard to the use of environment-compatible non-phosphate builders like zeolites, and the bleaching agents. In addition, enzyme suppliers and detergent manufacturers are actively pursuing the development of new enzyme activities that address the consumer-expressed need for improved cleaning, fabric care and antimicrobial benefits[160,187,188]. However, apart from their use in laundry detergents, they are also popular in the formulation of household dish- washing detergents and both industrial and institutional cleaning detergents[11,189].

1.14.6 Selection and evaluation of detergent protease performance

One of the important parameters for selection of detergent proteases is the pH value. It is known that detergent proteases perform best when the pH value of the detergent solution in which it works is approximately the same as the pH value for the enzyme. However, there are many more parameters involved in the selection of a good detergent protease, such as compatibility with detergent components, e.g. surfactants, perfumes and bleaches[190-192], good activity at relevant washing pH and temperature[193-195], compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and shelf life[196]. Over the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents. Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of alkaline proteases which are effective over a wide temperature range[195]. In addition, the current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the

use of low washing temperatures[197-199]. This has pushed enzyme manufacturers to look for novel enzyme that can act under low temperatures.

Further, a good detergent enzyme should also be stable in the presence of oxidizing agents and bleaches. In general, the majority of the commercially available enzymes are not stable in the presence of bleaching/oxidizing agents. The evaluation of detergent proteases is mainly dependent upon parameters such as the pH and ionic strength of the detergent solution, the washing temperature and pH, mechanical handling, level of soiling and the type of textile. In the case of laundry detergents, protease performance is evaluated by using soiled test-fabrics and the efficiency is measured either visually or by measuring the reflectance of light under standard conditions[11,160].

1.15 Major obstacles and future prospect of microbial proteases

Proteases are a complex group of enzymes which differ in their properties such as substrate specificity, active site, and catalytic mechanism. Their exquisite specificities provide a basis for their numerous physiological and commercial applications. Despite the extensive research on several aspects of proteases from ancient times, there are several gaps in our knowledge of these enzymes and there is tremendous scope of improving their properties to suit projected applications. The future lines of development would include (i) designing of an appropriate screening conditions for isolation of alkaline proteases producing promising microbes,(ii) development of the economical fermentation method(s), (iii) exploration of protease suitable for application in industries such as detergent, leather, food, pharmaceuticals.(iv) physiological role of protease isoenzymes in the growth and development of producing bacteria.

The cost of enzyme production is a major obstacle in the successful application of proteases in industry. Protease yields have been improved by screening for hyper producing strains and /or by

optimization of the fermentation medium. Strain improvements by either conventional mutagenesis or recombinant-DNA technology have been useful in improving the production of proteases.Increase in the yield of viral proteases are particularly important for developing therapeutic agents against devastating diseases such as malaria, cancer, and AIDS[23].

Industrial applications of proteases have posed several problems and challenges for their further improvements. The biodiversity represents an invaluable resource for biotechnological innovations and plays an important role in the search for improved strains of micro organisms used in the industry. A recent trend has involved conducting industrial reactions with enzymes reaped from exotic micro organisms that inhabit hot waters, freezing Arctic waters, saline waters, or extremely acidic or alkaline habitats. The proteases isolated from extremophilic organisms are likely to mimic some of the unnatural properties of the enzymes that are desirable for their commercial applications. Exploitation of biodiversity to provide micro organisms that produce proteases well suited for their diverse applications is considered to be one of the most promising future alternatives. Introduction of extremophiles proteases into industrial processes is hampered by the difficulties encountered in growing the extremophiles as laboratory cultures. A revolutionary robotic approach such as DNA shuffling is being developed to rationalize the use of enzymes from extremophiles. The existing knowledge about the structure-function relationship of proteases complies / suggests a fair chance of success, in the near future, in evolving proteases that were never made in nature and that would meet the requirements of the multitude of protease applications[23].

1.16 Aims and objectives of present study

Alkaline active and/or alkali stable protease(s) have gained commercial importance because of their potential applications in textile, paper/pulp, and detergent industries. North-eastern part of India is considered as

one of the mega biodiversity zones of the world. Exploration of microbial diversity of these regions is possible only by screening the soil /water samples for industrial purpose in the development of biotechnological products and processes. The present study was undertaken to isolate alkaline active and alkali stable protease (s) from the promising microbe(s) of NE India. Alkalothermophilic micro organisms growing in alkaline habitats in nature were screened for selection of promising cultures that can produce extra cellular proteases of desired properties. Another main segment of the present study was statistical optimization of culture conditions during fermentation, evaluation of the biochemical and pharmacological properties and assessment of biotechnological potential of the crude/purified alkaline proteases produced by promising bacterial strains isolated from natural habitat's of North-eastern India.

The objectives of the present investigation were as follows.

1. Screening of microorganism from various environmental samples collected from various regions of northeast India, for the production of alkaline protease under thermophilic condition.

2. Obtaining pure culture and taxonomic identification of protease producing potential microbes.

3. Optimization of culture conditions of potential microbes for maximizing protease production using solid state fermentation (SSF) and submerged fermentation (SmF) systems.

4. Isolation, downstream processing and biochemical characterization of an alkaline protease produced by the most promising strain(s).

5. To investigate the possible industrial application of the isolated alkaline proteases.

CHAPTER II

REVIEW OF LITERATURE

Chapter 2

Review of literature

2. Screening and detection of protease producing potential micro-organisms

Of the entire alkalophilic microorganism that have been screened for use in various industrial applications, members of the genus *Bacillus* are found to be a predominant and prolific source of alkaline proteases (Table 2.1). However, a few exceptions are *Nocardiopsis* sp.[200], *Salinivibrio* sp. strain AF-2004[201], *Clostridium* sp.[202], *Vibrio fluvialis* TKU-005[203], *Aureobasidium pullulans*[204], *Streptomyces* sp. CN902[205] etc., which are also recognized as promising microbes for alkaline protease production. In addition to above microbial strains, *Monascus purpureus* CCRC31499[206], *Exiguobacterium* sp. YS1[207], *Vibrio metschnikovii* J1[208], etc., were also characterized for alkaline protease production.

Doddapaneni and his colleague's[131] isolated an alkaline protease producing bacterial strain *Bacillus cereus* from slaughter house in Hyderabad city, Andhra pradesh, India. A halophilic strain *Bacillus proteolyticus* CFR3001 isolated for protease production from coastal region of Gujarat, Western India was reported by Patel *et al*[209]. Recently, Shanmughapriya *et al*[210] reported an alkaline protease producing gram-negative strain *Roseobacter* sp. (MMD040) isolated from marine sponge *Fasciospongia cavernosa* originated from peninsular coast of Vizhinjam, Kerala, India. *Halogeometricum* sp. TSS101 is a protease producing strain isolated by Vidyasagar and his college[211] from solar evaporated salt pond situated in Tuticorin, on the coast of Tamil nadu, India. Shikha *et al*[212] reported in the year of 2007, an alkaline protease producing strain *Bacillus pantotheneticus* isolated from saline-alkali soil of the Avadh region Uttar Pradesh, India (Table 2.2).

Table 2.1 Screening results of isolated alkaline proteases from different environmental samples reported from abroad.

Microorganisms	Sources	Geographical Location	References
	Internationally reported protease producing microbes		
Nocardiopsis sp.	Soil sample	Northeast of Brazil	Moreira *et al*[200]
Bacillus clausii	Heavily polluted tidal mud	Korean yellow sea near Inchon City, Korea	Kumar *et al*[213]
Bacillus polymyxa	Alkaline soil sample	Isfahan, Iran	Emtiazi *et al*[214]
Clostridium sp.	Lake sediment sample	Schirmacher oasis, Antarctica.	Alam *et al*[202]
Euphorbia amygdaloides	Land of Erzurum	Turkey	Demir *et al*[215]
Pseudomonas aeruginosa strain K	Contaminated soils of a wood factory	Selangor, Malaysia	Rahman *et al*[216]
Bacillus subtilis and *Bacillus cereus*	Waste -discharge area of the leather industry	Turkey	Orhan *et al*[217]
Aspergillus fumigatus	Marine food processing industry	Taiwan	Wang *et al*[218]
Dactylella shizishanna	Soil samples in Hubei Province	China	Wang *et al*[219]

Microorganisms	Sources	Geographical Location	References
Bacillus sp. TKU004	Soil sample	Taiwan	Wang *et al*[220]
Monascus purpureus CCRC31499	Marine food processing industry werewashed	Taiwan	Liang *et al*[206]
Bacillus sp.	Extreme alkaline conditions	Izmir, Turkey	Genckal and Tari[221]
B.clausii I-52	Heavily polluted tidal mud	West sea of Inchon, Korea	Joo and Chang[187]
Bacillus sp. HR-08 and KR-8102	Soil samples were collected from the rhizospher and rhizoplane zone	Hamadan, Iran	Moradian *et al*[222]
Aureobasidium pullulans	Seawater and sediment ; hypersaline sea water and sediments of the salterns around	Southern sea of China and the pacific ocean ; coastal line of Qingdao	Chi *et al*[223]
Geobacillus sp. YMTC 1049	Hotspring in Rehai, Tengchong, Yunnan Province,	China	Zhu *et al*[224]
Vibrio fluvialis TKU005	Seawater sample of Tamsui River near Tamkang University	Taipei, Taiwan	Wang *et al*[203]

Microorganisms	Sources	Geographical Location	References
Salinivibrio sp. strain AF-2004	Hypersaline lake with 17% (w/v) total salt	Baktegan south of Iran	Karbalaei-Heidari *et al*[201]
Bacillus sp.	Soil samples were collected from sandy soil, milk processing plant, the drainage of a slaughterhouse and	China	Chu,[204]
Bacillus cereus	Soil sample	Tehran, Iran	Oskouie *et al*[87]
Bacillus pumilus	Contaminated soils of a wood factory	Selangor, Malaysia	Rahman *et al*[173]
Vibrio fluvialis TKU-005	Seawater sample of Tamsui River	Tamkang University, Taipei	Wang *et al*[203]
Colwellia sp. NJ341	Not specified	China	Wang *et al*[225]
Bacillus cereus SV1	Oil sewage station from a fishing port	Sfax in Tunisia	Manni *et al*[226]
Bacillus sp. HS08	Soil sample	Toulufan crater, china	Guangrong *et al*[89]
Bacillus licheniformis BA17	-do-	-do-	Nikerel *et al*[227]
Pseudomonas aeruginosa MN7	Alkaline waste water of a tannery.	Sfax Tunisia	Jellouli *et al*[228]
Bacillus sp. RKY3 KCTC 10412BP	Soil	Republic of Korea	Reddy *et al*[90]

Microorganisms	Sources	Geographical Location	References
Pseudomonas aeruginosa	Soil samples were collected from a crude oil contaminated environment located	Jiangsu province, China	Tang *et al*[229]
Lactobacillus helveticus	Kefir	Greece	Valasaki *et al*[230]
Lactobacillus paracasei subsp *paracasei* TKU010	Infant vomited	Taiwan	Wang *et al*[231]
Chryseobacterium indologenes TKU014	Soil sample	Taiwan	Wang *et al*[232]
Chryseobacterium taeanense TKU001	Soils by using red-koji rice	Taiwan	Wang *et al*[233]
Bacillus cereus TKU006	Soil sample	Taiwan	Wang *et al*[234]
Bacillus pumilus	Not specified	Not specified	Wan *et al*[235]
Bacillus sp. HR-08	Soil samples were collected from rhizosphere and rhizoplane zone of	Hamadan, Iran.	Moradian *et al*[236]
Vibrio metschnikovii J1	Alkaline waste- water of soap industry in	Sfax, Tunisia	Jellouli *et al*[208]
Streptomyces sp. CN902	Soil sample	Tunisian	Lazim *et al*[205]
Exiguobacterium sp. YS1	Soil sample	South Korea	Lee *et al*[207]

Microorganisms	Sources	Geographical Location	References
Bacillus pseudofirmus Mn6	Olive filed soil sample	El-Menia governorate, Egypt	Abdel-Fattah *et al*[93]

Table 2.2 Protease producing microbes reported from India.

Microorganisms	Sources	Geographical Location	References
	Nationally reported protease producing microbes		
Arthrobacter ramosus, MCM B-351	Sediment sample of alkaline lake	Lonar	Nilegaonkar *et al*[237]
Bacillus pseudofirmus	Haloalkaliphilic bacteria (isolate Vel)	west coast of India	Gupta *et al*[238]
Bacillus sp.	-do-	-do-	Prakasham *et al*[239]
Conidiobolus coronatus	Isolated by overlaying fine particles of plant detritus	Anekal , Karnataka	Laxman *et al*[240]
Bacillus sp.	Soil samples were collected from Nellore	Andhra Pradesh	Naidu and Devi[241]
Halogeometricum sp. TSS101	Solar evaporated salt pond	Tuticorin, coastal area of Tamilnadu	Vidyasagar, *et al*[211]
Pseudomonas aeruginosa	Soil samples were collected from the proximity of a solvent extraction unit	New Delhi	Gupta and Khare[172]

Microorganisms	Sources	Geographical Location	References
Engyodontium album BTMFS10	Marine sediment	Cochin,	Chellappan *et al*[127]
Bacillus sp. Po2	Seawater sample of porbandar	Coastal region of the Gujarat, Western India.	Patel *et al*[209]
Bacillus pantotheneticus	Saline-alkali soil of the Avadh region	Uttar Pradesh	Shikha *et al*[212]
Exiguobacterium sp. SKPB5 (MTCC 7803)	Soil samples were	Western Himalayas (Himachal Pradesh)	Kasana *et al*[242]
Bacillus proteolyticus CFR3001	Fish processing wastes	India	Bhaskar *et al*[243]
Bacillus cereus MCM B-326	Not specified	Not specified	Zambare *et al*[244]
Bacillus sp	Soil sample	India	Saran *et al*[88]
Bacillus circulans	Not specified	Not specified	Jaswal *et al*[245]
Pseudomonas aeruginosa	The limed animal fleshing	Chennai	Kumar *et al*[246]
Halophilic bacterium MBIC3303	Sea water samples	Costal Gujarat	Joshi *et al*[247]
Pseudomonas aeruginosa PseA	Not specified	New Delhi	Mahanta *et al*[248]

Microorganisms	Sources	Geographical Location	References
Roseobacter sp. (MMD040)	Marine sponge *Fasciospongia cavernosa*,	peninsular coast of India (Vizhinjam)	Shanmughapriya *et al*[210]
Penicillium godlewskii SBSS 25	Soil samples	Southern Kerala	Sindhu *et al*[249]
Bacillus cereus	Slaughterhouse waste samples,	Hyderabad	Doddapaneni *et al*[131]

A survey of literature demonstrated that many protease producing microbes were isolated from various regions of India, ***but to the best of our knowledge there is no report on alkaline protease producing microbes from North east India,*** and this has prompted us to explore the industrially important microbes from North-Eastern region of India.

2.1 A brief review on alkaline protease production by solid-state fermentation (SSF) and submerged fermentation (SSF) system

The rapid development of biotechnology has impacted diverse sectors of the economy over the last several years. The industries most benefited are the agricultural, fine chemical, food processing, marine, and pharmaceutical. In order to continue revolutionizing the industries, new processes must be developed to transform current biotechnological research into viable market products. Specifically, attention must be directed toward the industrial processes of cultivation of microorganisms and enzymes.

Commercial production of protease is carried out in various ways, and different the environmental parameters essential for the optimum growth of micro-organism are being employed for enzymes production. These parameters include nutrient supplementation, pH of the medium, osmotic relationship, degree of aeration, temperature and the control of contamination during fermentation. Maintaining the purity of the medium is also a very important factor, especially when the fermentation is carried out under aerobic conditions. Though the details of the specific fermentation processes adopted by different manufacturers vary, two main methods are followed for protease production viz submerged fermentation and solid–state fermentation[250,251].

2.1.1 Solid-state fermentation (SSF) systems

The term solid state fermentation (SSF) is applied to the processes in which water insoluble materials are used for the microbial growth[252,253]. In the fermentative processes of this type, the quantity of water should

not exceed the capacity of saturation of the solid bed in which the microorganisms grow[252,253]. Water is essential for the microbial growth and enzyme production in SSF system, and it is present in thin layers and sometimes, absorbed inside the substrates [252,253].

Solid-state fermentation has gained interest from industrialists and researchers for the production of these enzymes in view of its several economic and technological advantages and has been often employed to produce protease. Some advantages and disadvantages of the SSF are presented in Table 2.3.

Table 2.3 Advantages and disadvantages of the SSF system.

Solid-state fermentation	
Advantages	**Disadvantages**
The culture media are simple. Some substrates can be used direclty as a solid media or enriched with nutrients	The used microorganisms are limited to those that grow in reduced levels of humidity
The product of interest is concentrated, this facilitates its purification	The determination of parameters such as humidity, pH, free oxygen and dioxide of carbon, constitute a problem due to the lack of monitoring devices
The used inoculum is the natural flora of the substrates, spores or cells	The scale up of SSF processes has been little studied and it presents several problems
The low humidity content and the great inoculum used in a SSF reduce the possibility of microbial contamination	
The quantity of waste generated is smaller than the SmF	
The enzymes show low sensitive to	

Solid-state fermentation	
Advantages	**Disadvantages**
catabolic repression or induction	

Apart from above mentioned advantages this system also displayed excellent low humidity level demonstrating formation of gradient temperature, nutrients and products and also, sporulation mechanisms as well as the production of enzymes and secondary metabolites in SSF systems.

2.1.2 Solid-supports in SSF systems

Two types of SSF systems can be distinguished depending on the nature of the soild phase used. The first and the most commonly used system involves cultivation on a natural material as a substrate (Table 2.4). The second system, which is not as frequently used, involves cultivation on an inert support impregnated with a liquid medium[254].

SSF cultivation on natural substrates uses natural materials as substrate that serve both as support and a nutrient source (Table 2.4). Substrate from agricultural or industrial wastes (wheat straw or barley, sugar cane bagasse, coffee pulp, grape wastes, copra pasta, among other) or inert materials (such as resins of ionic exchange, acreolite or poliurethane foam) can be used (Table 2.4). The pretreatments of these materials is really few, generally a milled previous wash.

Table 2.4 Use of solid substrates as support for protease production in SSF systems

Substrates	Applications	References
Wheat bran	Acidic protease production	Germano *et al*[255]
Wheat bran	Alkaline protease production	Agrawal *et al*[256]

Substrates	Applications	References
Wheat bran and lentil husk	Alkaline protease production	Uyar and Baysal[257]
Wheat bran	Neutral protease production	Sandhya *et al*[258]
Soybean	Alkaline protease production	Elibol and Moreira[259]
Soybean and wheat flours	Protease production	Wang *et al*[260]
Soy cake	Protease production	Valeria *et al*[261]
Wheat bran	-do-	Chellappan *et al*[127]
Green gram husk	-do-	Prakasham *et al*[262]
Pre -filtered palm oil mill effluent (POME)	Protease production	Wu *et al*[263]
Jatropha seed cake	Protease production	Mahanta *et al*[248]
Untanned proteinaceous tannery solid waste, animal fleshing (ANFL)	Acidic protease	Kumar *et al*[246]
Mirabilis jalapa tubers powder (MJTP),	Alkaline protease production	Hajji *et al*[128]
Dhal husk	Milk clotting protease production	Sathya *et al*[264]
Wheat bran (WB) with chopped date stones (CDS)	Alkaline protease production	Lazim *et al*[205]
Hulled grain of wheat (HGW)	Alkaline protease production	Haddar *et al*[265]

2.2 Protease production under SSF system using agro-industrial wastes

Protease production is a major area of research globally, and with the rejuvenated interest created due to their applications in different bio-

formulation sectors, several investigators are working worldwide on various aspect of microbial protease[266]. Production of low titers of protease has always been a major concern and thus several workers are trying to improve the production titers by adopting multifaceted approaches, which include the use of better bioprocess technologies, using cheaper or crude raw materials as substrates for enzyme production, bioengineering the microorganisms, etc[267].

In order to obtain high and commercially viable yields of protease, many workers have been using agro-industrial waste as a cheaper carbon and nitrogen sources for protease/keratinase production under solid-state fermentation system (Table 2.5 and Table 2.6). Among various agro-industrial waste, wheat bran supporting substrates influences the protease/keratinase production under SSF by many microbes as shown in Table 2.5 and Table 2.6. Hongzhang *et al*[268] reported protease production under SSF by *Bacillus Pumilus* AS 1.1625 using synthetic supporting material such as polyurethane foam incubated at 32°C for 96h. Mahanta and his colleague's[248] reported the use of *Jatropha curcas* seed cake as a solid support for protease production by *Pseudomonas aeruginosa* PseA strain under SSF system.

Recently in the year 2009, use of green gram husk for protease production by *Bacillus* sp., was studied by Prakasham and his colleague's[269]. *Bacillus Megaterium* NCIM-2087 displayed an excellent protease production in a medium supplemented with soy cake post 60h of incubation[270]. *Mucor circinelloides,* a well known fungal species supported a protease production in the presence of lentil (dhal) husk post 120h of incubation at 30°C[264]. Paranthaman *et al*[271] reproted neutral protease production using rice mill wastes by a fungal species *Aspergillus niger* post 96h of incubation at 35°C with a maximum yield of 67.7 U /gds (Table 2.5).

Table 2.5 Alkaline protease production under solid-state fermentation system.

Micro-organisms	Substrates	pH	Moisture level (In %)	Innoculum level (In %)	Incubation temperature (In °C)	Incubation time (h)	Enzyme activity (U/gds)	References
Penicillium sp.	Defatted soybean cake supplemented	5.0	55.0	108 spores/g dry substrate	28.0	48	43.0	Germano *et al*[255]
Aspergillus oryzae	Wheat bran	7.5	43.6	1.0 ml (8×10^8 spores)	30.0	72.0	31.2	Sandhya *et al*[258]
Beauveria felina	Wheat bran	7.0	120.0	Not specified	Not specified	168.0	20,000.0	Agrawal *et al*[272]
Bacillus pumilus AS 1.1625	Wheat bran	Not specified	65	-do-	-do-	Not specified	4300.0	Aijun *et al*[273]
Bacillus subtilis	Soy cake & babassu cake	5.0-10.0	70.0	25.0 ml	37.0	140.0	960.0	Valeria *et al*[261]

.....Continued

Table 2.5 Continued....

R.microsporus NRRL 3671	Rice bran	7.0	44.4	10^6 spores/ml	30.0	72 h	129.0	Sumantha et al[274]
Thermoactinomyces thalpophilus PEE 14	Wheat bran	10.0	80.0	20.0 %	55.0	72	2756.0	Divakar *et al*[275]
Engyodontium album BTMFS10	Wheat bran	11.0	60.0	Not specified	25.0	120	Not specified	Chellappan *et al*[127]
Bacillus Pumilus AS 1.1625.	Polyurethane foam	Not specified	Not specified	8.2 x 108	32.0	96	Not specified	Hongzhang *et al*[268]
Bacillus sp.	Green gram husk	9.0	140.0	3.0 %	33.0	60	35,000.0	Prakasham *et al*[262]
Aspergillus tamarii	Wheat bran	9.0	65.0	5.0 %	30.0	96	1.46 x 104 U/g	Anandan *et al*[276]
Pseudomonas aeruginosa PseA	Jatropha curcas seed cake	6.0-7.5	50.0	200.0 µl	30.0	72	1818.0	Mahanta *et al*[248]

Micro-organisms	Substrates	pH	Moisture level (In %)	Innoculum level (In %)	Incubation temperature (In °C)	Incubation time (h)	Enzyme activity (U/gds)	References
Streptomyces sp. CN902	Wheat bran	8.0	60.0	1 x 108 (spore / gm substrate	45.0	120.0	245.50	Lazim *et al*[205]
Mucor circinelloides	Dhal husk holds	7.0	20.0	30.0	30.0	120	8,573.0	Sathya *et al*[264]
Penicillium godlewskii SBSS 25	Wheat bran	9.0	60.0	5 x 10^7 (spores /ml)	35.0	96	235.0	Sindhu *et al*[249]
Bacillus Megaterium NCIM-2087	Soy cake substrate	9.0	100.0	3.0 ml	-do-	60.0	5961.0	Reddy *et al*[270]
Aspergillus niger	Rice mill wastes	7.0	Not specified	(10^6 spores/ml)	35.0	96.0	67.7	Paranthaman *et al*[271]
Aspergillus oryzae MTCC 5341:	Wheat bran	5.0	60.0	1 ml(10^5 spores/ml)	30.0	120.0	8.64 x 10^5	Vishwanatha *et al*[277]

.....Continued

Table 2.5. Continued...

Thermomucor indicae-seudaticae N31	Wheat bran and 20% of casein (WBC),	5.7	Not specified	Not specified	70.0	24.0	168.0	Merheb-Dini *et al*[278]

Table 2.6 Alkaline keratinase production under solid-state fermentation system.

Micro-organisms	Substrates	pH	Moisture level (In %)	Innoculum level (In %)	Incubation temperature (In °C)	Incubation time (h)	Enzyme activity (U/gds)	References
B.subtilis (MTCC9102)	Horn meal	7.0	100.0	50.0 %	37.0	48.0	15,972.0	Kumar *et al*[279]
Myrothecium verrucaria	Poultry feather (PF)	8.5	70–75	1.0 x 10^9 spores	28.0	144.0	189.0	Gioppo *et al*[280]
Penicillium sp.	Rice straw	6.0	80.0	10^5 spores/ ml	26.0	120.0	1,834.20	Morsy and Gendy[281]

2.3 Protease production under submerged fermentation system

Biotechnological application of proteases requires the production of these enzymes economically viable amounts for commercial purposes. Protease production is usually carried out in submerged fermentation (SmF), i.e fermentation in the presence of excess water; instead of SSF, due to better monitoring and ease of handling. Literature survey shows majority of the reports on microbial proteases production utilizes the submerged fermentation technology (SmF) and the widely studied organism used in protease production - *Bacillus* species also has been tested mostly in liquid media (Tables 2.5 and 2.6).

Considering the regular demands of industrial enzymes, many scientists have reported the enhancement of protease production using the supplementation of cheaper carbon and nitrogen sources under SmF. For example, Joo and Chang[187] reported the protease production using soybean, wheat flour and liquid maltose meal as cheaper carbon and nitrogen sources at pH 10.65 by *Bacillus clausii* I-52 with a maximum enzyme yield of 89,300.0 U / ml. Zambare and his colleagues[244] reported protease production using starch and wheat bran by *B. cereus* MCM B-326 at pH 9.0. A protease producing strain, *Teredinocabcter turnirae* displayed maximum protease yield of 1950.0 U /ml in presence of soybean as a substrate[259]. Recently, Bhaskar and his colleagues[243] demonstrated the use of fish processing waste material for alkaline protease production by *Bacillus proteolyticus* CFR3001 at 37°C incubation temperature. Ramesh *et al*[282] reported protease production by *Streptomyces fungicidicus* MML1614 using sucrose and peptone as the best carbon and nitrogen sources. Using wheat bran and maltose, Kalaiarasi and Sunitha[283] reported the maximum protease production of 0.9388 U/ ml by *Pseudomonas fluorescens* strain post 24h of incubation. Esakkiraj and his colleagues[284] reported the protease production using Tuna waste by *Bacillus cereus* with a maximum production of 259.83 U/ ml.

2.4 Statistical optimization of protease production

Media components were found to have great influence on extracellular protease production and are different for each microorganism. Therefore, the required constituents and their concentrations have to be optimized accordingly[87]. Industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better controlled process[285].

A number of optimization techniques can be used for this purpose. Statistical approaches offer ideal ways for process optimization studies in biotechnology. Time consuming, requirement of more experimental data sets[87].and missing the interactions among parameters are the obstacles in predicting the accurate results when the conventional optimization procedures like 'one-factor at a time' were applied[87]. On the contrary, statistical procedures have advantages basically due to utilization of fundamental principles of statistics, randomization, replication and duplication[87].

Several reports on the process optimization for protease production using different model and design are shown in Tables 2.5 and 2.6. Majority of scientists reported the application of Plackett-Burman factorial design for initial screening of factors influencing protease production followed by central composite design (CCD) using different softwares to see the interaction and effects of the chosen factors(Table 2.6). Zhou and his colleague's[286] reported the protease production optimization from *Laccocephalum strain* using MATLAB software package. Process optimization for protease production was performed by minitab 13v software[92,287,288] as shown in Table 2.7. For keratinase production majority of workers use design expert software and statistica package[289,290] as shown in Table 2.8.

Table 2.7 Statistical optimization of alkaline protease production

Micro-organisms	Fermentation system (SSF / SmF)	Software	Design	Protease Yield (U/ml or U /gds)	References
Bacillus sp.	SmF	Not specified	L16 OA	89.23 U / ml	Prakasham *et al*[239]
Bacillus sp. L21	SmF	Minitab statistical software (Release 13v)	response surface methodology (RSM)	269 U/ ml	Tari *et al*[287]
Microbacterium sp.	SmF	Statistic 5.0 software (Statsoft, USA).	Response surface method	202.7 U/ ml	Thys *et al*[291]
Pseudomonas aeruginosa (B-2)	SmF		Plackett-Burman and Central Composite Design (CCD)	793492 U / l	Khan *et al*[292]
Bacillus sp.	SmF	Design-Expert® 6.0 (Stat-Ease, Min neapolis, MN)	RSM: FCCCD as Experimental Design	3978 U/mL	Saran et al[88]

Micro-organisms	Fermentation system (SSF / SmF)	Software	Design	Protease Yield (U/ml or U /gds)	References
Colwellia sp. NJ341	SmF	Design Expert' software (Version 7.0, Stat-Ease Inc.,Min- neapolis, USA) was	Response surface methodology	183.21 U/mL	Wang *et al*[225]
Bacillus sp. RKY3	SmF	Design-Expert 7.1 (Stat- Ease, Inc., Minneapolis, MN, USA)	Plackett–Burman and response surface methodology	939 U /ml	Reddy *et al*[90]
Bacillus sp. HS08	SmF	Statistica, Version 7.0 (Statsoft Inc., Tulsa, OK	Central composite design	6804 U/ml	Guangrong *et al*[89]
B.clausii	SmF	Stat-Ease Inc., Minneapolis, MN, USA	Central composite design	3914 U/ ml	Oskouie *et al*[87]

.Continued

Table 2.7 Continued....

Aspergillus clavatus ES1	SmF	Design Expert® 7.0' Stat-Ease, Inc., Minneapolis, MN, USA,	Plackett–Burman and response surface methodology	770.66 U/ml	Hajji *et al*[128]
Laccocephalum mylittae	SmF	Matlab (Version 7.1, the MathWorks, Inc., Natick, MA	Central composite rotatable design (CCRD)	4557.22 U per gram mycelial dry weight (U/g)	Zhou *et al*[286]
Bacillus pseudofirmus SVB1	SmF	Minitab (Minitab Inc., State College, PA., USA)	Plackett-Burman experimental design. Central composite design	Not specified	Sen *et al*[92]
Bacillus pseudofirmus Mn6	SmF	Microsoft Excel 97	Box-Behnken factorial experimental design	3,213.0 U /ml	Abdel-Fattah *et al*[93]

Micro-organisms	Fermentation system (SSF / SmF)	Software	Design	Protease Yield (U/ml or U /gds)	References
Streptomyces sp. A6	SmF	Design Expert (version 7.1.6, State-Ease, Minneapolis, MN, U.S.A.).	Plackett–Burman design and CCD	129.02 U/ ml	Singh and Chhatpar[293]
Bacillus sphaericus DS11	SmF		Plackett-Burman design and Box-Behnken design	1182.68 U / ml	Liu *et al*[294]
Bacillus mojavensis A21	SmF	Design Expert® 7.0" Stat-Ease, Inc., Minneapolis, USA	Central composite design	1860.63U/ml	Haddar *et al*[265]
Mangifera Indica Cv. Chokanan	For extraction process	Minitab v.14 statistical package (Minitab Inc., PA, USA).	central composite design (CCD	Not specified	Amid *et al*[288]

Table 2.8 Statistical optimization of alkaline keratinase production

Micro-organisms	Fermentation system (SSF / SmF)	Software	Design	Protease Yield (U/ml or U /gds)	References
Bacillus licheniformis RG1	SmF	Design expert 6.0 software (Stat-Ease).	FCCCD	1295.0	Ramnani and Gupta [289]
Streptomyces sp7	SmF	Statistica 6.0 (Stat Soft)	Full -factorial rotatable CCD	95.0	Tatineni *et al*[295]
Bacillus subtilis strain, KDN2	SmF	Expert-Design software	Fractional factorial design (FFD); central composite design (CCD)	125	Cai and Zheng[290]

2.5 Purification and biochemical characterization of alkaline protease

The properties of microbial proteases may be diverse depending on the producer microorganism. These enzymes are predominantly extracellular, although cell-bound and intracellular enzymes have also been reported[6,296]. The purification, biochemical and physicochemical properties of some of the selected proteases are presented in Table 2.9.

In a broad-spectrum, all currently used detergent stable proteases are alkaline and thermostable in nature with a high pH optima (the pH of laundry detergents is generally in the range of 8.0-12.0) and have varying in thermostabilities at laundry temperature (50-70 °C). Therefore, most of the commercially available proteases are also active in the pH and temperature ranges 8-12 and 50-70°C respectively (Table 2.9). In addition, a recent trend in the detergent industry is a requirement for alkaline protease active at low washing temperatures to reduce the cost of heating the water to improve the wash performance. Alkaline proteases have board substrate specificity and are active against a number of natural proteins. However, literature conclusively suggests that they are much more active against casein than against other proteinous substrates (Table 2.9). Moreover, there are specific types of alkaline proteases such as collagenase, keratinase[296,297] which are active against specific types of keratinaceous wastes.

To develop an enzyme-based process, prior information about kinetic parameters of the enzyme in question is of utmost importance. To be precise, kinetic properties, like V_{max}, K_m, K_{cat} and E_s are important for substrate and environment specific for designing an enzyme reactors or quantifying the applications of the enzyme under different conditions[298,299]. For alkaline protease from *Salinivibrio* sp. strain AF-2004 decreasing the km value to 1.4 (mg/ml) lead in increase in Vmax value of 264 (U/mg) reported by Karbalaei-Heidari *et al*[201]. Singh and

his colleague's[285] reported the kinetic properties of alkaline protease *Crinum asiaticum* displaying increase in V_{max} value of 5 x 10^4 µM/min with a corresponding decrease in Km value of 31.7 µM. Literature survey suggests decrease in K_m value lead to increase in V_{max} value as demonstrated by purified protease from *Bacillus* sp. HR-08[236], *Laccocephalum mylittae*[300], *Sardinella aurita*[301], *Bacillus cereus*[302] etc.,

Table 2.9 Properties of few selected proteases from different micro-organisms.

Micro-organisms	pH optima	Temperature optima (In °C)	Substrates specificity	Molecular mass (kDa)	Specific activity (U/mg)	Other properties	References
Bacillus pseudofirmus	10.0–11.0	37.0	Casein	30.0–32.0	5142	Marginally inhibited by EDTA (1 mM) and PMSF (10 mM) ; Ca^{2+} and Mn^{2+} ;	Patel *et al*[209]
Engyodontium album BTMFS10	10.0	60.0	Casein	38.0	438	Not specified	Chellappan *et al*[127]
Salinivibrio sp. strain AF-2004	8.5	55.0	Casein	31.0	116.8	Complete inhibition by Pefabloc SC and PMSF ; Zn^{2+},Ca^{2+} and Ni^{2+} ; organic solvent stable protease	Karbalaei-Heidari, *et al*[201]
Aspergillus clavatus ES1	8.5	50.0	Casein	32.0	37600	inhibited by the serine protease inhibitor (PMSF) indicating	Hajji *et al*[128]
Serratia rubidaea	10 0	30.0	Casein	97.0 45.0	Not specified	Strong inhibition by EDTA ; Cu^{2+} dependent	Doddapaneni *et al*[303]

Micro-organisms	pH optima	Temperature optima (In °C)	Substrates specificity	Molecular mass (kDa)	Specific activity (U/mg)	Other properties	References
Botrytis cinerea	9.0–10.0	60.0	Casein	43.0	21,700	DTNB, 2- mercaptoethanol and DTT (thiol protease) ; Influence by Cu^{2+} ions.	Abidi *et al*[304]
Aspergillus nidulans PW1	8.5	40.0	Casein	37.0	5.620	Co^{2+} and Fe^{2+}; organic solvent stable ; thermostable	Pena-Montes *et al*[305]
Bacillus sp. PN-13	10.0	40.0	Casein and gelatin	30.0	65,700.0	Not specified	Ogino *et al* [181]
Bacillus licheniformis RSP-09-37	10.0	50.0	Casein	55.0	2983.0	Organic solvent stable protease; Ca^{2+}	Sareen and Mishra[306]
Penicillium chrysogenum FS010	9.0	35	Casein	41.0	36542.6	Strongly inhibited by the serine protease inhibitors PMSF and DFP ; Mg^{2+} and Ca^{2+}	Zhu *et al*[307]

Micro-organisms	pH optima	Temperature optima (In °C)	Substrates specificity	Molecular mass (kDa)	Specific activity (U/mg)	Other properties	References
B.circulans	11.0	70	Casein	39.5	9000	Ca^{2+},Mg^{2+} and Mn^{2+}	Rao *et al*[309]
Bacillus sp. HR-08	10.0	60.0	Casein	29.0	2,938	Inhibited by PMSF (serine alkaline protease) ; organic solvent stable; Influencing metal ions Ca^{2+},Na^{2+}, Cu^{2+} , 100% stable at 50 °C for 180 min	Moradian *et al*[236]
B.mojavensis A21	8.5	40.0	Casein	20.0	15,575.22	inhibited by the serine protease inhibitor (PMSF) indicating	Haddar *et al*[96]
Myrothecium verrucaria	8.3	37.0	feather keratin	22.0	12,851.8	Inhibited by PMSF; organic-solvent stable protease	Moreira, *et al*[309]
Bacillus sp. B001	10.0	30.0	Casein	28.0	75.0×10^3	strong inhibition by PMSF ; influence by Ca^{2+},Ba^{2+}, and Mg^{2+} ,	Deng *et al*[310]

The molecular mass of alkaline proteases ranges from 20 to 71.0 kDa[96,311] with few reports of higher molecular masses of 86.29 kDa [312] ,91.0 kDa[311], 97.0 kDa[303]; 115.0 kDa[313] and 124.0 kDa[218]. However, an enzyme from *Sardinella aurita* had an extremely low molecular weight of 14.2 kDa[301].

Literature survey demonstrated that majority of alkaline keratinase molecular mass ranges from 30.0 to 80 kDa[314,315] with few higher molecular mass 134.0 kDa[316] and 203.2 kDa [317] as shown in Table 2.10.In addition, the lowest molecular mass serine keratinase was reported from *Streptomyces albidoflavus* strain[318].

Table 2.10 Properties of few selected keratinase from different micro-organisms.

Micro-organisms	pH optima	Temperature optima (In °C)	Substrates specificity	Molecular mass (kDa)	Specific activity (U/mg)	Other properties	References
B licheniformis PWD-1	7.5	50	-	33	-	Serine	Lin *et al*[319]
Bacillus subtilis KS-1	7.5	-	-	25.4	-	Serine	Suh and Lee [320]
Bacillus licheniformis K-508	8.5	52	-	42	-	Thiolprotease	Rozs *et al*[321]
Bacillus sp. SCB-3	7	40	-	134	-	Metalloprotease	Lee *et al*[316]
Bacillus sp. SCB-3	7.0-8.0	60.0	Chicken feather	60.0	303.63	Activated by calcium and barium ions	Farag and Hassan[322]
Scopulariopsis brevicaulis	8.0	40.0	Not tested	39.0 and 36.0	70.6	Serine protease	Anbu *et al*[323]
Bacillus licheniformis FK14	8.5	60	-	35	-	Serine protease	Suntornsuk *et al*[324]

Micro-organism	pH optima	Temperature optima (In °C)	Substrates specificity	Molecular mass (kDa)	Specific activity (U/mg)	Other properties	Reference
B. licheniformis MSK103	9–10	60–70	-	26	-	Serine	Yoshioka *et al*[325]
B. subtilis MTCC (9102)	6.0	40.0	Soluble keratin,	64.0 and 69.0	4181.8	Cysteine protease	Balaji *et al*[326]
Chryseobacterium indologenes TKU014	10.0	30	Casein	40.0	0.23	Zn-metalloproteases	Wang *et al*[327]
Bacillus cereus DCUW	8.5	50	-	80	-	Serineprotease	Ghosh *et al*[314]
Bacillus pumilis	8.0	65	-	65	-	Serine	Kumar *et al*[246]
Bacillus subtilis KD-N2	8.5	55	-	30.5	-	Serine	Cai *et al*[328]
Bacillus licheniformis RPk	9.0	60	-	32	-	Serine	Fakhfakh *et al*[329]
Streptomyces sp. AB1	11.5	75	Keratin	30	67,000	Serineprotease	Jaouadi *et al*[315]
Streptomyces sp. strain 16	9	50	-	203.2	-	Serine	Xie *et al*[317]

Most of the alkaline proteases reported to date are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) as shown in Table 2.6. In this regard, PMSF sulfonates the essential serine residue in the active site resulting in the complete loss of activity[310,330]. This inhibition profile classifies these proteases as serine hydrolases[310]. In addition, some of the alkaline proteases were found to be metal ion dependent in view of their sensitivity to metal chelating agents, such as EDTA[303]. Iodo acetic acid a thiol inhibitor demonstrated a lesser amount of effect on alkaline proteases of *Botrytis cinerea* [304].

2.6 Immobilization of alkaline proteases

Immobilization is one of the efficient methods to improve enzyme stability. Many organic and inorganic substances have been used as the support materials. Using magnetic nanoparticles as the support of immobilized enzymes has the following advantages: (1) higher specific surface area was obtained for binding of a larger amount of enzymes, (2) lower mass transfer resistance and less fouling, and (3) the immobilized enzymes can be selectively separated from a reaction mixture by the application of a magnetic field[331].

2.6.1 Cell-free immobilization

Attachment of alkaline proteases to an insoluble carrier (by either physical adsorption or covalent coupling) is the most prevalent method of immobilization. Various carriers employed for the purpose include magnetic nanoparticle, bentonite, porous glass, nylon and vermiculite[6]. Although porous glass has been widely used, the relatively high cost of this support has been the limiting factor for industrial application. The method of immobilization of the alkaline proteases on these supports using glutaraldehyde involves covalent attachment of the amino groups of the enzyme to the available aldehyde groups present in the glutaraldehyde-activated support. In one study, Wu and his colleague's[332] successfully immobilized an alkaline protease onto a (3-aminopropl) triethoxysilane and modified magnetic nanoparticles with

the average diameter of 25.4 nm synthesized in water-phase co-precipitation method. Sadjadi *et al*[333] reported successful immobilization of alkaline protease Au–Ag nanoparticles (Au–Ag-bi-MNPs) have been prepared on amine functionalized Si-MCM-41 (NH_2–Si-MCM-41) particles through a reduction of $AgNO_3$ and $HAuCl_4$ by $NaBH_4$ at ambient conditions. Au–Ag-bi-MNPs loaded on the NH2–Si-MCM-41, provide a good biocompatible surface for immobilization of the enzyme alkaline protease. And also on TiO2 nanoparticles assembled on the porous MCM-41 (Mobile Crystalline Material No. 41) particles could provide an active biocatalyst.

2.7 Industrial application of proteases

Alkaline proteases are robust enzymes with considerable industrial potential in detergent formulations, leather processing, medical purposes, food processing, and waste treatment. These enzymes contribute to the development of high value-added applications or products by using enzyme-aided (partial) digestion. The different applications currently using alkaline proteases are:

2.7.1 Detergent industries

Current cleaning technology regimes state for lower washing temperature in addition to the alteration in the detergent compositions with regard to the use of environment-compatible non-phosphate builders like zeolites, and the bleaching agents[187]. Microbial alkaline proteases dominate commercial applications with a significant share of the market captured by subtilisins and/or alkaline proteases from *Bacillus* sp. for laundry detergent applications[96,279,312] as shown in Table 2.11. Alkaline proteases added to laundry detergents enable the release of proteinaceous material from stains[187]. The increased usage of these proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes in environmentally acceptable, non-phosphate detergents. In addition to improved washing efficiency, the use of enzymes allows lower wash temperatures and shorter periods of agitation, often after a preliminary period of soaking[124].

Literature survey demonstrated that majority of detergent stable proteases are isolated from *Bacillus* species (Table 2.11), with a few limited exception, such as *Serratia rubidaea*[303]; *Aspergillus nidulans*[305]; *Streptomyces fungicidicus* MML1614[282] and *Streptomyces* sp. strain AB1[315].

Table 2.11 Industrial application of different class of protease in various biotech sectors

Micro-organism	Class of protease	Industrial applications						References
		Detergents	Food industries	Peptide synthesis	Leather sector	Pharmaceutical sector	Waste-management	
Nesterenkonia sp. AL20	alkaline protease	Not tested	Not specified	Not tested	Not tested	Not tested	Suitable for poultry waste management	Bakhtiar *et al*[334]
Pseudomonas aeruginosa PseA	Alkaline protease	Not tested	Not specified	Tested for peptide synthesis purpose	Not tested	Not tested	Not tested	Gupta *et al*[130]
Bacillus pseudofirmus	Alkaline protease	Tested with surfactants suggesting it for detergent formulation	Not specified	Not tested	Not tested	-do-	-do-	Patel *et al*[209]

Micro-organisms	Class of protease	Industrial applications						References
		Detergents	Food industries	Peptide synthesis	Leather sector	Pharmaceutical sector	Waste-management	
Bacillus subtilis TP-6	-do-	-do-	Not specified	-do-	-do-	-do-	-do-	Kim *et al*[335]
Bacillus subtilis DC33	-do-	-do-	Not specified	-do-	-do-	-do-	-do-	Wang *et al*[336]
Bacillus clausii I-52	Alkaline protease	Suitable for laundry detergent formulation	Not tested	Not tested	Not tested	Not tested	Not tested	Joo and Chang[187]
Bacillus pumilus	Alkaline protease	Not tested	Not tested	Not tested	Tested for dehairing purpose	Not tested	Not tested	Wang *et al*[337]
Botrytis cinerea	Alkaline Protease	Tested for laundry detergent formulation	Not specified	Not specified	Not specified	Not specified	Not specified	Abidi *et al*[304]

Micro-organisms	Class of protease	Industrial applications						References
		Detergents	Food industries	Peptide synthesis	Leather sector	Pharmaceutical sector	Waste-management	
Bacillus laterosporus-AK1	serine Alkaline protease	-do-	Not specified	Not specified	-do-	-do-	-do-	Arulmani, *et al*[96]
Serratia rubidaea	Alkaline protease	-do-	Not specified	Not specified	-do-	-do-	-do-	Doddapaneni *et al*[303]
Salinivibrio sp. strain AF-2004	Alkaline protease	Not mentioned	Not specified	Prospective application of this protease in peptide synthesis	Not mentioned	Not mentioned	Not mentioned	Karbalaei-Heidari *et al*[201]
Bacillus vallismortis	Fibrinolytic Enzyme	-do-	Not specified	-do-	-do-	-do-	-do-	Kim, *et al*[338]

Micro-organisms	Class of protease	Industrial applications						References
		Detergents	Food industries	Peptide synthesis	Leather sector	Pharmaceutical sector	Waste-management	
Bacillus cereus MCM B-326	Alkaline protease	Not tested	Not specified	Not tested	Tested for dehairing purpose	Not tested	Not tested	Nilegaonkar *et al*[339]
B. licheniformis RP1	Alkaline protease	Suitable for commercial solid laundry detergent formulations	Not specified	Not tested	Not tested	Not tested	Not tested	Sellami-Kamoun *et al*[340]
Bacillus subtilis LD-8547	-do-	-do-	Not specified	-do-	-do-	-do-	-do-	Wang *et al*[341]
Bacillus sp. nov. SK006	Fibrinolytic Enzyme(Alkaline protease)	-do-	Not specified	-do-	-do-	-do-	-do-	Hua *et al*[342]

Micro-organisms	Class of protease	Industrial applications						References
		Detergents	Food industries	Peptide synthesis	Leather sector	Pharmaceutical sector	Waste-management	
Cordyceps militaris	Fibrinolytic enzyme (Alkaline protease)	Not tested	Not specified	Not tested	Not tested	Thrombolytic agents	Not tested	Cui *et al*[343]
Penicillium chrysogenum FS010	alkaline protease	Prospective application of the cold-active alkaline protease is extremely extensive, and widely used in detergents, feed, food, leather and many other industries.						Zhu *et al*[307]
Chryseobacterium indologenes TKU014	Metalloproteases	Not tested	Not specified	Not tested	Not tested	Not tested	Feather and hair degradation	Wang *et al*[232]
Aspergillus nidulans	alkaline protease	-do-	Not specified	Tested for peptide synthesis purpose	Not tested	-do-	-do-	Pena-Montes *et al*[305]

Micro-organisms	Class of protease	Industrial applications						References
		Detergents	Food industries	Peptide synthesis	Leather sector	Pharmaceutical sector	Waste-management	
Bacillus sp. PN-13	Protease	Not specified	Not specified	Not tested	Tested for leather dehairing purpose	-do-	-do-	Ogino *et al*[181]
Synergistes sp.	acid protease	Not tested	Not specified	Not specified	Not tested	Not tested	Tannery waste	Kumar *et al*[344]
Bacillus sp. HR-08	Alkaline Protease	Tested for laundry detergent formulation	Not specified	Tested for peptide synthesis purpose	-do-	-do-	-do-	Moradian *et al*[236]
Bacillus mojavensis A21	Alkaline serine protease	-do-	Not specified	Not specified	-do-	-do-	-do-	Haddar *et al*[96]
Bionectria sp.,	-do-	-do-	-do-	-do-	-do-	-do-	-do-	Rovati *et al*[345]

Micro-organisms	Class of protease	Industrial applications						References
		Detergents	**Food industries**	**Peptide synthesis**	**Leather sector**	**Pharmaceutical sector**	**Waste-management**	
Bacillus circulans	Alkaline protease	Tested for laundry detergent formulation purpose	Not specified	Not tested	Not tested	Not tested	Not tested	Rao *et al*[310]
Bacillus subtilis	Acid protease	Not tested	Suitable application in chesse making industries	Not tested	Not tested	Not tested	Not tested	Shieh *et al*[346]
Streptomyces fungicidicus MML1614	Alkaline protease	Suitable for detergent formulation purpose	Not tested	Not tested	Not tested	Not tested	Not tested	Ramesh *et al*[282]

Micro-organisms	Class of protease	Industrial applications						References
		Detergents	Food industries	Peptide synthesis	Leather sector	Pharmaceutical sector	Waste-management	
Bacillus subtilis (MTCC9102)	Alkaline protease	Not tested	Not tested	Not tested	Not tested	Not tested	Biowaste Management	Kumar *et al*[279]
Thermomucor indicae-seudaticae N31	Milk-clotting protease	Not tested	Suitable in cheese making industires	Not tested	Not tested	Not tested	Not tested	Dini *et al*[278]
Streptomyces sp. CS684	-do-	-do-	Not specified	-do-	-do-	-do-	-do-	Simkhada *et al*[347]
Neanthes japonica (Iznka)	Fibrinolytic enzyme(Alkaline protease)	-do-	Not specified	-do-	-do-	-do-	-do-	Deng *et al*[348]
Streptomyces sp. strain AB1	serine alkaline proteinase	-do-	Not specified	Not specified	Tested	-do-	-do-	Jaouadi *et al*[315]

2.7.2 Leather industry

Alkaline proteases possessing elastolytic and keratinolytic activity offer an effective biotreatment of leather, especially the dehairing and bating of skins and hides[349]. The alkaline conditions enable the swelling of hair roots and subsequent attack of proteases on the hair follicle protein allows for easy removal of the hair. Despite the strong alkaline conditions, this process is pleasant and safer than traditional methods using sodium sulfide treatment, which contributes to 100% of sulfide and over 80% of the suspended solids in tannery effluents[349]. The bating following the dehairing process involves the degradation of elastin and keratin, removal of hair residues, and the deswelling of collagen, which produces a good, soft leather mainly used for making leather clothes and goods.

In addition, studies conducted by different scientists have demonstrated the successful leather dehairing by using alkaline protease from *Bacillus* sp. PN-13[181], *Penicillium chrysogenum*[307], *Bacillus cereus* MCM B-326[339], and *Bacillus pumilus*[337] as shown in Table 2.11.

2.7.3 Pharmaceutical industry (with especial reference with fibrinolytic enzymes)

The microbial fibrinolytic enzymes, especially those from food-grade microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure cardiovascular diseases. NK has already been developed as drugs in the market, including Nattokinase NSK- SD, Jarrow NattoMax JR-154, and Natto-K. Development of other microbial fibrinolytic enzymes is still ongoing, some of which are mainly from *Bacillus* species such as *Bacillus subtilis* TP-6[335], *Bacillus subtilis* DC33[336], *Bacillus vallismortis*[338], *Bacillus subtilis* LD-8547[341] and *Bacillus* sp. nov. SK006[342]. With a few exceptions, for example *Cordyceps militaris*[343], *Bionectria* sp.,[345], ,*Streptomyces* sp. CS684[347], and *Neanthes japonica* (Iznka)[348] (Table 2.11). However, much work needs to be done intensively and extensively, especially concerning assessment of their thrombolytic effects *in vivo*.

2.7.4 Solid-waste management

Alkaline proteases provide potential application for the management of wastes from various food processing industries and household activities. These proteases can solubilize proteins in wastes through a multistep process to recover liquid concentrates or dry solids of nutritional value for fish or livestock[296]. In 2008, Wang and his colleagues[327] reported an enzymatic process using a *Chryseobacterium indologenes* TKU014 alkaline protease in the processing of waste feathers from poultry slaughterhouses (Table 2.12). Feathers constitute approximately 5% of the body weight of poultry and can be considered a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. Pretreatment with NaOH, mechanical disintegration, and enzymatic hydrolysis resulted in total solubilization of the feathers. The end product was a heavy, grayish powder with a very high protein content which could be used as a feed additive.

Similarly, many other keratinolytic alkaline proteases were used in feed technology[354,359] for the production of amino acids or peptides[328,358] for degrading waste keratinous material in household refuse, and as a depilatory agent to remove hair in bath tub drains, which caused bad odors in houses and in public places[355] as shown in Table 2.12.

Table 2.12 Industrial application of alkaline keratinase

Micro-organisms	Industrial application	References
Bacillus licheniformis PWD-1	Hydrolysis of Feather Keratin for feed formulation	Lin *et al*[350]
Vibrio sp. strain kr2	Solid-waste management	Riffel *et al*[351]
Doratomyces microsporus	Application in leather processing	Friedrich *et al*[352]
Pseudallescheria boydii	Sludge Liming	Ulfig[353]
Vibrio sp. strain kr2	Nutritional improvement of feather protein for live stock feed formulation	Grazziotin *et al*[354]
Bacillus subtilis	Solid-waste management	Cai *et al*[328]
Bacillus pumilus	Solid-waste management	Kumar *et al*[246]
Streptomyces	Leather, keratin waste treatment, animal feeding industry, and also cosmetic industry	Tapia and Simoes[355]
Bacillus licheniformis	Solid-waste management	Tamilmani *et al*[356]
Meiothermus ruber H328	Solid-waste management	Matsui *et al*[357]
Serratia sp. HPC 1383	Solid-waste management	Khardenavis *et al*[358]

Micro-organisms	Industrial application	References
Paecilomyces marquandii	Feather meal formulation	Vesela and Friedrich[359]
B. halodurans Strain PPKS-2	Solid-waste management ; Leather industry	Prakash *et al*[360]

CHAPTER III

MATERIALS AND METHODS

Chapter 3

Materials and methods

3.1 Materials

3.1.1 Plasticware / Glassware /Columns

50ml tube sterilized polystyrene tube was purchase from Tarson, India; 100, 250, 500 and 1000 ml Erlenmeyer flask were purchase from Borosil, Mumbai, India; Reverse –phase C_{18} nova pak column was purchased from Waters, USA; DEAE Sephadex A-50 column, sephacryl S-200 column, and CM-Cellulose column were purchased from Sigma-Aldrich, USA.

3.1.2 Chemicals

3.1.2.1 Analytical grade

Methanol,2-propanol, n-hexane, ethanol, glycine, Iron oxide, Tween-20, Triton-X-100, sodium dodecyl sulphate (SDS), Tris-HCl, sodium hydroxide, Sodium carbonate, potassium dihydrogen phosphate, coomassie brilliant blue, glycine, casein, Tween -20, di-sodium hydrogen phosphate, di-potassium hydrogen phosphate, acetonitrile, Folin-Ciocalteu's D-glucose, fructose, sucrose, galactose, maltose, lactose, cellulose, ammonium sulphate, ammonium nitrate, potassium nitrate, magnesium chloride, mercuric chloride, copper chloride, zinc sulphate, manganese chloride, calcium chloride, cadmium chloride, cobalt chloride, nickel chloride, sodium nitrate, ammonium chloride and EDTA were purchased from Merck, USA. 3-(aminopropyl) triethoxy silane, carbodimide / cyanimide, synthetic peptides, ethidium bromide, collagens, phenylmethylsulfonyl fluoride (PMSF), iodo acetic acid (IAA), 4-bromophenacyl bromide (4-BPB), dithiothreitol (DTT) were purchased from Sigma-Aldrich, USA.

3.1.2.2 Microbiological grade culture media/chemicals

Nutrient agar, nutrient broth, skim-milk agar, luria broth, yeast extract, beef extract, peptone, urea broth, litmus milk broth, phenol red dextrose broth, phenol red lactose broth, phenol red sucrose broth,

phenol red mannitol broth, gelatine, keratin powder, tryptone broth, simmon citrate broth, nitrate broth, indole nitrate broth, methyl red (MR)- voges proskaure (VP) broth, triple sugar iron agar , tributyrin agar, microbiological grade agar, bovine serum albumin, bovine serum globulin, bovine serum fibrinogen, hemoglobulin, carboxylmethyl cellulose were purchase from Himedia

3.1.2.3 Molecular biology grade chemicals / kits

Gel filtration molecular weight marker kits was purchase from Sigma-aldrich, USA., dNTP mix, 10X Taq buffer, magnesium chloride, Taq DNA polymerase, protein molecular weight marker were purchase from Fermentas, USA. AxyPrep bacterial genomic DNA miniprep kit was purchase from Axygen, USA. QIA quick gel extraction kit was purchased from Qiagen, Germany. HiIMVIC Biochemical test kits was purchased from Himedia, India.

3.1.2.4 Raw materials

Raw chicken feather, goat skin, goat blood, heart, lung, kidney, liver were collected from known local vendors. Agro-industrial wastes were collected from Tezpur University canteen, hostels as well as local shops.

3.1.2.5 Microbial strains

The protease producing bacterium *Bacillus subtilis* strain DM-04 was previously isolated from our laboratory and taxonomically identified[361]. *Klebsiella pneumoniae* , *Staphlyococcus aureus* and *E.coli* were obtained from IMTECH, Chandigarh, India.

3.2 Methods

3.2.1 Collection of environmental samples

Soil samples were collected at random in 50 ml blue cap polystyrene sterile tubes. From each location /site a minimum of five samples were collected. The collection areas were chosen at random within NER (North eastern region).They varied from natural habitats to extreme habitats like area around the hot springs, oil-fields, high-altitude mountains, rivers site, effluents from paper mills, slaughter house. Samples were collected from the different states of north-east India.

Collection of samples from near-surface soil/water was accomplished with tools such as spades, shovels, trowels, and scoops as described by Mason under EPA-600/4-83-020[363] with the following steps.

3.2.2 Sample collection from soil

(a) A minimum of five samples were taken from each location.

(b) The vegetation covering the soil at each site was completely removed

(c) A sterile shovel (or clean tool) was used to take the sample from the desired depth area in protocol. Incase of soil the average depth, and incase of water the samples were collected from the sub-surface clear layer.

(d) Each sample was packed in a perfectly clean plastic bag or cardboard container.

(e) Each sample was labeled providing all pertinent technical information.

(f) The soil samples were taken from an average depth of 90.0cm below surface. This was done to avoid surface bacteria that were likely to be totally aerobic.

(g) Collecting soil samples the following were avoided places where commercial fertilizer had been applied, or the ground fertilized with coffee pulp or compost within the last six months.

(h) Lands which had been recently burnt off.

(i) Samples were collected from different soils of different locations throughout Northeast India. The description of the place, soil treatment and kind of crop were registered on a sheet giving technical information; this was later transferred to files.

(j)The samples were air-dried for one week and sieved (2 mm mesh Fisher Scientific sieve). Humidity was determined from 10 grams of sample by drying at 80°C until constant weight was achieved.

(k)The pH was potentiometrically determined after suspending and homogenizing 1 g dry sample for 20 minutes in 10 ml distilled water.

3.2.3 Sample collection from water

(a) Water samples were collected in pre-cleaned new sample 50 ml polystyrene blue cap tubes that were appropriately sterilized.

(b) The sterilized sample container from the laboratory was then filled and emptied four times with water from the sampling source. The cap of the sample container was than similarly flushed out using the water from the sampling source. This flushing out of the sample container and cap eliminated any residual sterilant in the sample container and cap that could possibly sterilize or otherwise impact the results of the laboratory analysis for microbial life in the sample of fire sprinkler or domestic water.

(d) The sample container was then slowly filled with water from the water source, including allowing the meniscus to develop above the very top of the rim of the sampling container. Approximately twenty (20) seconds was allowed for any entrapped air to escape from the water. A cap full of sample water collected in the cap of the same sample container was then carefully and slowly poured into the top of the sample container to ensure that the meniscus remained. The objective of these procedures was to eliminate, as far as humanly possible, any air bubble being entrapped in the sample container.

3.3 Screening of alkaline protease producing thermophilic bacteria

For the dilution plate method, 1.0 gm of soil or 1.0 ml of water sample was mixed with 9.0 ml of 0.9% (w/v) sterile saline and serially diluted upto 10^{-6}. Then 100 µl aliquot of each dilution was plated on skim-milk agar (Appendix I-A) plate and incubated for 24-48 h at 50°C under static condition in an inverted position.

The following criteria's were adopted for the selection of alkaline protease producing promising bacterial isolates.

3.3.1 Measurement of zone of hydrolysis

A clear area surrounding the bacterial growth on agar plate containing skim- milk was positive reaction for extra cellular protease secretion while absence of clear zone around bacterial colonies was considered as a non-secretion of protease enzyme. The protease producing bacteria were counted in a Cubek colony counter and the zone of hydrolysis around the colonies was measured in mm.

3.3.2 Growth kinetics and protease production

For study the bacterial growth and alkaline protease production, bacteria were propagated at 45°C and pH 8.0 for different time intervals (24–120 h) in 0.1 l M9 media (Appendix I-B(i)) supplemented with 1.0 %, (w/v) casein, placed in a 0.5 l flask with constant shaking (200 rev /min) on a rotary shaker. At a regular time interval samples (5.0ml aliquots) were taken out aseptically, followed by assay of protease activity (section 3.11.1) and protein estimation of culture supernatant[363].

3.3.3 Thermo stability study

The thermo stability of the crude alkaline protease (cell free culture supernatant) was investigated by measuring the residual activity after incubating the enzyme (2.0 mg / ml) at 60°C from 15 to 120 minutes[364]. At designated time intervals 500µl was withdraw every 15 minutes and checked for protease activity (section 3.11.1).

3.3.4 Detergent compatibility study

The compatibility and stability of crude protease enzymes with some commercial laundry detergents available in the local market such as Surf excel®, Rin advanced®, (Hindustan Lever Ltd, India), Ghadi® (Calcutta detergent Pvt. Ltd, India), Safed® (Safechem Industry, India), Fena Ultra® (Jericho Detergent Pvt. Ltd, India), Wheel® (Hindustan Lever Ltd, India) and Tide® (Procter and Gamble, India) were examined as described by Mukherjee[365]. Detergents were individually dissolved in tap water at a concentration of 7 mg /ml (to simulate the washing conditions), and were pre-heated at 100°C for 60 min to destroy the endogenous protease activity, if any (which was reconfirmed by protease assay of heated detergent solution). Then, different concentrations of crude protease were added to the detergent solutions. The relative enzyme activity in the presence of detergent was expressed in percentage activity taking the activity of control (enzyme with buffer) as 100% Mukherjee[365].

3.3.5 Storage stability of protease enzyme

The crude protease was stored at 4°C and at a regular interval of time, a suitable volume was withdrawn to assay the protease activity. The

activity of enzyme at the beginning of experiment was considered as 100% activity and other values were compared against that.

3.4 Pure culture of alkaline protease secreting bacterial isolates

In order to obtain a pure culture of isolated bacterial isolates, a loopful of bacterial culture was inoculated in nutrient broth with pH adjusted to 8.0 (Appendix I-M). With a 0.5 growth OD at 600nm, 100µl of culture was mixed in 0.9% (w/v) sterile normal saline and serially diluted upto 10^{-7} with a final volume of 2.0 ml. 100 µl aliquot were spread over sterile nutrient agar plates (Appendix I-D), and kept for 24h at 45°C in order to obtained single distinct colonies. For isolation of single pure colonies which were required for further studies. The following techniques were used: *(i) Spread-plate method, (ii) Streak-plate* method, for isolation of single pure colony was achieved for further studies[366,367]

3.4.1 Spread plate technique

The spread-plate technique is used for the separation of a dilute, mixed population of micro-organisms so that individual colonies can be isolated. In this technique microorganisms were spread over the solidified agar medium with a sterile L-shaped glass rod while the Petri dish was spun on a turn table. The advantage of this technique is to allow cells to be separated from each other by a distance sufficient to allow the colonies that develop to be free from each other.

3.4.2 Streak-plate Technique

The streak-plate method offers a most practical method of obtaining discrete colonies and pure cultures.In this method, a sterilized loop or transfer needle was dipped into a suitable diluted suspension of organisms which was then streaked on the surface of an already solidified agar plate to make a series of parallel, non-overlapping streaks.

3.5 Routine maintenance and preservation of microorganisms

Pure cultures of bacteria were preserved at 4°C in nutrient agar slants and transferred to fresh slants at an interval of one month. Isolates were

also stored in 15 % (v/v) glycerol in nutrient broth and kept at -80°C for long term storage.

3.6 Taxonomic identification of alkaline protease producing bacteria

The selected protease producing, thermophilic bacteria were taxonomically identified by (a) standard biochemical test, (b) studying their morphological characteristics[367-369], (c) GC-FAME analysis[370], (c) Ribotyping[371] as described below.

3.6.1 *Morphological tests*

3.6.1.1 Simple staining

Two drops of bacterial culture were heat fixed over a clean glass slide and flooded by 5 drops of methylene blue stain for about 5 min. The stain was removed gently by placing the slide under running tap water. The slide was then dried by gently tapping with tissue paper and bacteria were observed under oil-immersion objective lens (1000 X).

3.6.1.2 Negative staining

One drop of nigrosine or Indian ink stain was placed at one end of clean glass slide with the help of pasteur pipette. Two drops of liquid culture was placed on it, and with the help of clean slide held at an angle of 30° the mixture was spread throughout the slide and kept at room temperature for drying. This was followed by visualization of bacteria under oil-immersion objective lens (1000 X).

3.6.1.3 Gram's staining

Over a clean glass slide using sterile inoculating loop, a loopful of 24h bacterial culture was spread over sterile glass slide and kept for 5-10 min for air drying followed by heat fixing. Few drops of primary stain were flooded over bacterial culture and kept for 1 min followed by washing the slide under tap water. Gram's iodine mordant was flooded over this after primary staining and kept at room temperature for 1 min. Removal of Gram's iodine mordant by washing under tap water was followed by addition of few drops of decolorizing agent (95% ethyl alcohol) over bacterial culture. It was washed again with tap water to remove the decolourizer, and followed by addition of counter stain

safranin for 45 seconds. After this step counterstain was removed with tap water and slide was kept at room temperature for complete air-drying. The bacteria were visualized under a light microscope (1000 X).

3.6.1.4 Spore staining

Using clean glass slide, loopful of 24h bacterial culture was air dry followed by heat fixing, Then few drops of malachite green were added and the slide was placed on a warm hot plate, allowing the preparation to steam for 2-3 mins. Slides from hot plate were removed and kept at room temperature for cooling and then washed under running tap water. Few drops of counter stain (safranin) were added over bacterial culture and kept for 30 seconds. This was followed by washing with tap water. Remaining water drops were absorbed by blotting paper and bacteria were examined under oil immersion objective lens (1000X).

3.6.2 Biochemical tests

3.6.2.1 Hydrolysis test for casein, starch, lipid and gelatin

Sterile nutrient agar plates (pH 8.0) containing with 1% (w/v) of casein, starch or triglyceride tributyrin (as a lipid substrate) were used for hydrolysis tests. 24h bacterial cultures were streaked with into plates using inoculating loop and kept in an inverted position for 24-36h at 45°C. In case of gelatine hydrolysis, 12% (w/v) of gelatin was supplemented in nutrient broth tubes and inoculated with a loopful of bacterial culture. The tubes were kept at 45°C for 48h followed by placing the tubes in refrigerator at 4°C for 30 minutes. The hydrolysis result was considered as positive on the basis of zone of hydrolysis around the colonies.

3.6.2.2 Carbohydrate fermentation test

Fermentation medium containing sugars was prepared as shown in appendix I-C(i). A loopful of 24h bacterial culture was inoculated into all tubes supplemented with respective sugars and incubated for 48h at 45°C. Change in medium colour and gas formation (if any) post 48h incubation were recorded. A control was also set up.

3.6.2.3 Triple sugar iron (TSI) agar test

Sucrose, lactose and glucose (0.1 %, w/v) were supplemented in nutrient agar slants (Appendix I-L), and a loopful of 24h bacterial culture was streak over TSI agar slants. The slants were kept at 37°C for 48h. Change in medium colour was observed over TSI agar slants. A control was also set up.

3.6.2.4 IMViC test

3.6.2.4.1. Indole production test

Sterile sulfur indole motility (SIM) agar deep tubes were streaked with 24h bacterial culture over agar surface and kept at 37°C for 48h. Change in medium colour after addition of Kovac's reagent was observed and recorded.

3.6.2.4.2. Methyl red - Voges-Proskauer (MR-VP) test

100 ml of sterile methyl red- Voges-Proskauer (MR-VP) broth was inoculated with 1.0 ml of 24h bacterial culture and kept at 37°C for 48h. MR-VP medium was separated into parts A and B. In part A, few drops of methyl red indicator was added for conformation of MR test. In part B, mixtures of Barritt A and B solutions were added in VP broth. In MR test, red colour formation after addition of methyl red indicator showed positive test and formation of yellow colour indicated negative test. In case of VP test, formation of pink colour complex indicated positive test.

3.6.2.4.3. Citrate Utilization test

10.0 ml of sterile Simmons citrate agar slants were prepared in streaked hard tubes with 24h bacterial cultures and kept at 37°C for 48h, followed by addition of bromo-thymol blue indicator over surface. Formation of blue colour complex was taken as a positive confirmation test for citrate.

3.6.2.4.4 Hydrogen sulfide test

10 ml of sterile sulfur indole motility (SIM) agar tubes was inoculated with a loopful of 24-h bacterial culture inside agar slants, and kept at 37°C for 48h. Formation of black colour indicated the production of hydrogen sulfide by bacterial culture.

3.6.2.4.5 Urease test

10 ml of sterile urea broth was inoculated with 0.1 ml of 24h bacterial culture and kept at 37°C for 48h.Change in medium color from red to pink indicated the presence of urease activity.

3.6.2.4.6 Litmus-milk reactions test

10.0 ml of sterile litmus milk broth was inoculated with 0.1 ml of 24h bacterial culture and kept at 37°C for 48h.Change in medium color, lactose fermentation, gas formation, curd formation, litmus reduction, peptonization and alkaline reaction if any, were observed post 48h of incubation.

3.6.2.4.7 Nitrate reduction test

100.0 ml of sterile trypticase nitrate broth was inoculated with 1.0 ml of 24h bacterial culture and kept at 37°C for 48h. To confirm the nitrate reduction capability of bacterial culture post 48h of incubation, solution A (sulfanilic acid) and solution B (alpha-naphthylamine) and amount of zinc powder were mixed with bacterial culture. Formation of cherry red colour indicated the reduction of nitrate (ability of bacterial cells to reduce nitrate).

3.6.2.4.8 Catalase test

10.0 ml of trypticase soy agar slant was streaked with a loopful of 24h bacterial culture and kept at 37°C for 48h. To confirm the presence of catalase activity, 3.0 % (v/v) hydrogen peroxide (H_2O_2) was flooded over it. If bubble formation was observed, it indicated the catalase positive test (production of catalase by bacteria).

3.6.2.4.9 Oxidase test

10.0 ml of trypticase soy agar slant was streak with a loopful of 24h bacterial culture and kept at 37°C for 48h. To confirm the presence of oxidase activity, p-aminodimethylaniline oxalate was added over the surface of bacterial culture. Development of pink color which finally turned on colony surface indicated the presence of oxidase activity.

3.6.3 Chemotaxonomic identification: analysis of bacterial cellular fatty acid methyl esters (FAME)

Chemotaxonomic identification based on cellular fatty acids content of bacteria was done using Microbial identification system (MIS) of institute of Microbial Technology (IMTECH) Chandigrah, India. The cellular fatty acids of bacteria were isolated and methylated before gas chromatography (GC) analysis[372]. The MIS system determines the cellular fatty acid profile of the bacterial isolates by high resolution GC analysis. This profile was then compared by utilizing the MIDI software which possesses the database of cellular fatty acid profile of known bacteria. A similarity index (SI) of 0.6 or higher indicated a very good match, where as SI index lower than 0.25 indicated a poor match[370,373]

3.6.4 Ribotyping using 16S rRNA gene amplification

3.6.4.1 Isolation of chromosomal DNA

3.6.4.1.1 DNA extraction by alkaline lysis

Genomic DNA from bacteria was prepared as described by Ausubel *et al*[374]. The cells were pelleted by spinning 4ml of culture at 9,450 x g for 10 min in a refrigerated Beckman Centrifuge, UK and supernatant was decanted. Pellet was resuspended in a 0.8 ml of solution-I (composition is given in appendix I-O(i)). To this resultant mixture, 160 µl of lysozyme (10 mg /ml) was added and incubated at room temperature (24°C) for 20 min. Subsequently, 44.5 µl of 10% (w/v) SDS solution was added and re-incubated for 10 min at 50°C .There after, 53.3 µl of RNase A (10 mg /ml) was added and incubated at 37°C for 90 min. This was followed by addition of 45.3 µl of Na-EDTA (0.1M, pH-8.0) and re-incubated at 50°C for 10 min. To remove the protein, 26.6 µl of proteinase K (5.0 mg /ml stock) was added and incubated at 50°C for 100 min. Equal volume of phenol (saturated with 0.1M Tris-HCl, pH-8.0) was added and mixed thoroughly. The mixture was centrifuged at 9,450 x g for 10 min, the upper (aqueous) phase was aspirated into sterile microfuge tube (Eppendorf) and lower phase was discarded. Then 700 µl of (1:1) phenol and chloroform-isoamylalcohol (24:1) were added and mixed thoroughly. After centrifugation at 9,450 x g for 10min, the upper phase was transferred to a sterile microfuge tube, then equal volume of chloroform-isoamylalcohol (24:1) was added and spun at 9,450 x g for 10 min. The upper phase was transferred to a

sterile microfuge tube and 1 / 10th volume of salt (Na-acetate 3M, pH-7.0) was added. The DNA was precipitated by adding 2 volumes of ice-cold absolute ethanol in the above solution and the DNA-pellet was recovered by centrifugation. After removal of alcohol, DNA was re-suspended in 10 mM Tris HCl-1 mM EDTA buffer (pH-8.0) at a final concentration of 1 µg /ml and was stored at 4°C until it was further used.

3.6.4.1.2 Direct chromosomal DNA extraction

A direct method of DNA extraction from the alkaline protease producing bacterial isolate was adopted. In brief, after removal of the colonies from the surface of agar plates, the bacteria were suspended in a microfuge tube containing 300 µl of sterile MilliQ water and then vortexed for 20 sec. The DNA was extracted after cell lysis by immersing the tubes in boiling water bath for 10 min. The cell debris was pelleted by centrifugation at 16,000 x g for 10 sec (Beckman, UK) and the supernatant containing DNA was removed and transferred to fresh micro centrifuge tube. The concentration of DNA was estimated by spectrophotometry at 260 nm (Beckman DU 530^{R} spectrophotometer).

3.6.4.1.3 PCR amplification of 16S-rRNA gene

For amplification of 16S rRNA gene of the bacteria following universal primers were used: forward primer (27F) 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer (1525R) 5'-AAGGAGGTGATCCAGCCGCA-3'[375]. DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA). Reaction mixture for the PCR contained 1 X PCR buffer (Fermentas, Canada), each deoxynucleotide triphosphate (dNTP) at a final concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a final concentration of 0.1 µM and 2.5 U of Taq DNA polymerase (Bangalore Genei, India) in a final volume of 100 µl. Standardized PCR condition with respect to each strain is shown in Table 3.1. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5µl) on a 1.0% agarose gel in 1X TAE (Tris-acetate-EDTA) buffer. 16S rDNA amplicon was gel eluted using QIAquick columns (Quiagen Inc., USA) and the sequence was generated by chain termination method using an Applied

Biosystem automated sequencer (Delhi University South Campus, Delhi, India).

Table 3.1 Optimal PCR reaction conditions for amplification of conserved region of 16S-rRNA gene of selected protease secreting bacterial strains.

PCR conditions	Bacterial strains			
	AS-S24-II	***AS-S20-I***	***AS-S24-I***	***AS-S10-II***
Pre-denaturation	94°C for 5 min	90 °C for 2 min	94 °C for 5 min	94 °C for 5 min
Denaturation	94°C for 1 min	92 °C for 1 min	94 °C for 1 min	94 °C for 30 sec
Annealing	55°C for 30 sec	44 °C for 30 sec	55 °C for 90 sec	54 °C for 30 sec
Synthesis	72°C for 2 min	72 °C for 2 min	72 °C for 90 sec	72 °C for 2 min
Final extension	72 °C for 7 min	72 °C for 6 min	72 °C for 7 min	72 °C for 5 min
Hold	2	2	2	2
Cycles	36	36	36	36

3.6.4.1.4 Phylogenetic analysis

The 16S rDNA sequence of bacteria under study was aligned with reference sequences showing sequence homology from the NCBI database (http://blast.ncbi.nlm.nih.gov) using the multiple sequence alignment programme of MEGA4[376]. Phylogenetic trees were constructed by distance matrix-based cluster algorithms viz. unweighted pair group method with averages (UPGMA), neighbour-joining[377] analyses. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The stability of trees obtained from above cluster analyses was assessed by using BOOTSTRAP programme in sets of 1000 resamplings (MEGA 4). The 16S rDNA gene sequence determined in this study was deposited in GenBank of NCBI data library (http://www.ncbi.nlm.nih.gov/Genbank) under different accession number with respect to each strain.

3.6.5. PCR-RFLP analysis of selected strains

For PCR-RFLP analysis of all the selected strains , PCR amplification with primers 16S/p1 and 23S/p2 by using the flanking terminal sequences of the 16S-23S rRNA genes was performed with the isolated chromosomal DNA[374].

3.6.5.1 Synthesis of 16S-23S Inter specific region (ISR) primers for *Bacillus subtilis,* and *Bacillus licheniformis* strains

For PCR-RFLP, 16S-23S ISR gene-based oligonucleotide probes were designed for detecting specific bacteria. The 16S-23S ISR gene sequences for each taxon were retrieved from GenBank database and aligned by ClustalW[378]. Sequences of highly variable regions were selected to design species- or genus specific probes. The specificity of the probes were evaluated *in silico* by using BLAST (Basic Local Alignment Search Tool) program. Oligonucleotide probes were commercially synthesized, and the 3' end of each probe was modified by nucleotide during the synthesis to enable the immobilization of the oligonucleotide to the CSS-1000 silylated glass slides (CEL). The 16S-23S ISR forward primers 16S/p1 (5'-AGTCTGCAACTCGACTGCGTG-3') and 23S/p2 (5'-CAACCCCAAGAGGCAAGCCTC-3')[379] were used for 16S rRNA gene amplification from the chromosomal DNA of above mentioned bacterial strains. For the specificity of the 16S-23S ISR amplification, the PCR-product was used as a template in a second PCR (Nested PCR) with primers 16S/p3 (5'-GGAAGGTGCGGCTGGATCACC-3') and 23S/p4 (5'-CCCGAAGCATATCGGTGTTCG-3'), which anneal to position 11334 to 11355 of the 16S-rRNA gene and position 11770 to 11791 of the 23S-rRNA gene respectively. The nested PCR amplification includes the entire ISR and apart of the flanking rDNA (28bp of 16S-rRNA and 83bp of 23S-rRNA).

3.6.6 16S-rRNA gene species-specific amplification of AS-S20-I and AS-S24-II

The *Kocuria*-specific forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTC-3' were used for amplification of 16S-rRNA gene[380].

The *Paenibacillus*-specific primers PAEN515F 5'-GCTCGGAGAGTGACGGTACCTGAGA-3' were used in conjunction with a reverse primer RNA1484R 5'-GGCATGCTGATCCGCGATTACTAGC-3' in PCR amplifications for presumptive identification as strain of this genus, and *B. subtilis* was used as negative control[381].

3.7 Optimization of culture condition for optimum growth and maximum alkaline protease production by selected bacteria under SSF and SmF systems

The following criteria were adopted for screening of parameters influencing protease yield in SmF and SSF conditions.

3.7.1 Alkaline protease production under submerged fermentation (SmF) system

The pre-screening of the media components influencing the protease yield was done using the M9 mineral salt media (see appendix I-B(i)for composition) and changing the individual component one at a time. The actual fermentation was carried out in 50 ml of the modified M9 media (in 250 ml E-M flask) inoculated with 1% (v/v) of the mid-logarithmic culture of cells (O.D. at 600 nm ~ 0.5) and incubated at 45°C and 200 rpm for 24-120 hours. At different time intervals, suitable volume was withdrawn to assay the protease activity, estimation of protein content of culture supernatants and determination of bacterial dry biomass[361].

3.7.2 Effect of various carbon sources on alkaline protease production

Various carbon sources at a final concentration of 10 % (w/v or v/v) such as - starch, carboxyl methyl cellulose (CMC), cellulose, maltose, lactose, sucrose, dextrose, galactose, casein, fructose, glycerol, mannitol, sorbitol and xylose was added in the M9 culture media to check the alkaline protease production by selected bacteria.

3.7.3 Effect of various inorganic and organic nitrogen sources on alkaline protease production

The effect of various organic and inorganic nitrogen sources (at a final concentration of 1.0 % (w/v or v/v) viz- beef extract, peptone, yeast extract, tryptone, casein, urea, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate and ammonium nitrate on alkaline protease production was examined by adding these compounds one at a time in M9 medium inoculated with bacteria. The cell-free culture supernatant was used for assay of proteolytic activity and protein content estimation.

3.7.4 Effect of pH on alkaline protease production

Influence of initial pH on alkaline protease production was studied in the pre-screening step by adjusting pH of the M9 mineral salt medium from 1-14.

3.7.5 Effect of agitation rate on alkaline protease production

The culture flasks were agitated in a temperature controlled orbital shaking incubator in the range of 50-300 rpm and protease production was monitored.

3.7.6 Effect of temperature on alkaline protease production

Influence of temperature on alkaline protease production was studied by incubating the culture flasks in the temperature range of 25-60°C in an orbital shaking incubator.

3.7.7 Effect of incubation time on alkaline protease production

Influence of incubation time on alkaline protease production was studied from 24-120h post incubation of flasks at a desired temperature.

3.8 Alkaline protease production under solid-state fermentation system

The underline method describes the protease production in SSF.

3.8.1 Process optimization of various parameters in SSF for alkaline protease production.

3.8.2 Preparation of substrates for alkaline protease production

Different agro-industrial waste materials viz. mustard-oil cakes (MOC), wheat bran (WB), rice bran (RB) were obtained from local market. The wild grass *Imperata cylindrica* (IC) (see Table 3.2 for description) and banana leaves (BL) were collected from Tezpur University campus. Potato peels (PP) and used tea leaves (TL) were collected from hostel canteens. These waste materials were washed first with tap water followed by distilled water to remove the adhered surface dust particles. Then blenching operation was carried out by immersing them in hot water (75-80 °C) for 20 min followed by oven drying at 45°C. The dried material was ground in a mixer grinder (Remi) then sterilized at 121°C, 15 lbs pressure for 15 min and stored at 4°C for further use.

Table 3.2. Taxonomic Description of the plant *Imperata cylindrica*

Taxonomic name	*Imperata cylindrica* (L.) Beauv
Synonyms	*Imperata Arundinaceae* Cirillo, *Lagurus cylindricus* L
Common name in Assam	*Ulukher*
Other common names	alang-alang, blady grass (English), Blutgras (German), carrizo, cogon grass (English), gi (Fijian), imperata cylindrique (France), japgrass (English), kunai, lalang, ngi (Fijian), paille de dys (Frence), paillotte(french), speargrass (English), ulukher (Assames)
Life form	Grass.
Description	A slender, flat linear-lanceolate stemless grass with a slightly off center white mid rib and silica bodies embeded in the leaf margins, reaching a height of 1-1.5 meters, arising from a thick underground mat of rhizomes
Habitat	Agricultural areas, coastland, disturbed areas, natural

forests, planted forests, range/grasslands, riparian zones, scrub/shrublands, urban areas, wetlands

---

3.8.3 Preparation of substrates for alkaline keratinase production

Broiler chicken-feathers were washed in boiling water for about 5min to remove any bloodstain and/or any other adhering materials. Feathers were then dried in oven at 50◦C until the feathers regained their original fluffiness. These were then cut into about 5–8mm sized structures using a pair of scissors. Before using as substrate, feathers were sterilized by heating under pressure at 120°C for 15min.

3.8.4 Solid-state fermentation and optimization of process conditions for protease production

To screen the various substrates / supports for alkaline protease production in SmF , initially 5.0 g of coarse substrate was taken in a 500ml Erlenmeyer flask and to this a predetermined quantity of 50 mM K- phosphate buffer, pH 7.4 was added, mixed thoroughly and autoclaved at 121°C, 15 lbs pressure for 15 min. The flasks were cooled to room temperature and then inoculated with 2.0 ml of 24 h grown bacterial culture (O.D. at 600nm between 0.49 - 0.51) under sterile conditions. This was incubated at optimum growth temperature for bacterial growth (45°C) for various time periods (24, 36, 48, 72, 96, and 120 h) To investigate the influence of other culture parameters on protease production, effects of initial moisture content of the substrate (50%, 100%, 200% and 300%), inoculum size (10, 20, 40, 60, 80, 100 % (w/w)), co-carbon source (glucose, fructose, galactose, maltose, sucrose, and lactose at 10.0 % w/w) and co-nitrogen source (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, beef extract, casein and peptone at a final concentration 10.0 % w/w) were studied. For initial moisture content, solid substrate was mixed with predetermined amount of water and 100% moisturization was achieved by adding 1.0 ml water to the substrate and vice-versa[262]. Un-inoculated flasks served as negative controls whereas the inoculated flasks without any co-carbon or co-nitrogen source served as positive control. Alkaline

protease production was expressed as mean and standard deviations based on the results obtained with triplicate flasks.

3.8.5 Solid-state fermentation and optimization of process conditions for alkaline keratinase production

For the initial determination of time-course of keratinase production, 0.2g of finely cut chicken-feather was taken in 250ml Erlenmeyer flasks and to this, 1.0ml of distilled water (adjusted to pH 8.0) was added, mixed thoroughly and autoclaved at 121°C, 15lbs pressure for 15min. The flasks were cooled to room temperature and then inoculated with 1.0ml of 24h grown bacterial culture (O.D. at 600nm between 0.49 and 0.51) under sterile conditions and incubated at 50°C temperature for various time period (24h, 48h, 72h, 96h and 120h). To investigate the influence of other culture parameters on keratinase production, effect of growth temperature (30–55°C), effects of initial moisture content of the substrate (50%, 75%, 100%, 150%, 200% and 300%), supplementation with co-carbon sources (glucose, fructose, galactose, maltose, sucrose, lactose and starch at 10.0%, w/w) and co-nitrogen sources (NH_4Cl, $NaNO_3$, yeast extract, beef extract, casein and peptone at 1.0%, w/w) were studied. For determining the effect of initial moisture content of the substrate on keratinase yield, chicken-feather was mixed with predetermined amount of water and 100% moisturization was achieved by adding 1.0ml water to 1.0g substrate and other ratios in similar lines. Influence of moistening liquids on keratinase yield was determined by using distilled water (pH 8.0) 100mM K-phosphate buffer, pH 8.0, 100mM Tris–HCl buffer, pH 9.0 and 100mM glycine–NaOH buffer, pH 10.0. Un-inoculated flasks served as negative controls where as the inoculated flasks without any co-carbon or co-nitrogen source served as positive control. Keratinase production was expressed as mean and standard deviations based on the results obtained with triplicate flasks.

3.8.6 Optimization of protease / keratinase extraction from fermented matter

For isolation of protease produced under SSF, a known quantity of fermented matter was mixed with distilled water (1:5, w/v) by stirring on a magnetic stirrer for 30 min at room temperature (~24°C). The slurry was then squeezed through a cheesecloth followed by centrifuging

the whole content at 10,000 x g for 10 min at 4°C to remove the insoluble materials. The clear supernatant was used for the protease assay and the protease recovery was expressed as total units (U) of crude protease obtained per gram of dry substrate (gds).

For optimizing the protease extraction, different extraction medium media were used. The first media contained 50mM K-phosphate buffer with a pH range of 7.0-9.0. The second medium contained Glycine-NaOH buffer with a pH range of 9.0-13.0. The third contained plane distilled water. The fourth media contained distilled water with pH adjusted to 8.0.And the final media contained every case; the media was adjusted to the pH that give maximum protease yield.

3.9 Statistical optimization of alkaline protease production in SmF and SSF

3.9.1 Screening of factors effecting protease production using Plackett-Burman design

Our preliminary screening study using the best sources of carbon, nitrogen and physical parameters like incubation time, agitation rate (rpm), pH of the M9 culture medium, and incubation temperature were subjected to, Plackett–Burman factorial design for screening the most significant factors effecting the protease production by strains under study. Based on Plackett–Burman factorial design, each parameter was examined at two levels: low level (-1) and +1 for high level. Simultaneously a center point was run to evaluate the linear and curvature effects of the variables[382]. Plackett–Burman experimental design is based on the following first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \text{ ------------------------------ } (3.1)$$

Where Y is the response (total alkaline protease activity in U), β_o is the model intercepts, β_I is the linear coefficient, and X_I is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. The protease activity assay was carried out in triplicate and the averages of these experimental values were taken as response Y. From the regression analysis of the variables, the

significant factors ($P < 0.05$) for alkaline protease production were further optimized by using response surface methodology (RSM).

3.9.2 Statistical optimization of alkaline protease production using Response surface method (RSM)

Response Surface Methodology (RSM) was used to estimate main effects on response i.e. the protease yield. Central composite design (CCD) consisting of three main critical independent variables, (i) (C_1), (ii) (C_2), and (iii) (C_3) were chosen as the independent variables capable of influencing the alkaline protease production (Y) by bacteria under study in SmF or SSF (Table 3.3). For each factor, a conventional level was set to zero as a coded level. These three factors, each with three coded levels consisting of 20 experimental runs, were used to analyze the experimental data including five replicates at the center point. The experimental data were fitted as a second order polynomial regression equation including individual and cross effect of each variable.

$$Y = a_0 + \sum_{i=1}^{3} a_i C_i + \sum_{i=1}^{3} a_{ii} C_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} a_{ij} C_i C_j \text{ ------- } (3.2)$$

Where, Y= predicted response (total protease activity in U /ml for SmF and U /gds for SSF), a_0 intercept term, ai = linear effect, a_{ii} = square effect, a_{ij} = interaction effect, and C_i and C_j = variables. The above equation was used to optimize the values of independent parameters for the response. The following equation was used for coding the variables

$$C_i = \frac{C_i - C_0}{\Delta C_i} \quad i = 1,2,3,\ldots, k \text{ --------------------- } (3.3)$$

Multiple regression analysis, response surface plots and analysis of variance (ANOVA) were performed using Minitab 15 Statistical Software® (Minitab Inc, PA, USA).

Table 3.3 Independent variables for alkaline protease / keratinase production with respect to each strain under SSF and SmF systems using central composite design.

Strains	Independent variables		
	C_1	C_2	C_3
		Solid-state fermentation	
B. subtilis RM-01	pH of best moistening liquid (distilled water)	Maltose level (% w/w)	Sodium nitrate level (% w/w)
		Submerged fermentation	
B. subtilis DM-04	IC:PP level (% w/v)	Beef extract level (% w/w)	Time interval (h)
Brevibacillus sp. AS-S10-II	pH of the M9 medium	Casein level (% w/w)	Potassium nitrate (% w/w)
Bacillus sp. AS-S20-I	-do-	-do-	Ammonium sulphate (% w/w)
B.licheniformis AS-S24-I	-do-	Glucose level (% w/w)	Yeast extract (% w/w)
P. tezpurensis sp. nov. AS-S24-II	-do-	Casein level (% w/w)	Ammonium sulphate (% w/w)

3.9.3 Validation of response surface: Batch fermentation under optimized conditions

3.9.3.1 Alkaline protease production under SSF systems

Batch fermentation for alkaline protease production under optimized conditions was performed using 100 g of either IC or PP or different combinations of IC and PP (either supplied or not supplied with co-carbon and co-nitrogen source) moistened with 5.0 ml of distilled water (optimum pH was adjusted with respect to each strain). The substrate

was taken in rectangular trays of approximate dimension 286 mm x 120 mm x 80 mm. The tray was incubated at 50°C temperature under static condition. The protease was extracted at different time period (24, 48, 60, 72 and 96 h post inoculation) and assayed. A set of control was run in parallel.

3.9.3.2 Alkaline keratinase production under SSF systems

5.0 gram of chicken-feather was cut into uniform small sized pieces, taken in rectangular trays of approximate dimension 286 x 120 x 80 mm and mixed thoroughly with 5.0 ml of distilled water (pH was adjusted to 8.0 with 0.1N NaOH). The tray was incubated at 50°C temperature under static condition and then the protease was extracted at different time periods (24, 48, 60, 72 and 96 h post inoculation). A set of control was run in parallel.

3.9.3.3 Alkaline protease production under SmF systems

Batch fermentation for optimized protease production (Table 3.4) was carried out in a 5-l Bioflow 110 Fermentor (New Brunswick Scientific, USA) with a working volume of 3 l, operating with foam/anti-foam probe system and the M9 medium composed of best carbon and nitrogen for protease production sources at a desired concentration (% w/v). The pH of medium and optimum temperature was adjusted according to the optimum pH and temperature requirements for each strain. The agitation speed was 200 rpm, provided by a centrifuge propeller. The O_2 and pH electrodes were used for controlling process conditions. The cells were harvested at different time period (24, 48, 60, 72 and 96 h post inoculation), and the cell-free clear supernatant was used to determine the protease yield.

Table 3.4 Validation of response surface for alkaline protease/keratinase production

Strains	pH of the medium	Optimized concentration of	
		Carbon sources (% w/v)	Nitrogen sources (% w/v)
B.subtilis DM-04	8.0	2.5	1.25
Brevibacillus sp. AS-S10-II	13.3	1.4	0.13
Bacillus sp. AS-S20-I	10.9	2.59	0.12
B.licheniformis AS-S24-I	12.3	0.4	0.30
Peanibacillus tezpurensis sp.nov. AS-S24-II	7.0	1.0	0.15
B.subtilis strain RM-01	8.0	10.0	1.25

3.10. Isolation and downstream processing of alkaline proteases / keratinase

Unless otherwise stated, all the fractionation was carried out at room temperature (~24°C).

3.10.1 Isolation and purification of Bsubap-I, an alkaline protease from *B. subtilis* DM-04

Cell-free culture supernatant (48 h post inoculation) equivalent to 100 mg protein was applied to a CM-cellulose column (1 cm x 20 cm), pre-equilibrated with 20 mM K-phosphate buffer pH 7.0. The unbound proteins (showing major protease activity) eluted from the column with wash buffer were loaded on a DEAE-Sephadex A-50 column (1.5 cm x 9.0 cm) and eluted stepwise using phosphate buffers of increasing molarities and decreasing pH values as indicated. (a) 100 mM potassium phosphate buffer, pH 7.5; (b) 100 mM potassium phosphate buffer, pH 8.0; (c) 150 mM potassium phosphate buffer,pH 8.0; (d) 200 mM potassium phosphate buffer, pH 8.0; (e) 300 mM Potassium phosphate buffer, pH 8.0; (f) 300 mM potassium phosphate buffer, pH 8.5. Individual fraction was screened for protease activity and the fractions showing high caseinolytic activity were pooled, desalted on a

pre-packed desalting column (16 cm×2.5 cm, Bangalore Genei, India), vaccum concentrated and further fractionated on a Sephacryl S-200 gel filtration column (1.5 cm x 60 cm) with 20 mM K-phosphate buffer (pH 7.0). One ml fraction was collected in each tube at a flow rate of 24ml h and fractions showing the high protease activity were pooled and protein content was determined[363]. The homogeneity of preparation and molecular mass of protein was determined by 12.5% SDS-PAGE (section 3.10.8) analysis, with or without reduction of protein(s) with β-mercaptoethanol[383]. Purity of protein (protease) preparation was also checked by reverse-phase high performance liquid chromatography (RP-HPLC) of gel-filtration fraction (GF-II) on Waters reverse-phase Nova-Pak C_{18} column (3.9 mm x 300 mm). Protein was eluted with a linear gradient from 5% to 70% acetonitrile containing 0.1% TFA and elution was monitored at 220nm. Fractions were collected manually and peak was checked for protease activity.

3.10.2 Isolation and purification of Alzwiprase (an alkaline protease) from *B. subtilis* DM-04

One hundred milliliter (equivalent to 240.0 mg protein) of cell-free culture supernatant (post 60 h incubation) was applied to a CM-cellulose column (1 cm x 20 cm) pre-equilibrated with 20 mM K-phosphate buffer, pH 7.0. The flow through (unbound proteins) was collected in a single tube and then the column was washed with two column volume of equilibration buffer to elute the non-specifically bound proteins. The combined wash fraction and flow-through fraction was applied to a DEAE-Sephadex A-50 column (1 cm x 20 cm) pre-equilibrated with 20 mM K-phosphate buffer, pH 7.0.The unbound proteins as well as proteins eluted post washing the column with equilibration buffer were collected in a single tube, desalted on a pre-packed 154 desalting column (16 cm x 2.5 cm, Bangalore Genei, India). The pre-chilled ethanol (final concentration 66%) was added and the mixture was kept at 4°C for 12 h. The mixture was centrifuged at 10,000 x g for 10 min. The pellet obtained was dried in vacuum, re-suspended in 20 mM K-phosphate buffer, pH 7.0 followed by re-fractionation on Waters Nova-Pak reverse-phase C_{18} column (3.0mm x 300mm).Elution of protein was monitored at 220nm and peaks were collected manually.

The peak showing maximum protease activity was checked for homogeneity by SDS-PAGE as described in section 3.10.8.

3.10.3 Isolation and purification of alkaline β-keratinase protease from *B. subtilis* RM-01

The clear cell-free extract was applied to a CM-cellulose column (3.5 cm x 10.0 cm), pre-equilibrated with 50 mM K-phosphate buffer (pH 7.0). After washing the column with twice the volume of equilibration buffer, bound proteins were eluted stepwise at a flow rate of 24 ml /h using phosphate buffers of following molarities and pH values at room temperature (~24°C): The fractions showing high β-keratinase activity were pooled, concentrated in a vacuum centrifuge (Maxi Dry Plus, Heto Holten, Denmark) and further fractionated on a Sephacryl S-200 gel-filtration column (1.0 cm × 64.0 cm) equilibrated with 20mM K-phosphate buffer (pH 7.0). The peaks showing the maximum keratinolytic activity were pooled, desalted on a pre-packed desalting column (16.0 cm × 2.5 cm, Bangalore Genei, India), and further separated on Waters Nova-Pak C_{18} reverse phase HPLC column (3.0 mm × 300.0 mm) with a linear gradient from 5 to 70% acetonitrile containing 0.1% TFA at a flow rate of 1.0 ml/min and monitored at 280 nm. Peaks were collected manually, dried in a vacuum centrifuge at −20°C, and then assayed for keratinase activity as well as protein content determination[363]. The homogeneity of protease peak was checked by SDS-PAGE analysis as described in section 3.10.8.

3.10.4 Isolation and purification of detergent-stable alkaline protease from *Peanibacillus tezpurensis* sp.nov. AS-S24-II

The clear cell-free culture supernatant was precipitated with different concentrations of ice-cold acetone (50–90%, v/v) at 4°C and kept at the same temperature for 4 h. The precipitate was recovered by centrifugation and dissolved in minimal volume of 20 mM Tris–HCl buffer, pH 8.0, and assayed for protease activity and detergent stability. The fraction displaying high protease activity and showing highest detergent stability was selected, and further fractionated on a Sephacryl S-200 gel filtration column (1.0 x 64 cm) equilibrated with 20 mM K phosphate buffer (pH 7.0). One-millilitre fraction was collected in each tube and tested for detergent stability at 37°C (section 3.3.4) using

Safed® detergent (against which the crude protease demonstrated maximum stability) and as well as assaying the protease activity. The fractions showing the alkaline protease activity as well as high detergent stability were pooled and desalted on a pre-packed desalting column (16 x 2.5 cm, Bangalore Genei, India). This fraction was further separated on Waters Nova-Pak C18 reverse-phase (RP) high-performance liquid chromatography (HPLC) column (3.0×300 mm) with a linear gradient of 5% to 95% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) at a flow rate of 1 ml/min, and monitored at 280 nm. Protein peaks were collected manually, dried in a vacuum centrifuge at −20°C and then assayed for alkaline protease activity, detergent stability and protein content determination[363]. The purity of HPLC fraction showing protease activity was determined by SDS-PAGE analysis (section 3.10.8).

3.10.5 Isolation and purification of Alkarnase (an oxidant and detergent-stable alkaline protease) from *Bacillus licheniformis* AS-S24-I

The clear cell-free culture supernatant (60 h post inoculation) was precipitated with different concentrations of ice-cold acetone (50–90%, v/v) at 4°C and kept at the same temperature for 4 h. The precipitate was recovered by centrifugation and dissolved in minimal volume of 20 mM K-phosphate buffer, pH 8.0 and assayed for keratinase activity (100 mM Glycine-NaOH, pH 10.0). The fraction displaying highest keratinase activity was selected and further fractionated on a Sephacryl S-200 gel filtration column (1.0 x 64 cm) equilibrated with 20 mM K-phosphate buffer (pH 8.0). The peak showing the highest β-keratinase activity was desalted on a pre-packed desalting column (16 X 2.5 cm, Bangalore Genei, India) and lyophilized. This protein preparation was further purified using Waters Nova-Pak C_{18} reverse-phase (RP) HPLC column (3.0×300 mm). Proteins were eluted with a linear gradient from 5% to 70% (v/v) acetonitrile containing 0.1% (v/v) TFA at a flow rate of 1.0 ml /min and elution was monitored at 280 nm. The HPLC peak showing keratinase activity was checked for homogeneity by 12.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of proteins under reducing as well as non-reducing conditions[383]. For details (see section 3.10.8)

3.10.6 Isolation and purification of Brevicarnase (detergent-stable alkaline protease) from *Brevibacillus* sp. AS-S10-II

The clear cell-free culture supernatant (48 h post inoculation) was precipitated with different concentrations of ice-cold acetone (50–90%, v/v) at 4°C and kept at the same temperature for 4 h. The precipitate was recovered by centrifugation and dissolved in minimal volume of 20 mM K-phosphate buffer, pH 8.0 and assayed for keratinase activity (100 mM Glycine-NaOH, pH 12.5). The fraction displaying highest activity was selected and further fractionated on a Sephacryl S-200 gel filtration column (1.0 x 64 cm) equilibrated with 20 mM K phosphate buffer (pH 8.0). The peak showing the highest β-keratinase activity was desalted on a pre-packed desalting column (16 X 2.5 cm, Bangalore Genei, India) and lyophilized. This protein preparation was checked for homogeneity by 12.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of proteins under reducing as well as non-reducing conditions (section 3.10.8). The purity of purified protein was further checked by using high-performance liquid chromatography on a Waters Nova-Pak C_{18} reverse-phase (RP) HPLC column (3.0×300 mm). Proteins were eluted with a linear gradient from 5% to 70% (v/v) acetonitrile containing 0.1% (v/v) TFA at a flow rate of 1.0 ml / min and elution was monitored at 280 nm. The protease zymography of the purified β-keratinase was done as suggested by Mazotto *et al*[384] using keratin as a substrate (section 3.10.8).

3.10.7 Partial purification of alkaline protease from *Bacillus* sp. AS-S20-I

The crude protease extract was precipitated with 70% ice-cold acetone and allowed to stand at -20°C for overnight. The resulting precipitate was collected by centrifugation at 10,000g for 20 min at 4°C. The pellet so obtained was suspended in 20 mM K-phosphate buffer, pH 7.0 subjected to protease activity and protein content determination. The analysis of partially purified fraction showing protease activity was done by SDS-PAGE analysis as shown below.

3.10.8 Criteria of purity and determination of molecular weight

The SDS-PAGE was carried out with or without reduction of proteins by β-mercaptoethanol as described by Laemmli[383]. Briefly, either defined

amount of crude protein or purified protease was loaded into 15% or 12.5 % separating gel containing 5% (v/v) glycerol (Appendix I-N(i)). Electrophoresis was carried out at a constant current of 15mA until the dye font (bromophenol blue) reached the bottom of the gel. Before staining, proteins were fixed by incubating the gel in 20% TCA for 30 min at room temperature followed by washing the gel thrice in distilled water. Proteins bands were visualized by staining the gel with 1% (w/v) Coomassie brilliant blue R 250 in methanol:acetic acid:water (4:1:5 v/v/v) and destained with methanol:acetic acid:water (4:1:5 v/v/v), and then photographed. Mobility of the purified protein was compared with the following molecular weight markers: phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soyabean trypsin inhibitor (20,000) and lysozyme (14,300). For determining molecular mass of unknown proteins, the migration profile of protein was measured and the $\log_{10}$ values of the molecular weights of the standard proteins versus their migration distance were plotted. Taking the logarithm R_f allows the data to be plotted as a straight line. The molecular weight of unknown protein was then calculated from the standard curve.

3.10.9 Determination of active alkaline protease/ fibrinolytic / keratinase activity using zymographic study

3.10.9.1 Casein zymographic study

Casein zymograms were carried out as described previously Thangam and Rajkumar[385]. Briefly, casein (1.0%, w/v) that was dissolved in a 1.5M Tris-HCl buffer (pH 8.8) was co-polymerized with 12% (w/v) acrylamide, 0.32% (w/v) bisacrylamide in order to make a running gel. Then 4% (w/ v) acrylamide, 0.11% (w/v) bisacrylamide, and 0.5 M Tris-HCl (pH 6.8) (no substrate) were used for the stacking gel, then poured into a mini-gel cast (Bio-Rad) (Appendix I-N(ii)). The samples (15 µg) that were obtained from the culture supernatant of all strains were prepared by a zymogram sample buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, and 0.5% bromophenol blue). After the prepared samples were loaded into the wells, an electrophoresis[383] was carried out in the cold room at a constant current of 12 mA. After the electrophoresis was completed, the gel was incubated for 20.0 min at 4°C temperature on a rotary shaker in buffers w.r.t purified enzyme, which contained 2.5%

Triton X-100. The gel was washed with distilled water to remove Triton X-100, and then incubated in a zymogram reaction buffer (w.r.t purified enzyme) at 37°C for 12 h. The gel was stained with Coomassie brilliant blue (0.5%) for 8h and then destained in the fixed volume (100 ml) of the destaining solution that contained 10% methanol and 5% acetic acid for a limited period of time. For quantification, the densities of the digested bands on the zymograms were analyzed by video densitometry using Bio 1D ver. 97.04 (Vilber Lourmat, France) image analyzer software using

3.10.9.2 Fibrin zymographic study

Fibrin zymography was performed as described previously Kim *et al*[386]. Bovine fibrinogen (0.12%, w/v, Sigma) dissolved in 20 mM sodium phosphate buffer (pH 7.4) and 100 µl of bovine thrombin (10 NIH unit /ml, Sigma) was co-polymerized with 12% (w/v) acrylamide, 0.32% (w/v) bisacrylamide and 1.5 M Tris/HCl (pH 8.8) in order to make the fibrin gel the running gel. Then 4% (w/v) acrylamide, 0.11% (w/v) bisacrylamide and 0.5 M Tris/HCl (pH 6.8) (no fibrinogen) were used for the stacking gel, which was poured into a mini-gel cast (Tarson, India). The samples for analysis were prepared by diluting the culture supernatant 5-fold with zymogram sample buffer (0.5MTris/HCl, pH 6.8, 20% glycerol and 0.5% Bromphenol Blue)[387].After the prepared samples (10 µl) were loaded into the wells, electrophoresis[383] was carried out in the cold room (4 °C) at a constant 12 mA. After electrophoresis, the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris/Cl (pH 7.4), which contained 2.5% Triton X-100. The gel was washed with distilled water to remove Triton X-100, and then incubated in zymogram reaction buffer (30 mM Tris/HCl, pH 7.4) at 37°Cfor 16 h. The gel was stained with Coomassie blue for 8 h and then destained. The digested bands were visualized as the *non-stained regions of the fibrin gel. For the quantification, the* densities of digested bands on the gel were analyzed by video densitometry using Bio 1D Ver. 97.04 (Vilber Lourmat, France)[387].

3.10.9.3 Keratin zymographic study

Activity staining of purified proteases was performed by keratin–SDS–PAGE zymography[388] using 10 mg/ml keratin in 12.5% polyacrylamide

mixture. After electrophoresis, the gels were soaked thrice for 20 min in 2.5% (v/v) Triton X-100 at room temperature. Enzyme activity was visualized by incubating the gel for 16 h in substrate buffer (0.1M Tris-HCl buffer pH 8.3) at room temperature. The gel was stained with 0.5% of Coomassie brilliant blue R-250 and bleached with methanol–glacial acetic acid–water (30:10:60).

3.10.10 Estimation of protein content

The protein content was estimated by Lowry method[363] using BSA as a protein standard. The protein content of the unknown sample was calculated from the standard curve BSA obtained by plotting optical density (660nm) Vs concentrations of BSA (0.1 to 1.0mg /ml).

3.11 Assay of proteolytic activity

Caseinolytic activity was evaluated colorimetrically as described by Mukherjee and Maity[391]. Briefly, 1% (w/v) of casein in 0.1M concentration of reaction buffer w.r.t purified enzymes was incubated with specific amount of crude/ purified protease for 30 min at optimum incubation temperature w.r.t to purified enzymes followed by addition of 0.5 ml of 10% (w/v) ice-cold TCA to stop the reaction. After centrifugation of the mixture, supernatant was transferred to a fresh tube containing 2.0 ml of 2 % (w/v) sodium carbonate in 0.1N sodium hydroxide. The reaction was allowed to continue for 10 min at room temperature followed by addition of 0.5 ml of Folin-Ciocalteus reagents (1:2 dilutions). After 30 min absorbance was measured at 660 nm. Caseinolytic activity of the crude/ purified protein was calculated from a standard tyrosine curve. One unit (U) of caseinolytic activity is defined as nanomole equivalent of tryrosine formed per min per ml of enzyme.

For keratinase activity assay, the same procedure was followed except the casein was replaced with keratin. The specific activity of enzyme was calculated as Units of protease activity per mg of protein (U /mg).

Fibrinolytic activity assay was done as described by Kim *et al*[386] with the following modifications. Briefly, 3 µl of thrombin (10 NIH mL^{-1}) was added to 40 µl of 0.6% (w/v) bovine fibrinogen solution (prepared in 100 mM K-phosphate, pH 7.4) and clot was allowed to form at room

temperature. Different concentrations of crude protease / purified protease were added to the clots and the reaction mixtures were incubated at 37°C for 30 min. The reaction was stopped by adding 10 µl of ice-cold trichloro acetic acid and the supernatant was used to determine the release of free amino acids (tyrosine) at 660 nm using Folin-Ciocalteus reagent. One unit (U) of fibrinolytic activity is defined as µg of tyrosine liberated per min per ml of enzyme.

3.12 Biochemical characterization of alkaline protease

3.12.1 Dose-dependent protease activity

To determine the effect of enzyme concentration on catalytic activity graded amounts of enzyme (0.5 to 10.0 µg) were added to the reaction mixture and the protease activity was assayed by the method as described in section 3.11.

3.12.2 Effect of pH on protease activity and stability

The protease was incubated in different pH buffers (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 and 13.0) and the protease activity in each buffer was determined. The following buffer (0.1M) systems were used: citrate- phosphate (pH 4.0), potassium-phosphate (pH 7.0), Tris-HCl (pH 9.0), glycine-NaOH (pH 10.5-11) and glycine-NaOH (pH 13.0). The optimum pH for alkaline protease activity was determined by standard alkaline protease assay using casein/keratin 1.0 % (w/v) as substrate dissolved in the buffer systems mentioned above. To study the protease stability as a function of pH, 2.0 ml of the crude enzyme or 0.1- 4.0 µg of purified protease was mixed with 2.0 ml of the buffer solutions mentioned above and incubated at 37°C for desired time period Thereafter, protease activity (%) was determined as discussed in section 3.11.

3.12.3 Determination of optimum temperature for protease activity

The optimum temperature for alkaline protease activity was determined by incubating the reaction mixture (containing protease and substrate) at optimum pH for 20 minutes, at different temperatures ranging from 30 to 80°C. Before the addition of enzymes, the substrate (1.0 % (w/v) casein) was pre-incubated at the respective temperature for 10 minutes. For each assay, a control was also setup with respective temperature .

3.12.4 Heat-inactivation study

Thermo-stability of purified / partially purified protease preparation was determined by following the procedure as described in section 3.3.3.

3.12.5 Influence of metal ions on protease activity

To investigate the effects of different divalent cations on protease activity, casein / keratin hydrolysis reaction was performed with 2.0 µg of protease in presence of different metal ions (Ni^{2+}, Co^{2+}, Mg^{2+}, Mo^{2+}, Fe^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Cd^{2+}). The enzyme activity without metal ions served as control and was considered as 100% activity.

3.12.6 Chemical modification of protease activity

Chemical modification of the histidine, cysteine and serine residues was performed by pre-incubating the purified protease with different concentration of 4-bromophenacyl bromide, iodoacetamide (IAA) and phenyl methyl sulfonyl fluoride (PMSF), respectively at room temperature (~24°C) for 30 min and then assaying the protease activity against appropriate controls[364].

3.12.7 Effect of surfactants, chelators, denaturing, oxidizing and bleaching agents

To investigate the effects of surfactants (ionic surfactants like SDS, non-ionic surfactants like Tween-20, 80, Trition –X-100), chelator (EDTA), denaturing agent (urea), oxidizing agent (H_2O_2), and bleaching agent (sodium perborate) on protease activity. 2.0 µg of purified protease was pre-incubated with these chemicals for 30 min at room temperature followed by assay of protease activity. The protease activity without surfactants, chelator, and denaturing, oxidizing and bleaching agents was considered as 100% activity (control) and other values were compared with that.

3.12.8 Organic-solvent stability

The organic solvent stability of protease preparation was studied by pre-incubating the purified enzyme with various organic solvents (20% v/v) viz xylene, n-hexane, 2-propanol, methanol, ethanol, acetonitrile, and benzene at room temperature (~25°C) for 120 min. Aliquots were

withdrawn at desired time intervals to test the remaining protease activity. The purified enzyme without added organic solvent was considered as control (100% activity) and other values were compared with that[173].

3.12.9 Substrate specificity

To determine the substrate specificity of purified/ partially purified alkaline protease, different proteolytic substrates viz., bovine serum albumin (BSA), bovine serum globulin (BSG), bovine serum fibrinogen (BSF), hemoglobulin, raw chicken-feather, human hair, collagen, gelatin, keratin and casein were used at a final concentration 1.0 mg / ml and incubated with 2.0 µg of crude/ 1.0 µg of purified alkaline protease at desired (optimum) temperature for 15 min. The protease activity was assayed as described in the section 3.11.

3.12.10 Enzyme kinetics

The K_m and V_{max} values of the protease preparation were calculated by Lineweaver–Burk plot using 0.1-2% (w/v) casein/keratin as a substrate[390]. The turnover number (K_{cat}) of the enzyme was determined by dividing the value of Vmax with the quantity of enzyme (E) used for driving the reaction [$K_{cat} = V_{max}/ E(T)$].

3.13 Immobilization of purified alkaline keratinase onto iron-oxide magnetic nanoparticle for improvement of catalytic activity and biotechnological potential of enzyme

3.13.1 Preparation of iron-oxide magnetic nanoparticle (MNPs)

Iron-oxide MNPs were prepared as described by Rossi *et al*[391]. Briefly, 1.0 M KOH solution was added drop wise to 0.1N $FeCl_2$ (25 ml), prepared in Millipore water, with constant stirring at room temperature till pH 7.2 was attained, when a characteristic dark green colour was observed. Addition of 250 µl of 3 % (v/v) H_2O_2 to the above dark green suspension yielded a black precipitate that was attracted by a permanent magnet. After separating the particles by magnetic decantation, they were washed thrice with 20 ml distilled water thrice, followed by washing with 20 ml acetone (twice). After drying overnight at room temperature, the yielded MNPs were weighed.

3.13.2 Optimization of enzyme coupling to magnetic nanoparticle

The following protocol was executed to find the best method (aminofunctionalisation vs. carbodiimide activation) and the best buffer for activation of the MNP for efficient protease binding.

3.13.3 Preparation of aminofunctionalized MNP

100 mg of iron-oxide nanoparticles were redispersed in 100 ml of ethanol by sonication. After adding 70 µl of 3-(aminopropyl) triethoxy silane, the solution was mechanically stirred at room temperature. Next day, the black precipitate was separated by centrifugation and washed with 20 ml ethanol twice and dried in air at room temperature.

3.13.4 Covalent immobilization of keratinase from *B.subtilis* strain RM-01 on Fe_3O_4 MNP

Four sets of 2.5 mg amino-functionalised MNP were subjected to sonication in the following buffers : (i) 10 % glutaraldehyde-K-phosphate buffer (20 mM) solution, pH 7.4 (ii) Tris-HCl (50 mM), pH 7.5 (iii) Tris-HCl (50 mM), pH 8.0 and (iv) Tris-HCl (50 mM), pH 8.5 for 1 hour. The MNP were separated by magnetic decantation, followed by washing with 1 ml 20mM K-phosphate buffer pH 7.4, and then resuspending in 0.5 ml of the 20mM K-phosphate buffers, pH 7.4. Then 0.5 ml of purified keratinase (5.0 mg / ml) was added, stirred gently and kept at 4°C for overnight. The enzyme coupled MNP were separated by magnetic decantation followed by washing and re-suspending in 2.0 ml of the respective buffers. This was followed by assay of protease and keratinase activity and the best buffer in terms of maximal MNP-bound keratinase activity was selected.

3.13.5 Attachment of purified enzyme onto MNP by carbodimide/cyanimide activation

50 mg of MNP were suspended in any of the following buffer (i) 10 % w/v glutaraldehyde- K-phosphate buffer (20 mM) solution, pH 7.4 (ii) 10 % w/v glutaraldehyde Tris-HCl (50 mM), pH 7.5 (iii) 10 % w/v glutaraldehyde Tris-HCl (50 mM), pH 8.0 and (iv) 10 % w/v glutaraldehyde Tris-HCl (50 mM), pH 8.5. 0.5ml of freshly prepared carbodiimide solution (prepared by dissolving 20 mg / ml of the respective buffers) was added to the above sets. This was followed by

sonication for 15 minutes at room temperature and then, 0.5 ml (concentration 5.0 mg / ml) of purified keratinase enzyme was added. Sonication was done for 30 minutes at 4°C (ice-bath), followed by centrifugation of the sonication mixture at 3000 rpm for 10 minutes. The supernatant was collected in a fresh microfuge tube (post-precipitate supernatant). The precipitate was washed twice and finally suspended in 0.5 ml of the respective buffers. Protein content determination (section 3.10.10) and keratinase activity assay (section 3.11) were performed to determine the best buffer in terms of maximal MNP-bound keratinase activity.

3.13.6 Binding of MNP binding to purified keratinase enzyme: dose-dependent study

After finding the best buffer and best method for MNP activation and enzyme binding, dose dependent study was performed by varying the amount of MNP while the volume of enzyme added was kept constant.

3.13.7 Statistical procedure for optimizing conditions for maximal coupling of keratinase on to iron-oxide MNP

Response surface methodology (RSM) was used to estimate main effects on response i.e. the enzyme coupling to the iron oxide MNP. Two factors with three levels consisting of 13 experimental runs, were used to analyze the experimental data including four replicates at the centre point to allow better estimate of the experimental error and to provide extra information about the specific activity of the coupled enzyme in the interior of the experimental region[392]. The amount of MNP used for binding (C_1) and pH of the buffer system used (C_2) were chosen as the experimental factors or the independent variables capable of influencing the studied specific activity of MNP-coupled keratinase (Y).

The correspondence between the coded values and natural values was obtained by the following formula :

$$C_i = \frac{C_i - C_0}{\Delta C_i}, \quad i = 1,2,3,\ldots, k \qquad \text{(3.4)}$$

Where Z is the coded value, C_i is the corresponding natural value, C• is the natural value in the center of the domain and ΔC is the increment of C corresponding to one unit of Z. Using this design, the experimental data were fitted according to the Eq. (3.5) as a second order polynomial regression equation including individual and cross effect of each variable.

$$Y = a_0 + \sum_{i=1}^{3} a_i C_i + \sum_{i=1}^{3} a_{ii} C_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} a_{ij} C_i C_j \quad \text{.......... (3.5)}$$

Where, Y = Specific activity of the enzyme coupled to MNP (response), a_0 intercept term, a_i = linear effect, a_{ii} = square effect, a_{ij} = interaction effect, and C_i and C_j = variables.

Analyses were carried out in triplicate and the data tabulated were the average of the three measurements. The above model was used to optimize the values of independent parameters for the response. Multiple regression analysis and response surface plots were generated using Minitab 15 Statistical Software® (Minitab Inc, PA, USA).

3.14 Characterization of the enzyme-coupled iron-oxide magnetic nanoparticle

3.14.1 XRD analysis for determination of nanocrystalllite size

X-ray diffractometer 'Miniflex', (Rigaku corporation Japan), was used for the powder XRD analysis. The data was fed into the software, X-PERT, for determination of the nanocrystallite size, based on Scherrer's formula:

$$D_v = \frac{K\lambda}{\beta \cos\theta} \quad \text{.......... (3.6)}$$

Where, D_v = Volume weighted crystallite size

K = Scherrer constant (0.9 in the present study)

λ = the wavelength of the radiation (1.574×10^{-10} m in the present study)

β = the integral breadth of a reflection (in radians 2θ) located at 2θ

3.14.2 FTIR analysis

FTIR spectrometer for Nicolet USA (Model Impact-410) was used to check the binding of the enzyme coupled to the MNPs. The experiment range of the FTIR used was from 400 to 4000 cm^{-1}. The sample was mixed with KBr (Merck, India) uniformly (0.01%) and a pellet was prepared with a handy press equipped with a pellet holder of 50 nnm thickness, placed in the sample holder and the spectrum was collected.

3.14.3 SEM analysis for determination of particle size

Uncoated carbodiimide functionalized and enzyme bound MNP were examined with a JEOL JSM-6390 LV scanning electron microscope. The samples were platinum coated using JEOL JFC-1600 Auto fine coater.

3.14.4 Comparison of stability storage of free and MNP-bound β-keratinase

The storage stabilities of MNP-bound and free β-keratinase were examined by assaying their residual activity post storage at 4°C for a predetermined time period. The activity of free or Fe_3O_4 MNP-bound enzyme at the beginning of experiment was considered as 100% activity and other values were compared with this.

3.15 Industrial application of purified / partially purified alkaline proteases / keratinase

3.15.1 Blood clot lysis (thrombolytic) activity

Clot lysis was studied with both natural and synthetic blood clot *in vitro* condition. The mixture of 50 ml fibrinogen (0.6% w/v) and 5 ml thrombin was incubated at room temperature for 1 h, washed and vortexed for dispersion. One hundred micro- litre of enzyme (20 mg) was added into this solution, and incubated at 37°C for 16 h. At different time intervals, aliquots were taken from the reaction mixture for analysis[393].

3.15.2 Detergent compatibility test

Detergent compatibility and stability study was performed as explained in section 3.3.4.

3.15.3 Wash performance test

Wash performance of crude / purified protease was evaluated by subjecting the blood stain removal test from cotton fabrics as described by Mukherjee[365] with the following modifications. Briefly, white cotton cloth was cut into 5.0 cm^2 pieces, and each piece was stained with 1.0 ml of fresh goat blood (obtained from slaughter house) and then dried at 40°C for overnight. To test the wash performance, each piece of stained cloth was dipped in any one of the following flasks containing: (a) 25 ml of tap water (control), (b) 20 ml of tap water and 5 ml of heated detergent (7 mg/ ml), (c) 20 ml of tap water and 5 ml of unheated detergent (7 mg /ml), (d) 20 ml of tap water and 5 ml of heated detergent (7 mg / ml) containing 2-8 µg / ml protease, (e) 20 ml of tap water and 5 ml of unheated detergent (7 mg /ml) containing 2-8 µg/ ml protease and (f) 20 ml of tap water and 5.0 ml of 2.0 to 8.0 µg / ml protease. Flasks were kept at 25°C, 37°C and 45°C for 10 min followed by removal of the cloth pieces, and the left over washes were used to determine the quantity of blood (haemoglobin) removed from the cloths[365].The dried cloths were visually examined for blood stain removal and then photographed.

3.15.4 Pharmacological characterization of proteases

3.15.4.1 Interference in blood coagulation

Interference in blood coagulation (coagulant or anticoagulant effect) by crude / purified protease was assayed as described by Doley and Mukherjee[394]. Briefly, platelet poor plasma (PPP) from goat was prepared by centrifuging (2,500 X g at a Remi Centrifuge) the citrated blood (1:9) twice for 15 min at 4°C and used within 4h of collection. To assay the recalcification time, specific amount of crude / purified proteases (in a final volume of 30 µl) was added to 300 µl of PPP pre-incubated at 37°C. The mixture was incubated for 3 min at 37 °C and 40 µl of 250 mM $CaCl_2$ was added to this reaction mixture . The clotting time of plasma was recorded with the help of stopwatch based on the first appearance of a fibrin clot. As a control, plasma aliquot was incubated with 30 µl of phosphate buffer saline (PBS) and coagulation time was determined identically.

3.15.4.2 Assay of haemolytic activity

Direct haemolytic activity was assayed as described by Doley and Mukherjee[394]. Briefly, graded concentrations of protease solutions were added to 5 % (v/v) of human erythrocyte suspension (in 100 mM phosphate buffer, pH 7.4) and final volume was adjusted to 3 ml. After incubating for 60 min at 37°C, the reaction mixture was centrifuged and released haemoglobin was measured spectrophotometrically at 540 nm (U-2001 UV/Vis Spectrophotometer, Hitachi, Japan). For cent percent (100 %) hemolysis, RBC suspension was incubated with 0.1 % (v/v) Triton-X-100.

3.15.4.3 In-vitro tissue damaging activity

For the assay of in-vitro tissue damaging activity, procedure of Doley and Mukherjee[394] was followed with the following modifications. Fresh chicken liver, heart and lungs were washed with 0.9%sodium chloride solution, cut into small uniform sized pieces, patted dry with tissue paper and 300 ± 10 mg pieces were weighed. The tissues were pre-incubated with 1 ml of 0.2 M K-phosphate buffer, pH 7.4, for 45 min at 37°C. The tissues were then washed twice with the same buffer and incubated with 25 mg of either crude venom or purified protein in a final volume of 2.5 ml in 0.2M K-phosphate buffer, pH 7.4 for 5 h at 37°C. After incubation, reaction mixtures were centrifuged for 5 min at 3000 rpm and the absorbance of the supernatant was read at 540 nm by an UV–VIS Spectrophotometer (Hitachi, Japan). The percentage of haemoglobin released was calculated with respect to tissues incubated with 0.1% Triton X-100 solution (100% activity).

3.15.5 Dehairing activity

Goat skin obtained from a slaughter house was cut into 5 cm^2 pieces and kept in a disposable sterile perti dish. To this purified alkaline protease (all prepared in sterile medium) was added and the final volume was adjusted to 10.0 ml with sterile deionized water. Then the petri dish was sealed with parafilm and incubation was carried out for 12 h at 37 °C followed by virtually analyzing skin pieces for dehairing activity as described by Huang *et al*[395]. Briefly, the hair of the skin was pulled by hand to test whether the hair was separated from the skin easily. Since there is no quantitative method to determine the effect of

dehairing; therefore, we define the dehairing effect to be "no" or "yes" or "easily."

3.15.6 Antibacterial property of purified alkaline protease

Antibacterial activity was measured by a liquid growth inhibition assay, performed in 96-wells microtiter plates (Microplates, Flat-bottom, sterile, Axygen Scientific Pvt. Ltd USA)[396,397]. Ten ml aliquots of the purified protease were added to 100 ml of a logarithmic phase (O.D.630nm ≈ 0.3) cultures of *Escherichia coli, Bacillus licheniformis, Kocuria varians, Peanibacillus polymyxa, Staphylococcus aureus* and *Klebsiella pneumoniae* of the tested bacteria which was diluted to 10^5 - 10^6 colony-forming units (cfu) per ml. Controls (bacterial cultures with or without antibiotics) were run in parallel and inhibition of bacterial growth (if any) by purified protease enzyme. Final concentrations of the purified protease in the test were used 0.25 mg /ml. Microbial growth was measured as an increase of optical density at 630 nm by a microplate reader (Multiskan, Thermo electron corporation, China) after incubation at 37°C for 16 h. Minimal inhibitory concentrations (MIC) are expressed as intervals of concentrations: [a]-[b]; where [a] is the highest concentration tested at which the bacteria are growing and [b] being the lowest concentration causing 100% of growth inhibition (no change in O.D. at 630nm)[397]. After 24 h, 80 ml of the test well which showed no change in OD630 was plated, either directly or diluted (1:100) in medium, to define whether the cells were lysed or impeded to grow. Each concentration was tested in triplicates[398].

3.15.7 Feather degradation using MNP-Bound β-keratinase

Evaluation of chicken-feather hydrolysis by either free or iron-oxide MNP-bound keratinase was carried out by using 2% (w/v) washed-dried and finely cut chicken-feathers suspended in 100 mM Tris-HCl buffer pH-9.0. To this mixture, a predetermined amount of either free or iron-oxide MNP- bound β-keratinase was added and incubated at 45°C for different time periods under shaking at 100 rpm. Sodium sulphite at a final concentration of 2 mM was added to each reaction mixture to accelerate the reaction rate[399]. At the end of desired time period, the hydrolyzate was filtered through a pre-weighed Whatman® filter paper to isolate the non-degraded (undigested) chicken-feather. Filter papers

were dried under the vacuum, weighed and the mass of non-degraded chicken-feather was estimated against appropriate control. The filtrate was analyzed for amino acids and free fatty acids contents as described below.

3.15.8 Release of free-amino acids from the chicken-feather

A predetermined volume of feather-hydrolisate was withdrawn at a regular interval (24 h to 40 h), passed through four layers of cheesecloth, centrifuged at 10,000 X *g* for 5 min at 4°C and the supernatant was collected in a fresh tube. Two hundred microliter of the supernatant was extracted in methanol and then analyzed for free-amino acids by high performance liquid chromatography (HPLC) using C_{18} column[400]. The standard amino acids used were cysteine, threonine, valine, methionine, isoleucine, phenylalanine and lysine.

3.15.9 Analysis of liberated free fatty acids from chicken-feather by β-keratinase

For quantitative analysis of FFA released post treatment of MNP bound β-keratinase, 5.0 ml of methanolic NaOH solution (45.0 gm (% w/v) NaOH in 150.0 ml of methanol) was added to a measured amount of lipid extract (in a round bottomed flask) and the mixture was refluxed for 30 min. Then 5.0 ml of deionised water and 1.0 ml of concentrated HCl were added, mixed well and the mixture was extracted with 10.0 ml of petroleum ether. The process was repeated twice and the combined samples were dried in a rotary vacuum evaporator. To these FFAs, 10.0 ml of methyl alcohol and few drops of concentrated H_2SO_4 were added. The mixture was again refluxed for 3 hours followed by dilution with 10 ml of water and then re-extracted with 10.0 ml of petroleum ether. The extraction procedure was repeated twice. All the petroleum-ether extracted fractions were pooled and allowed to dry in a dessicator for analysis by GC.

The dry sample (fatty acid methyl esters) was dissolved in a minimum quantity of chloroform and analyzed on a GC-MS (Varian 3800, Saturn 2000) system. The samples were injected using a split ratio of 100:1 into a fused silica GC column CP-Sil 8 CB low bleed (30m x 0.25mm x 0.25 µm) coupled with CP-Sil 5C low bleed / MS (30m x 0.25mm x 0.25

μm) column with helium as the carrier gas. The system was equipped with flame ionization detector. The initial oven temperature was 40°C and a temperature program of 3°C min^{-1} began at injection and continued to a final oven temperature of 240°C , which was held isothermal for 5 min. The injector port and detector temperature were set at 280°C. The mass spectrometric data were acquired in electron ionization mode (70eV). Mass spectra of unknown methylated fatty acids were identified by comparing the retention time of authenticated fatty acids, using the Saturn 2000 MS library search where 99% matching was observed.

CHAPTER IV

RESULTS

Sudhir K Rai

PhD Thesis, Tezpur University, 2010

Chapter 4

Screening of micro-organisms from different habitats of North-east India (with special emphasis on extreme habitats) for the production of protease with particular reference to the alkaline, thermophilic enzymes

4.1 Collection of environmental samples

From different environmental samples, 52 soil samples were collected at random in sterile tubes, minimum five, from a field including area around the hot springs, oil-fields, high altitude mountains, etc.

Fifteen (15) water samples were collected in sterile screw cap tubes from rivers, hot-springs effluents from paper mills, were collected and kept at 4 °C until further use. A total of 67 samples were collected from the five states of north east India as shown in Table 4.1.

Table 4.1 Collection of soil samples from various regions of North-East India

S. No.	North-Eastern States	Samples collected Soil	Samples collected Water	Total
1	Assam	30	6	36
2	Arunachal Pradesh	10	8	18
3	Manipur	4	Nil	4
4	Meghalaya	5	1	6
5	Tripura	3	Nil	3
		Total: 67		

Out of the 33 bacterial cultures obtained from screening the environmental samples of NE India, only 12 isolates showed zone of hydrolysis ≥ 25.0 mm (Tables 4.2 and 4.3). Pure cultures of such isolates were obtained for further studies.

Table 4.2 Screening for alkaline protease producing alkalophilic bacteria from different samples of North- East India. The medium used was skim-milk agar, pH 8.0.

S. N	Sample code	District	Type of sample (soil / water)	pH of sample	Zone of hydrolysis in mm (No. of CFU)
		State : Assam			
1	AS-S02a	Golaghat	Soil	7.14	17.0,26.8 (02)
2	AS-S03a	-do-	-do-	5.04	7.0 ; 24.0 ; 40.0 (03)
3	AS-S03b	-do-	-do-	4.28	29.0 ; 16.0 (02)
4	AS-S05	Sivasagar	-do-	8.09	14.0; 16.0 (02)
5	AS-S06	-do-	Soil	8.31	7.0; 5.0 (02)
6	AS-S08	-do-	-do-	6.61	17.0 (01)
7	AS-S09	-do-	-do-	8.09	13.0; 8.0 (02)
8	AS-S10	-do-	-do-	5.30	34.0; 24.0 (02)
9	AS-S13	-do-	-do-	7.85	16.0 (01)
10	AS-S14	-do-	-do-	7.22	19.0 (01)

S. N	Sample code	District	Type of sample (soil / water)	pH of sample	Zone of hydrolysis in mm (No. of CFU)
		State : Assam			
11	AS-S15	Sivagar	Soil	7.77	(22-CFU)- very small
12	AS-S18	Cachar	-do-	9.39	27.0; 24.0 (02)
13	AS-S20	-do-	-do-	5.93	20.0; 19.0; 26.0 (02); 27.0 (05)
14	AS-S21	Sonitpur	-do-	5.85	21.0; 6.0; 40.0 (02)
15	AS-S24	-do-	-do-	3.94	29.0; 22.0; 30.0 (03)
16	AS-W01	Golaghat	Water	6.77	23.0 (01)
17	AS-W02	Sonitpur	Water	7.95	21.0 (01)
		State : Arunachal Pradesh			
18	AR-S01	Papum Pare	Soil	4.48	6.0 (01)
19	AR-S02	-do-	-do-	6.22	5; 12.0 (02)
20	AR-S04	-do-	-do-	4.48	10; 15.0 (02)
21	AR-W01	-do-	Water	7.02	6.0 (02)
22	AR-W02	-do-	-do-	6.65	5.0, 6.0 (02)

...Continued

Table 4.2 Continued....

23	AR-W04	Papum Pare	Water	5.56	5.0; 12.0 (02)
		State : Manipur			
24	MN-S01	Imphal West	Soil	4.90	15.0 (01)
25	MN-S02	Thoubal	-do-	4.97	6.0 (01)
26	MN-S03	Imphal West	-do-	6.74	7.0 (03)
27	MN-S04	-do-	-do-	8.24	10; 24.0 (02)
		State : Meghalaya			
28	ML-S01	East Khasi Hills	Soil	6.18	19.0 ; 20.0 (02)
29	ML-S02	-do-	-do-	4.99	3.6; 7.0 ; 8.0 (03)
30	ML-S03	-do-	-do-	4.61	7.0 ; 7.0 ; 7.0 (03)
		State : Tripura			
31	TR-S01	West Tripura	-do-	5.93	22.0 (01)
32	TR-S02	-do-	-do-	4.38	20; 22.0 (02)
33	TR-S03	-do-	-do-	4.35	5.0 (01)

These twelve isolates were further characterized based on their growth and protease yield in SmF (Table 4.4). On the basis of protease production, only 7 isolates (shown in bold letter) were considered as alkaline protease producing potential bacteria (Table 4.4).

Table 4.3 Selection of alkaline protease producing 12 bacteria having zone of hydrolysis ≥ 25.0 mm (see Table 4.2)

S.No	Sample code	District	Type of sample (Soil / Water)	pH of sample	Zone of hydrolysis (in mm)
		State : Assam			
1	AS-S-02a	Golaghat	Soil	4.31	26.8
2	AS-S03a	-do-	-do-	5.04	40.0
3	AS-S03b	-do-	-do-	4.28	29.0
4	AS-S10 I	Sivasagar	-do-	5.30	25.0
5	AS-S10 II	-do-	-do-	5.30	34.0
6	AS-S18	Cacher	-do-	9.39	27.0
7	AS-S20-I	-do-	-do-	5.39	26.0
8	AS-S20-II	-do-	-do-	5.39	26 0
9	AS-S20-III	-do-	-do-	5 39	27.0
10	AS-S21	Sonitpur	-do-	5.85	40.0
11	AS-S24-I	-do-	-do-	3.94	29.0
12	AS-S24-II	-do-	-do-	3.94	30.0

Table 4.4 Selection of alkaline protease producing potential bacteria on the basis of alkaline protease production in culture medium. Results represent mean ± S.D of three individual experiments.

Bacterial Isolates	Protease yield (Units)	Growth (OD at 600 nm)
AS-S-02a	12.0 ± 0.6	0.421 ± 0.02
AS-S03a	15.0 ± 0.8	0.340 ± 0.02
AS-S03b	20.0 ± 1.0	0.246 ± 0.01
AS-S10 I	20.0 ± 1.0	0.310 ± 0.02
AS-S21	22.0 ± 1.1	0.424 ± 0.02
AS-S20-III	**20.3 ± 1.0**	**0.388 ± 0.02**
AS-S20-II	**22.0 ± 1.1**	**0.346 ± 0.02**
AS-S18	**35.0 ± 1.8**	**0.358 ± 0.02**
AS-S10-II	**62.1 ± 3.1**	**0.254 ± 0.01**
AS-S24-I	**63.9 ± 3.2**	**0.325 ± 0.02**
AS-S24-II	**64.1 ± 3.2**	**0.410 ± 0.02**
AS-S20-I	**66.6 ± 3.3**	**0.367 ± 0.02**

The next criterion of selection was based on some of the biochemical properties of the crude protease enzyme from the selected bacteria, for example, their stability and compatibility with commercial laundry detergents (Table 4.5), thermo-stability (Table 4.6), and storage stability at 4°C (Fig.4.1). Results showed that protease from five isolates viz. **AS-S10-II, AS-S18, AS-S20-I, AS-S24-I and AS-S24-II** displayed superior properties as compared to same properties displayed by crude protease enzyme from others three strains. These four strains were selected for taxonomic identification, optimization of protease production, isolation, purification and characterization of extracellular protease enzyme(s).

Table 4.5 Detergent compatibility study of crude protease pre-incubated at 45°C. Enzyme activity in absence of detergent was considered as 100% activity and other values were compared with that. Results represent mean ± S.D of three individual experiments.

Commercial laundry detergents (7.0 mg /ml)	**Isolates**						
	AS-S10-II	**AS-S18**	**AS-S20-I**	**AS-S20-II**	**AS-S20-III**	**AS-S24-I**	**AS-S24-II**
	Remaining protease activity (%)						
Ariel	96.3 ± 4.8	93.1 ±4.7	119.7 ± 6.0	86.5 ± 4.0	96.9 ± 4.8	78.5 ± 3.9	131.6 ±6.6
Henko	165.8 ± 8.3	86.3 ± 4.3	94.3 ± 4.8	82.0 ± 4.1	90.0 ± 4.5	123.4 ± 6.2	140.0 ± 7.0
Rin advanced	115.8 ± 5.8	95.8 ± 4.8	115.4 ± 5.6	98.5 ± 5.0	104.5 ± 5.2	124.4 ± 6.2	116.6 ± 5.8
Surf excel	102.4 ± 5.1	105.4 ± 5.3	111.2 ± 5.6	97.0 ± 4.9	98.7 ± 4.9	74.4 ± 3.7	156.6 ± 7.8
Tide	93.9 ± 4.7	98.6 ± 4.9	119.7 ± 6.0	97.0 ± 4.9	106.0 ± 5.3	95.9 ± 4.8	160.0 ± 8.0
Wheel	90 ± 4.5	95.8 ± 4.8	97.1 ± 4.9	95.5 ± 4.8	95.4 ± 4.8	122.4 ± 6.1	158.3 ± 7.9
Control	100	100	100	100	100	100	100

Table 4.6 Thermo stability study of protease from selected bacterial isolates. The crude protease (2 mg/ml) was heated at 60°C for 15 to 60 mins followed by assay of protease activity. Results represent mean ± S.D of three individual experiments

Incubation time (In mins)	**AS-S10-II**	**AS-S18**	**AS-S20-I**	**AS-S20-II**	**AS-S20-III**	**AS-S24-I**	**AS-S24-II**
15	100.0 ± 2.0	100.0 ± 2.0	100.0 ± 2.0	97.0± 1.9	95.0 ± 1.9	105.0 ± 2.1	101.0 ± 2.0
30	100.0 ± 2.0	94.0 ± 1.9	99.0 ±1.9	92.0± 1.8	90.0 ± 1.8	100.0 ± 2.0	99.0 ±1.9
45	97.0 ±1.9	88.0 ± 1.8	94.0 ± 1.9	83.0 ± 1.7	87.0 ± 1.7	99.0 ±1.9	97.0± 1.9
60	95.0 ± 1.9	72.0 ±1.7	91.0 ± 1.8	70.0 ± 1.6	80.0±1.6	97.0± 1.9	92.0± 1.8
Control(0)	100	100	100	100	100	100	100

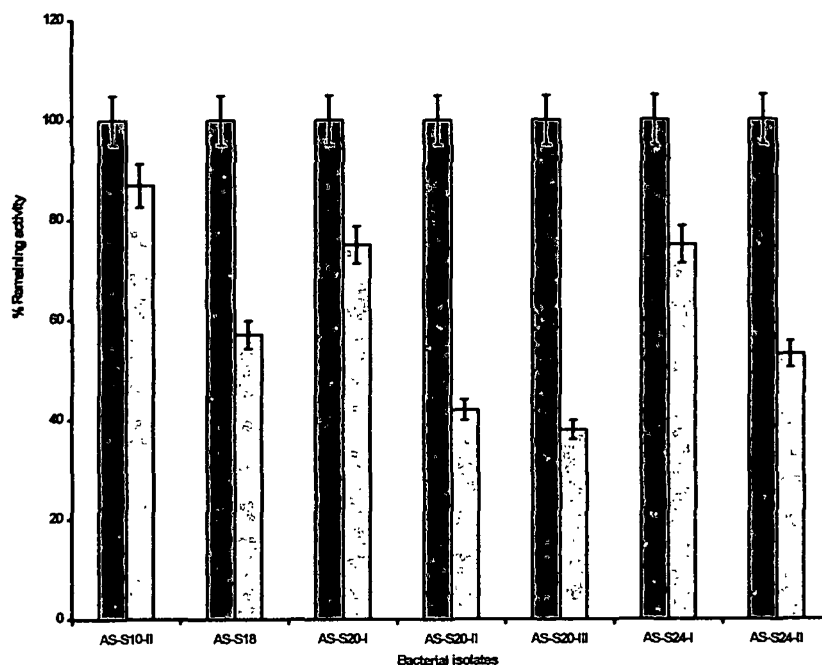


Fig.4.1 Storage stability of crude protease from selected bacteria at zero day (■) and post 30 days of storage at 4°C (□). Results represent mean ± S.D of three individual experiments.

4.2 Taxonomic identification of pure culture of bacterial isolates

The results of taxonomic identification by polyphasic approach are shown below.

4.2.1 Biochemical study of alkaline protease producing microbes

The results of the biochemical and morphological tests of the selected bacterial isolates are shown in Tables 4.7 to 4.10.

Bacterial morphology and the results of the biochemical tests suggested that AS-S10-II belongs *Bacillus* sp. genus, , AS-S18 belongs to *Bacillus* genus, AS-S20-I belongs to the *Kocuria* genus, AS-S24-I belonged to genus *Paenibacillus,* AS-S24-II belonged to the genus *Bacillus licheniformis* (Bergey's manual of systematic bacteriology, 1999,2001,2005).

Table 4.7 Biochemical and morphological tests of AS-S10 II. Experiments were repeated thrice to assure the reproducibility

Morphology	Rod shaped, motile, Gram negative, border circular, White color, single
Growth	
Spore	No endospore
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –10.0, optimum at pH 8.0-8.5
Temperature	Growth range 30-60°C, optimum at 45-50 °C
Catalase	Positive
Voges-Proskauer Test	Positive
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
Sucrose	Negative
Lactose	Negative
D- Mannitol	Positive
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Negative
Starch	Positive
Urease activity	Negative
Utilization of Citrate	Negative
Formation of Indole	Positive
Nitrate reduction	Positive
H_2S production	Positive
Lipid hydrolysis	Positive
Litmus milk reaction	Peptonization

Table 4.8 Biochemical and morphological tests AS-S18. Experiments were repeated thrice to assure the reproducibility.

Morphology	Rod shaped single cell with 1-2 μm in diameter, motile, Gram positive, border circular, white color
Growth	
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –11.0, optimum at pH 8.5-9.0
Temperature	Growth range 30-60 °C, optimum at 45 °C
Catalase	Positive
Voges-Proskauer Test	Positive
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
Lactose	Positive
Sucrose	Positive
D- Mannitol	Positive
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Positive
Starch	Positive
Utilization of Citrate	Positive
Formation of Indole	Negative
Nitrate reduction	Negative
Litmus milk reaction	Peptonization

Table 4.9 Biochemical and morphological tests of bacteria AS-S20-I. Experiments were repeated thrice to assure the reproducibility.

Morphology	Long rod shaped, motile, Gram negative, border irregular, surface raised, white colour glistening colonies
Growth	
Spore	No endospore
Agar	Abundant, White colour
Broth	Good growth, with sediment
pH	4.0 –10.0, optimum at 8.0
Temperature	Range 30-60 °C, optimum at 45 °C
Catalase	Positive
Voges-Proskauer Test	Not determined
Methyl Red Test	Positive
Acid from	
D- Glucose	Positive
Sucrose	Positive
Lactose	Positive
D- Mannitol	Positive
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Negative
Starch	Positive
Utilization of Citrate	Positive
Formation of Indole	Negative
Nitrate reduction	Negative
H_2S production	Positive
Urease activity	Negative
Lipid hydrolysis	Positive
Litmus milk reaction	Peptonization

Table 4.10 Biochemical and morphological tests of AS-S24-I. Experiments were repeated thrice to assure the reproducibility.

Morphology Rod shaped with 1-2 μm in diameter, motile, Gram negative, border circular, arrangement in chains, white color, glistering

Growth	
Spore	No endospore
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –10.0, optimum at pH 8.0
Temperature	Growth range 30-60 °C, optimum at 45 °C
Catalase	Negative
Voges-Proskauer Test	Positive
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
Sucrose	Negative
Lactose	Negative
D- Mannitol	Negative
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Negative
Starch	Positive
Urease activity	Negative
Utilization of Citrate	Negative
Formation of Indole	Positive
Nitrate reduction	Positive
H_2S production	Positive
Lipid hydrolysis	Negative
Litmus milk reaction	Peptonization

Table 4.11 Biochemical and morphological tests AS-S24-II. Experiments were repeated thrice to assure the reproducibility.

Morphology	Rod shaped single cell with 1-2 μm in diameter, motile, Gram positive, border circular, white color
Growth	
Spore	Endospore formation
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –11.0, optimum at pH 8.0
Temperature	Growth range 30-60 °C, optimum at 45 °C
Catalase	Positive
Voges-Proskauer Test	Positive
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
Lactose	Positive
Sucrose	Positive
D- Mannitol	Negative
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Negative
Starch	Positive
Urease activity	Negative
Utilization of Citrate	Negative
Formation of Indole	Positive
Nitrate reduction	Positive
H_2S production	Positive
Lipid hydrolysis	Negative
Litmus milk reaction	Peptonization

4.2.3 FAME analysis

The similarity index (SI) and species identification of these four bacterial strains on the basis of cellular fatty acids composition are shown in Tables 4.12 and 4.13, respectively.

4.2.3.1 AS-S10-II

The predominant fatty acids of AS-S10-II were anteiso-branched $C_{15\,0}$ (44.77%) >anteiso-branched $C_{17\,0}$ (16.76%) >iso-branched $C_{15:0}$ (25.78%) >iso-branched $C_{17\,0}$ (12.70%).

4.2.3.2 AS-S18

Cellular fatty acids composition of AS-S18 bacterial isolates demonstrated that majority of fatty acids straight chain $C_{16\,0}$ (32.98%) > $C_{17\,0}$(15.17%) > $C_{14\,0}$(6.49%) > $C_{12\,0}$(3.27%)> $C_{18\,0}$ (0.90%), whereas from iso-branched family only $C_{18\,1}$(1.50%) chain was determined. In addition, no major fatty acids was observed in AS-S18 isolate.

4.2.3.4 AS-S20-I

The predominant fatty acids of AS-S20-I were anteiso-branched $C_{15\,0}$ (45.20%) >anteiso-branched $C_{17\,0}$ (16.6%) > $C_{16\,0}$ (14.29 %) > iso-branched $C_{16\,0}$ (8.47%) >iso-branched $C_{15\,0}$ (6.89%) >iso-branched $C_{17\,0}$ (4.5%) > $C_{14\,0}$ (2.1%).

4.2.3.5 AS-S24-I

The cellular fatty acid distribution of AS-S24-I demonstrated that predominant fatty acids were anteiso-branched $C_{15\,0}$ (67.86%), followed by iso-branched $C_{15\,0}$ (15.39%)> iso-branched $C_{16\,0}$ (8.82%) > anteiso-branched $C_{17\,0}$ (7.93%).

4.2.3.6 AS-S24-II

The predominant fatty acids of AS-S24-II were anteiso-branched C15:0 (46.54%) > anteiso-branched C17:0 (16.7%) > C16:0 (14.3%) > iso-branched C16:0 (8.47%) > iso-branched C15:0 (6.89%) > iso-branched C17:0 (4.5%) > C14:0 (2.1%).

The Similarity Index (SI) of FAME of all protease secreting potential microbes is represented in Table 4.13.

Table 4.12 Composition of cellular fatty acids content of selected bacterial strains.

Organism/ strain	MTCC No	Straight chain					Iso-branched						Anteiso-branched	
		$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{15:0}$	$C_{17:0}$
AS-S10-II	8960	-	-	-	-	-	-	25.78	-	12.70	-	-	44.77	16.76
AS-S20-I	8961	-	2.06	14.3	-	1.04	0.87	6.89	8.47	4.52	-	-	45.20	16.7
AS-S24-I	9129	-	-	-	-	-	-	15.4	8.8	-	-	-	67.9	7.9
AS-S24-II	8959	-	2.06	14.29	-	1.04	0.87	6.89	8.47	4.52	-	-	45.2	16.66
AS-S18	No	3.27	6.49	32.98	15.17	0.90	-	-	-	-	-	1.50	-	-

Table 4.13 A similarity index of FAME analysis of protease producing selected bacteria.

Sl.No	Selected isolates	Similarity Index	Matching strains based on SI	MTCC No
1.	AS-S10-II	0.551	*Bacillus subtilis*	8960
2.	AS-S20-I	0.689	*Kocuria varians*	8961
3.	AS-S24-I	0.263	*Peanibacillus polymyxa*	9129
4.	AS-S24-II	0.623	*Bacillus licheniformis*	8959
5.	AS-S18	0.860	*Bacillus subtilis*	Not deposited yet

4.2.4 Phylogenetic analysis of bacterial isolates based on sequencing the conserved region of 16S rDNA

The 16S rRNA genes of all the protease secreting bacterial isolates were amplified, sequenced and the sequences are shown in Tables 4.14.

Table 4.14 Partial DNA sequence of conserved region of 16S-rRNA gene of all bacterial isolates selected in present study.

Bacterial isolates	16S-rRNA gene sequence (amplified region)	Length of sequence (bp)
AS-S10-II (Using universal primer sequence)	AAGTCGAGCGAGTCCCTTCGGGGGCTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCGTAAGCTCGGGATAAC ATGGGGAAACTCATGCTAATACCGGATAGGGTCTTCTCTCGCATGAGAGGAGACGGAAAGGTGGCGCAAGCTACCACTTA CGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGT GACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATTTTCCACAATGGACGAAAG TCTGATGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATTGTAAAGTTCTGTTGTCAGAGACGAACAAGTACCGTTC GAACAGGGCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTATGTAAGTCTGGTGTTAAAGCCCGGGGCTCAAC CCCGGTTCGCATCGGAAACTGTGTAGCTTGAGTGCAGAAGAGGAAAGCGGTATTCCACGTGTAGCGGTGAAATGCGTAGA GATGTGGAGGAACACCAGTGGCGAAAGCGGCTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACA GGATTAGAT	729
AS-S20-I (Using	TGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTG TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGC	621

universal	TTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTA	
primer	GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT	
sequence)	CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGA	
	AGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG	
	CGGTAATACGTAAGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGA	
	AAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGA	
AS-S20-I	TGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTG	622
(Using	TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGC	
species-	TTTTAGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTA	
specific	GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT	
primers)	CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGA	
	AGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG	
	CGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGA	
	AAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAG	
	TCCCACCTTCGGCGGCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTG	
AS-S24-I	TGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTG	
(Using	CAGACTGCGATCCGAACTGAGAACAGATTTGTGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTG	745
universal	TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGT	

primer sequence)	CACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACG ACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAGAGGAT GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC TTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCT CTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCA GCGTCAGTTACAGACCAGAGAGTCG	
AS-S24-II (Using universal primer sequence)	ATGGAGTGCTTGCACTCCTGATGCTTAGCGGCGGACGGGTGAGTAATACGTAGGTAACCTGCCCTTAAGACCGGGATAACT CACGGAAACGTGGGCTAATACCGGATAGGCGATTTCCTCGCATGAGGGAATCGGGAAAGGCGGAGCAATCTGCCGCTTATG GATGGACCTACGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAT CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCAAGTCTG ACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGCTATGGAGAGTAACT GTTCCATAGGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCG TTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCATGTAAGTCTGGTGTTTAAACCCGGGGCTCAACTCCGGGTC GCATCGGAAACTGTGTGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAG GAACACCAGTGGCGAAGGCGACTTTCTGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGC	714

4.2.4.1 Phylogeny of isolate strain AS-S10-II

A homologous search result of strain AS-S10-II demonstrated that 95-99% similarity of 16S-rDNA sequence was observed with other species of the genus *Brevibacillus* as shown in Table 4.15. The phylogenetic tree constructed from the sequence data by the neighbour-joining method showed that *Brevibacillus strain* DUT005 (FJ197026) showing 95% 16S rDNA sequence identity represented the closest phylogenetic neighbour of the strain AS-S10-II (Fig.4.2).

Table 4.15 Homologous search results of 16S-rRNA gene partial sequence of strain AS-S10-II using Basic Local Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI). The 16S-rDNA sequences from microbes showing upto 99% identity are shown.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ982663.1	*Brevibacillus borstelensis* strain JBE0014 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
EU376399.1	*Bacillales* bacterium 01QDX 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
FJ529038.1	*Brevibacillus* sp. ES-SL-1 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
FJ417406.1	*Brevibacillus borstelensis* strain P35 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
EU816694.1	*Brevibacillus borstelensis* clone K11 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
EU714903.1	*Brevibacillus borstelensis* strain SRDTh2 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
EU714902.1	*Brevibacillus borstelensis* strain SRDTh1 partial sequence	1341	1341	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB364960.1	*Brevibacillus borstelensis* gene for 16S rRNA, partial sequence	1341	1341	100%	0.0	99%
DQ232878.1	*Brevibacillus* sp. D10 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
DQ140185.1	*Brevibacillus* sp. D4 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
AB215102.1	*Brevibacillus borstelensis* gene for 16S ribosomal RNA, partial sequence, strain: T2-1	1341	1341	100%	0.0	99%
AF378230.1	*Brevibacillus borstelensis* strain LMG 15536 16S ribosomal RNA gene, partial sequence	1341	1341	100%	0.0	99%
AB112721.1	*Brevibacillus borstelensis* gene for 16S rRNA, partial sequence, strain:DSM 6347T	1341	1341	100%	0.0	99%
AB116134.1	*Brevibacillus borstelensis* gene for 16S ribosomal RNA, partial sequence, strain: M63	1341	1341	100%	0.0	99%
FJ268957.1	*Brevibacillus* sp. B2(2008) 16S ribosomal RNA gene, partial sequence	1339	1339	99%	0.0	99%
EF139656.1	*Brevibacillus borstelensis* strain AR9 16S ribosomal RNA gene, partial sequence	1339	1339	99%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM910272.1	*Bacillus* sp. R-30914 partial 16S rRNA gene, strain R-30914	1338	1338	100%	0.0	99%
D78456.1	*Brevibacillus borstelensis* DNA for 16S ribosomal RNA	1338	1338	100%	0.0	99%
DQ350830.1	*Brevibacillus borstelensis* strain MH301 16S ribosomal RNA gene, complete sequence	1336	1336	100%	0.0	99%
DQ350827.1	*Brevibacillus borstelensis* strain IPH701 16S ribosomal RNA gene, complete sequence	1336	1336	100%	0.0	99%
AF252328.1	*Brevibacillus* sp. HC6 16S ribosomal RNA gene, partial sequence	1336	1336	100%	0.0	99%
AY373322.1	*Brevibacillus borstelensis* strain R-7201 16S ribosomal RNA gene, partial sequence	1334	1334	100%	0.0	99%
EU376400.1	*Bacillales* bacterium 01QDY 16S ribosomal RNA gene, partial sequence	1332	1332	99%	0.0	99%
FJ529040.1	*Brevibacillus* sp. ES-SL-3 16S ribosomal RNA gene, partial sequence	1330	1330	100%	0.0	99%
EU816699.1	*Brevibacillus borstelensis* clone US12 16S ribosomal RNA gene, partial sequence	1330	1330	100%	0.0	99%
FJ821653.1	*Brevibacillus* sp. TB3 16S ribosomal RNA gene, partial sequence	1328	1328	99%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ821617.1	*Brevibacillus* sp. TB2 16S ribosomal RNA gene, partial sequence	1328	1328	99%	0.0	99%
EU816695.1	*Brevibacillus borstelensis* clone DQ-1 16S ribosomal RNA gene, partial sequence	1325	1325	100%	0.0	99%
EU714904.1	*Brevibacillus borstelensis* strain SRDTh3 16S ribosomal RNA gene, partial sequence	1325	1325	100%	0.0	99%
DQ350837.1	*Brevibacillus borstelensis* strain U404 16S ribosomal RNA gene, complete sequence	1325	1325	100%	0.0	99%
DQ141602.1	*Brevibacillus* sp. E 16S ribosomal RNA gene, partial sequence	1321	1321	98%	0.0	99%
DQ350828.1	*Brevibacillus borstelensis* strain IPH801 16S ribosomal RNA gene, complete sequence	1321	1321	100%	0.0	99%
EF439668.1	*Brevibacillus borstelensis* strain S3 16S ribosomal RNA gene, partial sequence	1317	1317	98%	0.0	99%
FJ441049.1	*Brevibacillus* sp. B67 16S ribosomal RNA gene, partial sequence	1315	1315	98%	0.0	99%
EF409324.1	*Brevibacillus* sp. 'Brevi. M1a' 16S ribosomal RNA gene, partial sequence	1314	1314	98%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ971855.1	*Brevibacillus* sp. Am 18 partial 16S rRNA gene, isolate Am 18	1314	1314	99%	0.0	99%
FJ382938.1	*Brevibacillus* sp. enrichment culture clone 11W_3 16S ribosomal RNA gene, partial sequence	1312	1312	99%	0.0	99%
AJ586382.1	*Brevibacillus borstelensis* partial 16S rRNA gene, strain R-16402	1301	1301	96%	0.0	99%

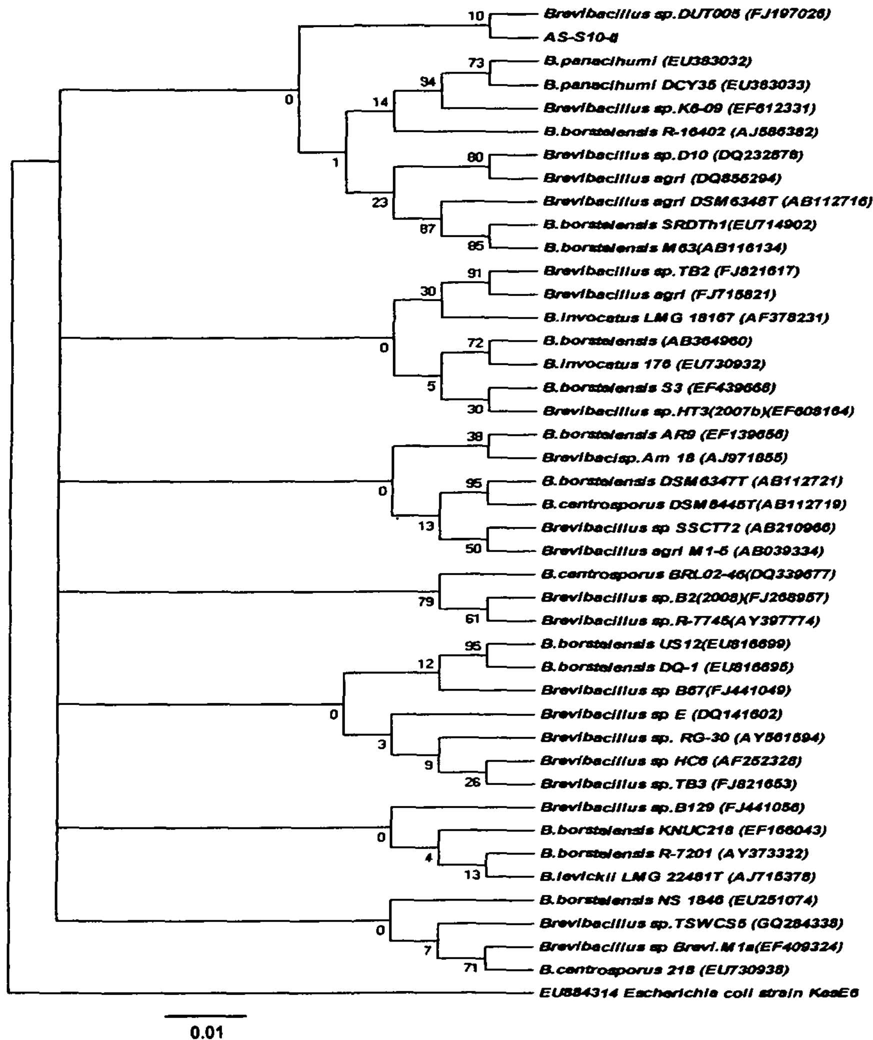


Fig.4.2 (a) Phylogenetic relationships of strain AS-S10-II and other closely related *Brevibacillus* species based on 16S rDNA sequencing. The tree was generated using the neighbour-joining method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

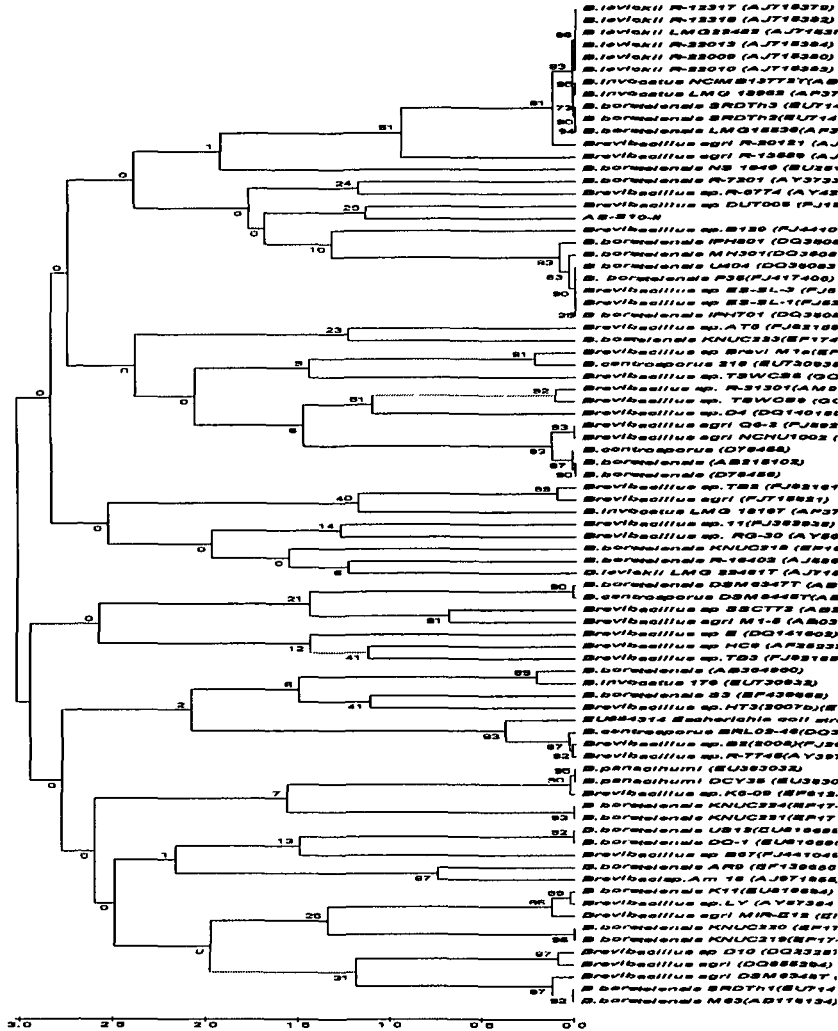


Fig.4.2 (b) Phylogenetic relationships of strain AS-S10-II and other closely related *Brevibacillus* species based on 16S rDNA sequencing. The tree was generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

4.2.4.2 Phylogeny of strain AS-S20-I

A homologous search result of 16S –rDNA sequence of strain AS-S20-I demonstrated that 99-100% sequence similarity was observed with other species of the genus *Bacillus* sp. as shown in Table 4.16. The phylogenetic tree was constructed from the 16S-rDNA partial sequence data by the neighbour-joining as well as UPGMA (Fig 4.3a amd 4.3b) methods. It was observed that *Bacillus.* sp *strain* BCL 23-1(EF026994) showing 99% 16S rDNA sequence identity represented the closest phylogenetic neighbours of the strain AS-S20-I (Fig.4.3).

Table 4.16 A Homologous search results of 16S-rRNA gene partial sequence of AS-S20-I using Basic Local Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI).

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB374301.1	*Bacillus* sp. PT101 gene for 16S rRNA, partial sequence	1142	1142	100%	0.0	99%
GU458871.1	Uncultured *Bacillus* sp. clone T0501 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
GU191906.1	*Bacillus licheniformis* strain SB 3181 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
GQ375232.1	*Bacillus licheniformis* strain CICC 10087 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
FN433039.1	*Bacillus licheniformis* partial 16S rRNA gene, isolate CCM28B	1142	1142	100%	0.0	99%
FN393821.1	*Bacillus licheniformis* partial 16S rRNA gene, strain ES-188-4	1142	1142	100%	0.0	99%
FJ641027.1	*Bacillus licheniformis* strain IMAUB1014 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ641023.1	*Bacillus licheniformis* strain IMAUB1009 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
FM958173.1	*Bacillus* sp. 3LF 16T partial 16S rRNA gene, strain 3LF 16T	1142	1142	100%	0.0	99%
AM913944.1	*Bacillus* sp. L240 partial 16S rRNA gene, isolate L240	1142	1142	100%	0.0	99%
AM913932.1	*Bacillus* sp. L164 partial 16S rRNA gene, isolate L164	1142	1142	100%	0.0	99%
FM877591.1	*Bacillus* sp. AG07-20 partial 16S rRNA gene, isolate AG07-20	1142	1142	100%	0.0	99%
FJ432006.1	*Bacillus licheniformis* strain BM-Y6 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
EU675997.1	*Bacillus* sp. Epbas6 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
EU384246.1	*Bacillus* sp. By138(B) Ydz-ss 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
EU368761.1	*Bacillus* sp. By157 Ydz-ds 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
EU344793.1	*Bacillus licheniformis* strain MML2501 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
EF026994.1	*Bacillus* sp. BCL23-1 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99 %

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF433410.1	*Bacillus licheniformis* strain BCRC 11702 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
DQ981799.1	Uncultured bacterium clone 1-gw1-su4-18 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
DQ981798.1	Uncultured bacterium clone 1-gw2-su4-10 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
AY786999.1	*Bacillus licheniformis* strain CICC 10219 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
AY750906.1	*Bacillus licheniformis* strain EHD 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
AY631057.1	*Bacillus licheniformis* strain TKW3 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
AY186036.1	*Bacillus licheniformis* strain EBI2 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ372686.1	*Bacillus licheniformis* 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
AB219153.1	*Bacillus licheniformis* gene for 16S rRNA, partial sequence, strain:SSH4	1142	1142	100%	0.0	99%
DQ082996.1	*Bacillus licheniformis* strain CICC 10100 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
DQ082995.1	*Bacillus licheniformis* strain CICC 10087 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
AB039328.1	*Bacillus licheniformis* gene for 16S rRNA, strain:M1-1	1142	1142	100%	0.0	99%
AY871103.1	*Bacillus licheniformis* strain CICC 10084 16S ribosomal RNA gene, partial sequence	1142	1142	100%	0.0	99%
AB055006.1	*Bacillus licheniformis* gene for 16S rRNA	1142	1142	100%	0.0	99%
X68416.1	*B.licheniformis* gene for 16S rRNA	1142	1142	100%	0.0	99%
FJ517542.1	*Bacillus licheniformis* strain AR1 16S ribosomal RNA gene, partial sequence	1140	1140	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB425370.1	*Bacillus* sp. SG607 gene for 16S rRNA, partial sequence, Version 1 of two types of sequence	1138	1138	100%	0.0	99%
AB374306.1	*Bacillus* sp. RT105 gene for 16S rRNA, partial sequence	1138	1138	100%	0.0	99%
FJ517543.1	*Bacillus licheniformis* strain P3 16S ribosomal RNA gene, partial sequence	1138	1138	100%	0.0	99%
FJ413051.1	*Bacillus* sp. LAMI 011 16S ribosomal RNA gene, partial sequence	1138	1138	99%	0.0	99%
EU869249.1	*Bacillus licheniformis* strain BG-B11 16S ribosomal RNA (rrs) gene, partial sequence	1138	1138	100%	0.0	99%
AY553106.1	*Bacillus* sp. GSP63 16S ribosomal RNA gene, partial sequence	1138	1138	100%	0.0	99%
AB374322.1	*Bacillus* sp. TT402 gene for 16S rRNA, partial sequence, Version 1 of two types of sequence	1136	1136	100%	0.0	99%
GU458873.1	Uncultured *Bacillus* sp. clone T0552 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU191905.1	*Bacillus licheniformis* strain SB 3180 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
GU086446.1	*Bacillus licheniformis* strain G7A 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
GU086434.1	*Bacillus* sp. N35 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
AB513628.1	*Bacillus licheniformis* gene for 16S rRNA, partial sequence	1136	1136	100%	0.0	99%
GQ871450.1	*Bacillus licheniformis* strain KK2 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
GQ470399.1	*Bacillus licheniformis* strain DQgbc4 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
GQ247891.1	*Bacillus licheniformis* strain x8 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
GQ152136.1	*Bacillus* sp. WT143 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
GQ131306.1	*Bacillus* sp. G-1 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ808719.1	*Bacillus licheniformis* strain P79 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
FJ641025.1	*Bacillus licheniformis* strain IMAUB1012 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
FJ641018.1	*Bacillus licheniformis* strain IMAUB1002 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
FJ615520.1	*Bacillus* sp. 24KZ 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
FJ614258.1	*Bacillus licheniformis* strain pK 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
FJ655808.1	*Bacillus licheniformis* isolate D14A3 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
FJ655795.1	*Bacillus licheniformis* isolate D6AO2 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
FM958163.1	*Bacillus* sp. 5.5LF 34TD partial 16S rRNA gene, strain 5.5LF 34TD	1136	1136	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ417403.1	*Bacillus licheniformis* strain P13 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
AM990718.1	*Bacillus* sp. MOLA 451 partial 16S rRNA gene, culture collection MOLA:451	1136	1136	100%	0.0	99%
EU626409.1	*Bacillus licheniformis* strain biosXds2 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EU373344.1	*Bacillus licheniformis* strain HNL09 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
AM932277.1	*Bacillus licheniformis* partial 16S rRNA gene, strain S22-52	1136	1136	100%	0.0	99%
EU256501.1	*Bacillus licheniformis* strain G7-2 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EU281636.1	*Bacillus* sp. D6(2007) 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EU257697.1	*Bacillus licheniformis* strain DC3-1 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU257696.1	*Bacillus licheniformis* strain F1 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
AB364961.1	*Bacillus licheniformis* gene for 16S rRNA, partial sequence	1136	1136	100%	0.0	99%
EU231623.1	*Bacillus licheniformis* strain TCCC11009 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EU200968.1	*Bacillus licheniformis* strain SPRI-536 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EU117278.1	*Bacillus licheniformis* strain B8 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF702094.1	Uncultured *Bacilli* bacterium clone MS058A1-H09 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF702068.1	Uncultured *Bacilli* bacterium clone MS058A1-E12 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF701192.1	Uncultured *Bacilli* bacterium clone MS028A1-G09 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF700893.1	Uncultured *Bacilli* bacterium clone MS002A1-G01 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF698606.1	Uncultured *Bacilli* bacterium clone MS113A1-B08 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF698407.1	Uncultured *Bacilli* bacterium clone MS106A1-G06 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF698199.1	Uncultured *Bacilli* bacterium clone MS098A1-B05 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF698123.1	Uncultured *Bacilli* bacterium clone MS094A1-A05 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF698075.1	Uncultured *Bacilli* bacterium clone MS087A1-D08 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF697586.1	Uncultured *Bacilli* bacterium clone MS074A1-H08 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF697503.1	Uncultured *Bacilli* bacterium clone MS073A1-H06 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF697012.1	Uncultured *Bacilli* bacterium clone MS056A1-D10 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF696572.1	Uncultured *Bacilli* bacterium clone MS050A1-G02 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF696555.1	Uncultured *Bacilli* bacterium clone MS050A1-E06 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF696532.1	Uncultured *Bacilli* bacterium clone MS050A1-C06 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF696530.1	Uncultured *Bacilli* bacterium clone MS050A1-C04 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF696523.1	Uncultured *Bacilli* bacterium clone MS050A1-B09 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF696509.1	Uncultured *Bacilli* bacterium clone MS050A1-A07 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF696130.1	Uncultured *Bacilli* bacterium clone MS019A1-H02 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF696095.1	Uncultured *Bacilli* bacterium clone MS019A1-D06 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
EF695530.1	Uncultured *Bacilli* bacterium clone MS003A1-H06 16S ribosomal RNA gene, partial sequence	1136	1136	100%	0.0	99%
AB374320.1	*Bacillus* sp. TT307 gene for 16S rRNA, partial sequence	1134	1134	100%	0.0	99%
AB374318.1	*Bacillus* sp. TT104 gene for 16S rRNA, partial sequence, Version 1 of two types of sequence	1134	1134	100%	0.0	99%
AB374302.1	*Bacillus* sp. PT104 gene for 16S rRNA, partial sequence	1134	1134	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM910346.1	*Bacillus* sp. R-33575 partial 16S rRNA gene, strain R-33575	1134	1134	100%	0.0	99%
GQ911552.1	*Bacillus licheniformis* strain ATCC 14580 16S ribosomal RNA gene, partial sequence	1131	1131	100%	0.0	99%
FJ549007.1	*Bacillus licheniformis* strain SH-B2 16S ribosomal RNA (rrs) gene, partial sequence	1131	1131	100%	0.0	99%
EU869262.1	*Bacillus licheniformis* strain BG-B63 16S ribosomal RNA (rrs) gene, partial sequence	1127	1127	100%	0.0	99%

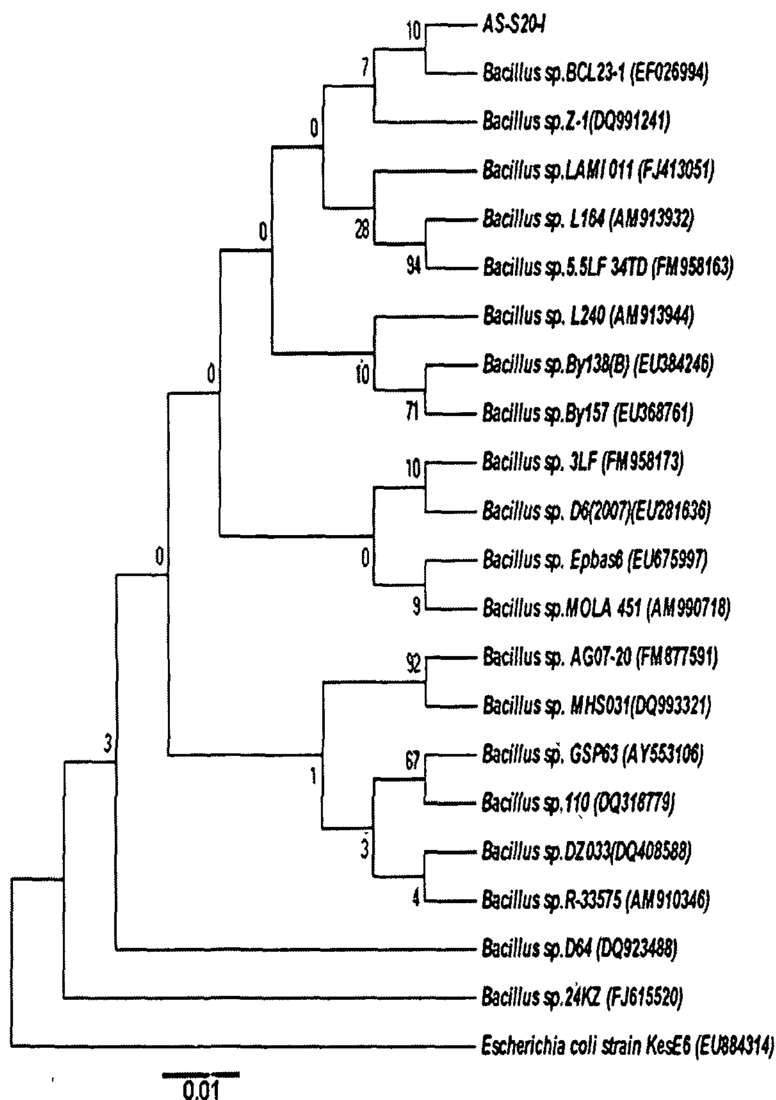


Fig.4.3(a) Phylogenetic relationships of strain AS-S20-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the neighbour-joining method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

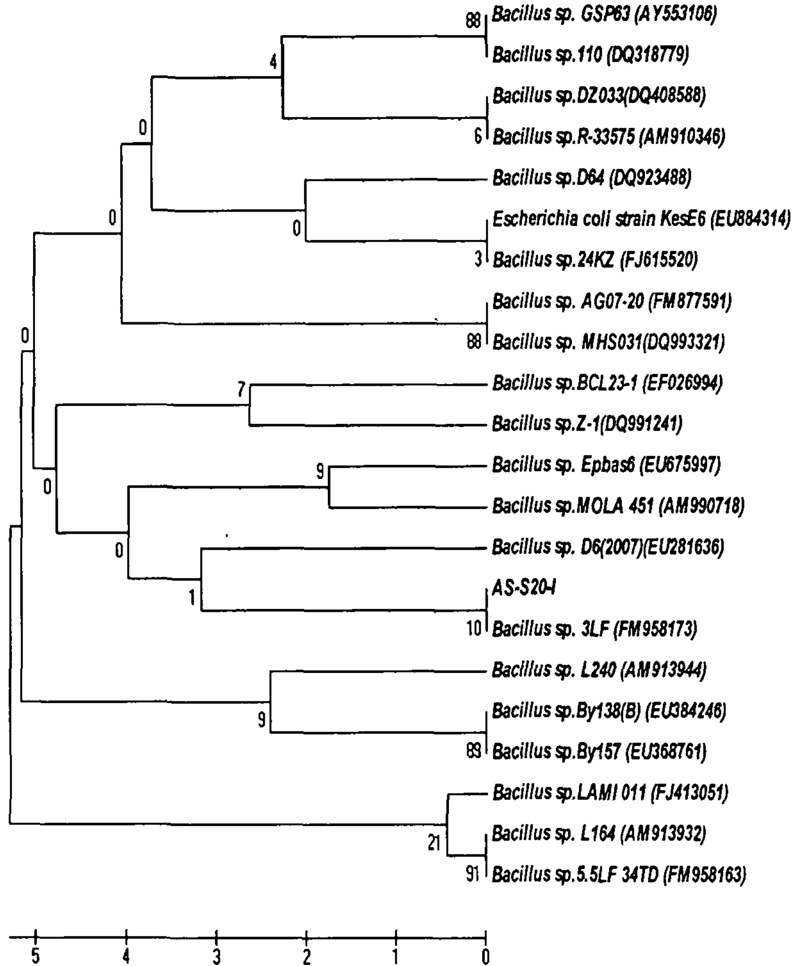


Fig.4.3(b) Phylogenetic relationships of strain AS-S20-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

4.2.4.3 Phylogeny of strain AS-S24-I

A homologous search result of 16S- rDNA partial sequence of AS-S24-I demonstrated that 95-99% similarity was observed with other species of the genus *Bacillus* (Table 4.17). The phylogenetic tree constructed from the sequence data by the neighbour-joining method (Fig 4.4a) and by UPGMA method (Fig 4.4b) demonstrated that *B. licheniformis* (Accession no DQ171720) showing 100% 16S rDNA sequence coverage represented the closest phylogenetic neighbours of the strain AS-S24-I.

Table 4.17 Homologous search results of 16S-rRNA partial sequence gene of strain AS-S24-I using BLAST from NCBI database.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ375235.1	*Bacillus licheniformis* strain CICC 10181 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
GQ375234.1	*Bacillus licheniformis* strain CICC 10101 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
GQ375232.1	*Bacillus licheniformis* strain CICC 10087 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
GQ375230.1	*Bacillus licheniformis* strain CICC 10085 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AB508877.1	*Bacillus* sp. TSH3 gene for 16S ribosomal RNA, partial sequence	1376	1376	100%	0.0	100%
GQ152136.1	*Bacillus* sp. WT143 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
GQ153850.1	*Bacillus licheniformis* strain T7-2 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ957804.1	Uncultured *Bacillus* sp. clone JPL-4_G11 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ957781.1	Uncultured *Bacillus* sp. clone JPL-4_A13 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ957553.1	Uncultured *Bacillus* sp. clone JPL-2_E24 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU718490.1	*Bacillus licheniformis* strain MS5-14 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ641027.1	*Bacillus licheniformis* strain IMAUB1014 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ641025.1	*Bacillus licheniformis* strain IMAUB1012 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ641023.1	*Bacillus licheniformis* strain IMAUB1009 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ641018.1	*Bacillus licheniformis* strain IMAUB1002 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ615520.1	*Bacillus* sp. 24KZ 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU371573.1	Uncultured *Bacillus* sp. clone CBIOS-09 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ655803.1	*Bacillus licheniformis* isolate D13AO6 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ655802.1	*Bacillus licheniformis* isolate D13A4 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ655801.1	*Bacillus licheniformis* isolate D13A5 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ549007.1	*Bacillus licheniformis* strain SH-B2 16S ribosomal RNA (rrs) gene, partial sequence	1376	1376	100%	0.0	100%
AM913932.1	*Bacillus* sp. L164 partial 16S rRNA gene, isolate L164	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM913927.1	*Bacillus* sp. L157 partial 16S rRNA gene, isolate L157	1376	1376	100%	0.0	100%
FJ458451.1	*Bacillus licheniformis* strain ycsd01 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ458450.1	*Bacillus licheniformis* strain ycsd02 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU308302.1	*Bacillus* sp. 50B11-3 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU308301.1	*Bacillus* sp. 50B11-2 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU308300.1	*Bacillus* sp. 50B11-1 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
FJ357337.1	*Gluconacetobacter diazotrophicus* isolate H5 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU870503.1	*Bacillus licheniformis* strain Sua-BAC006 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU870499.1	*Bacillus licheniformis* strain Sua-BAC002 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU362149.1	*Bacillus* sp. B3(2008) 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU869262.1	*Bacillus licheniformis* strain BG-B63 16S ribosomal RNA (rrs) gene, partial sequence	1376	1376	100%	0.0	100%
EU869249.1	*Bacillus licheniformis* strain BG-B11 16S ribosomal RNA (rrs) gene, partial sequence	1376	1376	100%	0.0	100%
EU710720.1	Bacterium B-WPhG12 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU816690.1	*Bacillus licheniformis* clone S-3 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AM910346.1	*Bacillus* sp. R-33575 partial 16S rRNA gene, strain R-33575	1376	1376	100%	0.0	100%
EU742145.1	*Bacillus licheniformis* strain SKU 3 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU373408.1	*Bacillus licheniformis* strain YRL03 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU373344.1	*Bacillus licheniformis* strain HNL09 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU445292.1	*Bacillus licheniformis* strain RPk 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU344793.1	*Bacillus licheniformis* strain MML2501 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EU281636.1	*Bacillus* sp. D6(2007) 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AB363734.1	*Bacillus licheniformis* gene for 16S rRNA, partial sequence, strain: NBRC 12202	1376	1376	100%	0.0	100%
AB354236.1	*Bacillus licheniformis* gene for 16S rRNA, partial sequence, strain: NBRC 12107	1376	1376	100%	0.0	100%
EU031767.1	*Bacillus* sp. BS17 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EF635428.1	*Bacillus licheniformis* strain MY75 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF591780.1	*Bacillus* sp. XJ1-05 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
CP000002.3	*Bacillus licheniformis* ATCC 14580, complete genome	1376	9627	100%	0.0	100%
DQ870721.1	*Bacillus licheniformis* strain LMAtotj1 16S ribosomal RNA (rrn) gene, partial sequence	1376	1376	100%	0.0	100%
EF472268.1	*Bacillus licheniformis* strain LQ98 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EF471917.1	*Bacillus* sp. J24 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EF433410.1	*Bacillus licheniformis* strain BCRC 11702 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EF423609.1	*Bacillus licheniformis* strain BCRC 14353 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EF423608.1	*Bacillus licheniformis* strain BCRC 12826 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AB275356.1	*Bacillus licheniformis* gene for 16S rRNA, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF101734.1	*Bacillus licheniformis* strain HU14 16S ribosomal RNA (rrnE) gene, partial sequence	1376	1376	100%	0.0	100%
EF059752.1	*Bacillus licheniformis* 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EF026994.1	*Bacillus* sp. BCL23-1 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
EF026995.1	*Bacillus* sp. BCL23-2 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ990042.1	Bacterium 8-gw2-5 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ981823.1	Uncultured bacterium clone 9-gw1-su4-1 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ981804.1	Uncultured bacterium clone 1-gw2-su4-210 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ981799.1	Uncultured bacterium clone 1-gw1-su4-18 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ981798.1	Uncultured bacterium clone 1-gw2-su4-10 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ993676.1	*Bacillus licheniformis* strain BCRC 15413 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ480087.1	*Bacillus licheniformis* strain PLLA-2 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ351930.2	*Bacillus licheniformis* strain K10 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AM237383.1	*Bacillus licheniformis* partial 16S rRNA gene, isolate OS-116	1376	1376	100%	0.0	100%
AY842874.1	*Bacillus licheniformis* strain CICC10093 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY842873.1	*Bacillus licheniformis* strain CICC10094 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY842871.1	*Bacillus licheniformis* strain CICC10181 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY842869.1	*Bacillus licheniformis* strain CICC10085 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY839858.1	*Bacillus licheniformis* strain CICC10098 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY786999.1	*Bacillus licheniformis* strain CICC 10219 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ212969.1	*Bacillus licheniformis* strain CICC10103 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY750906.1	*Bacillus licheniformis* strain EHD 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AE017333.1	*Bacillus licheniformis* DSM 13, complete genome	1376	9627	100%	0.0	100%
AY635049.1	*Bacillus* sp. SAB 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY553109.1	*Bacillus* sp. MO16 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY553107.1	*Bacillus* sp. MO14 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY553105.1	*Bacillus* sp. MO12 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY553103.1	*Bacillus* sp. MO10 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY553102.1	*Bacillus* sp. MO9 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY553099.1	*Bacillus* sp. MO6 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY601721.1	*Bacillus licheniformis* isolate YB-42 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
DQ171720.1	*Bacillus licheniformis* strain CICC10099 16S ribosomal RNA gene, complete sequence	1376	1376	100%	0.0	100%
AY587808.1	Bacterium Te21R 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY505509.1	*Bacillus licheniformis* strain GSP30 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AB189317.1	*Bacillus* sp. SD-B2 gene for 16S rRNA, partial sequence	1376	1376	100%	0.0	100%
AJ717380.1	*Bacillus licheniformis* 16S rRNA gene, isolate CV60	1376	1376	100%	0.0	100%
AJ920002.1	*Bacillus* sp. CK10 partial 16S rRNA gene, strain CK10	1376	1376	100%	0.0	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ831843.1	*Bacillus aerius* partial 16S rRNA gene, type strain 24K	1376	1376	100%	0.0	100%
AJ586363.1	*Bacillus sonorensis* partial 16S rRNA gene, strain R-19056	1376	1376	100%	0.0	100%
AY871102.1	*Bacillus licheniformis* strain CICC 10037 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AY859477.1	*Bacillus licheniformis* strain CICC 10101 16S ribosomal RNA gene, partial sequence	1376	1376	100%	0.0	100%
AJ582723.1	*Bacillus licheniformis* partial 16S rRNA gene, isolate R-13646	1376	1376	100%	0.0	100%
FJ957659.1	Uncultured *Bacillus* sp. clone JPL-S4_C05 16S ribosomal RNA gene, partial sequence	1373	1373	100%	0.0	99%
FJ549021.1	*Bacillus licheniformis* strain SH-B35 16S ribosomal RNA (rrs) gene, partial sequence	1373	1373	100%	0.0	99%
AY553108.1	*Bacillus* sp. MO15 16S ribosomal RNA gene, partial sequence	1373	1373	100%	0.0	99%
AY553104.1	*Bacillus* sp. MO11 16S ribosomal RNA gene, partial sequence	1373	1373	100%	0.0	99%

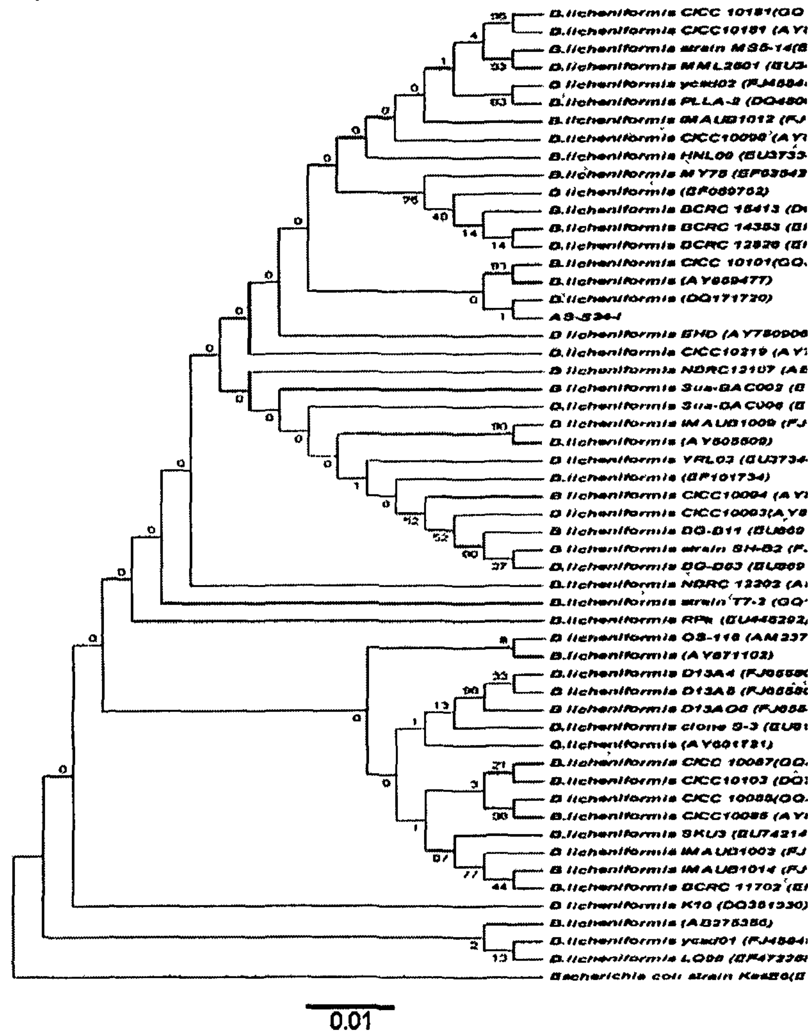


Fig.4.4(a) Phylogenetic relationships of strain AS-S24-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the neighbour-joining method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the *nodes. Bar, 0.01 substitutions per site.*

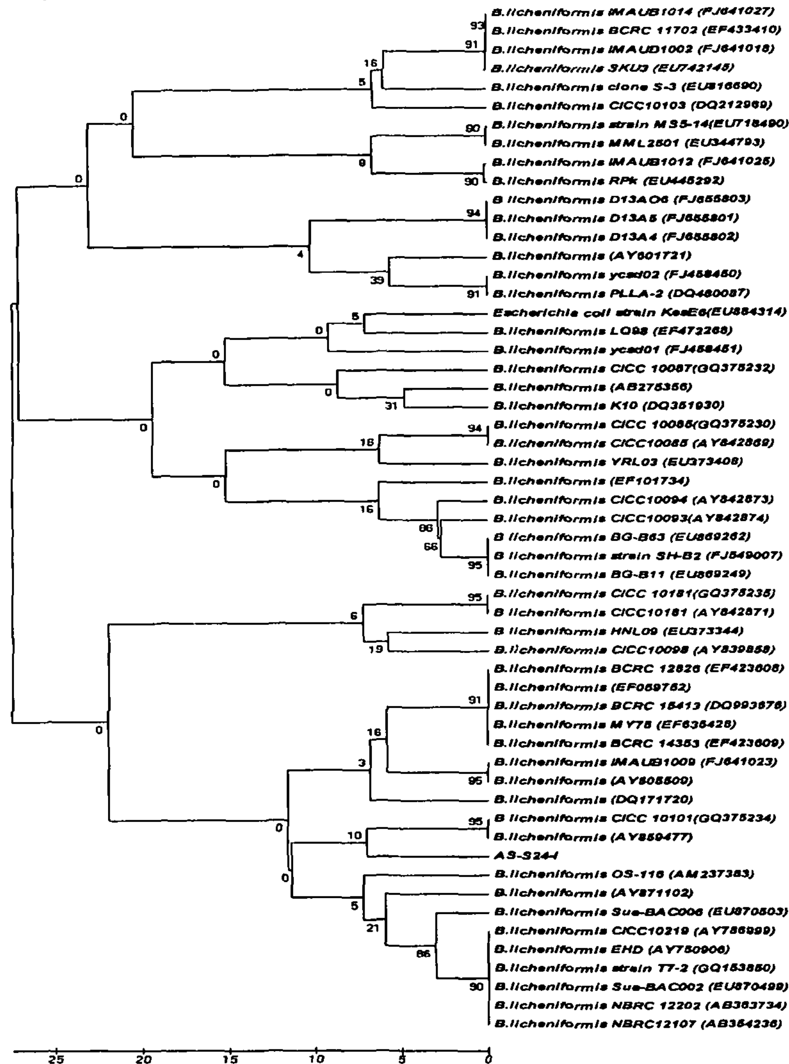


Fig.4.4(b) A phylogenetic relationships of strain AS-S24-I and other closely related *Bacillus* species based on 16S rDNA sequencing. The tree was generated using the unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was re-sampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

4.2.4.4 Phylogeny of strain AS-S24-II

A homologous search result of 16S-rDNA gene partial sequence of strain AS-S24-II demonstrated that 95-99% 16S-rDNA sequence similarity with other species of the genus *Paenibacillus* (Table 4.18). The phylogenetic tree constructed from the sequence data by the neighbour-joining method (Fig 4.5a) and UPGMA method (Fig 4.5b) displayed that *Paenibacillus lentimorbus* strain DNG-14 *and Paenibacillus lentimorbus* strain DNG-16 showing 93.0-99.0% 16S rDNA sequence identity respectively represented the closest phylogenetic neighbours of the strain AS-S24-II (Fig.4.5).

Table 4.17 Homologous search results of 16S-rRNA partial gene of sequence of AS-S24-II using Basic Local Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI).

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ804507.1	*Paenibacillus* sp. AS-S24-II 16S ribosomal RNA gene, partial sequence	1319	1319	100%	0.0	100%
GU117660.1	*Paenibacillus dendritiformis* strain OIV 870 16S ribosomal RNA gene, partial sequence	1308	1308	100%	0.0	99%
FJ821592.1	*Bacillus sp.* AT6 16S ribosomal RNA gene, partial sequence	1308	1308	100%	0.0	99%
AY359885.1	*Paenibacillus dendritiformis* 16S ribosomal RNA gene, partial sequence	1308	1308	100%	0.0	99%
GQ900693.1	*Paenibacillus* sp. G9-12 16S ribosomal RNA gene, partial sequence	1299	1299	100%	0.0	99%
AB045092.1	*Paenibacillus dendritiformis* gene for 16S rRNA, partial sequence	1299	1299	100%	0.0	99%
Y16129.1	*Paenibacillus* sp. C-168 16S rRNA gene	1299	1299	100%	0.0	99%
GU458945.1	Uncultured *Paenibacillus* sp. clone T0275 16S ribosomal RNA gene, partial sequence	1277	1277	100%	0.0	98%
Y16128.1	*Paenibacillus* sp. T-168 16S rRNA gene	1277	1277	100%	0.0	98%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU330645.1	*Paenibacillus thiaminolyticus* strain 8118 16S ribosomal RNA gene, partial sequence	1262	1262	96%	0.0	99%
EF190490.1	*Paenibacillus popilliae* strain Dutky 1S 16S ribosomal RNA gene, partial sequence	1258	1258	96%	0.0	99%
EU420075.1	*Paenibacillus popilliae* 16S ribosomal RNA gene, partial sequence	1256	1256	96%	0.0	99%
AB073197.1	*Paenibacillus thiaminolyticus* gene for 16S rRNA, partial sequence	1256	1256	96%	0.0	99%
EF190493.1	*Paenibacillus popilliae* strain DNG 9 16S ribosomal RNA gene, partial sequence	1253	1253	96%	0.0	99%
EF190491.1	*Paenibacillus popilliae* strain KLN 3 16S ribosomal RNA gene, partial sequence	1253	1253	96%	0.0	99%
EF190488.1	*Paenibacillus popilliae* strain Pj1 16S ribosomal RNA gene, partial sequence	1253	1253	96%	0.0	99%
EF190487.1	*Paenibacillus popilliae* strain NRRL B-2309 16S ribosomal RNA gene, partial sequence	1253	1253	96%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ854976.1	*Paenibacillus* sp. GPTSA9 16S ribosomal RNA gene, partial sequence	1253	1253	96%	0.0	99%
AB073198.1	*Paenibacillus popilliae* gene for 16S rRNA, partial sequence	1253	1253	96%	0.0	99%
AF071859.1	*Paenibacillus popilliae* strain ATCC14706(T) 16S ribosomal RNA gene, partial sequence	1253	1253	96%	0.0	99%
FJ821654.1	*Paenibacillus* sp. TB12 16S ribosomal RNA gene, partial sequence	1251	1251	100%	0.0	98%
EF190489.1	*Paenibacillus popilliae* strain NRRL B-2524 16S ribosomal RNA gene, partial sequence	1251	1251	96%	0.0	99%
EF190494.1	*Paenibacillus popilliae* strain DNG 12 16S ribosomal RNA gene, partial sequence	1249	1249	96%	0.0	99%
EF190492.1	*Paenibacillus popilliae* strain BPFR 16S ribosomal RNA gene, partial sequence	1249	1249	96%	0.0	99%
GQ288412.1	*Paenibacillus* sp. enrichment culture clone S16 16S ribosomal RNA gene, partial sequence	1247	1247	96%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF190495.1	*Paenibacillus popilliae* strain BPHD 16S ribosomal RNA gene, partial sequence	1247	1247	96%	0.0	99%
AF071860.1	*Paenibacillus popilliae* strain NRRL B-4081 16S ribosomal RNA gene, complete sequence	1247	1247	96%	0.0	99%
AJ320490.1	*Paenibacillus thiaminolyticus* partial 16S rRNA gene, strain DSM 7262T	1242	1242	96%	0.0	98%
FJ538210.1	*Paenibacillus thiaminolyticus* strain IPSr108 16S ribosomal RNA gene, partial sequence	1227	1227	96%	0.0	98%
AF039408.1	*Bacillus tipchiralis* 16S ribosomal RNA gene, complete sequence	1212	1212	100%	0.0	96%
AF071861.1	*Paenibacillus lentimorbus* 16S ribosomal RNA gene, complete sequence	1199	1199	96%	0.0	97%
AB073199.1	*Paenibacillus lentimorbus*,gene for 16S rRNA, partial sequence	1195	1195	96%	0.0	97%
EF190497.1	*Paenibacillus lentimorbus* strain KLN 2 16S ribosomal RNA gene, partial sequence	1194	1194	96%	0.0	97%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF190506.1	*Paenibacillus lentimorbus* strain Cb1 16S ribosomal RNA gene, partial sequence	1188	1188	96%	0.0	97%
EF190505.1	*Paenibacillus lentimorbus* strain Cb2 16S ribosomal RNA gene, partial sequence	1188	1188	96%	0.0	97%
EF190504.1	*Paenibacillus lentimorbus* strain Pa1 16S ribosomal RNA gene, partial sequence	1188	1188	96%	0.0	97%
EF190503.1	*Paenibacillus lentimorbus* strain DNG 16 16S ribosomal RNA gene, partial sequence	1188	1188	96%	0.0	97%
EF190502.1	*Paenibacillus lentimorbus* strain DNG 15 16S ribosomal RNA gene, partial sequence	1188	1188	96%	0.0	97%
EF190501.1	*Paenibacillus lentimorbus* strain DNG 14 16S ribosomal RNA gene, partial sequence	1188	1188	96%	0.0	97%
AB110988.1	*Paenibacillus lentimorbus* gene for 16S ribosomal RNA, complete sequence	1182	1182	96%	0.0	97%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF190498.1	*Paenibacillus lentimorbus* strain NRRL B-2521 16S ribosomal RNA gene, partial sequence	1181	1181	96%	0.0	97%
EF190499.1	*Paenibacillus lentimorbus* strain DNG 10 16S ribosomal RNA gene, partial sequence	1177	1177	96%	0.0	97%
EF190496.1	*Paenibacillus lentimorbus* strain NRRL B-2522 16S ribosomal RNA gene, partial sequence	1177	1177	96%	0.0	96%
EF190500.1	*Paenibacillus lentimorbus* strain DNG 21 16S ribosomal RNA gene, partial sequence	1166	1166	96%	0.0	96%
D88513.1	*Paenibacillus thiaminolyticus* DNA for 16S rRNA, partial sequence	1151	1151	96%	0.0	96%
FJ821595.1	*Paenibacillus* sp. AT3 16S ribosomal RNA gene, partial sequence	1118	1211	95%	0.0	99%
DQ404713.1	Uncultured bacterium clone 655927 16S ribosomal RNA gene, partial sequence	1110	1110	99%	0.0	94%
AB073200.1	*Paenibacillus alvei* gene for 16S rRNA, partial sequence	1109	1109	100%	0.0	94%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ288407.1	*Paenibacillus* sp. enrichment culture clone S25 16S ribosomal RNA gene, partial sequence	1103	1103	100%	0.0	94%
EU497636.1	*Paenibacillus* sp. Dg-904 16S ribosomal RNA gene, partial sequence	1103	1103	100%	0.0	94%
AY862508.1	*Paenibacillus alvei* 16S ribosomal RNA gene, partial sequence	1103	1103	100%	0.0	94%
AJ320491.1	*Paenibacillus alvei* partial 16S rRNA gene, strain DSM 29T	1103	1103	100%	0.0	94%
FJ178786.1	*Paenibacillus* sp. B1 16S ribosomal RNA gene, partial sequence	1099	1099	100%	0.0	94%
AB265205.1	*Paenibacillus thailandensis* gene for 16S rRNA, partial sequence	1099	1099	100%	0.0	94%
DQ435022.1	*Paenibacillus thiaminolyticus* strain ITRC BK4 16S ribosomal RNA gene, partial sequence	1099	1099	96%	0.0	95%
AM162340.1	*Paenibacillus* sp. JS01-05 partial 16S rRNA gene	1099	1099	100%	0.0	94%
GQ927307.2	*Paenibacillus* sp. RKJ14 16S ribosomal RNA gene, partial sequence	1098	1098	100%	0.0	94%
EF426449.1	*Paenibacillus* sp. L5 16S ribosomal RNA gene, partial sequence	1098	1098	100%	0.0	94%
AB377108.1	*Paenibacillus alvei* gene for 16S rRNA, partial sequence, strain: AUG6	1098	1098	100%	0.0	94%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU982912.1	*Paenibacillus apiarius* strain CK30 16S ribosomal RNA gene, partial sequence	1092	1092	100%	0.0	94%
AM162320.1	*Paenibacillus* sp. GT08-03 partial 16S rRNA gene	1092	1092	100%	0.0	94%
FJ151508.1	*Paenibacillus alvei* strain NP75 16S ribosomal RNA gene, partial sequence	1088	1088	99%	0.0	94%
EU435385.1	*Paenibacillus alvei* strain V1 16S ribosomal RNA gene, partial sequence	1086	1086	100%	0.0	94%
DQ196464.1	*Paenibacillus* sp. L55 16S ribosomal RNA gene, partial sequence	1086	1086	100%	0.0	94%
EU620450.1	Uncultured bacterium clone CS4-7 16S ribosomal RNA gene, partial sequence	1085	1085	100%	0.0	94%
D78475.1	*Paenibacillus thiaminolyticus* DNA for 16S rRNA	1085	1085	96%	0.0	94%
AY839868.1	*Paenibacillus xinjiangensis* strain B538 16S ribosomal RNA gene, partial sequence	1083	1083	100%	0.0	94%
AJ345018.1	*Paenibacillus* sp. DSM 6358 partial 16S rRNA gene, strain DSM 6358	1083	1083	99%	0.0	94%
FN433032.1	*Paenibacillus alvei* partial 16S rRNA gene, isolate CCM2B	1081	1081	100%	0.0	94%
EF090420.1	*Paenibacillus* sp. GB 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	94%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM162296.1	*Paenibacillus* sp. GT05-08 partial 16S rRNA gene	1081	1081	100%	0.0	94%
FJ172088.1	*Paenibacillus* sp. Tir6E 16S ribosomal RNA gene, partial sequence	1077	1077	99%	0.0	94%
AY827560.1	*Paenibacillus* sp. MY03 16S ribosomal RNA gene, partial sequence	1077	1077	99%	0.0	94%
EU571189.1	*Paenibacillus* sp. 23-13 16S ribosomal RNA gene, partial sequence	1075	1075	100%	0.0	93%
DQ250001.1	*Paenibacillus* sp. L63 16S ribosomal RNA gene, partial sequence	1075	1075	100%	0.0	93%
AB073201.1	*Paenibacillus apiarius* gene for 16S rRNA, partial sequence	1074	1074	99%	0.0	93%
FJ172087.1	*Paenibacillus* sp. Tir10E 16S ribosomal RNA gene, partial sequence	1072	1072	99%	0.0	93%
AB362827.1	*Paenibacillus* sp. YT0001 gene for 16S rRNA, partial sequence	1072	1072	99%	0.0	93%
GU186911.1	*Frankia* sp. NTA03 16S ribosomal RNA gene, partial sequence	1070	1070	99%	0.0	93%
GU124638.1	*Bacillus* sp. Td3 16S ribosomal RNA gene, partial sequence	1070	1070	100%	0.0	93%
DQ870731.1	*Paenibacillus mendelii* strain JSCtot7-1 16S ribosomal RNA (rrn) gene, partial sequence	1070	1070	100%	0.0	93%
DQ196465.1	*Paenibacillus* sp. L32 16S ribosomal RNA gene, partial sequence	1070	1070	100%	0.0	93%
DQ023221.2	*Paenibacillus* sp. GPTSA 19T 16S ribosomal RNA gene, partial sequence	1070	1070	100%	0.0	93%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU029572.1	Uncultured *Paenibacillus* sp. clone T8143 16S ribosomal RNA gene, partial sequence	1066	1066	99%	0.0	93%
EU029562.1	Uncultured *Paenibacillus* sp. clone T53107 16S ribosomal RNA gene, partial sequence	1066	1066	99%	0.0	93%
NR_025490.1	*Paenibacillus agaridevorans* strain DSM 1355 16S ribosomal RNA, partial sequence >emb|AJ345023.1| *Paenibacillus agaridevorans* partial 16S rRNA gene, strain DSM 1355	1066	1066	99%	0.0	93%
GU187432.1	*Paenibacillus* sp. CAU 9038 16S ribosomal RNA gene, partial sequence	1064	1064	99%	0.0	93%
EU570162.1	*Paenibacillus* sp. RMV1 16S ribosomal RNA gene, partial sequence	1064	1064	96%	0.0	94%
AB362824.1	*Paenibacillus* sp. YT0073 gene for 16S rRNA, partial sequence	1064	1064	100%	0.0	93%
AB295646.1	*Paenibacillus montaniterrae* gene for 16S rRNA, partial sequence	1062	1062	98%	0.0	93%
AY387396.1	Uncultured *Paenibacillaceae* bacterium clone 8F316r 16S ribosomal RNA gene, partial sequence	1062	1062	98%	0.0	94%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU029574.1	Uncultured *Paenibacillus* sp. clone T8152 16S ribosomal RNA gene, partial sequence	1061	1061	99%	0.0	93%
EU029569.1	Uncultured *Paenibacillus* sp. clone T8111 16S ribosomal RNA gene, partial sequence	1061	1061	99%	0.0	93%
EU029331.1	Uncultured *Paenibacillus* sp. clone M1108 16S ribosomal RNA gene, partial sequence	1061	1061	99%	0.0	93%
EU029318.1	Uncultured *Paenibacillus* sp. clone M175 16S ribosomal RNA gene, partial sequence	1061	1061	99%	0.0	93%
AB045097.1	*Bacillus* sp. HSCC 1649T gene for 16S rRNA, partial sequence	1061	1061	99%	0.0	93%
NR_025489.1	*Paenibacillus agarexedens* strain DSM 1327 16S ribosomal RNA, partial sequence >emb|AJ345020.1| *Paenibacillus agarexedens* partial 16S rRNA gene, strain DSM 1327	1061	1061	96%	0.0	94%
AM162351.1	*Paenibacillus* sp. YO4-17 partial 16S rRNA gene	1059	1059	96%	0.0	94%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM162329.1	*Paenibacillus* sp. L47 partial 16S rRNA gene	1059	1059	96%	0.0	94%
AM745262.1	*Paenibacillus anaericanus* partial 16S rRNA gene, type strain LMG 23878T	1055	1055	100%	0.0	93%

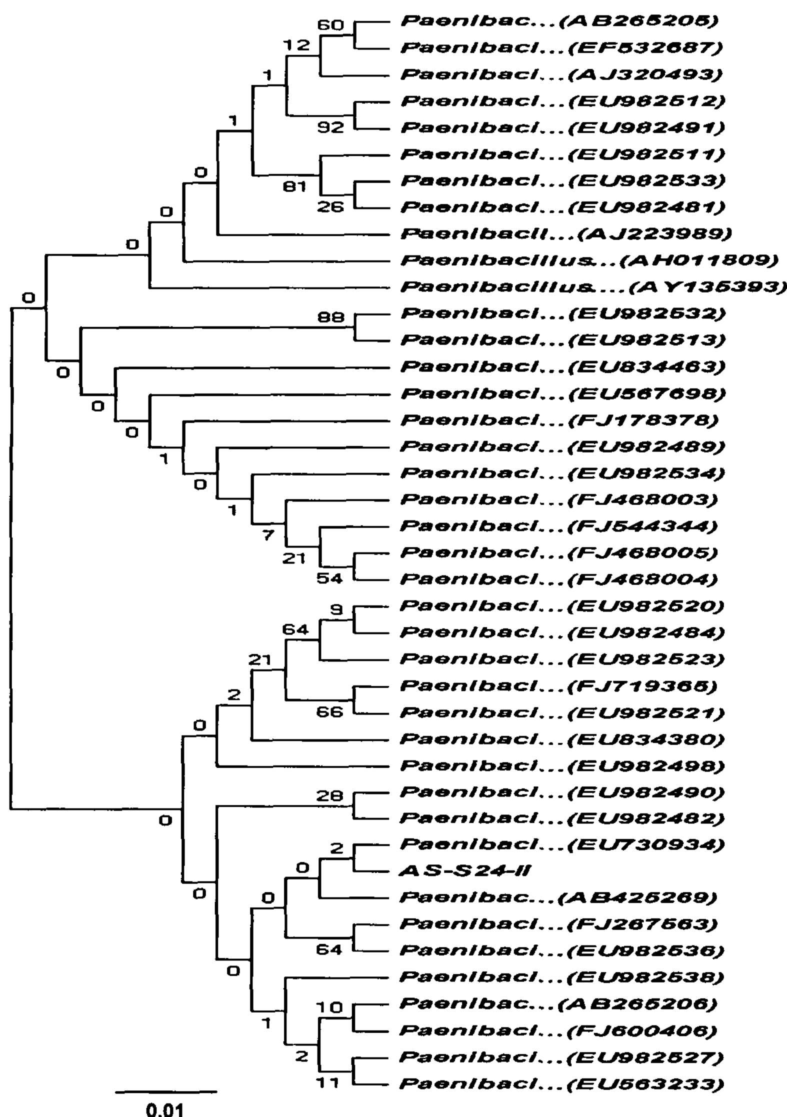


Fig. 4.5(a) Phylogenetic relationships of strain AS-S24-II and other closely related *Paenibacillus* species based on 16S rDNA sequencing. The tree was generated using the neighbour-joining method and the sequence from *Bacillus* sp. HSCC 1649 T (accession no. AB045097) was considered as out-group. The data set was resampled 1,000 times by using the bootstrap option, and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

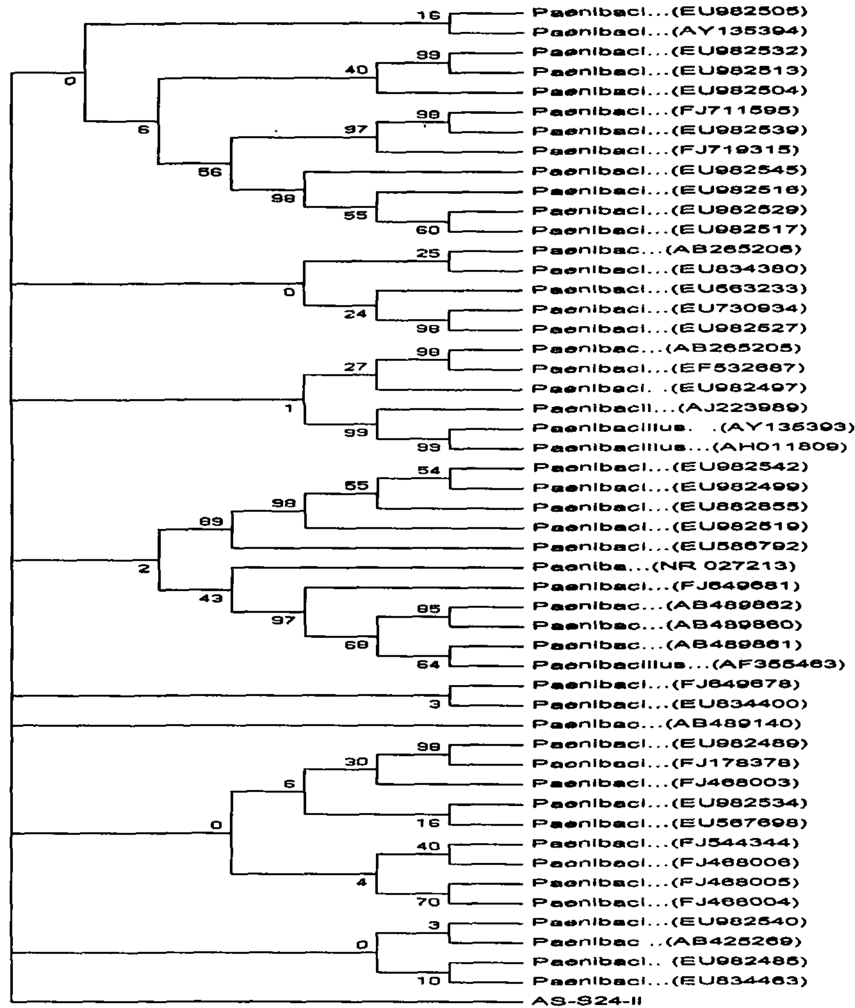
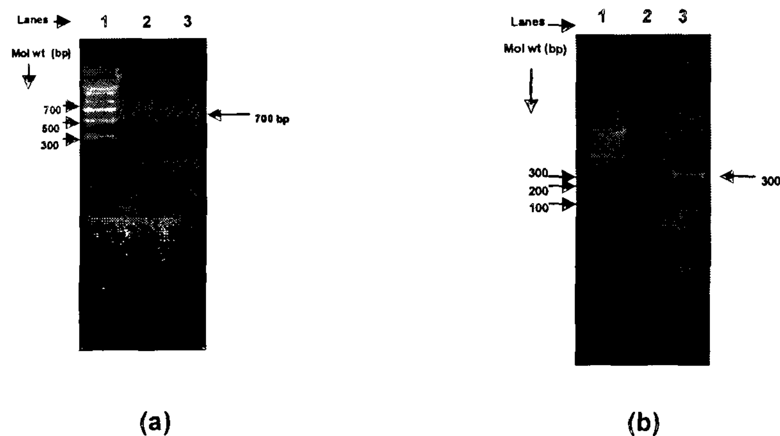


Fig.4.4(b) Phylogenetic relationships of strain AS-S24-II and other closely related *Paenibacillus* species based on 16S rDNA sequencing. The tree was generated using the unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the sequence from *Bacillus* sp. HSCC 1649 T (accession no. AB045097) was considered as out-group. The data set was resampled 1,000 times by using the bootstrap option, and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

4.2.5 PCR-Restriction fragment length polymorphism (PCR-RFLP) analysis

4.2.5.1 PCR-RFLP of strain *Bacillus subtilis* AS-S10-II

PCR amplification with p1 and p2 primers of a segment of the ISR of 16S-23S rRNA gene of strain AS-S20-II yielded 0.7 kb and 0.3 kb products as shown in Fig. 4.6 (a) and 4.6(b), respectively. The nested PCR amplification of 0.7 kb fragment with primers 16S/ p3 and 23S/ p4 resulted in amplification of 0.3 kb fragment (as shown in Fig. 4.6b).

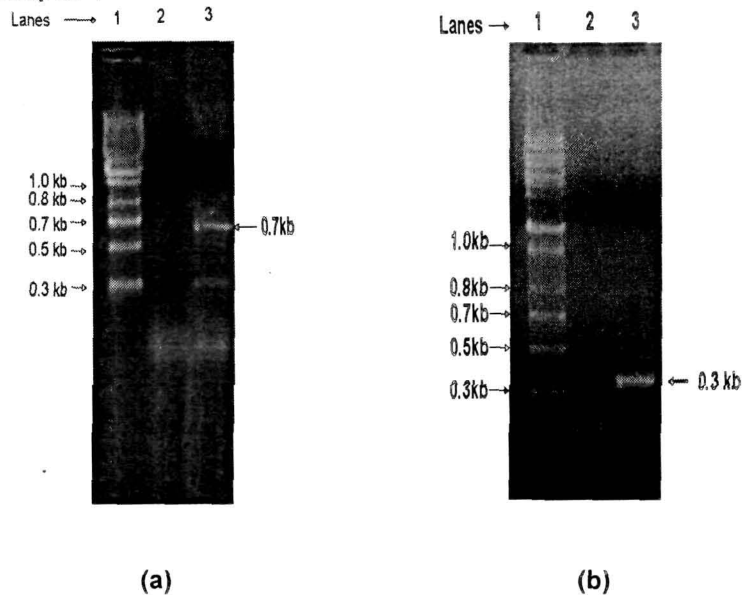


(a) (b)

Fig. 4.6 PCR amplification of ISR of 16S-23S r RNA gene of strain AS-S10-IILane 1 molecular weight marker in kbp [(a) 1.0 kb and (b) 0.5 kbp)], Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-S10-II with primers (p1/p2) in Fig. (a) and in Fig. (b) nested PCR of 0.7kb fragment with primer p3/p4.

4.2.5.2 PCR-RFLP of strain *Bacillus* sp. AS-S20-I

PCR amplification with p1 and p2 primers of a segment of the ISR of 16S-23S rRNA gene of strain AS-S20-I yielded 0.68 kb and 0.3 kb products [shown Fig. 4.7(a) and (b)]. The nested PCR amplification of 0.7 kb fragment with primers 16S/ p3 and 23S/ p4 resulted in amplification of 0.3 kb fragment (Fig.4.7b).

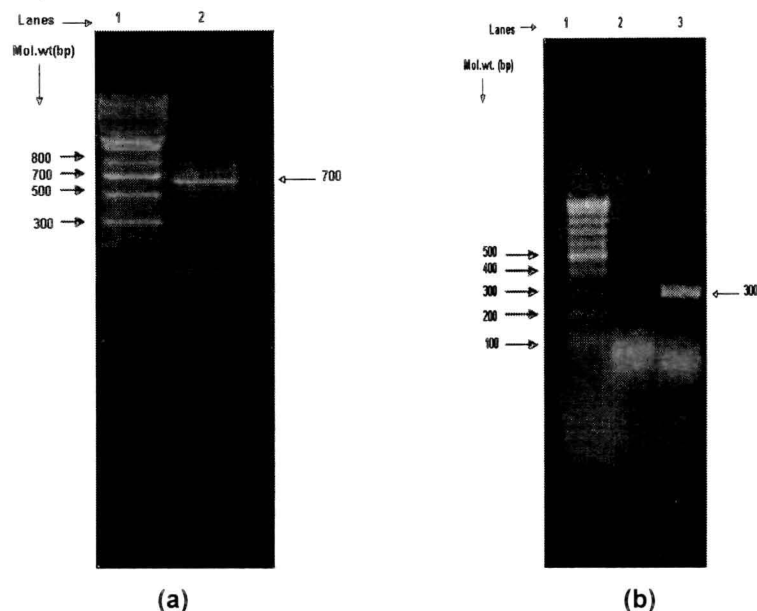


(a) (b)

Fig. 4.7 PCR amplification of ISR of 16S-23S r RNA gene of strain AS-S20-I: Lane 1 molecular weight marker in kbp ((a) 1.0 kb and (b) 0.5 kbp), Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-S20-I with primers (p1/p2) in fig. (a) and in fig. (b) nested PCR of 0.7kb fragment with primer p3/p4.

4.2.5.3 PCR-RFLP of *Bacillus subtilis* strain AS-S18

PCR amplification of a segment of the ISR of 16S-23S rRNA genes of strain AS-S18 yielded 0.7 kb and 0.3 kb products (Figs.4.8a and 4.8b).



(a) (b)

Fig. 4.8 PCR amplification of ISR of 16S-23S r RNA gene of strain AS-S18: Lane 1 molecular weight marker in kbp ((a) 1.0 kb and (b) 0.5 kbp), Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-S18 with primers (p1/p2) in fig. (a) and in fig. (b) nested PCR of 0.7kb fragment with primer p3/p4.

4.2.5.4 PCR-RFLP of strain *Bacillus licheniformis* AS-S24-II

The PCR amplification with *Bacillus*-specific oligonucleotide primers targeted to amplify the unique regions of the 16S rRNA gene showed the predicted product of around 1 kb only with strain AS-S24-II. PCR amplification with p1 and p2 primers of a segment of the ISR of 16S–23S rRNA gene of AS-S24-II yielded 0.7-kb product and the nested PCR amplification of this fragment with primers 16S/p3 and 23S/p4 resulted in amplification of 0.3 kb (Fig. 4.9).

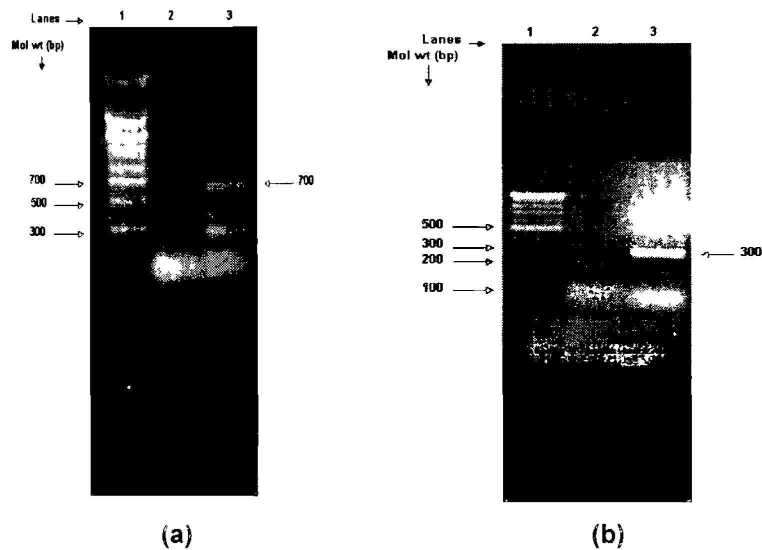


Fig. 4.9 PCR amplification of ISR of 16S-23S r RNA genes of strain AS-S24-I. Lane 1 molecular weight marker in kbp ((a) 1.0 kb and (b) 0.5 kbp), Lane 2 control with DNA template; Lane 3 amplified ISR of strain AS-S24-I with primers (p1/p2) in fig. (a) and in fig. (b) nested PCR of 0.7kb fragment with primer p3/p4.

4.3 Bacterial identification and re-designation

On the basis of phenotypic, chemotypic and genotypic properties the isolates were taxonomically identified as shown in Table 4.19 and were re-designated as shown in Table 4.20.

Table 4.19 A taxonomic classification of alkaline protease producing potential bacteria.

Bacterial isolates	Phenotypic analysis	GC-FAME analysis	16s rDNA sequencing (ribotyping)	GenBank ID of partial 16S-rDNA sequence
AS-S10-II	*Bacillus* sp.	*Bacillus subtilis* (MTCC 8960)	*Brevibacillus* sp.	GU332637
AS-S20-I	*Kocuria genus*	*Kocuria varian* (MTCC8961)	*Bacillus* sp.	GU001817
AS-S24-I	*Paenibacillus* sp.	*Peanibacillus polymyxa* (MTCC 9129)	*Bacillus licheniformis*	GU269542
AS-S24-II	*Bacillus licheniformis*	*Bacillus licheniformis* (MTCC 8959)	*Peanibacillus* sp.	FJ804507
AS-S18	*Bacillus*	*Bacillus subtilis*	N.D.	-

N.D. = Not determined

Table 4.20 Taxonomic identification and nomenclature of selected bacterial strains

Bacterial isolates	Taxonomic identity	Designated nomenclature
AS-S10-II	*Brevibacillus* sp.	*Brevibacillus* sp. strain AS-S10-II
AS-S20-I	*Bacillus* sp.	*Bacillus* sp.strain AS-S20-I
AS-S24-I	*Bacillus licheniformis*	*Bacillus licheniformis* strain AS-S24-I
AS-S24-II	*Peanibacillus* sp.	*Peanibacillus tezpurensis* sp.nov. strain AS-S24-II
AS-S18	*Bacillus subtilis*	*Bacillus subtilis* strain RM-01

CHAPTER V

RESULTS

Chapter 5

Process optimization, isolation, purification, biochemical characterization and industrial applications of alkaline proteases isolated from *Bacillus subtilis* DM-04

5.1 Alkaline protease production under solid-state fermentation

5.1.1 Screening of different agro-industrial and waste materials for alkaline protease production

The results showed that protease production by *B. subtilis* DM-04 varied with type of the substrate and also dependent on incubation time (Fig.5.1). Maximum enzyme production was observed with potato peel (341.81 U/gds) followed by IC (221.8 U/gds) as substrate post 24 h of incubation. Minimum protease production (1.818 U/gds) was observed when waste TL was used as substrate/ support material (Fig.5.1). The protein content of the cell-free extract as well as the bacterial dry biomass followed the similar trend like protease production on different substrates (Fig.5.2).

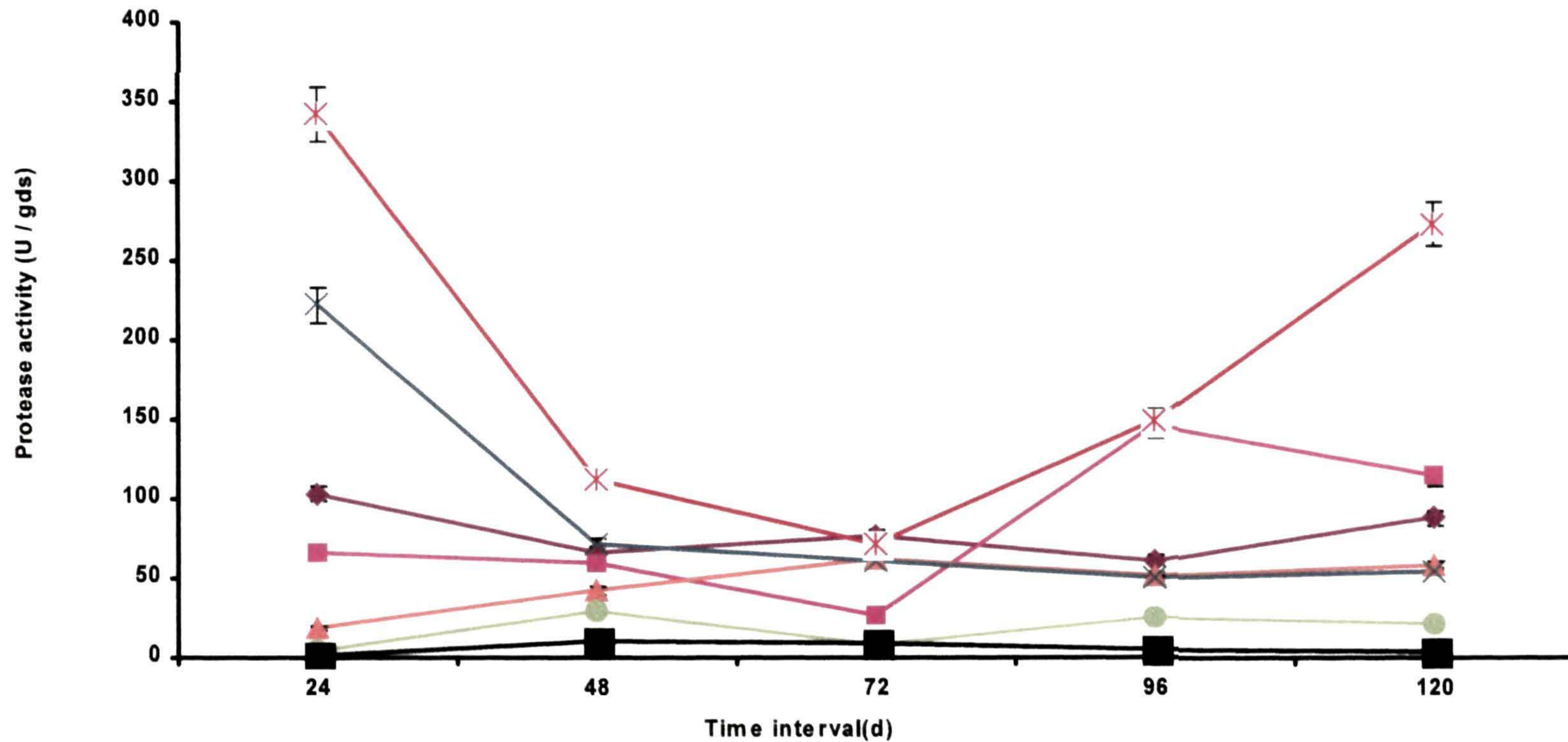


Fig.5.1 Screening of different waste residues such as MOC (◆), WB (■), RB (▲), IC(X), PP (⊠), BL (•), and TL (■) for the production of alkaline protease by *B.subtilis* strainDM-04 at different time intervals. Values are mean ± S.D. of three experiments.

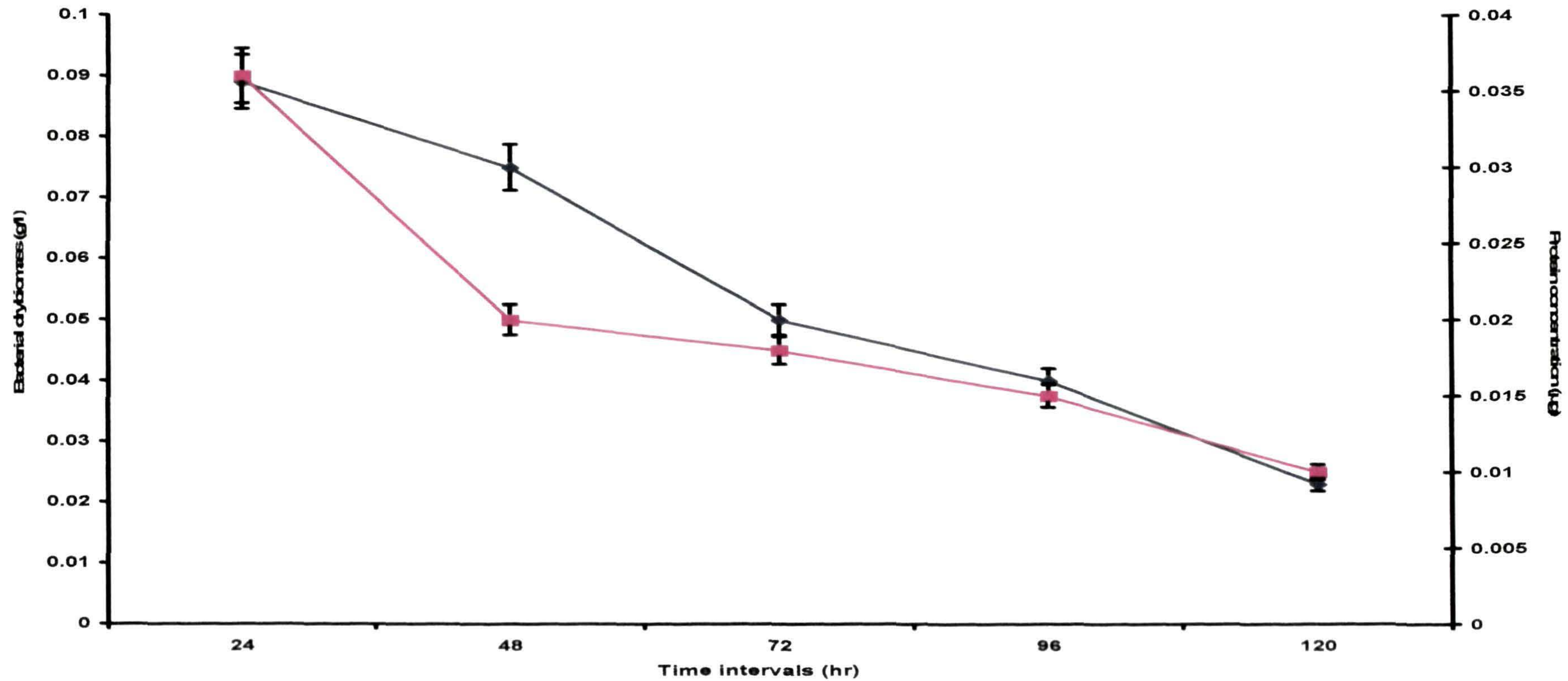


Fig.5.2 Time course of alkaline protease production by *Bacillus subtilis* strain DM-04. Legends show bacterial dry biomass (◆) and protein concentration of cell free extract (■). Values are mean ± S.D. of three experiments.

5.1.2 Effect of initial moisture content of the substrate and moistening agent on protease production

As shown in Fig. 5.3, initial moisture content of the either substrate (IC or PP) has a great influence on protease production by *B. subtilis* strain DM-04. With an increase in the initial moisture content of the substrate from 25 to 100 % alkaline protease production was concomitantly enhanced, and further increase in the moisture content of substrate resulted in a steady decline in protease yield. The nature of the moistening agent also played a crucial role in protease production by *B. subtilis* strain DM-04 under SSF condition. Distilled H_2O adjusted to pH 8.0 was most efficient moistening agent for protease production as compared to other moistening agents (Fig. 5.4).

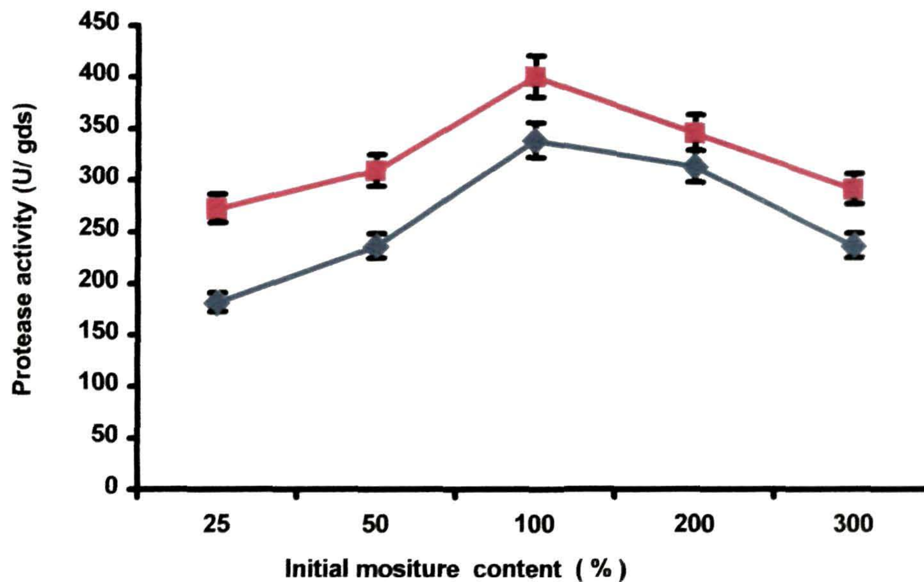


Fig.5.3 Influence of initial moisture content of the substrates [PP (■) or IC (♦)] on alkaline protease production by *B.subtilis* strain DM-04. Values are mean ± S.D. of three determinations post 24h incubation at 50°C. Moistening agent was distilled water, pH adjusted to 8.0.

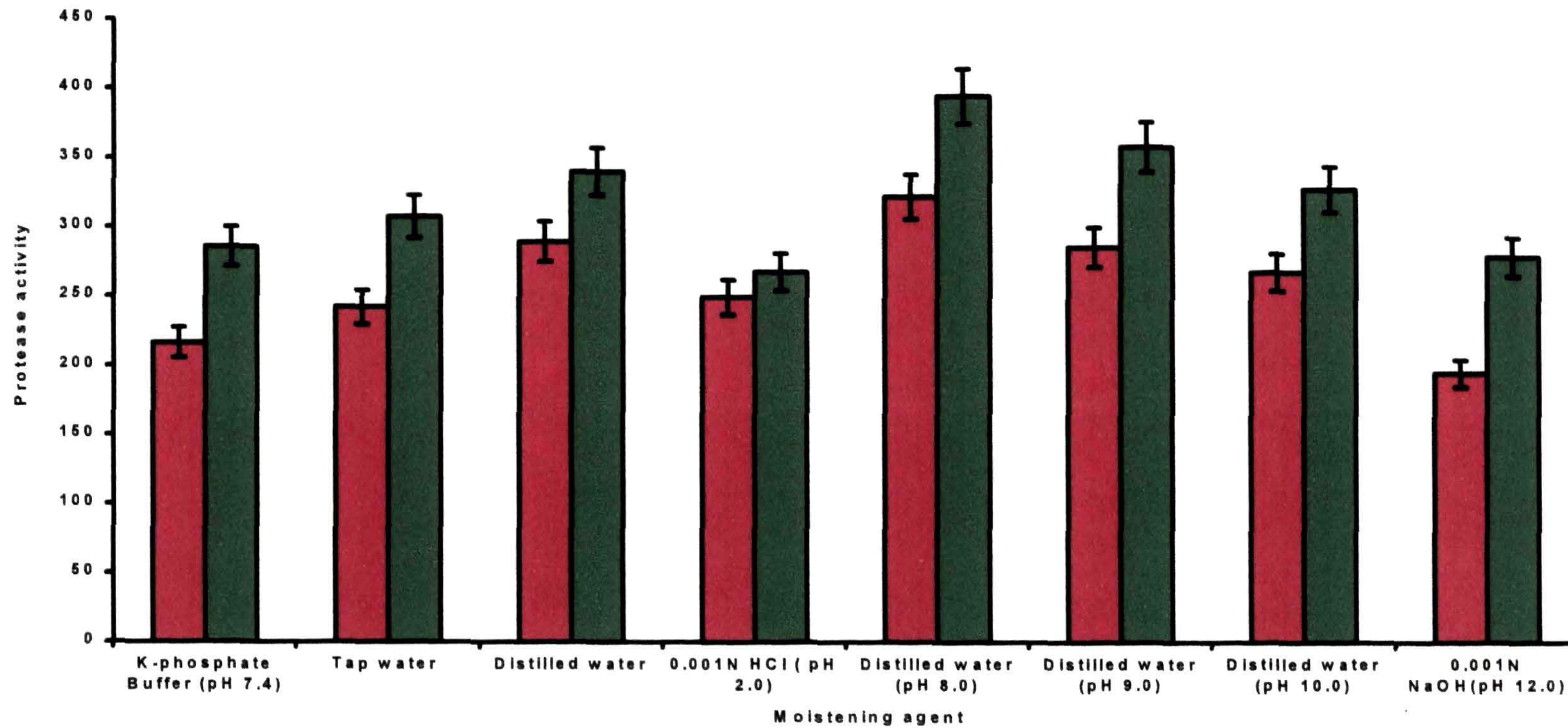


Fig.5.4 Influence of moistening agents on alkaline protease production by *B.subtilis* strain DM-04 using either IC (■) or PP (■) as a substrate. Values represent mean ± S.D of three experiments post 24h of incubation of *B.subtilis* strain DM-04 at 50°C.

5.1.3 Effect of inoculum's size on protease production

As shown in Fig.5.5, with an increase in inoculum's size from 0.5 ml to 2.0 ml (5.0 g substrate), protease production by *B. subtilis* strain DM-04 was enhanced linearly, while increasing the inoculum's size from 2.0 ml to 5.0 ml did not have a significant impact in enhancing the protease yield ($p>0.05$). However, an increase in inoculum's size beyond 5.0 ml resulted in a steady decline in protease production by *B. subtilis* strain DM-04 (Fig.5.5).

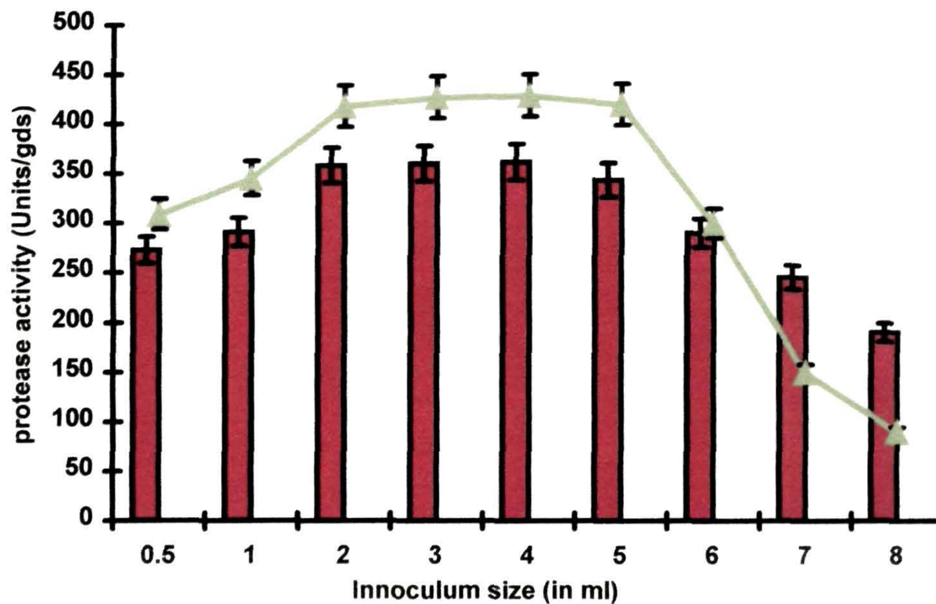


Fig.5.5 Influence of inoculum size on alkaline protease production by *B.subtilis* strain DM-04 using PP (▲) or IC (■) as substrate under SSF. Values are mean ± S.D. of three determinations.

5.1.4 Effect of supplementation of co-carbon and co-nitrogen sources on protease production

Maximum yield of protease was observed when the medium was supplemented with PP (1600 U/ gds) followed by maltose (1103.63 U/ gds) as co-carbon sources along with IC as main substrate (Fig.5.6). Among the tested nitrogen compounds, 0.1 % (w/w) beef extract followed by yeast extract served as the best co-nitrogen source for protease production by *B. subtilis* strain DM-04 strain on IC (Fig.5.7).

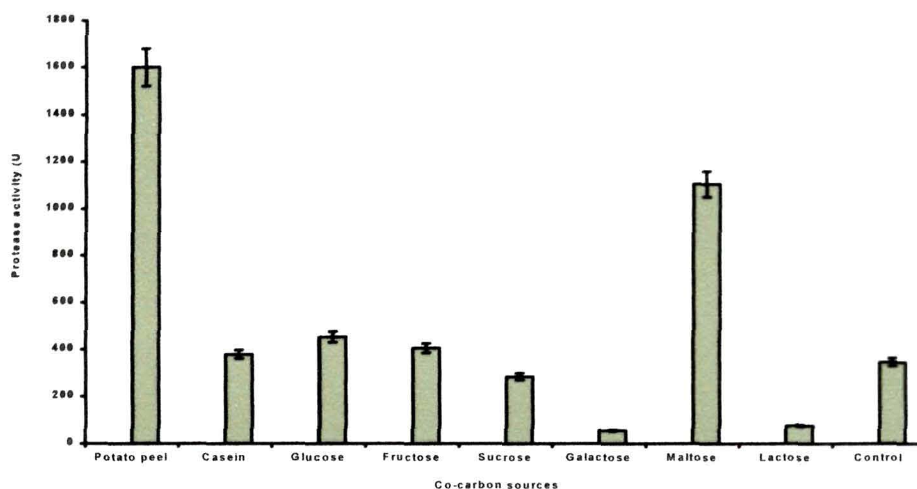


Fig. 5.6 Effect of supplementation of co-carbon sources (10 % w/w) to IC (substrate) on alkaline protease production by *B.subtilis* strain DM-04.Values are means ± S.D. of three determinations.

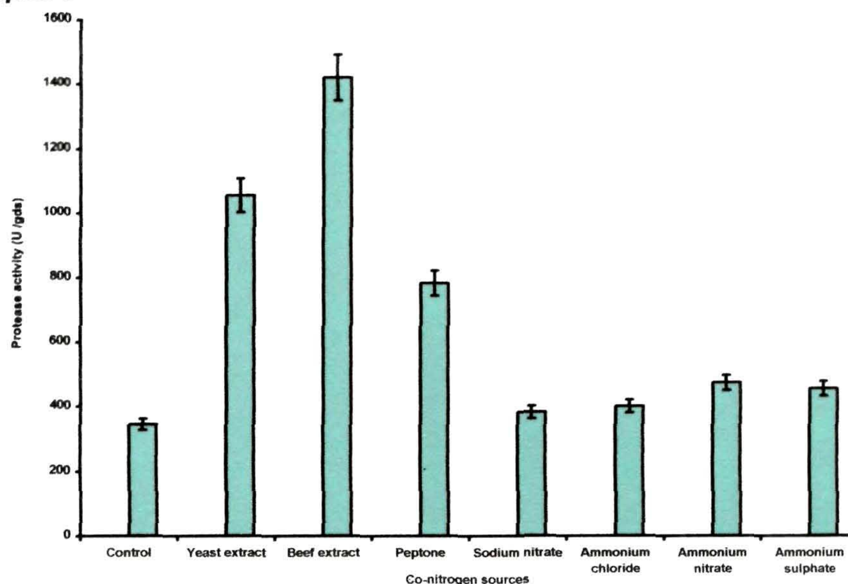


Fig.5.7 Effect of supplementation of co-nitrogen sources (1.0% w/w) to IC (99 % w/w) on alkaline protease production by *B.subtilis* strain DM-04.Values are means ± S.D. of three determinations.

5.1.5 Protease extraction from fermented matter

Among the different extractions medium used for the recovery of protease from the fermented matter, distilled water containing 0.1%(v/v) triton X-100, pH 8.0 served as best extraction medium (Fig.5.8).

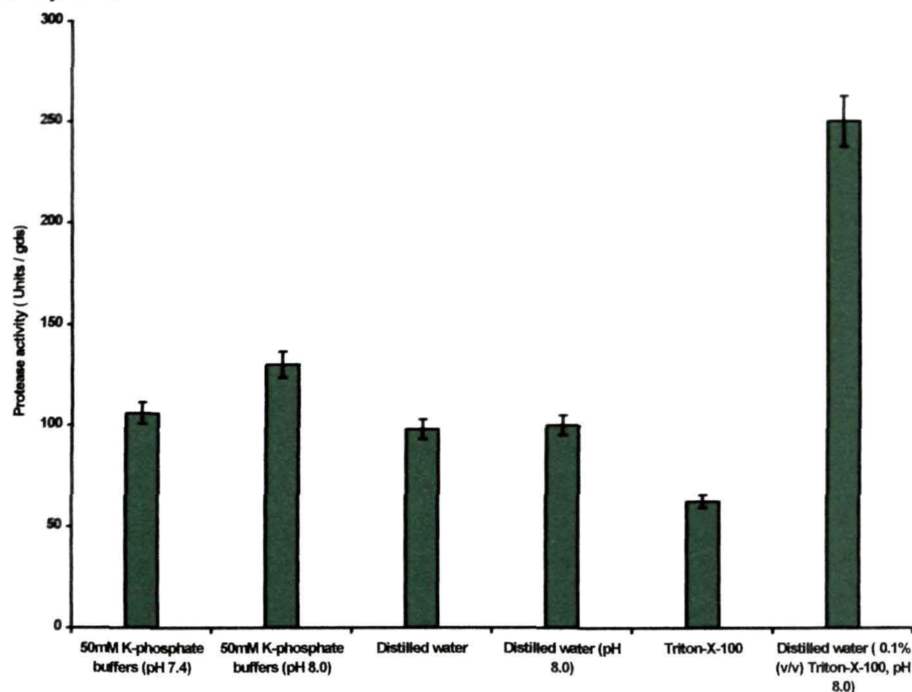


Fig. 5.8 Screening of best extraction medium for alkaline protease extraction from the fermented matter. Values are means ± S.D. of three determinations.

5.1.6 Batch fermentation

The influence of various combinations of IC and PP along with supplementation of co-carbon and co-nitrogen sources on protease production showed that when IC and PP powders were mixed in a ratio of 1:1 without any additional co- carbon and nitrogen sources, a significant increase in protease yield ($p < 0.05$) could be achieved (Table 5.1).

Table 5.1 Influence of different combinations of co-substrates on alkaline protease production by *B. subtilis* strain DM-04 under solid-state fermentation. Values are mean ± S.D of three determinations.

Substrate(s)	Protease activity (U/gds)	Carbon (%)	Nitrogen (%)	C: N (%)
l.cylindrica (100%)	221.8 ± 11.09	54.7	1.2	45.6
Potato peel (100%)	341.81 ± 17.1	51.7	2.6	19.9
l.cylindrica (89%w/w)+ Maltose (10 % w/w) + Beef extract (1% w/w)	1600.00 ± 32.0	ND	ND	ND
l.cylindrica (90%w/w) + Potato peel (10%)	1709.0 ± 21.0	54.4	1.34	40.5
l.cylindrica (80%w/w) + Potato peel (20 %w/w)	2036.0 ± 39.0	54.1	1.48	36.5
l.cylindrica (50%w/w) + Potato peel (50%w/w)	2382.0 ± 40.0	53.2	1.9	28.0
Potato peel (89% w/w) + Maltose (10%) + Beef extract (1%)	1146.0 ± 14.0	ND	ND	ND

ND : Not Determined

5.2 Alkaline protease production under submerged fermentation

5.2.1 Influence of carbon source on protease production

Different carbon sources were screened for alkaline protease production by *B.subtilis* strain DM-04 under submerged fermentation. Maximum alkaline protease production was observed in presence of 1% (w/v) maltose followed by casein as substrate (Fig.5.9).

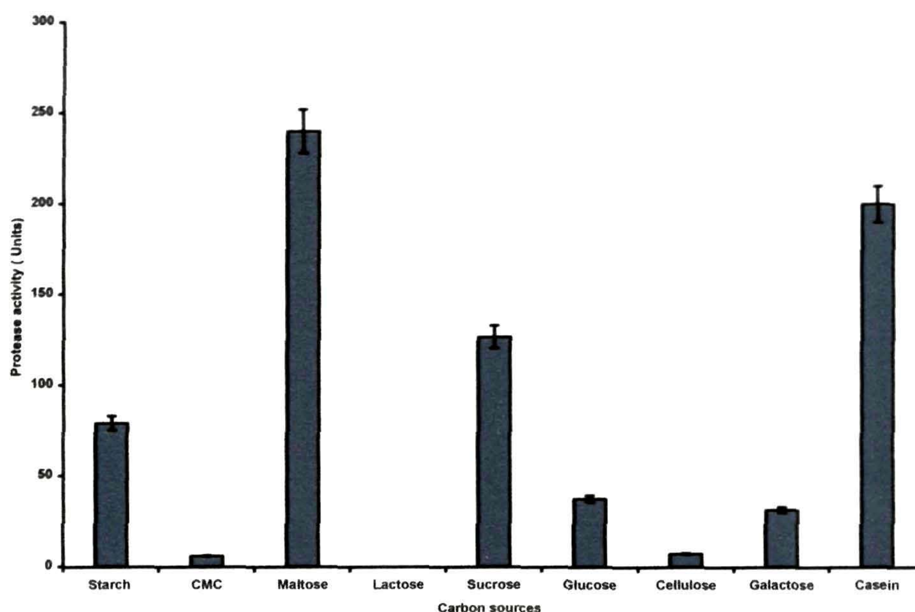


Fig.5.9 Effect of different carbon sources on alkaline protease production from *B.subtilis* strain DM-04 under SmF. Values are mean ± S.D of three determinations.

5.2.2 Effect of nitrogen source on protease production

Amongst the various nitrogen sources were tested for alkaline protease production, maximum alkaline production was observed in presence of 0.1% (w/v) beef extract as shown in Fig. 5.10.

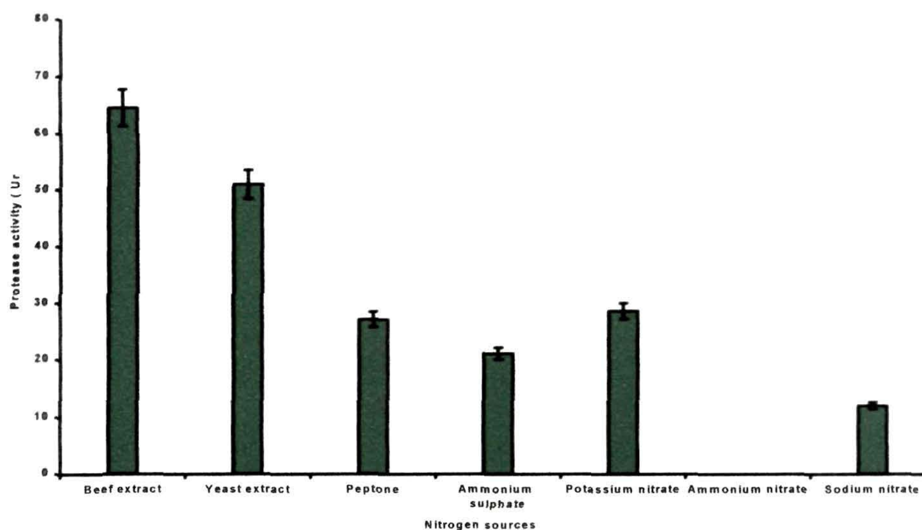


Fig.5.10 Effect of various nitrogen sources on alkaline protease production from *B.subtilis* strain DM-04 under SmF. Values are mean ± S.D of three determinations.

5.2.3 Effect of pH on protease production

The effect of pH on alkaline protease production from *B.subtilis* strain DM-04 was determined by adjusting the medium pH and assaying the enzyme production in that particular pH. Results showed that with an increase in the pH of the medium from 6.0 to 8.0, the protease production was enhanced significantly. The production remained constant from pH 8.0-10.0. However, a further increase in pH (beyond pH 10.0) resulted in decline in protease yield (Fig.5.11).

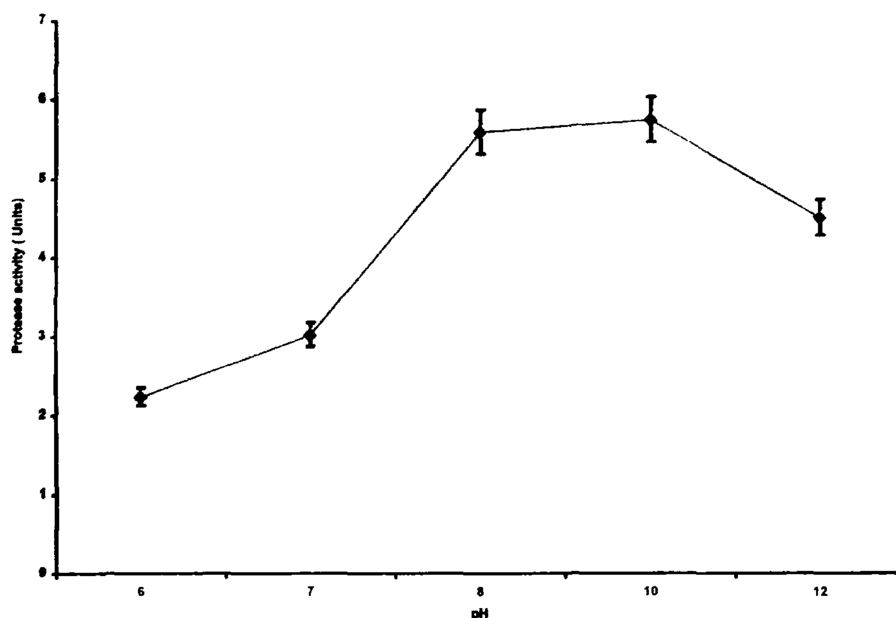


Fig.5.11 Effect of pH on alkaline protease production from *B.subtilis* strain DM-04. Values are mean ± S.D of three determinations.

5.2.4 Effect of temperature on protease production

The effect of temperature on alkaline protease production by *B.subtilis* strain DM-04 showed that protease production was significantly increased from 25 to 45°C and beyond this temperature protease production declined (Fig. 5.12). Therefore, 45°C was considered as the optimum temperature for protease production by *B.subtilis* strain DM-04.

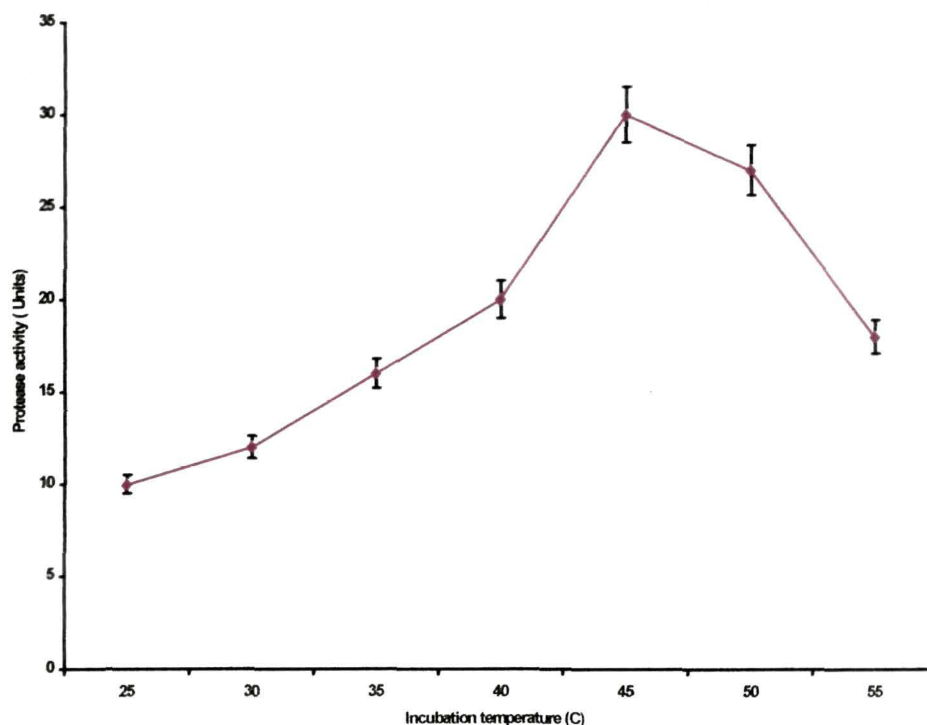


Fig.5.12 Effect of incubation temperature on alkaline protease production from *B.subtilis* strain DM-04. Values are mean ± S.D of three determinations.

5.2.5 Kinetics of protease production

Result showed that maximum alkaline protease production was achieved post 72h of incubation at 45.0°C and pH 8.0 (Fig. 5.13). Thereafter, a sharp fall in protease production was observed. Bacterial growth curve also followed the similar trend.

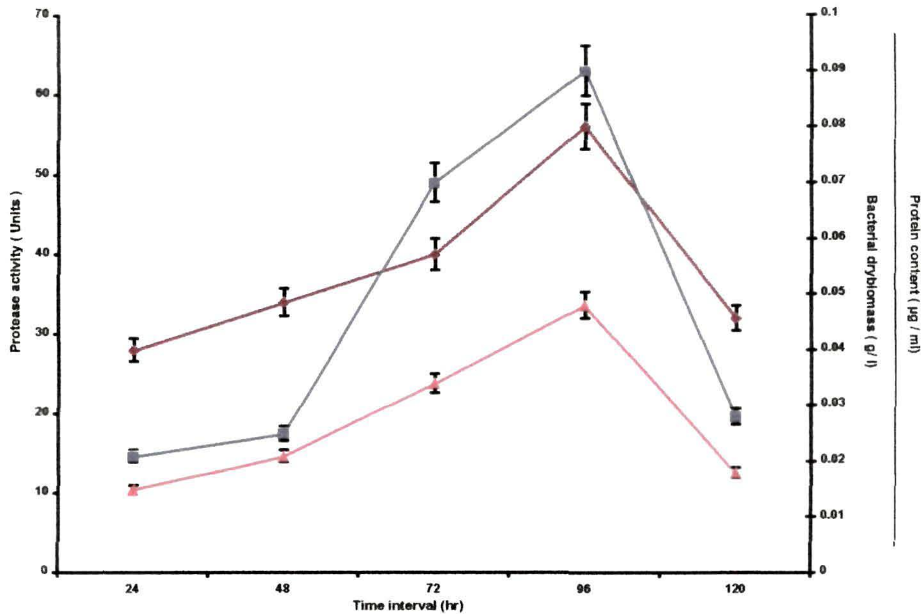


Fig. 5.13 Effect of incubation time on alkaline protease production (◆), bacterial dry biomass (■) and protein content (▲) of culture supernatant from *B.subtilis* strain DM-04 under submerged fermentation condition. The pH and temperature of the medium were adjusted to 8.0 and 45°C, respectively. Values are mean ± S.D of three determinations.

5.2.6 Effect of agitation rate on protease production

An examination of the effect of the agitation speed on the protease production showed that optimum alkaline protease production was observed at 200 rpm (Fig. 5.14).

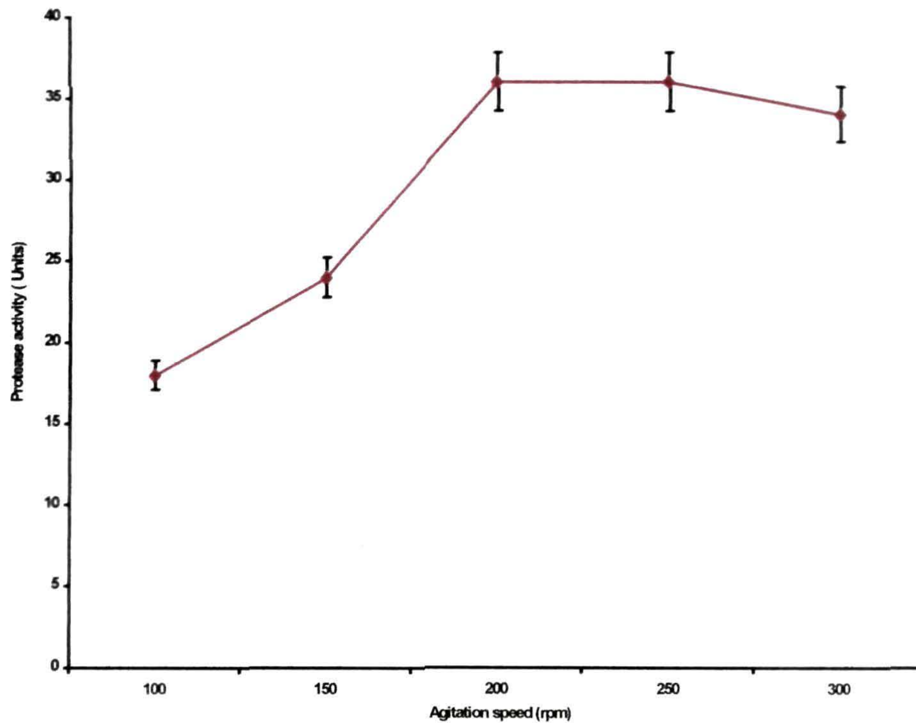


Fig.5.14 Effect of agitation rate on alkaline protease production from *B.subtilis* strain DM-04 under submerged fermentation. Values are mean ± S.D of three determinations.

5.3. Statistical optimization of alkaline protease production under submerged fermentation

5.3.1 Preliminary screening of factors influencing protease production by using Placket-Burman design

PB design was used to identify the variables which have significant effects on alkaline protease production. Protease activity was estimated in the cell free extract of *B.subtilis* strain DM-04 culture broth at 72 h post incubation, as protease production was maximum at 72 h. All the assays were carried out in triplicate and the average values are reported. The data in Table 5.2

indicated that there was a wide variation of protease activity from 80.100 U/ml to 194.460 U/ml in the fifteen trials. This variation reflected the significance of factors on the enzyme activity. The analysis of regression coefficients and t-value of six ingredients are shown in Table 5.3. It was observed that IC: PP, beef extract, and incubation time (h), displayed a positive effect for enzyme production where as, agitation rate (rpm), innoculum level (% v/v), and pH of the medium displayed a negative effect on protease production. Neglecting the variables which were insignificant, the model equation for protease production can be written as

$$Y = 135.580 + 9.405 X_1 + 18.495 X_2 + 16.485 X_3 \quad \text{---------(5.1)}$$

Where, X_1 = incubation time (h), X_2 = beef extract (% w/v), X_3 = IC:PP (% w/v)

On the basis of calculated t-values (Table 5.3), concentration of IC:PP, beef extract and incubation time(h) were chosen for further optimization, since these factors significantly effected protease production in SSF. Agitation rate and innoculum level were kept at middle level

Table 5.2 Plackett–Burman store design showing six variables with coded values along with the observed results for protease production by *B.subtilis* DM-04 strain in SmF. Values (protease yield) are mean of triplicate determinations.

IC:PP (% w/v)	Beef extract level (% w/v)	Incubation time (h)	Agitation rate (rpm)	pH of the medium	Innoculum size (% v/v)	Protease yield (Units)
1 (2.5)	-1(1.20)	1 (96)	-1 (150)	-1 (9.0)	-1 (1.5)	80.100
1 (2.5)	1 (1.30)	-1 (48)	1 (250)	-1 (9.0)	-1 (1.5)	110.460
-1 (1.5)	1 (1.30)	1 (96)	- (150)	1 (11.0)	-1 (1.5)	140.400

...Continued

Table 5.2 Continued.....

1 (2.5)	-1 (1.20)	1 (96)	1 (250)	-1 (9.0)	1 (5.0)	125.400
1 (2.5)	1 (1.30)	-1 (48)	1 (250)	1 (11.0)	-1 (1.5)	147.480
1 (2.5)	1 (1.30)	1 (96)	-1 (150)	1 (11.0)	1 (5.0)	154.500
-1 (1.5)	1 (1.30)	1 (96)	1 (250)	-1 (9.0)	1 (5.0)	149.460
-1 (1.5)	-1 (1.20)	1 (96)	1 (250)	1 (11.0)	-1 (1.5)	148.500
-1 (1.5)	-1 (1.20)	-1 (48)	1 (250)	1 (11.0)	1 (5.0)	194.460
1 (2.5)	-1 (1.20)	-1 (48)	-1 (150)	1 (11.0)	1 (5.0)	162.480
-1 (1.5)	1 (1.30)	-1 (48)	-1 (150)	-1 (9.0)	1 (5.0)	149.460
-1 (1.5)	-1 (1.20)	-1 (48)	- 1 (150)	-1 (9.0)	-1 (1.5)	111.000
0 (2.0)	0 (1.25)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	120.000
0 (2.0)	0 (1.25)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	120.000
0 (2.0)	0 (1.25)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	120.000

Table 5.3 Statistical analysis of Plackett–Burman design showing coefficient values, t- and p-value for each variable for protease production (p-value <0.05).

Variables	**Protease yield (Units)**			
	Co-efficient	**SE Coef**	**t-Stat**	**p-value**
Intercept	135.580	2.75483	49.2155	0.000
Incubation time (h)	9.405	3.07999	3.0536	0.016
Innoculum level (%v/v)	2.485	3.07999	0.8068	0.443
pH of the medium	-6.415	3.07999	-2.0828	0.071
Agitation rate (rpm)	6.485	3.07999	2.1055	0.068

Variables	Protease yield (Units)			
	Co-efficient	SE Coef	t-Stat	p-value
Beef extract (% w/v)	18.495	3.07999	6.0049	0.000
IC:PP level (% w/v)	16.485	3.07999	5.3523	0.001

5.3.2 RSM regression equation and model analytics

The results of the observed and predicted values for alkaline protease yield (response) in submerged fermentation (SmF) as function of the chosen variables with reference to the experiments performed according to the CCD is shown in Table 5.4. Maximum protease yield (508 U) was obtained with the data set of 2.5 % (w/v) of IC: PP (1:1), 1.25% (w/v) of beef extract post 72 h of incubation. The parameters of Eq. (3.2) (section 3.9.2) were determined by multiple regression analysis by the application of RSM. The overall second-order polynomial regression equation showing the empirical relationship between protease activity (Y) and three test variables in coded units is represented by equation 5.2

$$Y = 465.820 + 11.698C_1 + 14.323C_2 + 11.016C_3 - 0.117C_1^2 + 0.258C_2^2$$
$$+ 1.085C_3^2 + 1.395C_1C_2 - 0.105C_1C_3 - 0.105C_2C_3 \qquad \text{..........(5.2)}$$

Multiple regression model assumes a linear relationship between some variable Y (dependent variable) and independent variables C_1, C_2, C_3---- C_n. Based on the result obtained with the multiple regression analysis, it was observed that interaction of incubation time with the fermentation

substrates had a negative impact on protease production. The analysis of variance (ANOVA) by Fisher's statistical test was conducted for the second-order response surface model and the result showed that the computed F value for linear regression was much greater than the tabulated (P) > F value (Table 5.5); however, the F values for the square and interaction effects were less than the tabulated (P) > F value (Table 5.6). Therefore, the model terms C_1, C_2 and C_3 were found to be the significant and not their interaction effects (Figs.5.15 to 5.16).

Table 5.4 Observed responses and predicted values of alkaline protease produced by *B.subtilis* strain DM-04. Boundaries of the experimental domain and spacing of levels are expressed in coded (within parenthesis) and natural units. C_1, IC: PP level (% w/v); C_2, beef-extract level (% w/w); C_3, incubation time (h).

Run no.	Independent Variables			Y Response (Protease yield in U)		Residual value
	C_1	C_2	C_3	Observed value	Predicted value	
1	1.5 (-1)	0.75 (-1)	48 (-1)	428.129	431.193	-3.065
2	2.5 (+1)	0.75 (-1)	48 (-1)	452.629	452.008	0.621
3	1.5 (-1)	0.75 (-1)	48 (-1)	458.629	457.258	1.371
4	2.5 (+1)	0.75 (-1)	48 (-1)	484.129	483.653	0.476
5	1.5 (-1)	0.75 (-1)	72 (+1)	453.129	453.645	-0.516
6	2.5 (+1)	0.75 (-1)	72 (+1)	472.629	474.040	-1.411
7	1.5 (-1)	1.25 (+1)	72 (+1)	478.629	479.290	-0.661
8	**2.5 (+1)**	**1.25 (+1)**	**72 (+1)**	**508.290**	**505.266**	**3.024**
9	2.0 (0)	1.0 (0)	48 (-1)	456.486	455.888	0.598
10	2.0 (0)	1.0 (0)	72 (+1)	477.486	477.920	-0.435
11	1.0 (-2)	1.0 (0)	60 (0)	443.371	441.954	1.416

...Continued

Table 5.4 Continued

12	3.0 (+2)	1.0 (0)	60 (0)	487.370	488.744	-1.375
13	2.0 (0)	0.5 (-2)	60 (0)	440.370	438.204	2.166
14	2.0 (0)	1.5 (+2)	60 (0)	493.371	495.494	-2.125
15	2.0 (0)	1.0 (0)	60 (0)	466.086	465.819	-2.125
16	2.0 (0)	1.0 (0)	60 (0)	460.270	465.819	-5.550
17	2.0 (0)	1.0 (0)	60 (0)	463.492	465.819	-2.330
18	2.0 (0)	1.0 (0)	60 (0)	467.211	465.819	1.390
19	2.0 (0)	1.0 (0)	60 (0)	466.871	465.819	1.050
20	2.0 (0)	1.0 (0)	60 (0)	470.910	465.819	5.090

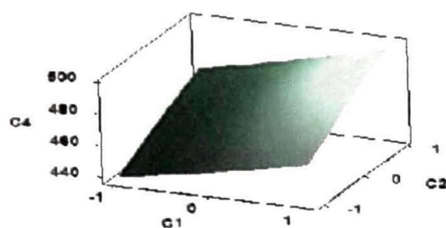
Table 5.5 Analysis of Variance (ANOVA) of alkaline protease produced by *B.subtilis* DM-04.

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	9	6709.15	6709.15	745.46	72.65	0.000
Linear	3	6685.04	6685.04	2228.35	217.16	0.000
Square	3	8.35	8.35	2.78	0.27	0.845
Interaction	3	15.75	15.75	5.25	0.51	0.683
Residual error	10	102.61	102.61	10.26	0.57	0.722
Lack-of-Fit	5	37.37	37.37	7.47		
Pure error	5	65.24	65.24	13.05		
Total	19	6811.76				

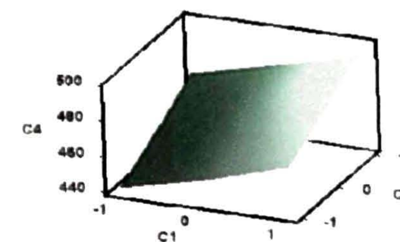
Table 5.6 Model coefficients estimated by multiple linear regressions (significance of regression coefficients) *Bacillus subtilis* DM-04 in SmF under shake-flask study.

Factor	Coefficient	SE coefficient	Computed t-value	p-value
Constant	465.820	1.2698	366.837	0.000
C_1	11.698	0.8008	14.607	0.000
C_2	14.323	0.8008	17.885	0.000
C_3	11.016	1.0130	10.875	0.000
C_1^2	-0.117	0.6240	-0.188	0.854
C_2^2	0.258	0.6240	0.413	0.689
C_3^2	1.085	1.4326	0.757	0.466
$C_1 C_2$	1.395	1.1325	1.232	0.246
$C_1 C_3$	-0.105	1.1325	-0.093	0.928
$C_2 C_3$	-0.105	1.1325	-0.093	0.928

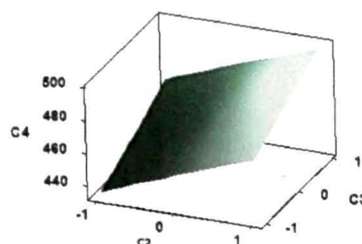
R^2 = 98.49%, R^2 (pred) = 92.1%, R^2 (adj) = 97.14%



(a)

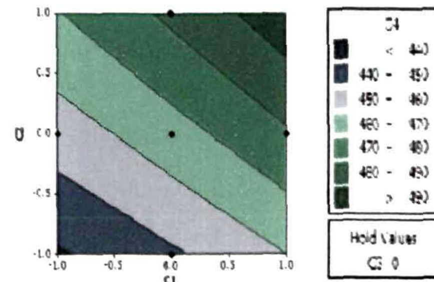


(b)

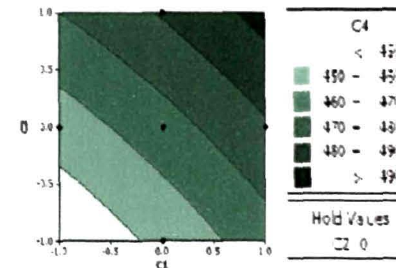


(c)

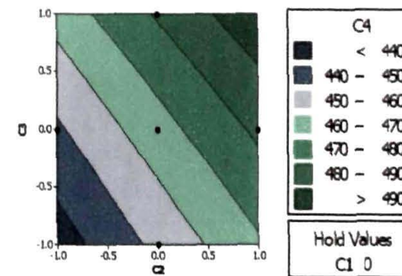
Fig. 5.15 Response surface plots for alkaline protease production by *B. subtilis* strain DM-04. The interaction between (a) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and concentration (%, w/v) of beef-extract level (C_2), hold value incubation time (C_3) = 0; (b) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and incubation time (C_3),; hold value beef extract level (C_2) =0; (c) concentration (%, w/v) of beef extract (C_2) and incubation time (C_3), hold value IC:PP level (C_1)= 0.



(a)



(b)



(c)

Fig.5.16 Countor surface plots for alkaline protease production by *B. subtilis* strain DM-04. The interaction between (a) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and concentration (%, w/v) of beef-extract level (C_2), hold value incubation time (C_3) = 0; (b) concentration (%, w/v) of (1:1, w/w) IC:PP (C_1) and incubation time (C_3),; hold value beef extract level (C_2) =0; (c) concentration (%, w/v) of beef extract (C_2) and incubation time (C_3), hold value IC:PP level (C_1)= 0.

5.3.4 Response surface plots

The three-dimensional response surface plots as a function of two factors at a time, maintaining the third factor at the minimum (zero) level, are depicted in Figs. 5.15a–c. A small trough in response surface plot (Fig. 5.15a) indicated an initial decrease in protease production with the initial increase of beef extract level. Figure 5.15b shows that protease production was increased with an initial increase of IC: PP concentration; however, further increase of this beef extract resulted in decrease in protease yield by bacteria. Interaction of beef extract level and incubation time *demonstrated that an increase in level of both the parameters significantly* enhance the prctease yield (Fig. 5.15c).

5.3.5 Countor plots

The maximum predicted value is indicated by the smallest ellipse in the contour plots Figs.5.16a–b show the 2D contour plots for protease production for each pair of variables by keeping the third variable constant at its middle level. It could be seen that the protease production was increased upon increasing the concentration of IC:PP from 2.5% (% w/v), but a further increase in its concentration values resulted in decrease in protease production. Similarly, the protease production was increased with an increase in the concentration of beef extract, and further increasing the beef extract concentration resulted in decreased protease production. Therefore, the optimal level of beef extract was determined as 1.25 % (w/v).

5.3.6 Batch fermentation

The validation of the statistically optimized condition for the production of alkaline protease by *B. subtilis* DM-04 was verified by carrying out batch fermentation in a 5-l fermenter. It was interesting to observe that maximum

protease yield of 518 U obtained by batch culture post 60 h of incubation was slightly higher than the observed highest experimental value in shake –flask study (195.0 U) as well as the predicted value of the protease yield by response surface method (508.0 U). Furthermore, the optimal time requirement for maximum protease production in a process-controlled fermenter was less than that observed under shake-flask study (60 h Vs 72 h).

5.4. Isolation and purification of an alkaline protease (Bsubap-I)

Fractionation of crude protease on a DEAE-Sephadex A-50 column resolved into three peaks DE-I to DE-III (Fig. 5.17). On the basis of maximum specific activity, the DE-I fraction were pooled. Fractionation of peak DE-I (eluted with 20 mM K-phosphate buffer, pH 6.5) on gel-filtration column resolved it into two peaks GF-I and GF-II (Fig.5.18). The peak GF-II, containing higher protease activity compared to GF-I, was found to be homogenous by SDS-PAGE as it displayed a single band of 33.1 kDa under both reduced and non-reduced conditions (Fig. 5.19). The protease activity of 33.1 kDa purified protein was further confirmed by casein zymography (Fig. 5.19). By RP-HPLC, the GF-II fraction was eluted as a single, sharp peak with the retention time of 10.97 min showing purity of the protease preparation (Fig.5.20). A summary of purification of *B. subtilis* DM-04 anionic protease (Bsubap- I) is shown in Table 5.7.

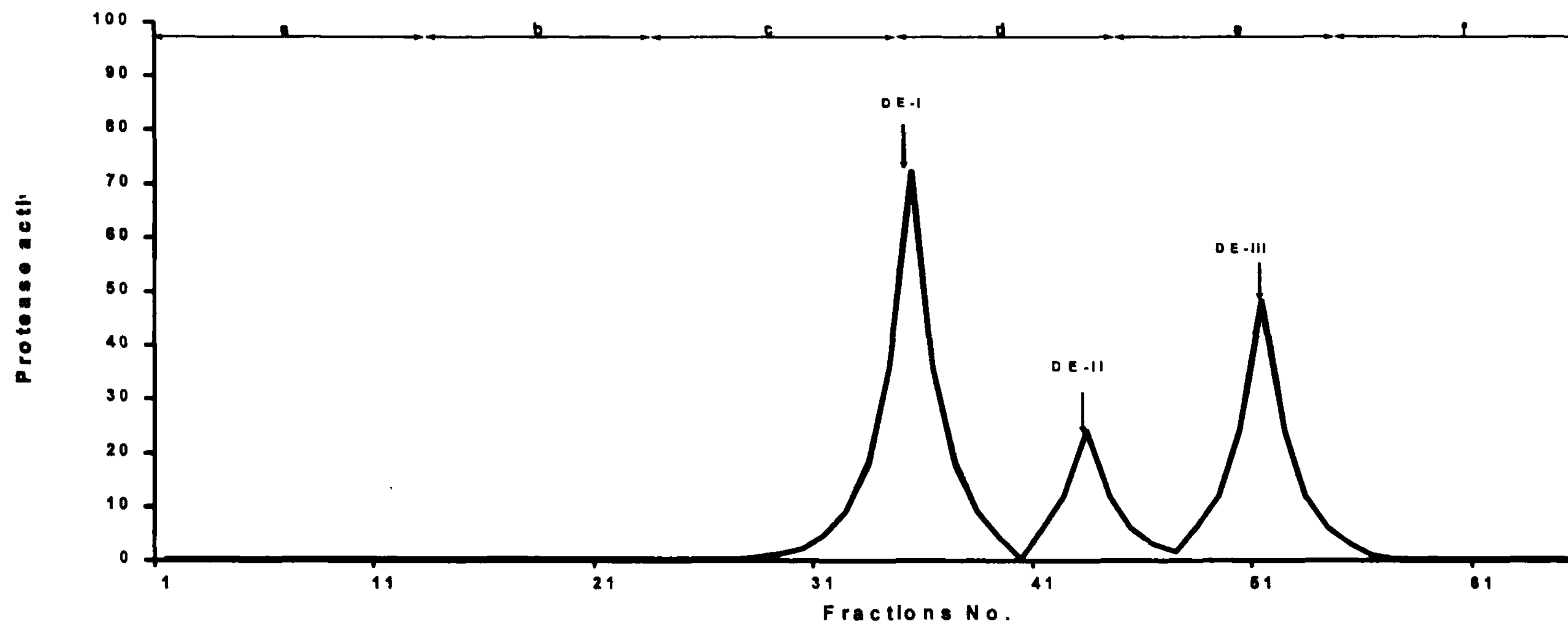


Fig.5.17 Fractionation of anionic proteases secreted from *B.subtilis* strain DM-04 by DEAE Sephadex A-50 column. Elution was performed stepwise with phosphate buffers of the following molarities and pH values: (a) 20 mM K-phosphate, pH 7.0); (b) 20 mM K-phosphate (pH 6.5); (c) 20 mM K-phosphate (pH 6.0); (d) 20 mM K-phosphate (pH 5.5); (e) 100 mM K-phosphate (pH 5.0); (f) 120 mM K-phosphate (pH 5.0). Data represent a typical experiment.

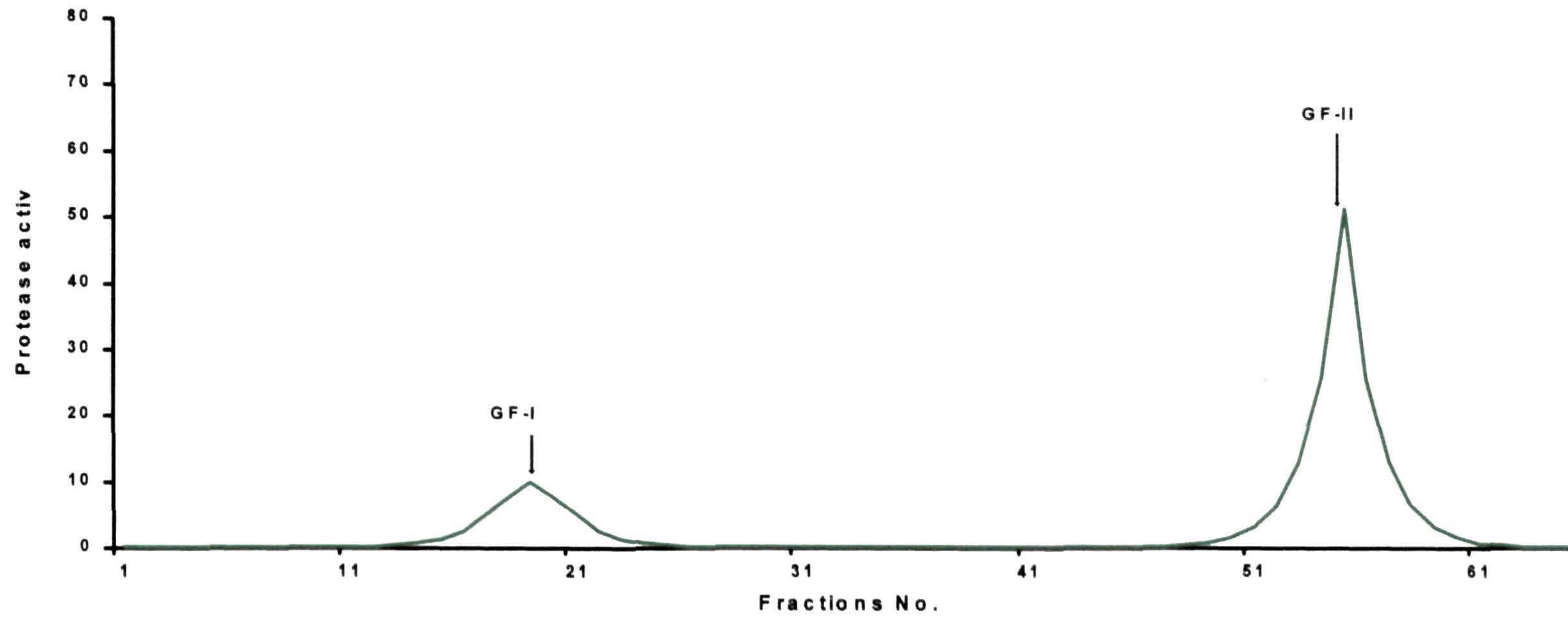


Fig.5.18 Sephacryl S-200 gel filtration profile of DE-I fraction. Data represent a typical experiment.

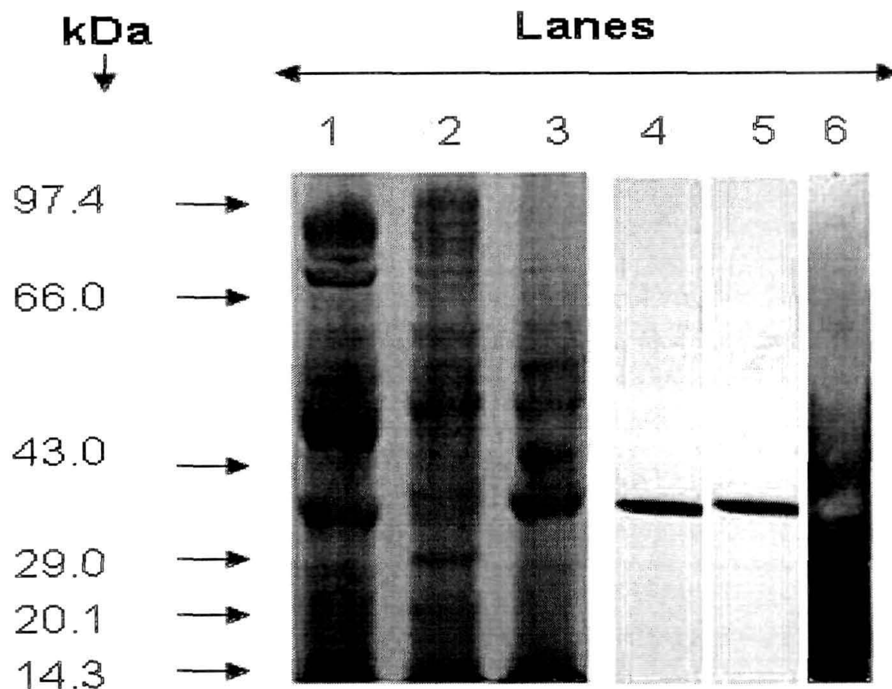


Fig. 5.19 12.5 % SDS-PAGE of crude protease and Bsubap-I from *B. subtilis* strain DM-04. Molecular weight markers are phosphorylase b (97,400 Da), BSA (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), soybean trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da); Lane 1: crude protease (50 µg); Lane 2: DEAE-Sephadex A-50 flow through fraction (50 µg); Lane 3: DE-01 fraction (50 µg); Lane 4: Bsubap-I under reduced condition (10 µg); Lane 5: Bsubap-I under non-reduced condition (10 µg), Lane 6: Casein zymograph of Bsubap-I (20 µg) post incubation at 45 °C for 4 h.

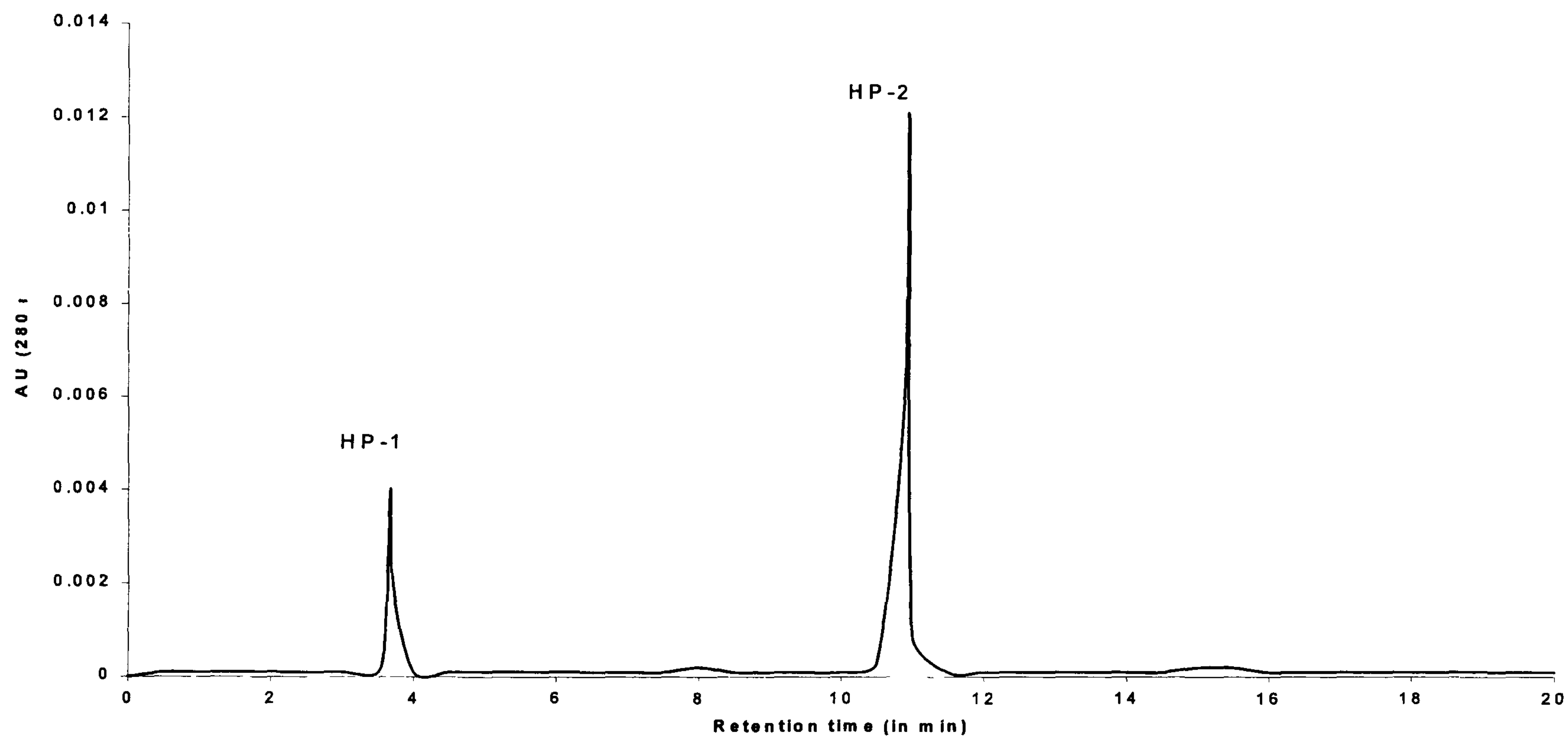


Fig.5.20 RP-HPLC of GF-II fraction. HP-I represents solvent peak.

Table 5.7 Summary of purification of Bsubap-I from *Bacillus subtilis* strain DM-04. Values are from a typical experiment.

Purification steps	Total protein (mg)	Yield (%)	Total enzyme activity (U)	Specific activity (U / mg)	Purification fold
Cell-free supernatant	100.0	100.0	2.1×10^4	210.0	1
DE – I	5.9	27.0	5.7×10^3	964.0	4.6
GF-II (Bsubap-I)	2.0	25.0	5.3×10^3	2650.0	12.6

5.5 Biochemical characterization

The optimum activity of Bsubap-I was detected at pH range of 10.0–10.5 and at 45°C temperature (Table 5.8).

Table 5.8 Biochemical characterization of Bsubap-I purified protease.

Parameters	Values
Optimum pH	10.0-10.5
Optimum incubation temperature (°C)	45.0
Optimum incubation time (min)	30.0

5.5.1 Substrate specificity study

Casein was found to be the most preferred substrate for Bsubap-I [specific activity (S.A) 2650 U/ mg] followed by gelatin [S.A. 729 U/ mg], chicken-feather keratin [S.A. 419 U/ mg], bovine serum albumin [S.A. 352.0], whereas collagen and human hair was not hydrolyzed by this protease.

5.5.2 Kinetics of substrate hydrolysis

The K_m and V_{max} values of Bsubap-I towards casein was determined as 6.5 mg / ml and 320.0 µmol / min / mg respectively (Fig.5.21); the K_{cat} and K_{cat}/K_m values were calculated as 7.0 x 10^3 / sec and 1.1 x 10^2 / sec / µM, respectively.

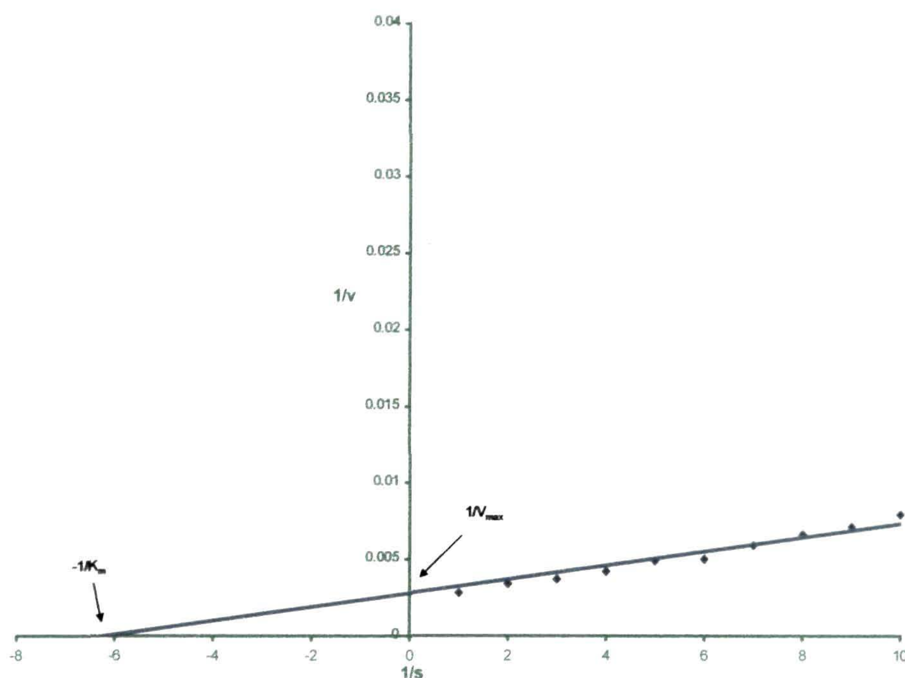


Fig. 5.21 Lineweaver-Burk plot to determine the K_m and V_{max} values for Bsubap-I using casein as a substrate. Values are mean of triplicate determinations.

5.5.3 Thermo-stability study of Bsubap-I

Heating the Bsubap-I at 60°C for 90 min and 120 min resulted in reduction of 71% and 89% of its original protease activity, respectively (Fig.5.22).

The residual protease activity post heating of Bsubap-I at 60°C for 120 min in presence of polyols was observed in the following order: glycerol (150% activity)> mannitol (132% activity) > sorbitol (97% activity) > xylitol (82% activity) (Fig.5.23). The activity of control was considered as 100% activity.

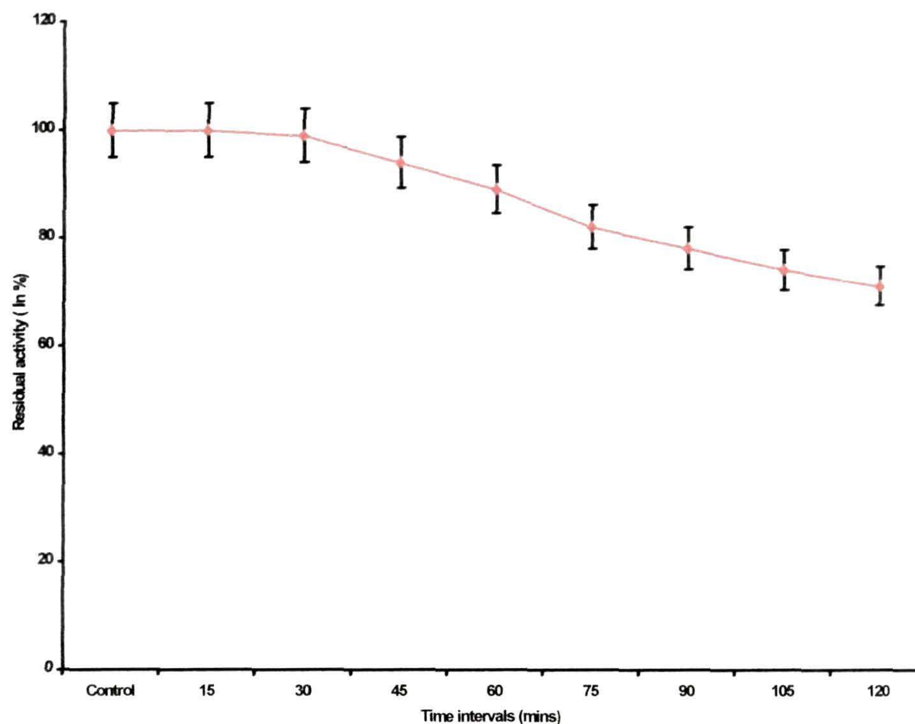


Fig.5.22 Thermo stability study of Bsubap-I from *B.subtilis* strain DM-04. Values are mean ± S. D. of three determinations.

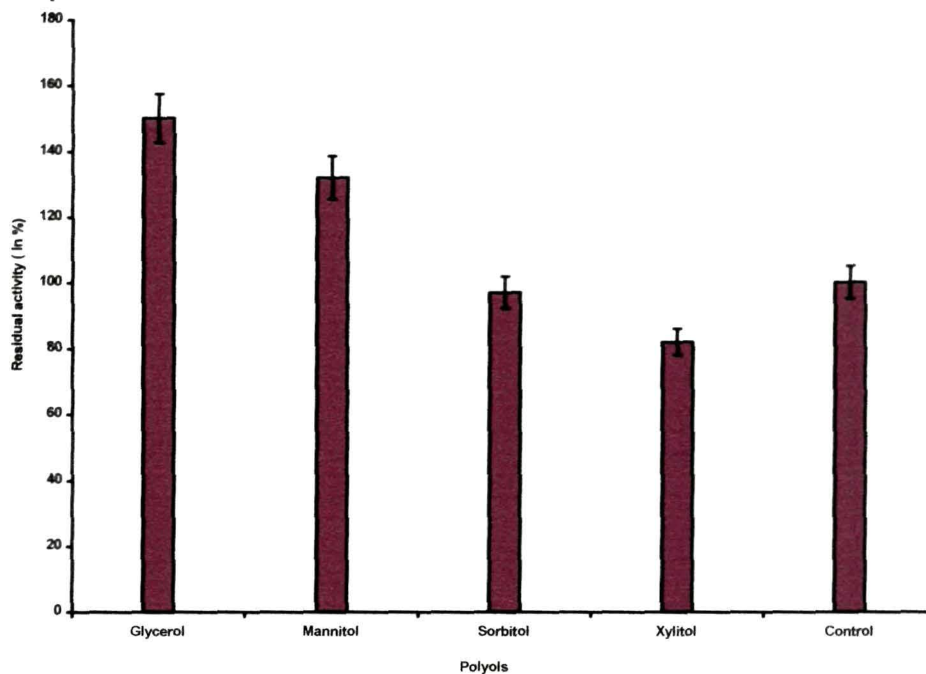


Fig.5.23 Effect of polyols on Bsubap-I stability at 60°C for 120 min . Values are mean ± S. D. of three determinations.

5.5.4 Effect of metal ions on protease activity of Bsubap-I

It was observed that Fe^{2+} stimulated the protease activity to 1.5-folds of its original activity. whereas the enzyme activity was significantly reduced (10–25% of the original activity) in presence of Hg^{2+}, Mn^{2+}, Ca^{2+} and Cd^{2+}(Fig.5.24) The enzymatic activity of Bsubap-I was completely inhibited by Ni^{2+}, Mg^{2+} and Cu^{2+} (Fig.5.24).

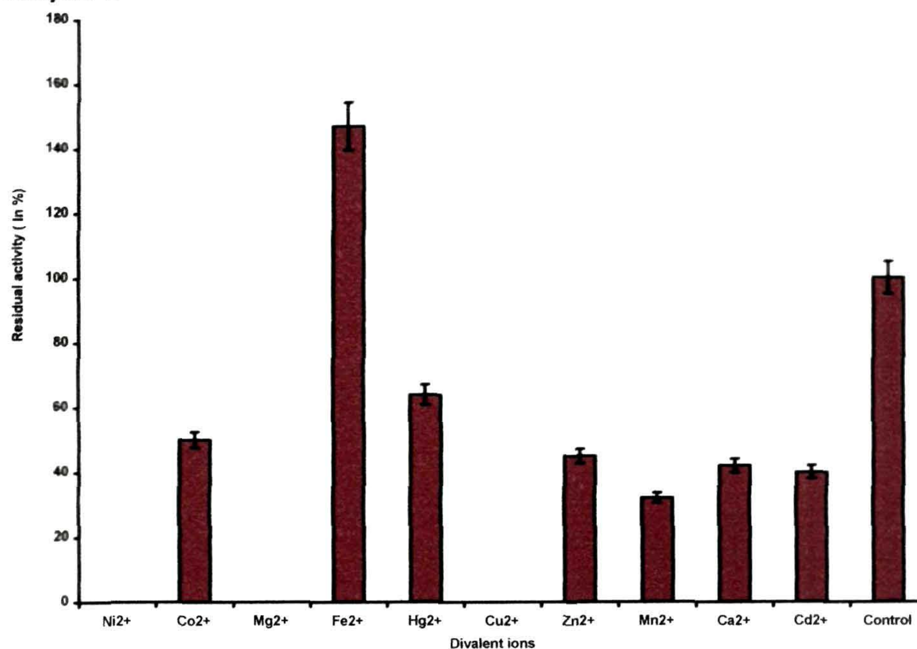


Fig.5.24 Effect of divalent metal ions on catalytic activity of Bsubap-I. Values are mean ± S. D. of three determinations.

5.5.5 Effect of surfactants, chelators, inhibitors, denaturing, oxidizing and bleaching agents on Bsubap-I

Chemical modification of serine and histidine residues of Bsubap-I resulted in a loss of about 97% and 88% of its original enzyme activity, respectively advocating this was a trypsin like serine protease (Ser-His-Asp triad). Iodoacetate, TPCK, SDS, EDTA, urea, H_2O_2, sodium perborate and Tween-20, and Triton X-100 failed to modulate the enzyme activity, where as TPCKand TLCK inhibited the protease activity of Bsubap-I to 4% and 17% respectively as compared to the control (Table 5.9).

Table 5.9 Effect of surfactants, chelators, inhibitors, denaturing, oxidizing and bleaching agents on enzymatic activity of Bsubap-I . Values are mean ± S. D. of three determinations.

Effectors	Relative Protease activity of Bsubap-I (%)
Control	**100**
Surfactants	
SDS (20 mM)	97.0 ± 2.1
Urea (6 M)	119.0 ± 3.1
Tween -20 (1% v/v)	95.1 ± 3.4
Triton-X-100 (1% v/v)	100
Chelators (5 mM)	
EDTA	92.0 ± 1.8
Reducing agent (2 mM)	
DTT	90.0 ± 3.2
Inhibitors (2 mM)	
PMSF	3.1 ± 1.2
pBPB	12.1 ± 0.8
Iodoacetate	100
TLCK	83 ± 3.3
TPCK	96 ± 1.9
Oxidizing agent	
H_2O_2 (% v/v)	
5	100.0± 2.
10	98.0 ± 1.4
Bleaching agent	
Sodium perborate (% v/v)	
0.5	110.0± 6.1
1.0	100.0 ± 5.1

5.5.6 Effect of organic solvents on Bsubap-I

The Bsubap-I demonstrated organic solvent stability in the following order: n-hexane ≥ methanol > xylene > 2-propanol > acetonitrile > ethanol > benzene (Fig.5.25). More than 90% of original activity of Bsubap-I was retained even after pre-incubation for 7 days at 25°C in the presence of 20% (v/v) of tested organic solvents, except benzene (Fig.5.25).

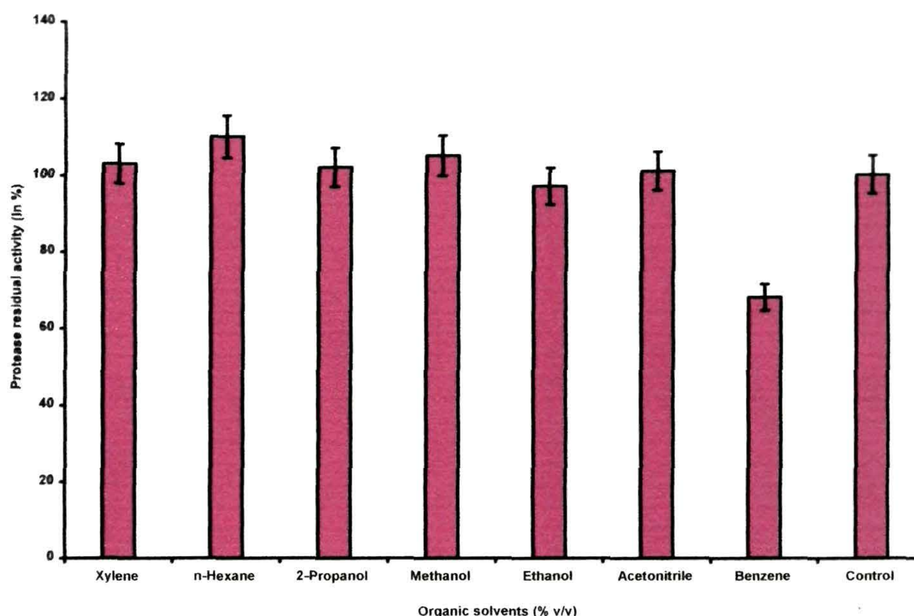


Fig.5.25 Organic solvent stability of Bsubap-I. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.

5.5.7 Pharmacological properties of Bsubap-I

Bsubap-I from *B.subtilis* DM-04 at a dose of 15 μg/ml induced 2.8 % hemolysis of the washed human erythrocytes, and did not display any detrimental effect on goat liver, heart, lungs and kidney tissues at the tested dose (Table 5.10). Further, as shown in Table 5.10, Bsubap-I did

not interfere with the normal clotting time of goat platelet- poor plasma (PPP).

Table 5.10 Pharmacological properties of Bsubap-I from *B.subtilis* DM-04. Values are mean± S.D. of three experiments.

Pharmacological properties	Values
Hemolysis (% Hb released / 15.0 µg of Bsubap-I 90 min post incubation at 37 °C)	
Control (without Bsubap-I)	1.4 ± 0.1
Treatment	2.8 ± 0.2
Ca-Clotting time (s)	
Control (without Bsubap-I)	128.0 ± 6.4
Treated (with 15.0 µg / ml of Bsubap-I)	131.0 ± 6.6
In vitro tissue damaging activity (% hemoglobin release by 15.0 µg / ml of Bsubap-I 5 h post incubation at 37 °C)	
a) ***Heart***	
Control (without Bsubap-I)	0.10 ± 0.01
Treatment	0.25 ± 0.01
b) ***Lung***	
Control (without Bsubap-I)	0.12 ± 0.01
Treatment	0.33 ± 0.01
c) ***Liver***	
Control (without Bsubap-I)	0.15 ± 0.06
Treatment	0.26 ± 0.01
d) ***Kidney***	
Control (without Bsubap-I)	0.11 ± 0.05
Treatment	0.28 ± 0.01

5.6 Some industrial applications of Bsubap-I

5.6.1 Detergent compatibility and stain removal potency of Bsubap-I

The stability and compatibility study of Bsubap-I (7.0 µg / ml) with most of the commercial laundry detergents at 37°C temperature was observed in the following order: Ghadi® > Rin advanced® = Safed® > Surf excel® > Tide® = Wheel® > Fena ultra® > Aerial® > Henko® (Fig. 5.26). The blood stain removal from cotton fabrics shown by a 1:1 (v/v) mixture of detergent (7.0 mg / ml): Bsubap-I (7.0 µg / ml) solution was significantly higher ($p < 0.05$) compared to the detergents solutions or purified protease solution alone in the tested temperature. For example, about 27% and 23% increase in removal of blood stain was observed post washing the stained cotton fabrics with Ghadi® and Rin advanced® detergents, respectively supplemented with Bsubap-I (Fig. 5.27).

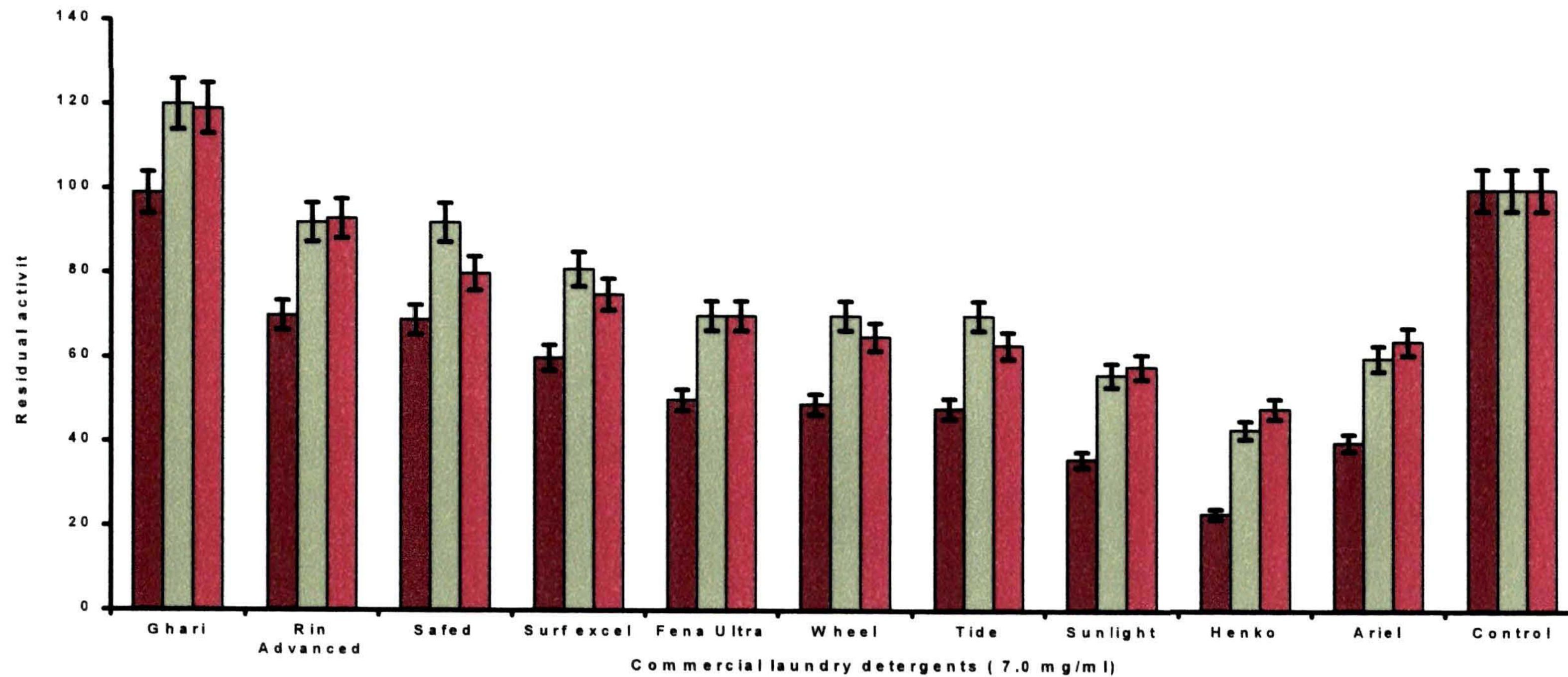


Fig.5.26 Detergent stability and compatibility of Bsubap-I (7.0 µg / ml) pre-incubated at 25 (■), 37(■), and 45°C (■). Enzyme activity in the absence of detergent was considered as 100% activity and other values were compared with that. The values represent mean ± S.D. of three determinations.

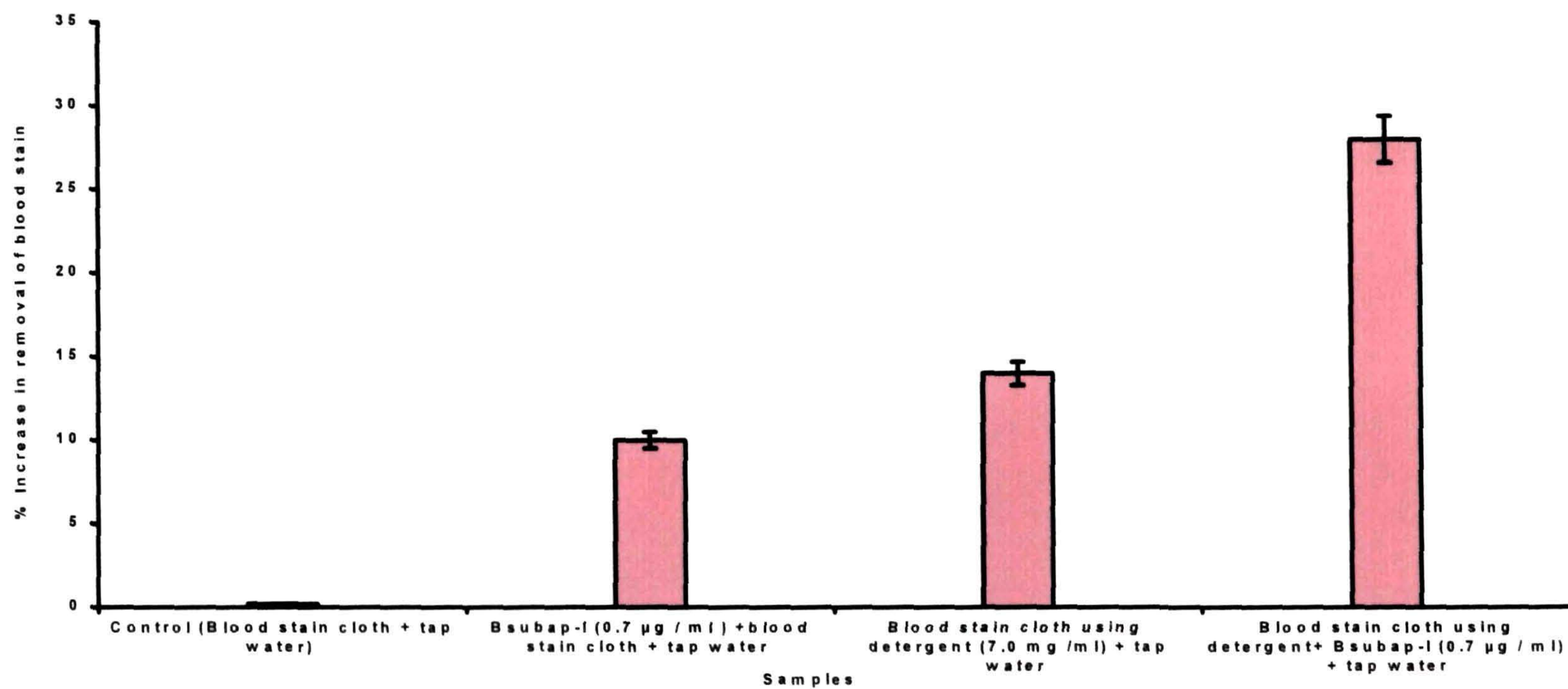


Fig.5.27 Wash performance test of Bsubap-I at 37°C. The values represent mean ± S.D. of three determinations.

5.6.2 Antibacterial activity

The Bsubap-I inhibited the growth of E. coli to 20% and 33% of its original growth (in absence of Bsubap-I) post 12 and 24 h of incubation, respectively at 37°C. The growth of *Bacillus licheniformis, Kocuria varians, Paenibacillus polymyxa, Klebsiella. pneumoniae* and *Stapylococcus. aureus* was inhibited by Bsubap-I to 22%, 25%, 27%, 3% and 0%, respectively, post 24 h of incubation at 37°C (Fig. 5.28).

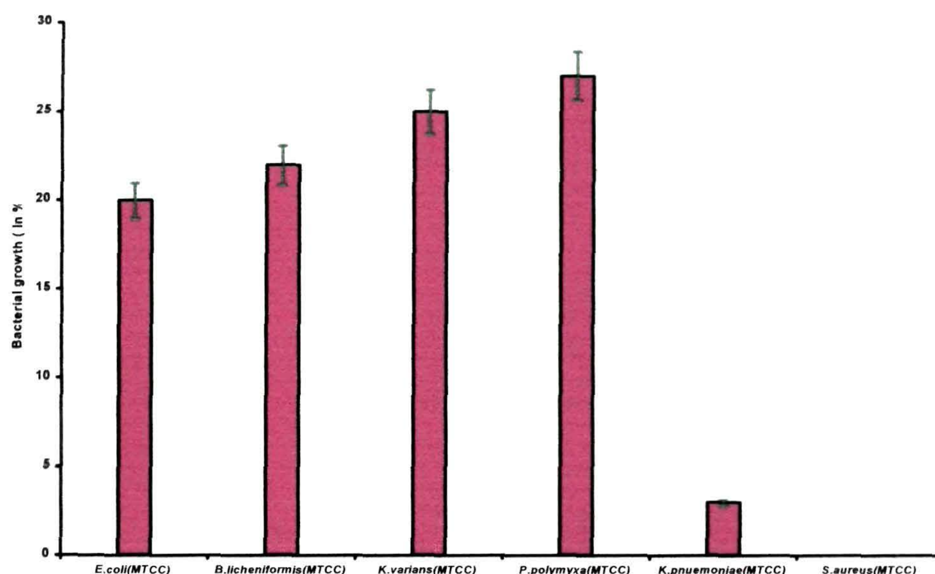
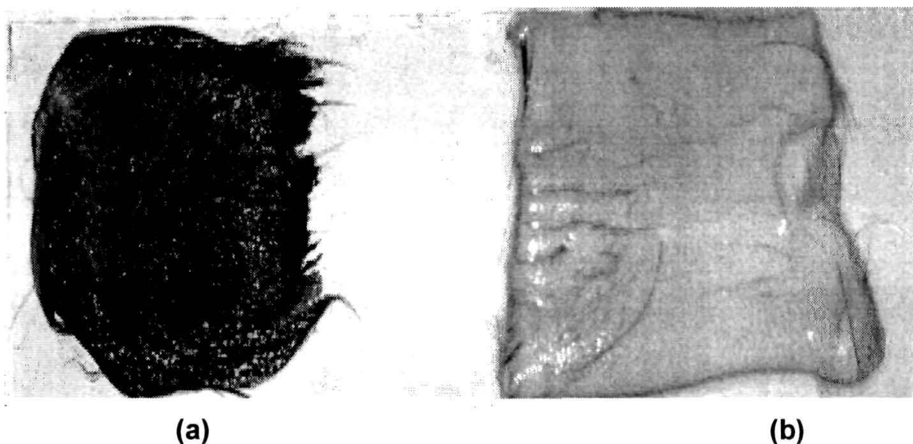


Fig.5.28 Antibacterial activity of purified Bsubap-I (0.25 mg /ml) from *B.subtilis* strain DM-04. Values represent mean ± S.D of three experiments.

5.6.3 Dehairing activity

The Bsubap-I from *B.subtilis* strain DM-04 demonstrated appreciable dehairing activity (Fig. 5.29). This effect was in accordance with the protease activity of Bsubap-I. When the raw skin was incubated with the purified protease at 37°C for 12 h, the hairs could be pulled out easily.



(a) **(b)**

Fig. 5.29 Dehairing activity of Bsubap-I (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with Bsubap-I (50 U / ml) for 12 h at 37°C.

5.7 Isolation and purification of a zwitterionic protease (Alzwiprase)

The CM-Cellulose and DEAE-Sephadex A-50 unbound fraction (DEAE-Sephadex A-50 flow-through) containing the neutral/zwitterionic proteases at pH 7.0 represented 57.0% of total extracellular proteases of *B. subtilis* strain DM-04. As shown in Fig 5.30, when this neutral fraction (at pH 7.0) was loaded into a sephacryl S-200 column, the proteins were eluted as a major peak (GF-I). The ethanol precipitated GF-I fraction was separated by C_{18} Nova pak RP-HPLC column into five major peaks (HP-I to HP-V).The

HP-I fraction with a retention time of 5.371min (Fig. 5.31) demonstrated maximum protease specific activity (5 X 10^3 U / mg) and was found to be homogenous by 12.5 % SDS-PAGE (Fig. 5.32). The SDS-PAGE of about 15 µg protein under both reduced and non-reduced conditions displayed a single band of 16.9 kDa (Fig.5.32) suggesting a monomer protease. This protease was named as Alzwiprase (Alkaline zwitterionic protease). A summary of purification of Alzwiprase is presented in Table 5.11.

Table 5.11 Summary of purification of Alzwiprase from *Bacillus subtilis* strain DM-04. Values are from a typical experiment.

Purification step	Total protein (mg)	Enzyme yield (%)	Total enzyme activity (U)	Specific activity (U / mg)	Purification fold
Cell-free supernatant	240.0	100	5.1 x 10^4	213	1
CM-Cellulose flow throw	153.0	66.7	3.4 x 10^4	222.0	1.04
DEAE Sephadex A-50 flow throw	73.0	57.0	2.9 x 10^4	397.0	1.9
Ethanol precipitate (GF-I)	18.0	45.0	2.3 x 10^4	1278.0	6.0
HP-I (Alzwiprase)	3.0	29.0	1.5 x 10^4	5,000.0	23.5

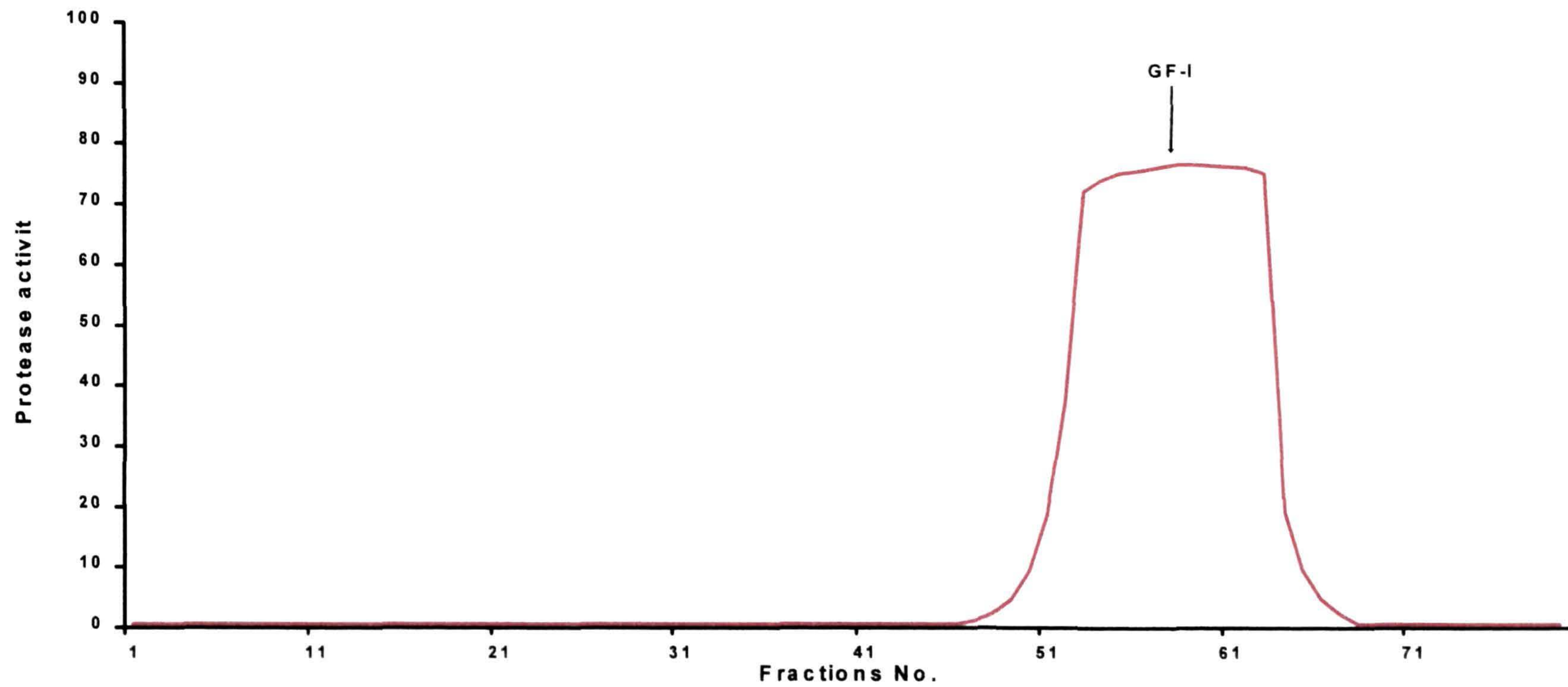


Fig. 5.30 Gel filtration profile of zwitterionic proteins from *B.subtilis* strain DM-04.

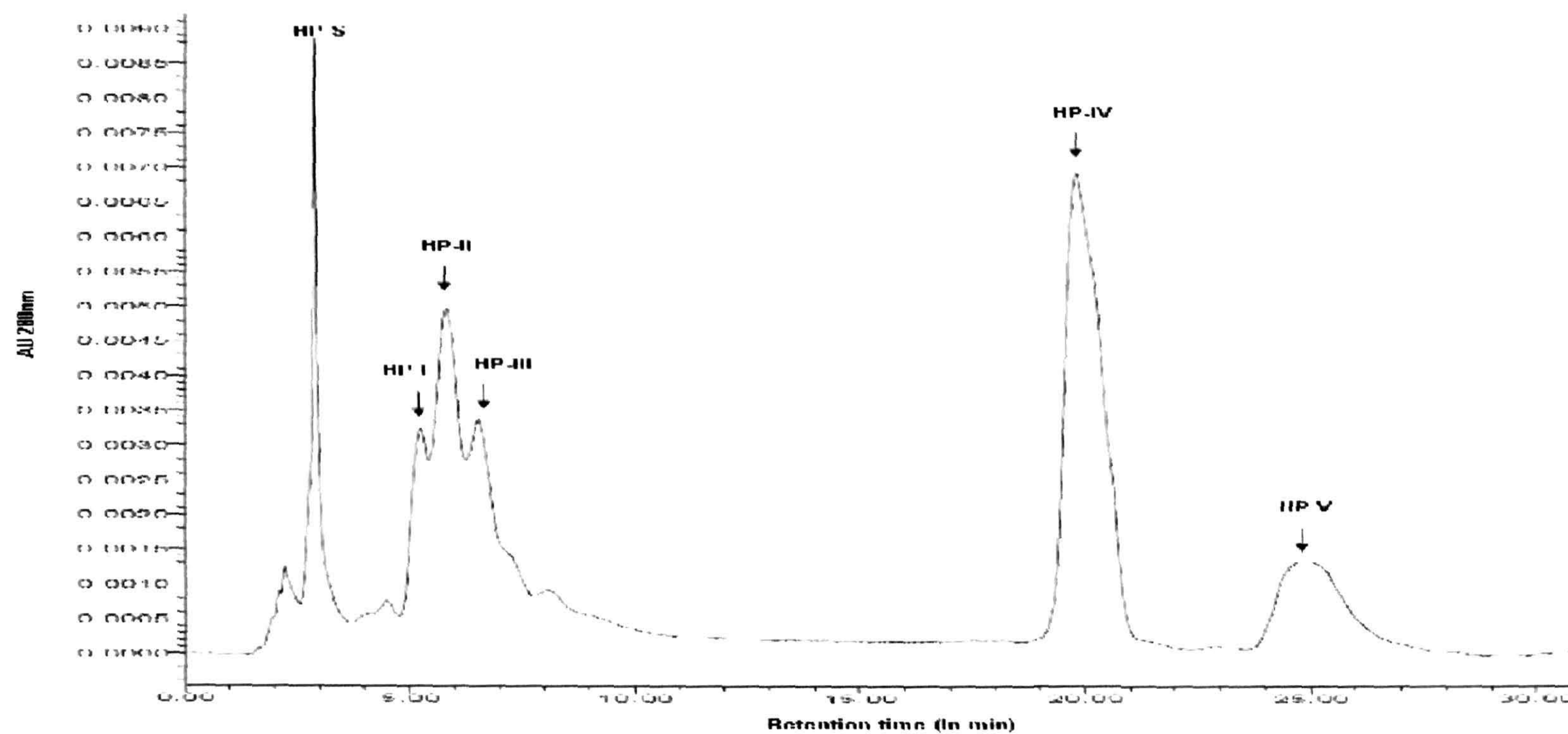


Fig.5.31 Reverse-phase HPLC of GF-I fraction. HP-S stand for solvent peak .

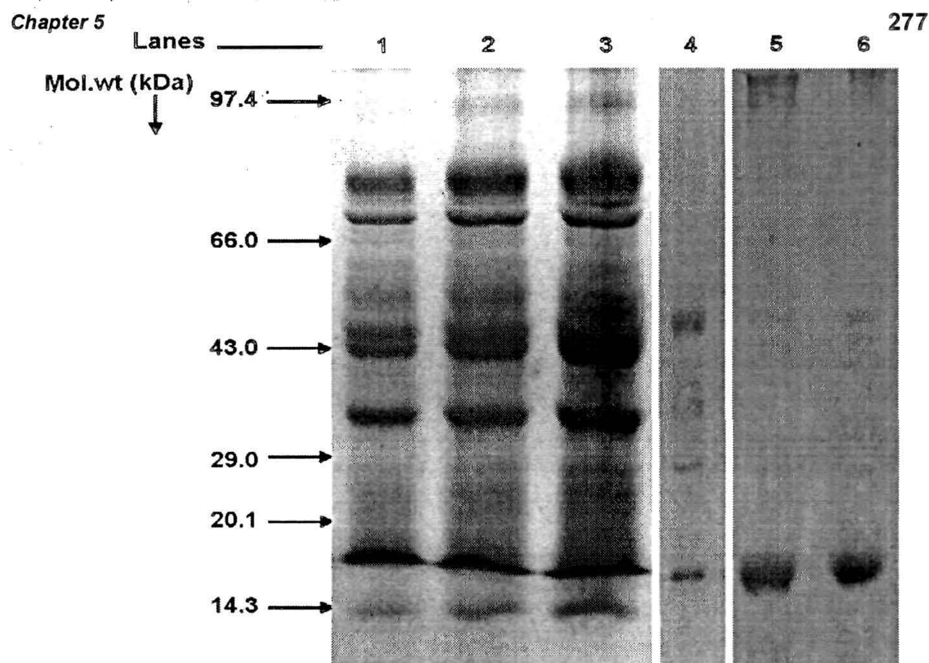


Fig.5.32 12.5% SDS-polyacrylamide gel electrophoresis of purified protease. Molecular weight markers were phosphorylase b (97,400 Da), BSA (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), soybean trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da); lane 1, crude protease (40 µg); lane 2, CM-Cellulose flow through (CMFT) (30 µg); lane 3, DEAE-Sephadex A-50 Flow through (DEFT) (30 µg); lane 4, ethanol precipitated fraction (8 µg); lane 5, HP-I fraction under reduced condition (15.0 µg); lane 6, HP-I fraction under non-reduced condition (15.0 µg).

5.8 Biochemical characterization of Alzwiprase

Some of the biochemical properties of Alzwiprase are shown in Table 5.12.

5.8.1 Effect of pH and incubation temperature

Alzwiprase showed activity at alkaline range of pH (8.0-12.0) and in a broad range of temperature (30-55°C); however, optimum activity was observed at 45°C (Fig. 5.33) and at pH 10.0 to 10.5 (Fig. 5.34).

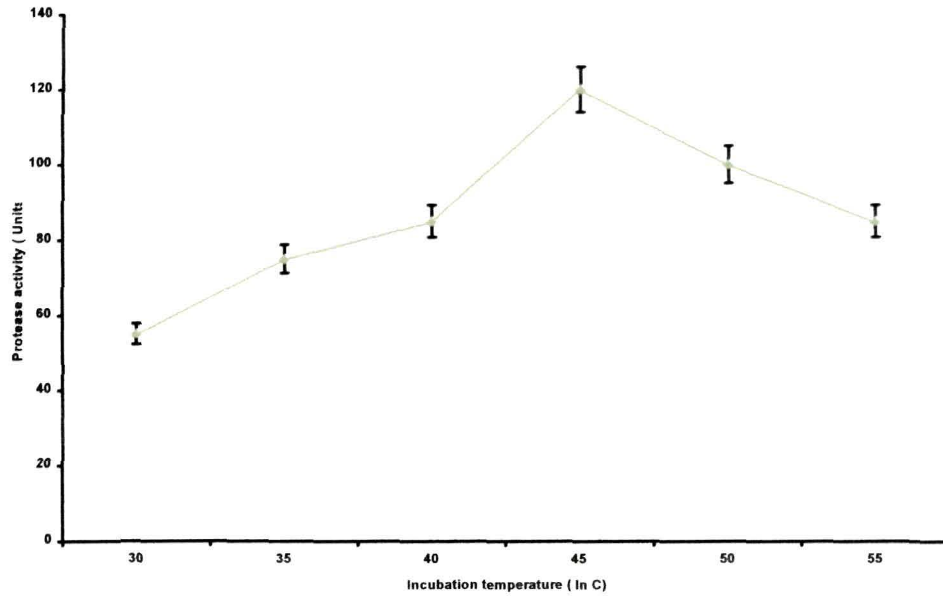


Fig. 5.33 Effect of incubation temperature on Alzwiprase activity. Each value represents mean ± S.D. of three experiments.

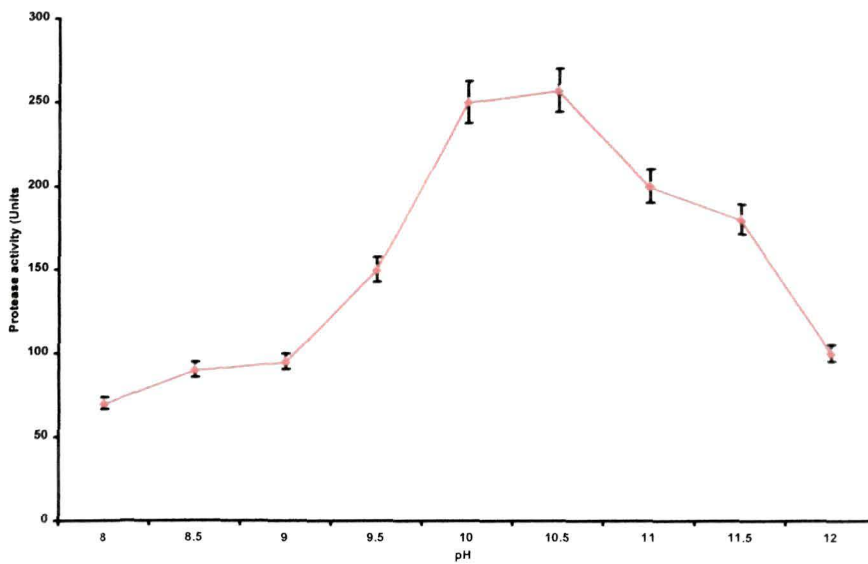


Fig.5.34 Effect of pH on Alzwiprase activity. Each value represents mean ± S.D. of three experiments.

5.8.2 Substrate specificity study

Casein was found to be the most preferred substrate for Alzwiprase followed by hemoglobin, gelatin, chicken-feather keratin (β-keratin), and bovine serum albumin; however human hair (α-keratin) and collagen were not hydrolyzed by this enzyme (Table 5.12).

Table 5.12 Biochemical properties and substrate specificity of Alzwiprase from *Bacillus subtilis* strain DM-04. Substrate specificity values are mean ± S.D of three experiments.

Biochemical property	Value
Optimum pH	10.0
Optimum temperature	45°C
K_m (μM) for casein	59.0
V_{max} (μmol / mg / min) for casein	336.0
Substrate specificity (unit mg^{-1}protein) (mean ± S.D., n=3)	
(a) Casein	5000.0 ± 100
(b) Hemoglobin	2560.0 ± 51.0
(c) Fibrin	1954.0 ± 39.1
(d) Gelatin	1300.0 ± 26.0
(e) Bovine serum fibrinogen	988.0 ± 20.0
(f) Chicken-feather keratin	433.0 ± 9.0
(g) Bovine serum albumin	149.0 ± 11.0
(h) Human-hair keratin	0.0
(i) Collagen	0.0

5.8.3 Thermostability study

Heating the Alzwiprase at 60 °C for 15 min did not affect its protease activity; however heating it for 90 min and 120 min at the same temperature resulted in reduction of 75% and 92% of protease activity, respectively (Fig.5.35).

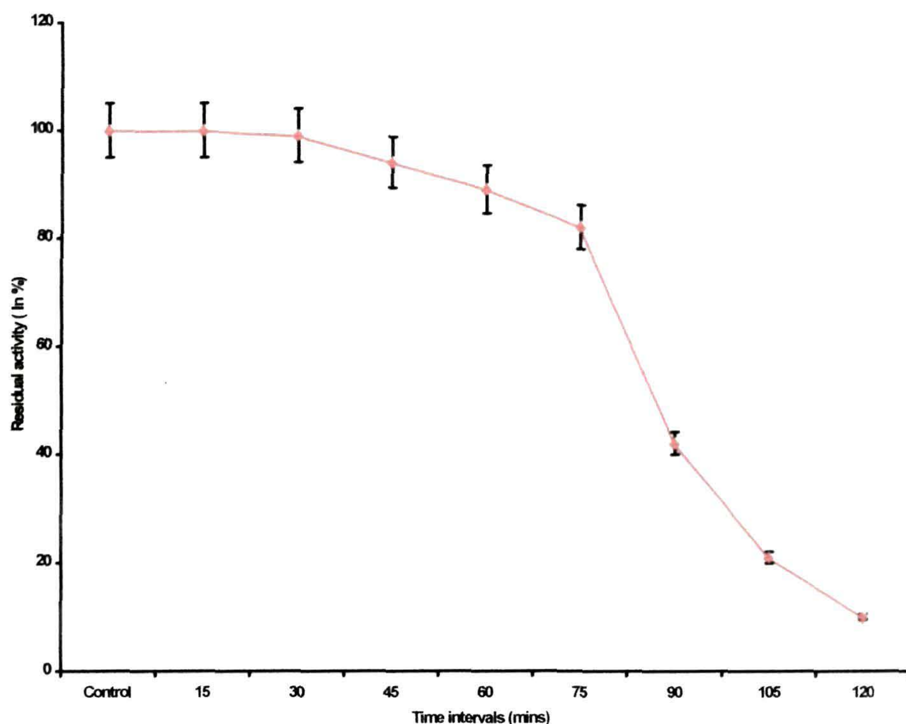


Fig.5.35 Thermo-stability study of Alzwiprase from *B.subtilis* strain DM-04 at 60°C. Each value represents mean ± S.D. of three experiments.

5.8.4 Effect of surfactants, chelators, denaturing agents, oxidizing and bleaching agents, and chemicals inhibitors on Alzwiprase

The effects of different chemicals, surfactant and urea on enzyme catalyzed reaction are shown in Table 5.13. The purified protease retained 92.0 ± 1.8% of its original activity in presence of 2 mM EDTA, indicating the metallic ions were not necessary for enzyme activity. Further, other chemicals such as that SDS (40 mM), urea (6M), tween-20 (1% v/v),

triton-X 100 (1% v/v), H_2O_2 (5 .0 and 10.0 % (v/v)) and sodium perborate [(0.5 and 1.0 % (v/v))] did not affect proteolytic activity of Alzwiprase. In fact, in presence of urea bleaching and oxidizing agents, the Alzwiprase activity was enhanced (Table 5.13). The serine protease inhibitor PMSF at a concentration of 2 mM significantly reduced the activity of Alzwiprase. Whereas, TPCK and TLCK, the inhibitors of chymotrypsin and trypsin- like serine protease, respectively at 2mM concentration could not modulate the activity of this enzyme. The Alzwiprase retained 90.0 ± 3.0 % of initial activity in presence of histidine inhibitor *p*BPB (2 mM). The partial inhibition by DTT (2 mM) might be due to reduction of intra-molecular disulfide bonds require to maintain the activity and stability of Alzwiprase (Table 5.13). Failure in inhibition of protease activity of Alzwiprase by p-BPB indicated absence of histidine residue in the active site of the enzyme.

Table 5.13 Effect of surfactants, inhibitors, EDTA, denaturing agents, oxidizing and bleaching agents on protease activity of Alzwiprase from *B. subtilis* strain DM-04. Assay was carried out at pH 10.0 and 45°C. Values are mean ± S.D. of three determinations.

Reagents/ effectors	Residual activity (%)
Control	100
Inhibitors	
PMSF (2 mM)	34.0 ± 0.7
(4 mM)	12.0 ± 0.2
4-Bromophenacyl bromide (2 mM)	90.0 ± 3.0
(4 mM)	89.0± 2.5
IAA (2 mM)	75 ± 4.0
(4 mM)	51.0 ± 3.0

Reagents/ effectors	Residual activity (%)
TLCK (2 mM)	100
(4 mM)	97 ± 1.0
TPCK (2 mM)	**100**
(4 mM)	98 ± 1.2
Chelators	
EDTA (2 mM)	92.0 ± 5.0
(4 mM)	89 ± 3.4
Surfactants	
SDS (40 mM)	97.6 ± 2.0
Trition-X-100 (1% v/v)	**100**
Tween 20 (1% v/v)	95.1 ± 5.0
Tween 80 (1% v/v)	95.1 ± 5.0
Denaturing agent	
Urea (6 M)	126.0 ± 6.0
Oxidizing agent	
H_2O_2 (% v/v)	
5.0	130.0 ± 2.6
10.0	121.0 ± 2.4
Bleaching agent	
Sodium perborate (% v/v)	
0.5	122.0 ± 2.4
1.0	**114.0 ± 2.3**
Disulfide bond reducing agent	
DTT(2 mM)	90.0 ± 5.0
(4 mM)	10.0 ± 1.0

5.8.5 Effect of metal ions on catalytic activity of Alzwiprase

Study on the influence of metal ions on activity of Alzwiprase showed that all the tested cations viz. Ni^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+} and Zn^{2+} inhibited the enzyme activity, to a various extents (Fig.5.36). Maximum (100%) inhibition of protease activity was observed in presence of Mg^{2+} , Ni^{2+} and Cu^{2+} (Fig.5.36). In presence of Fe^{2+}, the proteolytic activity of Alzwiprase was enhanced (Fig.5.36).

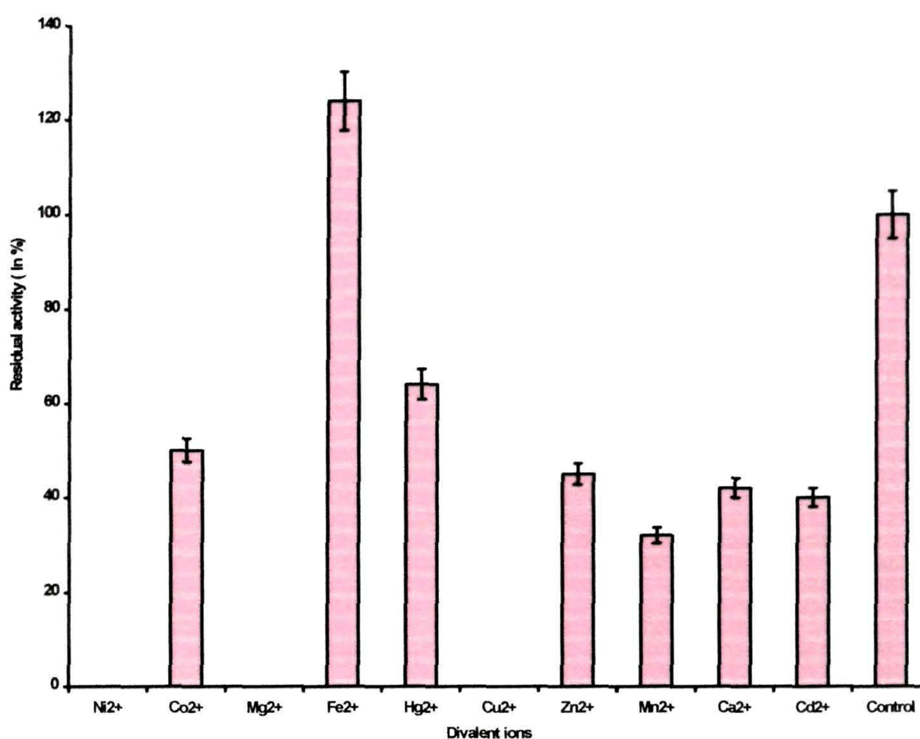


Fig.5.36 Effect of divalent ions on Alzwiprase from *B.subtilis* strain DM-04. Each value represents mean ± S.D. of three experiments.

5.8.6 Effect of polyols on thermostability of Alzwiprase

The residual protease activity remaining after heating of Alzwiprase at 60°C for 120 min in presence of polyols is displayed in Fig.5.37. It was observed that in presence of glycerol, the stability of enzyme was enhanced to 2 fold. The xylitol was found to be least influencing in stabilizing the enzyme against heat-denaturation.

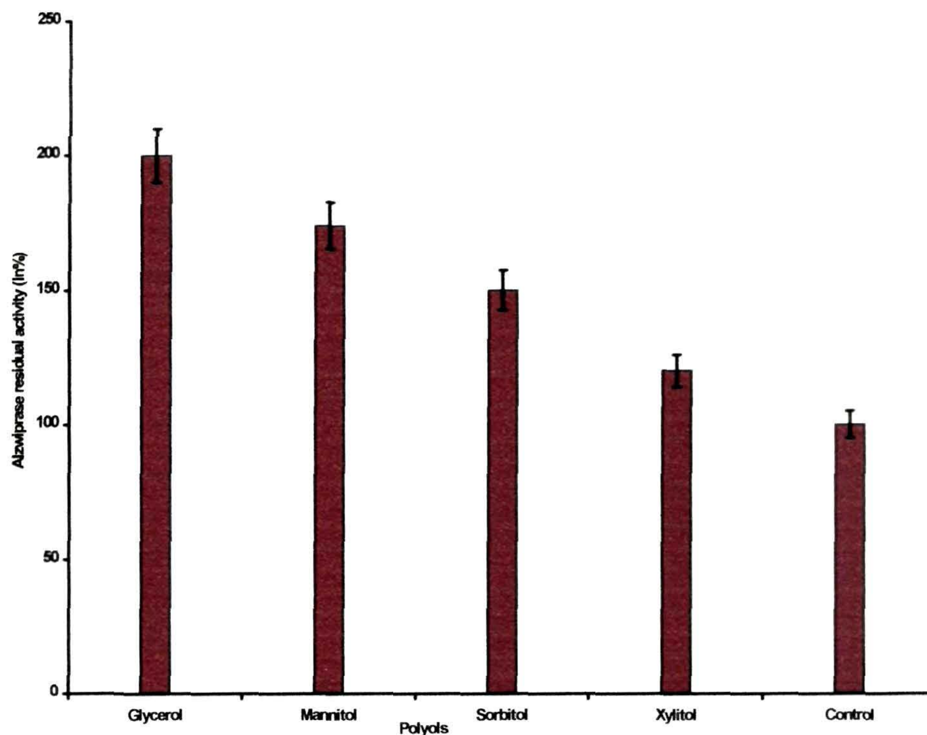


Fig. 5.37 Effect of polyols against heat-denaturation of catalytic activity of Alzwiprase. The activity of enzyme in absence of polyols was considered as 100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.

5.8.7 K_m and V_{max}

The kinetic constant of the enzyme for hydrolysis of casein was calculated which shows a K_m value of 5.9 mg /ml and a V_{max} value of 333.0 µmol / min/ mg (Fig 5.38).

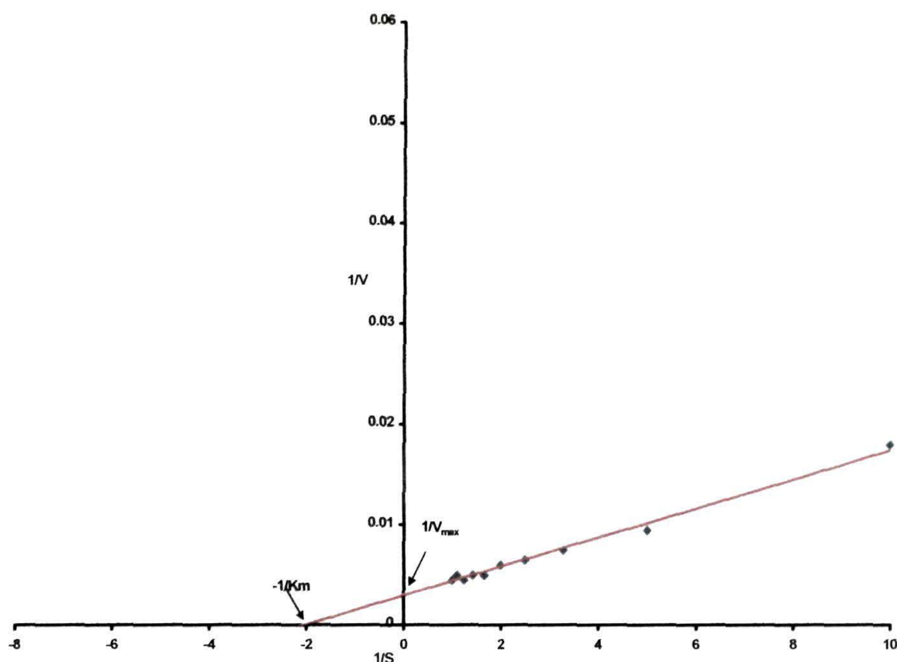


Fig. 5.38 Lineweaver-Burk plot to determine the K_m and V_{max} values of Alzwiprase using casein as a substrate. Each value represents mean of three experiments.

5.8.8 Effect of organic solvents on catalytic activity of Alzwiprase

The organic-solvent (at a final concentration of 20% v/v) stability of Alzwiprase is shown in Fig. 5.39. It was observed that enzyme retained its stability in the following order: n-hexane (Log P 3.5) > methanol (Log P -0.764) > xylene (Log P 3.1) > ethanol (Log P -0.235) ~ acetonitrile (Log P -0.394) > benzene (Log P 2.0). Ethanol and 2-propanol did not influence

the enzyme activity but benzene had a negative effect on enzyme activity of Alzwiprase.

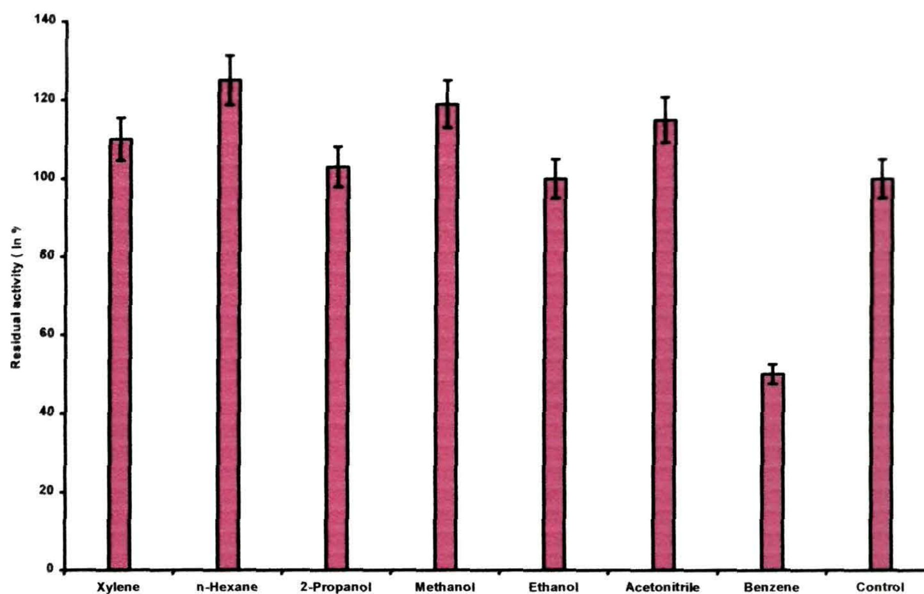


Fig. 5.39 Organic solvent stability of Alzwiprase. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.

5.8.9 Pharmacological properties of Alzwiprase

It was observed that Alzwiprase at a dose of 15 µg/ml caused 3% hemolysis of washed human erythrocytes and further, it did not damage the goat liver, heart, lungs and kidney tissues under the tested conditions (Table 5.14). Further, as shown in Table 5.14, Alzwiprase did not interfere with the normal clotting time of goat platelet- poor plasma (PPP).

Table 5.14 Pharmacological properties of Alzwiprase from *B.subtilis* strain DM-04. Values are mean ± S. D. of three determinations.

Pharmacological properties	Values
Hemolysis (% Hb released / 15.0 µg of Alzwiprase 90 min post incubation at 37 °C)	
Control (without Alzwiprase)	1.4 ± 0.1
Treatment	3.0 ± 0.5
Ca-Clotting time (s)	
Control (without Alzwiprase)	129.0 ± 6.5
Treated (with 15.0 µg / ml of Alzwiprase)	132.0 ± 7.0
In vitro tissue damaging activity (% hemoglobin release by 15.0 µg / ml of Alzwiprase 5 h post incubation at 37 °C)	
a) ***Heart***	
Control (without Alzwiprase)	0.09 ± 0.01
Treatment	0.27 ± 0.01
b) ***Lung***	
Control (without Alzwiprase)	0.13 ± 0.01
Treatment	0.36 ± 0.02
c) ***Liver***	
Control (without Alzwiprase)	0.16 ± 0.08
Treatment	0.28 ± 0.01
d) ***Kidney***	
Control (without Alzwiprase)	0.12 ± 0.05
Treatment	0.27 ± 0.01

5.9 Industrial applications of Alzwiprase

5.9.1 Detergent compatibility study

The Alzwiprase at concentration of 0.1 mg / ml demonstrated significant stability and compatibility with all the tested commercial laundry detergents at 37°C (Fig. 5.40). This excellent laundry detergent stability of Alzwiprase prompted us to evaluate its stain removal potency for application in commercial laundry detergent formulations. It was observed that Alzwiprase at a concentration of 0.1mg /ml could remove 28 ±2.1% (mean ± S.D., n = 3) of blood stain from cotton fabrics (Fig 5.41).

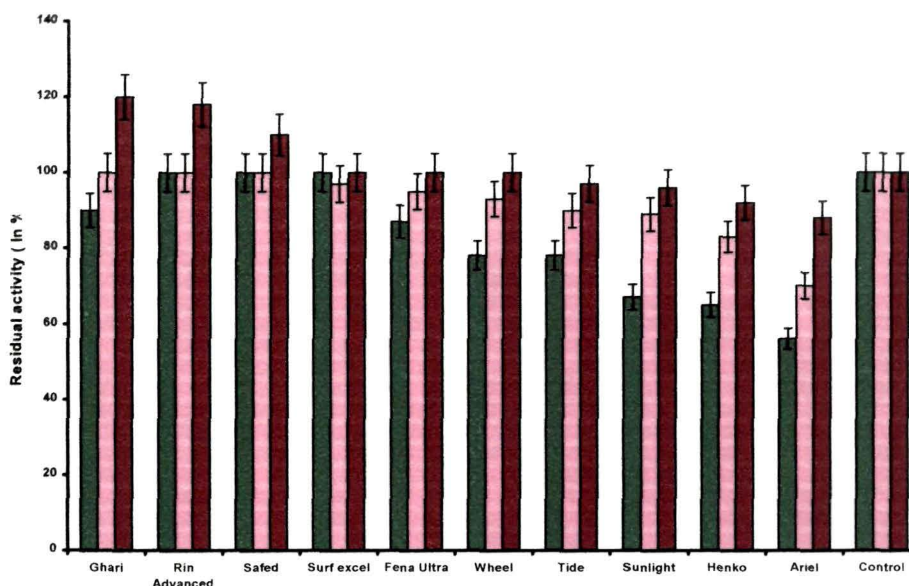


Fig.5.40 Detergent stability and compatibility of Alzwiprase at pre-incubation temperatures of 25(■), 37 (■) and 45 (■)°C for 60 min. Enzyme activity in the absence of detergent was considered as 100% activity and other values were compared with that. The values represent mean ± S.D. of three determinations.

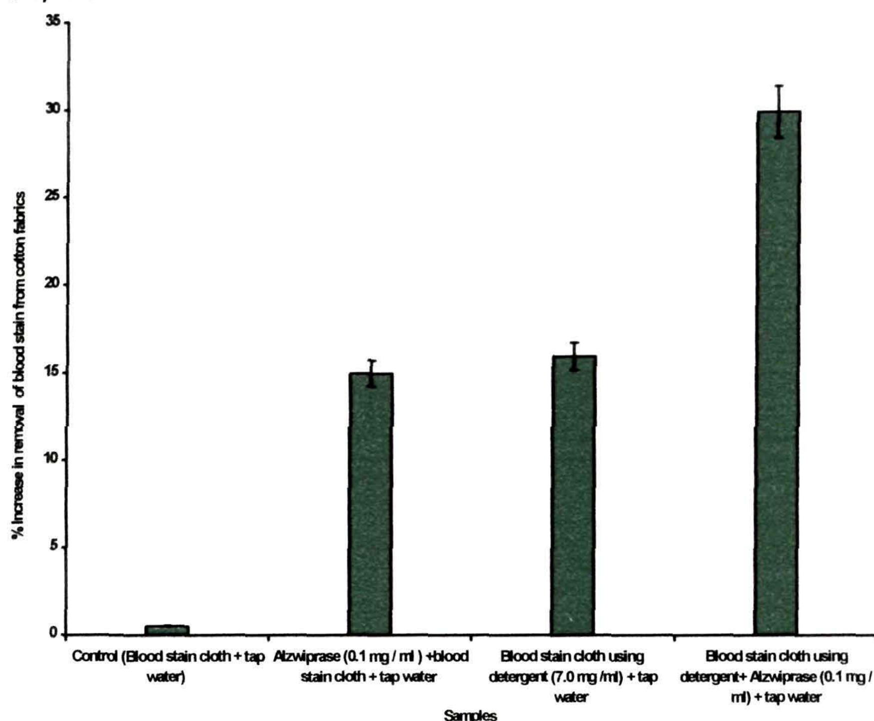


Fig.5.41 Wash performance study of Alzwiprase at 37°C.The values represents mean ± S.D. of three determinations.

5.9.2 Antibacterial activity

Staphylococcus aureus and *Klebseilla pneumoniae* displayed a fast antibacterial response when treated with a dose of 0.30 mg / ml concentrations of Alzwiprase for post 12h of incubation at 37°C (Fig. 5.42). In contrast, Alzwiprase exerted minimum inhibition of growth for *Bacillus licheniformis*, *Kocuria varian*, and *Peanibacillus polymyxa* strains (Fig. 5.42).

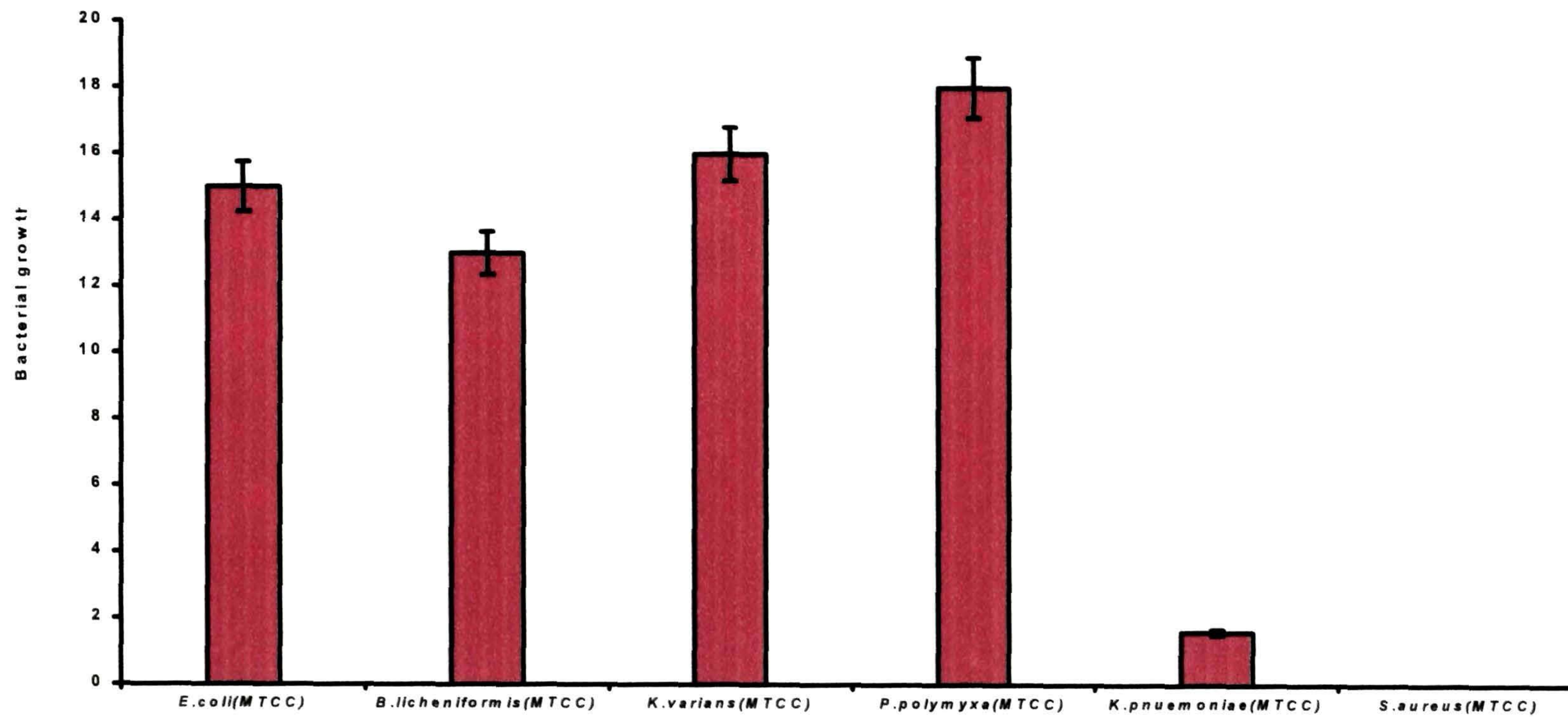


Fig.5.42 Antibacterial study of Alzwiprase (0.30 mg /ml) from *B.subtilis* strain DM-04 post 12h of incubation at 37°C. Values represent mean ± S.D of three experiments.

5.9.3 Dehairing activity

The Alzwiprase from *B.subtilis* strain DM-04 demonstrated appreciable dehairing activity (Fig. 5.43). The treated skins and controls showed no visible differences after incubation. No colour change was observed, although the presence of depilated areas was noted in the skins treated with the Alzwiprase. When the hairs were pulled with a forcep, they were very easily released in enzyme-treated pelts. After incubation intact hairs could be taken out of the skins easily by simple scraping. In controls, hair loosening was not observed, even by the mechanical action of a forcep.

(a) **(b)**

Fig. 5.43 Dehairing activity of Alzwiprase (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with Alzwiprase (50 U / ml) for 12 h at 37°C.

CHAPTER VI

RESULTS

Chapter 6

Process optimization, isolation, purification, biochemical characterization and industrial applications of alkaline protease isolated from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II

6.1 Alkaline protease production under solid-state fermentation

6.1.1 Screening of different agro-industrial and waste materials for alkaline protease production

In SSF, the selection of a suitable solid substrate for fermentation process is a critical factor and thus involves the screening of a number of agro-industrial and kitchen waste materials for microbial growth and product formation. All the substrates used in this except tea leaves study supported the growth and enzyme production by *P.tezpurensis* sp.nov. strain AS-S24-II in SSF condition; the maximum protease production was obtained with PP (356.0 U/gds) followed by IC (321.0 U/gds) as solid substrate post 24 h of incubation at 45°C. The minimum protease production (2.0 U/gds) was observed when waste TL was used as substrate/ support material (Fig. 6.1). The protein content of the cell free extract as well as bacterial growth followed the similar trend like protease production on dïfferent solid substrates (Fig.6.2).

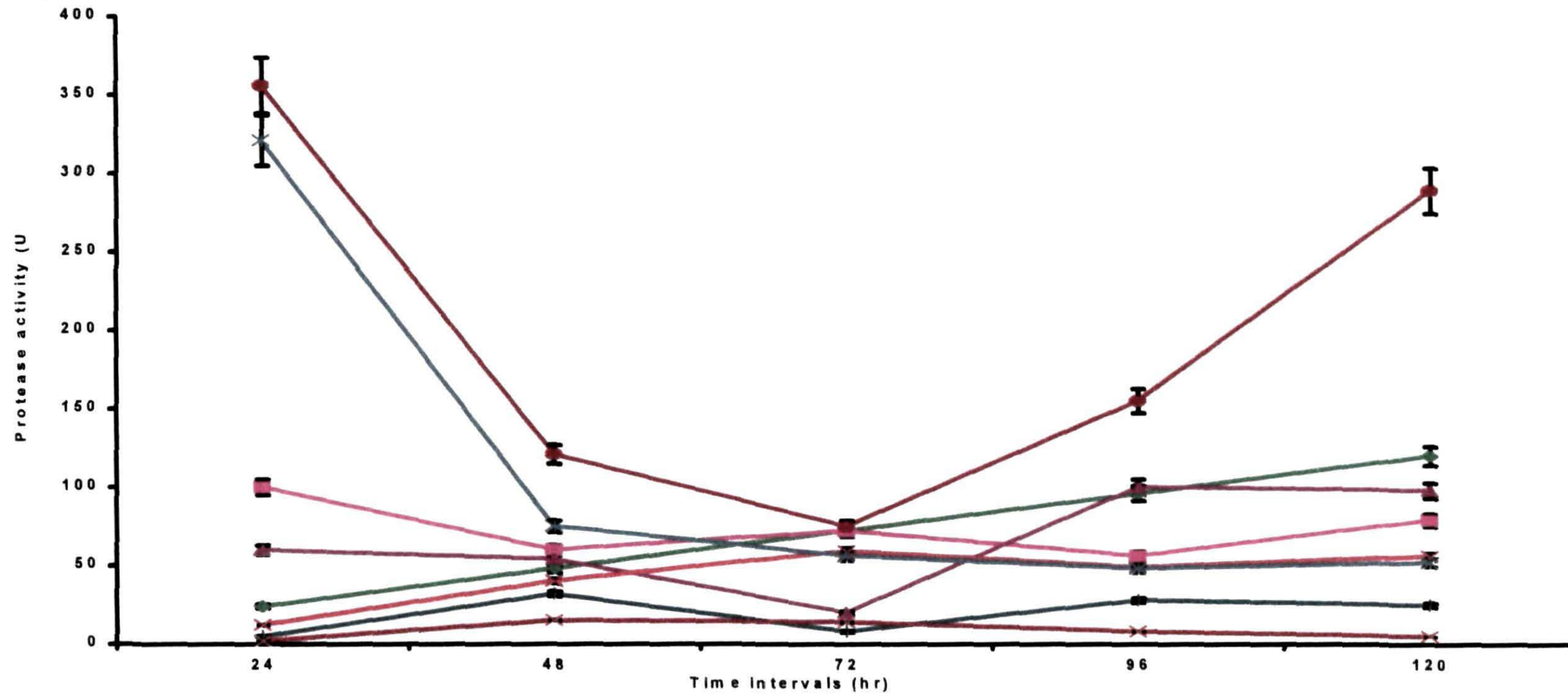


Fig.6.1 Screening of different waste residues such as MOC (◆), WB (■), RB (▲), IC(X), PP(●), BL(●), and TL(+) for the production of alkaline protease by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II at different time intervals. Values are mean ± S.D. of three experiments.

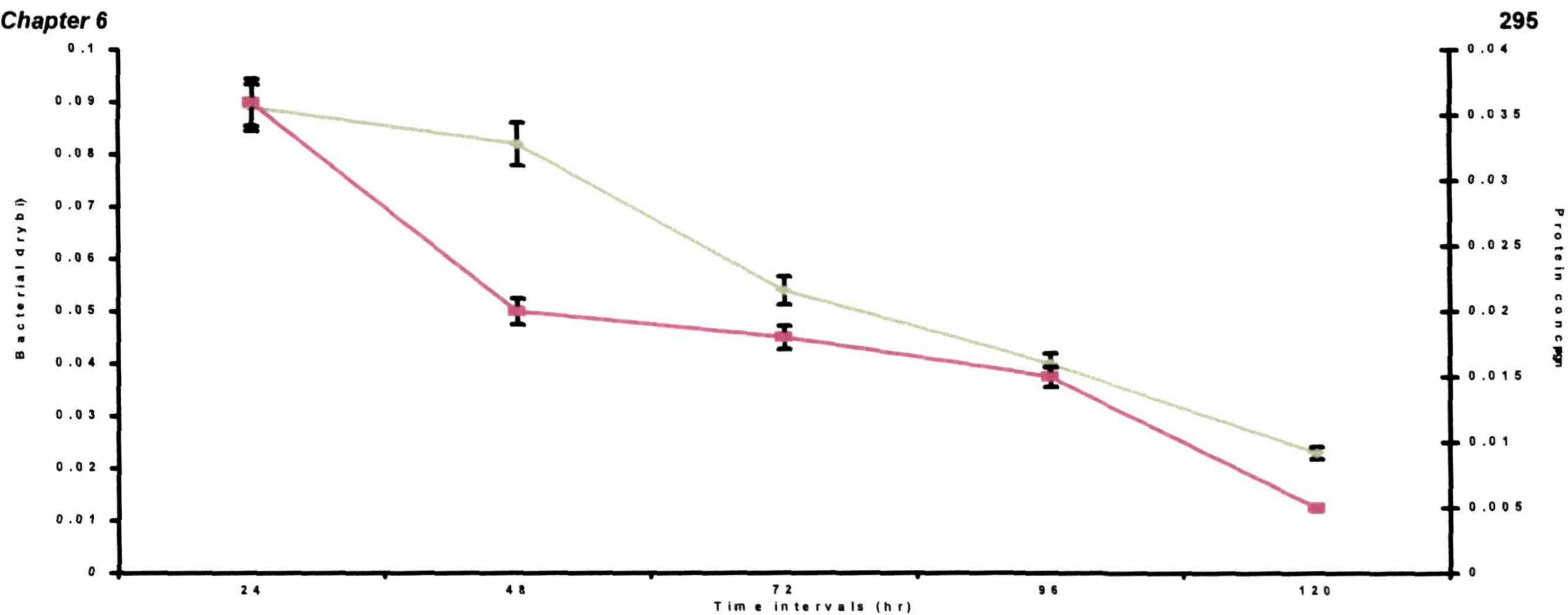


Fig.6.2 Kinetics of alkaline protease production by *P.tezpurensis* sp.nov. strain AS-S24-II. Legends show bacterial dry biomass (◆) and protein concentration of cell free extract (■).Values are mean ± S.D. of three experiments.

6.1.2. Effects of initial moisture content of the substrate and moistening agent on protease production

Optimum level of protease production (298 U/gds) was attained when the initial moisture level of the substrate was adjusted to 100 %, (Fig 6 3) Amongst the tested moistening agents, distilled H_2O adjusted to pH 8 0 supported maximum protease yield (Fig 6 4)

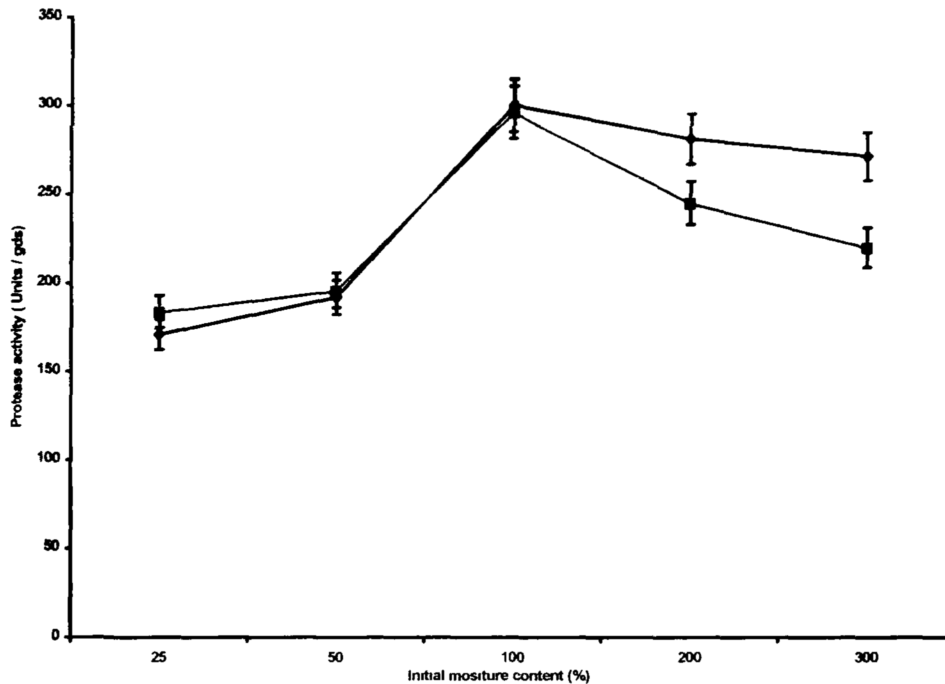


Fig.6.3 Influence of initial moisture content of the substrates [PP (■) or IC (♦)] on protease production Values are mean ± S D of triplicate determinations post 24h incubation at 45°C Moistening agent was distilled water, adjusted to pH 8 0 with 0 01N NaOH

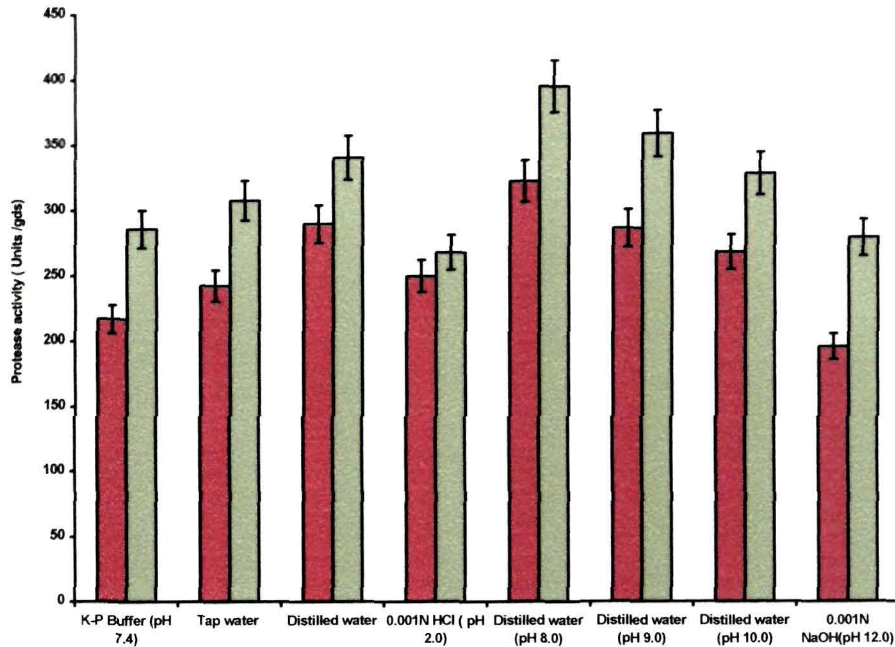


Fig.6.4 Influence of moistening agents on alkaline protease production by *P.tezpurensis* sp.nov. strain AS-S24-II in SSF using either IC (■) or PP (■) as a solid substrate. Values represent mean ± S.D of three experiments post 24h at 45°C.

6.1.3 Effect of inoculum's size on protease production

Inoculum level of bacterium was also found as an important parameter influencing the production of protease in SSF. As shown in Fig.6.5, with an increase in inoculum size from 0.5 ml to 2.0 ml (5.0 g substrate), protease production was also enhanced significantly (Fig.6.5), while increasing the inoculum's size from 2.0 ml to 5.0 ml did not have a significant impact in enhancing the protease yield ($p>0.05$). However, an increase in inoculum's size beyond 5.0 ml resulted in a steady decline in protease production by *P.tezpurensis* sp.nov. strain AS-S24-II (Fig. 6.5).

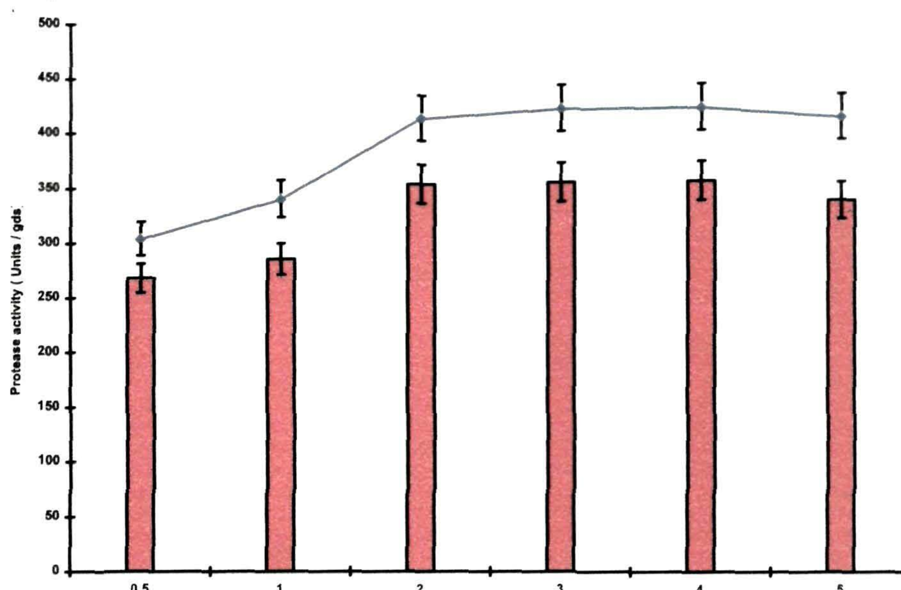


Fig.6.5 Influence of inoculum size on protease production by *P.tezpurensis* sp.nov. strain AS-S24-II strain using PP (◆) or IC (■) under SSF. Values are mean ± S.D. of three determinations.

6.1.4 Effect of supplementation of co-carbon and co-nitrogen sources on protease production

Several sources of carbon such as casein, glucose, fructose, xylose, galactose, maltose, sucrose, galactose, glucose and lactose were supplemented to solid medium at 1% (w/w) level. It was observed that potato peel alone supported maximum protease production (1500.0 U/gds) followed by casein (1103.0 U / gds), whereas least protease production was recorded in presence of galactose 45.0 U / gds (Fig.6.6) Among the tested nitrogen compounds, 0.1 % (w/w) ammonium sulphate followed by potassium nitrate served as the best co-nitrogen sources for alkaline protease production by *P.tezpurensis* sp.nov. strain AS-S24-II strain on IC as main substrate (Fig.6.7).

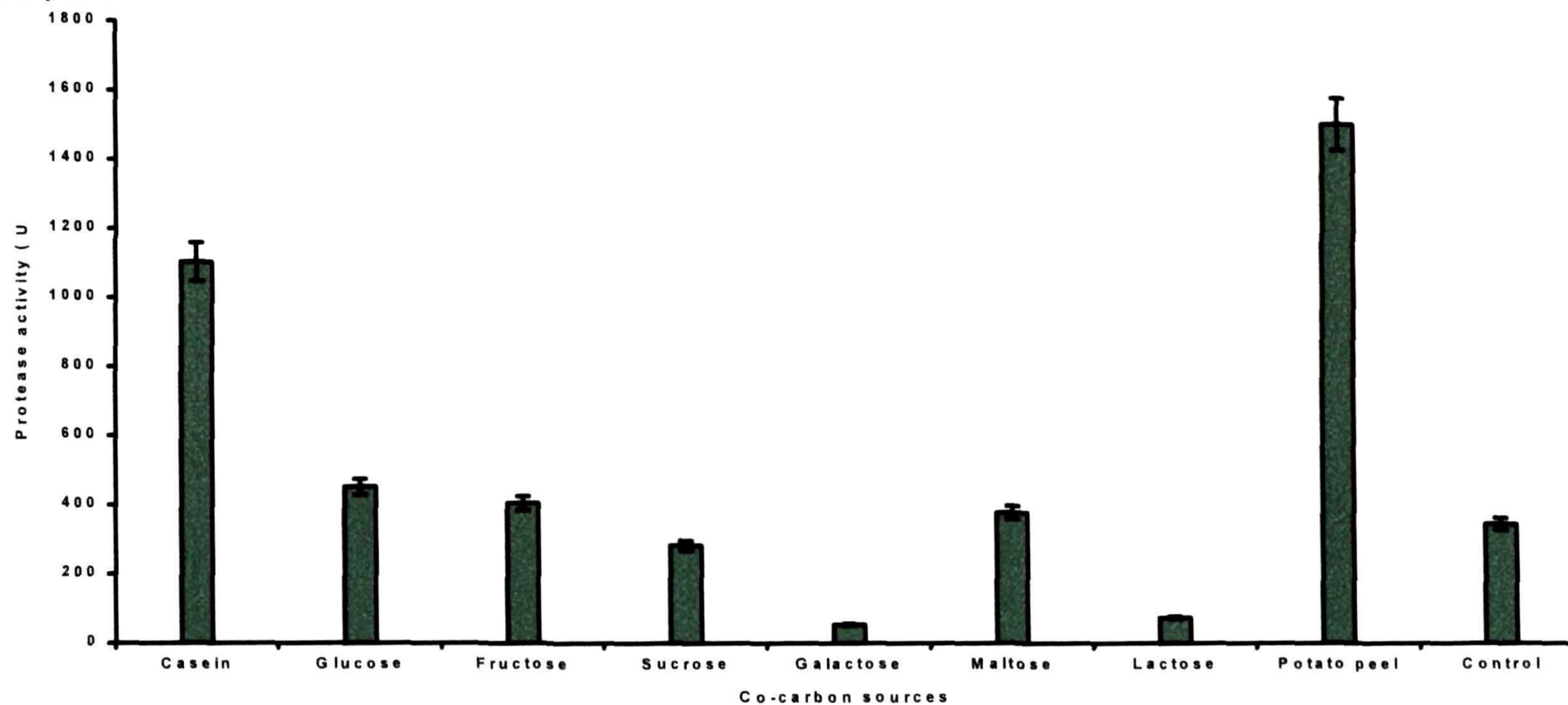


Fig.6.6 Effect of supplementation of co-carbon sources (10 % w/w) to IC (90 % w/w) on alkaline protease production by *P.tezpurensis* sp.nov. strain AS-S24-II. Values are means ± S.D. of three determinations.

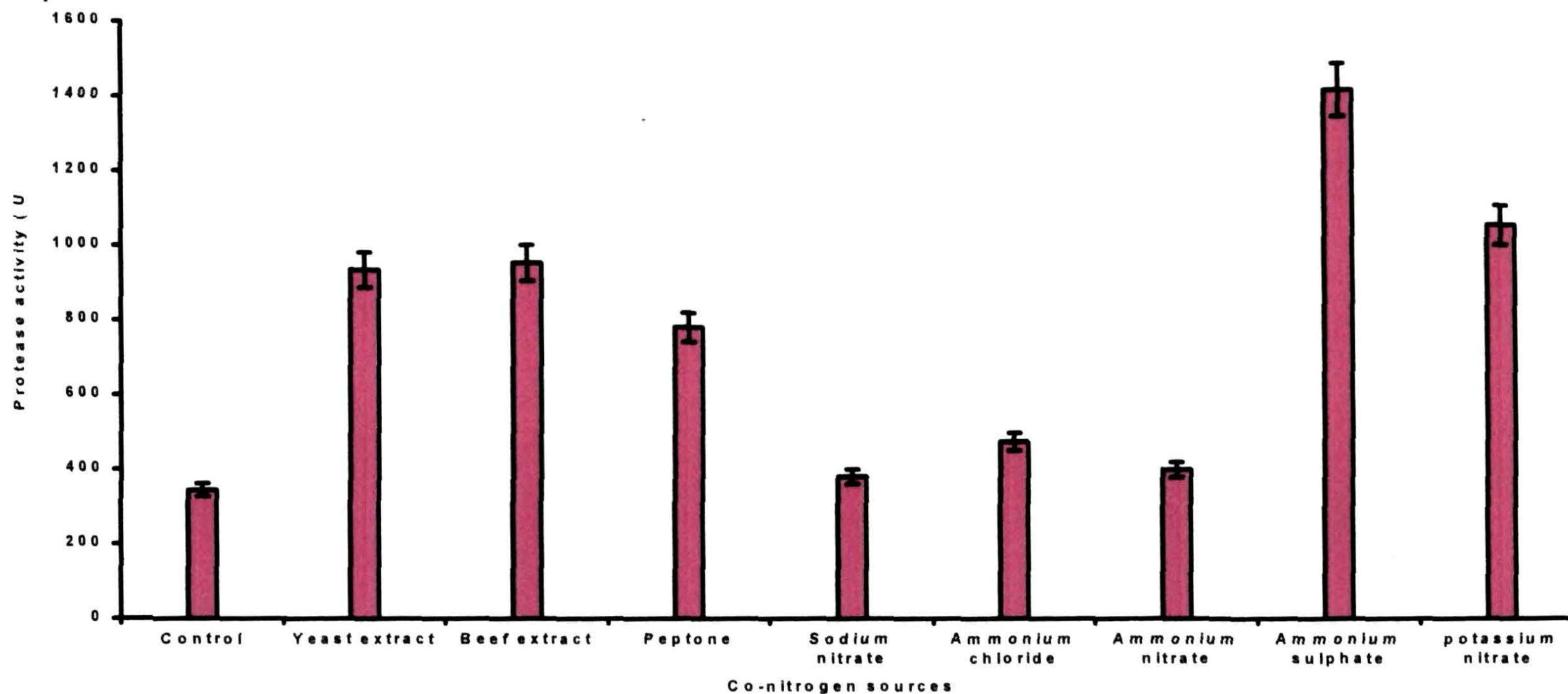


Fig.6.7 Effect of supplementation of co-nitrogen sources (1.0% w/w) to IC (99% w/w) (substrate) on protease production by *P.tezpurensis* sp.nov. strain AS-S24-II. Values are means ± S.D. of three determinations.

6.1.5 Protease extraction from fermented matter

Among the different extractions medium used for the recovery of protease from the fermented matter (IC), distilled water containing 0.1%(v/v) triton X-100, pH 8.0 served as best extraction medium (Fig.6.8).

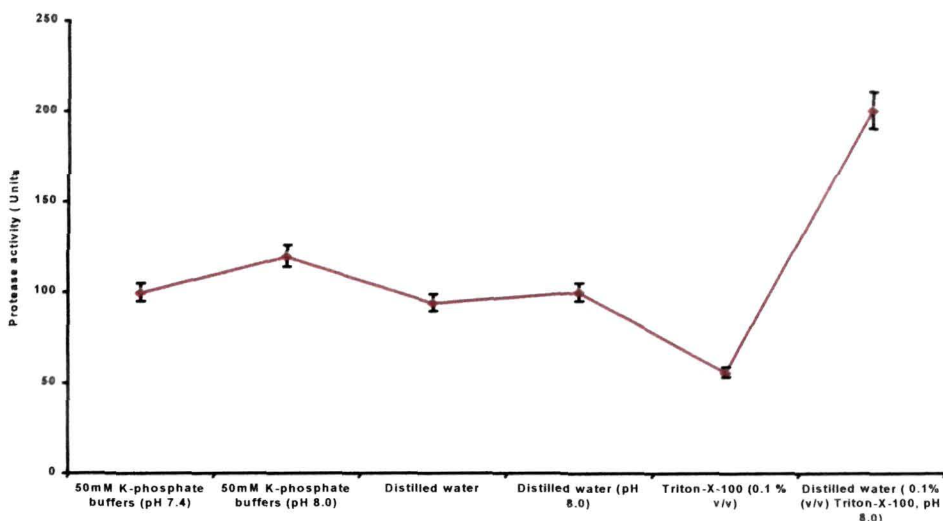


Fig.6.8 Screening of extraction mediums for maximum protease extraction from fermented substrate (IC). Values are means ± S.D. of three determinations.

6.1.6 Batch fermentation

The influence of various combinations of IC and PP along with supplementation of co-carbon and co-nitrogen sources on protease production showed that when IC and PP powders were mixed in a ratio of 1:1 without any co- carbon and nitrogen sources, a significant increase in protease yield ($p < 0.05$) could be achieved (Table 6.1).

Table 6.1 Influence of different combinations of co-substrates on alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II under solid-state fermentation. Values are mean± S.D of three determinations.

Substrate(s)	Protease activity (U/gds)
l.cylindrica (100%)	321.0 ± 16.1
Potato peel (100%)	356.0 ± 17.8
l.cylindrica (89%w/w)+ casein (10 % w/w) + ammonium sulphate (1% w/w)	1500.0 ± 75.0
l.cylindrica (90%w/w) + Potato peel (10%)	1699.0 ±84.95
l.cylindrica (80%w/w) + Potato peel (20 %w/w)	2016.0 ±100.8
l.cylindrica (50%w/w) + Potato peel (50%w/w)	2362.0 ± 118.1
Potato peel (89% w/w) + casein (10 % w/w) + ammonium sulphate (1% w/w)	1126.0 ± 56.3

6.2 Alkaline protease production under submerged fermentation

6.2.1 Effect of different carbon source on protease production

Different carbon sources were screened for alkaline protease production under submerged fermentation. Maximum bacterial growth as well as alkaline protease production was observed in presence of casein (50 U), while the least growth and alkaline protease production was observed in presence of lactose (8 U). The two carbonic compounds viz cellulose and carboxylmethyl cellulose could not support bacterial growth as well as protease production (Fig.6.9).

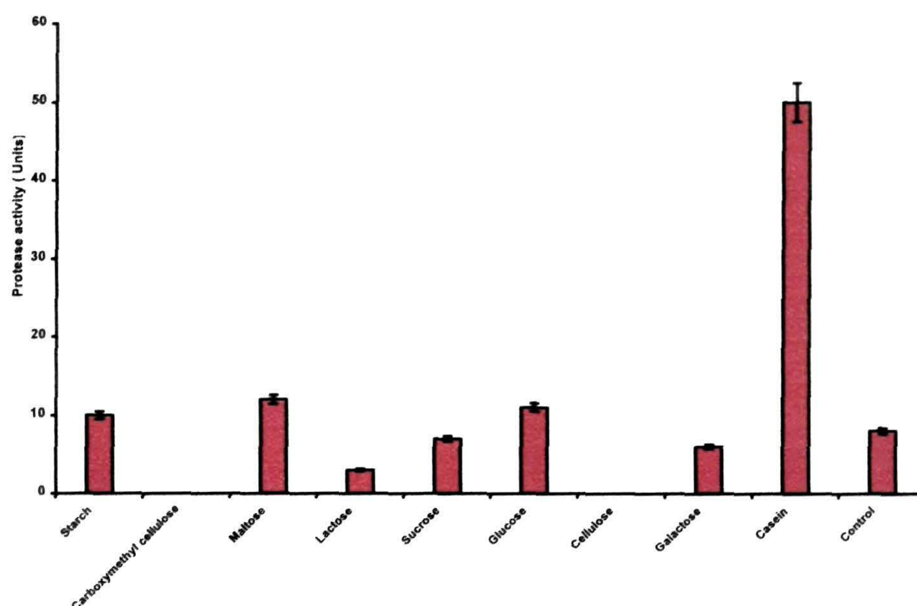


Fig.6.9 Effect of different carbon sources on alkaline protease production from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II under SmF. Values are means ± S.D. of three determinations.

6.2.2 Effect of nitrogen source on protease production

Various nitrogen sources were tested for alkaline protease production and maximum alkaline protease production was observed in presence of 0.1%

(w/v) ammonium sulphate followed by potassium nitrate. The least bacterial growth and protease production was observed in presence of peptone (Fig.6.10), demonstrating the bacteria have preference for inorganic nitrogen source compared to organic nitrogen compounds for protease production.

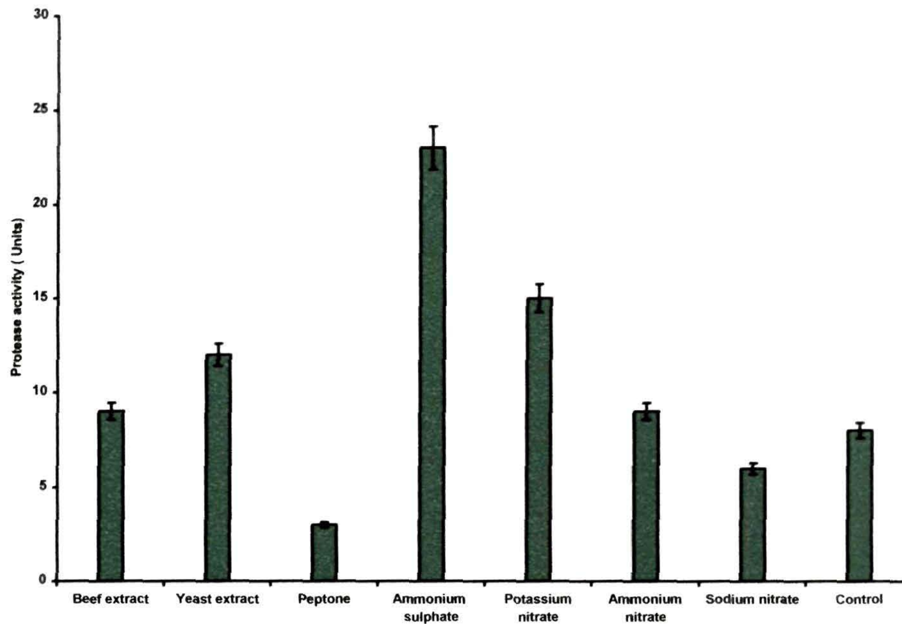


Fig.6.10 Effect of various nitrogen sources on alkaline protease production from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. Values are means ± S.D. of three determinations.

6.2.3 Effect of pH on protease production

The effect of pH on alkaline protease production from *P.tezpurensis* sp.nov. strain AS-S24-II was determined by adjusting the medium pH and assaying the enzyme activity. Result showed that with an increase in the pH of the medium from 6.0 to 8.0, the protease production was also enhanced linearly;and than the protease production remained constant

upto pH 9.0 of the medium. However, a further increase in pH beyond 9.0 resulted in a sharp decrease in protease yield (Fig.6.11).

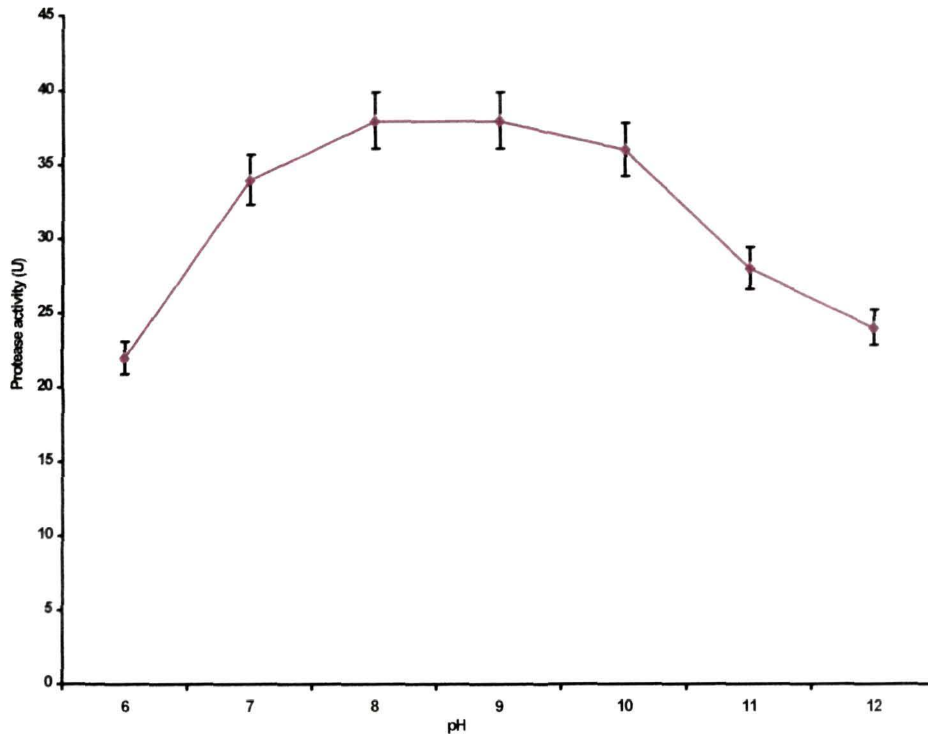


Fig.6.11 Effect of pH on alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. Values are means ± S.D. of three determinations.

6.2.4 Effect of temperature on protease production

The effect of temperature on alkaline protease production from *P.tezpurensis* sp.nov. strain AS-S24-II showed that alkaline protease production was linearly increased from 25 to 45°C and beyond this temperature protease production declined (Fig.6.12). Therefore, 45°C was considered as the optimum temperature for protease production by *P.tezpurensis* sp.nov. strain AS-S24-II in SmF at pH 8.0.

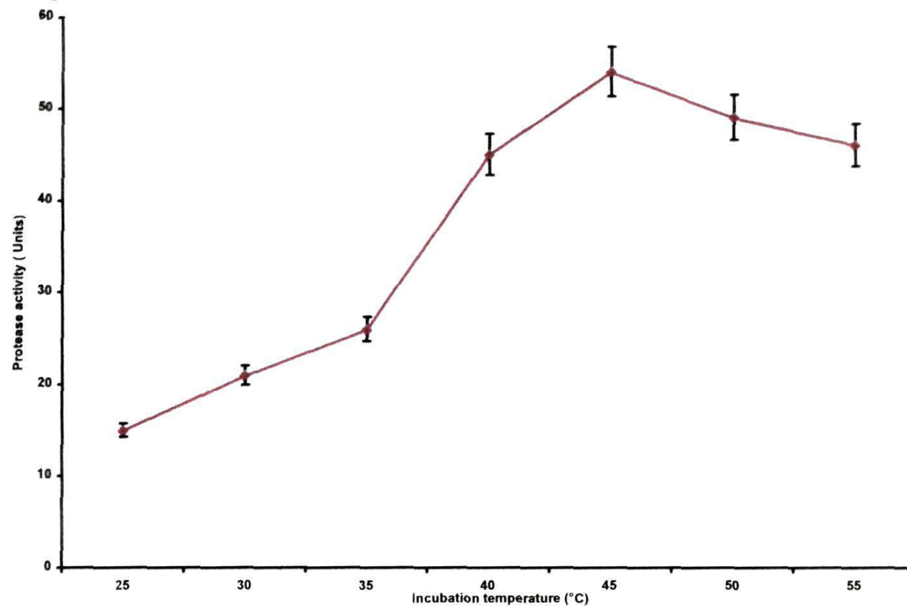


Fig.6.12 Effect of incubation temperature on alkaline protease production from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. The pH of the medium was adjusted to 8.0. Values are means ± S.D. of three determinations.

6.2.5 Kinetics of protease production

Result showed that optimum alkaline protease production was achieved after 48h of incubation (Fig. 6.13). Thereafter, a sharp fall in protease production was recorded.

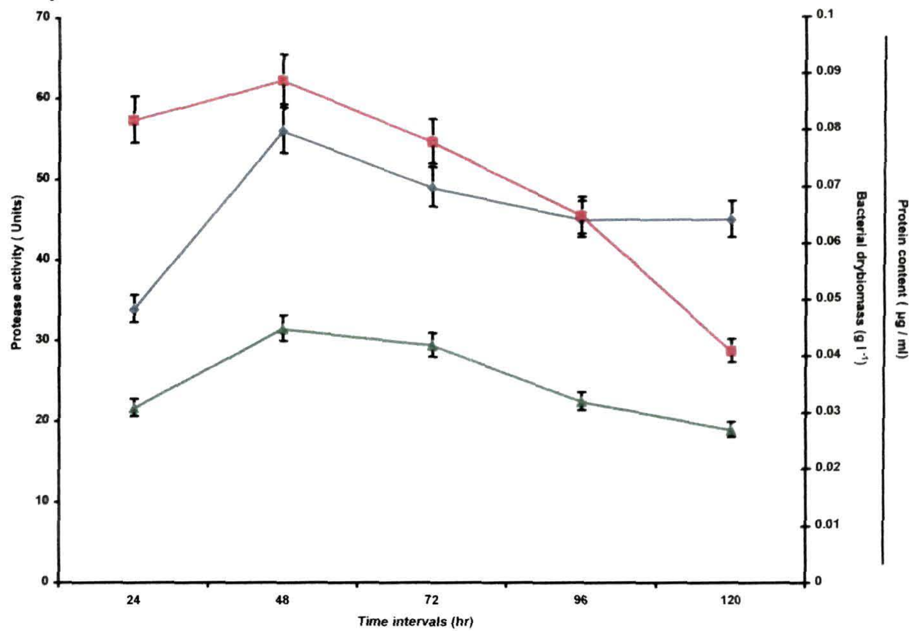


Fig. 6.13 Effect of incubation time on alkaline protease production by *P.tezpurensis* sp.nov. strain AS-S24-II in SmF. Legends show alkaline protease production (◆) bacterial dry biomass (■) and protein content (▲). Values are means ± S.D. of three determinations.

6.2.6 Effect of agitation rate on protease production

The effect of the agitation speed on the protease production showed that maximum alkaline protease production took place when the culture flasks were aerated at 200-250 rpm (Fig. 6.14).

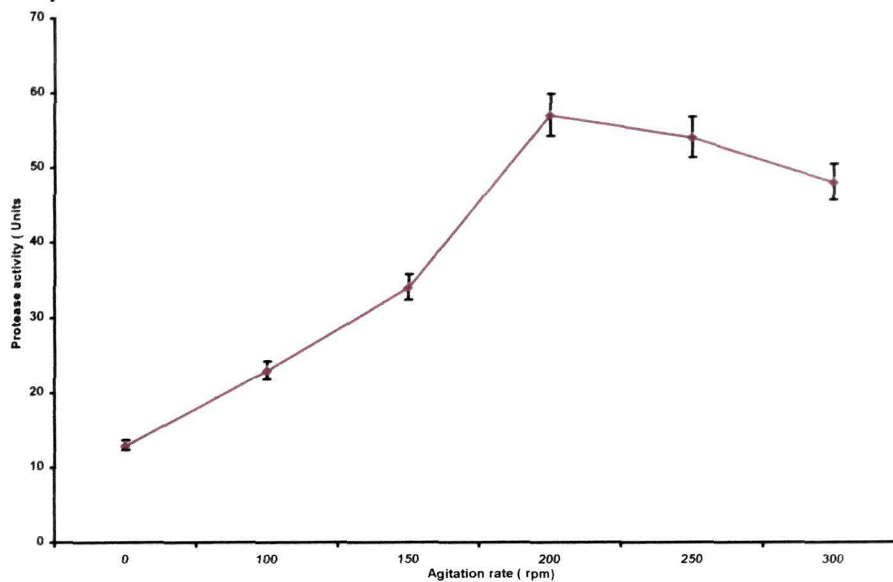


Fig.6.14 Effect of agitation rate on alkaline protease production from *P.tezpurensis* sp.nov. strain AS-S24-II under SmF. Values are means ± S.D. of three determinations.

6.3 Statistical optimization of alkaline protease production under submerged fermentation

6.3.1 Screening of process parameters significantly influencing the alkaline protease production in SmF using Plackett-Burman design

In this study, the factors significantly affecting the alkaline protease production by *P.tezpurensis* sp.nov. strain AS-S24-II was identified using a Plackett-Burman statistical design. The 15 sets of experiments were carried out according to the experimental matrix presented in Table 6.2, where the alkaline protease (U/ml/min) was the measured response. A wide variation in response (protease production) was found among the different trials (7.6 to 75.0 U/ml/min), as shown in Table 6.2, thereby emphasizing the importance of medium optimization to attain a higher

productivity. Thus, by neglecting the terms that were insignificant (p>0.05), the model equation for the alkaline protease production was written as equation 6.1

$$Y = 7.7 + 41.7X_1 + 10.0X_2 + 113.6X_3 \text{ ----- } (6.1)$$

Where, Y = (protease activity U), X_1 = Ammonium sulphate (% w/v), X_2= pH of the medium, and X_3= casein level (% w/v), respectively.
On the basis of calculated t-values (Table 6.3), ammonium sulphate, pH of the medium and casein level, were chosen for further optimization, since these factors significantly effected the protease production. Agitation rate, innoculum level and incubation time were kept at middle level.

Table 6.2 Plackett–Burman store design showing six variables with coded values along with the observed results for alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF.

Casein (% w/v)	Ammonium sulphate level (% w/v)	Incubation time (h)	Agitation rate (rpm)	pH of the medium	Innoculum size (% v/v)	Protease yield (Units)
1 (2.5)	-1(0.05)	1 (96)	-1 (150)	-1 (7.0)	-1 (1.5)	21.0
1 (2.5)	1 (1.5)	-1 (48)	1 (250)	-1 (7.0)	-1 (1.5)	28.0
-1 (1.5)	1 (1.5)	1 (96)	- (150)	1 (9.0)	-1 (1.5)	18.0
1 (2.5)	-1 (0.05)	1 (96)	1 (250)	-1 (7.0)	1 (5.0)	14.0
1 (2.5)	1 (1.5)	-1 (48)	1 (250)	1 (9.0)	-1 (1.5)	12.0
1 (2.5)	1 (1.5)	1 (96)	-1 (150)	1 (9.0)	1 (5.0)	8.0
-1 (1.5)	1 (1.30)	1 (96)	1 (250)	-1 (7.0)	1 (5.0)	37.0
-1 (1.5)	-1 (1.20)	1 (96)	1 (250)	1 (9.0)	-1 (1.5)	43.0
-1 (1.5)	-1 (1.20)	-1 (48)	1 (250)	1 (9.0)	1 (5.0)	75.0

1 (2.5)	-1 (1.20)	-1 (48)	-1 (150)	1 (9.0)	1 (5.0)	41.0
-1 (1.5)	1 (1.30)	-1 (48)	-1 (150)	-1 (7.0)	1 (5.0)	74.0
-1 (1.5)	-1 (1.20)	-1 (48)	- 1 (150)	-1 (7.0)	-1 (1.5)	31.0
0 (2.0)	0 (1.25)	0 (72)	0 (200)	0 (8.0)	0 (2.5)	7.6
0 (2.0)	0 (1.25)	0 (72)	0 (200)	0 (8.0)	0 (2.5)	7.6
0 (2.0)	0 (1.25)	0 (72)	0 (200)	0 (8.0)	0 (2.5)	7.6

Table 6.3 Statistical analysis of Plackett–Burman design showing coefficient, t- and p-values for each variable (p-value <0.05).

Variable	**Protease yield (U)**			
	Co-efficient	**SE Coef**	**t-Stat**	**p-value**
Intercept	7.7	1.2019	6.38	0.024
Ammonium sulphate level (% w/v)	41.7	1.5023	27.76	0.001
Innoculum level (%v/v)	-24.6	1.5899	-15.46	0.004
pH of the medium	10.0	1.5899	6.26	0.025
Agitation rate (rpm)	-107.5	5.5158	-19.48	0.003
Incubation time (h)	-0.9	0.9014	-0.97	0.434
Casein level (% w/v)	113.6	5.5403	20.50	0.002

6.3.2 Protease production optimization by RSM

Pursing the initial screening process, it was observed that the protease production was highly influenced by three variables, viz. concentration (% w/v) of casein, concentration (% w/v) of ammonium sulphate and pH of the M9 medium (Table 6.3). The results of the observed and predicted values of alkaline protease yield (response) as a function of the chosen variables with reference to the experiments performed according to the CCD is shown in Table 6.4. The parameters of Eq. 3.2 (see section 3.9.2) were determined by multiple-regression analysis by the application of RSM. The overall second-order polynomial regression equation showing the empirical relationship between protease activity (Y) and three test variables in coded units can be represented by Eq. 6.2.

$$\begin{aligned} Y &= 534.875 + 14.57C_1 - 10.03C_2 + 11.39C_3 - 30.386C_1^2 \\ &+ 38.914C_2^2 - 39.786C_3^2 - 4.738C_1C_2 + 2.263C_1C_3 - 4.388C_2C_3 \end{aligned} \quad \text{-------- (6.2)}$$

The coefficients of the model including the significance of each coefficient as determined by t test and p values in Table 6.5. Results showed that all the effects of C_1, C_2 and C_3, except quadratic effects of C_3 (C_1C_3 and C_2C_3) are significant ($p<0.001$). It was found that C_1 (pH of the medium) followed by C_3 (ammonium sulphate level) had significant effect ($p<0.001$) on alkaline protease production by bacterium under study (Table 6.5).The ANOVA of the quadratic regression model demonstrated that the computed F value for linear regression was much greater than the tabulated (P)>F value (Table 6.6). The coefficient (R^2) and adjusted R^2 values were calculated as 99.40% and 98.86%, respectively.

Table 6.4 Observed and predicted values of alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II post 48 h of incubation at 45°C and 200 rpm. The observed values are mean ± S.D of triplicate determinations. Boundaries of the experimental domain and spacing of levels are expressed in coded (within parenthesis) and natural units C_1, pH of the medium; C_2, casein level (% w/v); C_3, ammonium sulphate (% w/v).

Run no.	Independent Variables			Y Response (Protease activity in U/ml)		Residual value
	C_1	C_2	C_3	Observed value	Predicted value	
1	-1 (7.5)	-1(0.5)	-1(0.05)	480.0	480.823	-0.823
2	1(8.5)	-1(0.75)	-1(0.1)	516.4	514.913	1.487
3	-1(7.5)	1(1.25)	-1(0.1)	480.5	479.013	1.487
4	1(8.5)	1(1.25)	-1(0.1)	490.0	494.153	-4.153
5	-1(7.5)	-1(0.75)	1(0.20)	511.8	507.853	3.947
6	1(8.5)	-1(0.75)	1(0.20)	549.3	550.993	-1.693
7	-1(7.5)	1(1.25)	1(0.20)	486.8	488.493	-1.693
8	1(8.5)	1(1.25)	1(0.20)	513.3	512.683	0.617
9	0(8.0)	0(1.0)	-1(0.1)	487.0	489.918	-2.918
10	0(8.0)	0(1.0)	1(0.20)	522.8	519.058	3.742
11	-2 (7.0)	0(1.0)	0(0.15)	580.9	583.818	-2.918
12	2(9.0)	0(1.0)	0(0.15)	567.5	563.758	3.742
13	0(8.0)	-2(0.5)	0(0.15)	485.7	483.698	2.002
14	0(8.0)	2(1.5)	0(0.15)	505.3	506.478	-1.178
15	0(8.0)	0(1.0)	0(0.15)	534.6	534.875	-0.275
16	0(8.0)	0(1.0)	0(0.15)	534.6	534.875	-0.275
17	0(8.0)	0(1.0)	0(0.15)	534.6	534.875	-0.275

...Continued

Table 6.4 continued .....

18	0(8.0)	0(1.0)	0(0.15)	534.6	534.875	-0.275
19	0(8.0)	0(1.0)	0(0.15)	534.6	534.875	-0.275
20	0(8.0)	0(1.0)	0(0.15)	534.6	534.875	-0.275

Table 6.5 Analysis of Variance (ANOVA) of alkaline protease produced by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II.

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	9	15773.2	15773.2	1752.58	184.64	0.000
Linear	3	4426.2	4426.2	1475.39	155.43	0.000
Square	3	10972.5	10972.5	3657.50	385.32	0.000
Interaction	3	374.5	374.5	124.83	13.15	0.001
Residual error	10	94.9	94.9	9.49		
Lack-of-Fit	5	94.9	94.9	18.98		
Pure error	5	0.0	0.0	0.00		
Total	19	15868.1				

Table 6.6 Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF under shake-flask study.

Factor	Coefficient	SE coefficient	Computed t-value	p-value
Constant	534.875	1.0591	505.006	0.0*
C_1	14.570	0.9743	14.955	0.0

...Continued

Table 6.6 Continued...

C_2	-10.030	0.9743	-10.295	0.0
C_3	11.390	0.9743	11.691	0.0
C_1^2	-30.386	1.8579	-16.356	0.0
C_2^2	38.914	1.8579	20.945	0.0
C_3^2	-39.786	1.8579	-21.415	0.0
$C_1 C_2$	-4.738	1.0893	-4.349	0.001
$C_1 C_3$	2.263	1.0893	2.077	0.065
$C_2 C_3$	-4.388	1.0893	-4.028	0.002

R^2 = 99.40%, R^2 (pred) = 92.28%, R^2 (adj) = 98.86 %

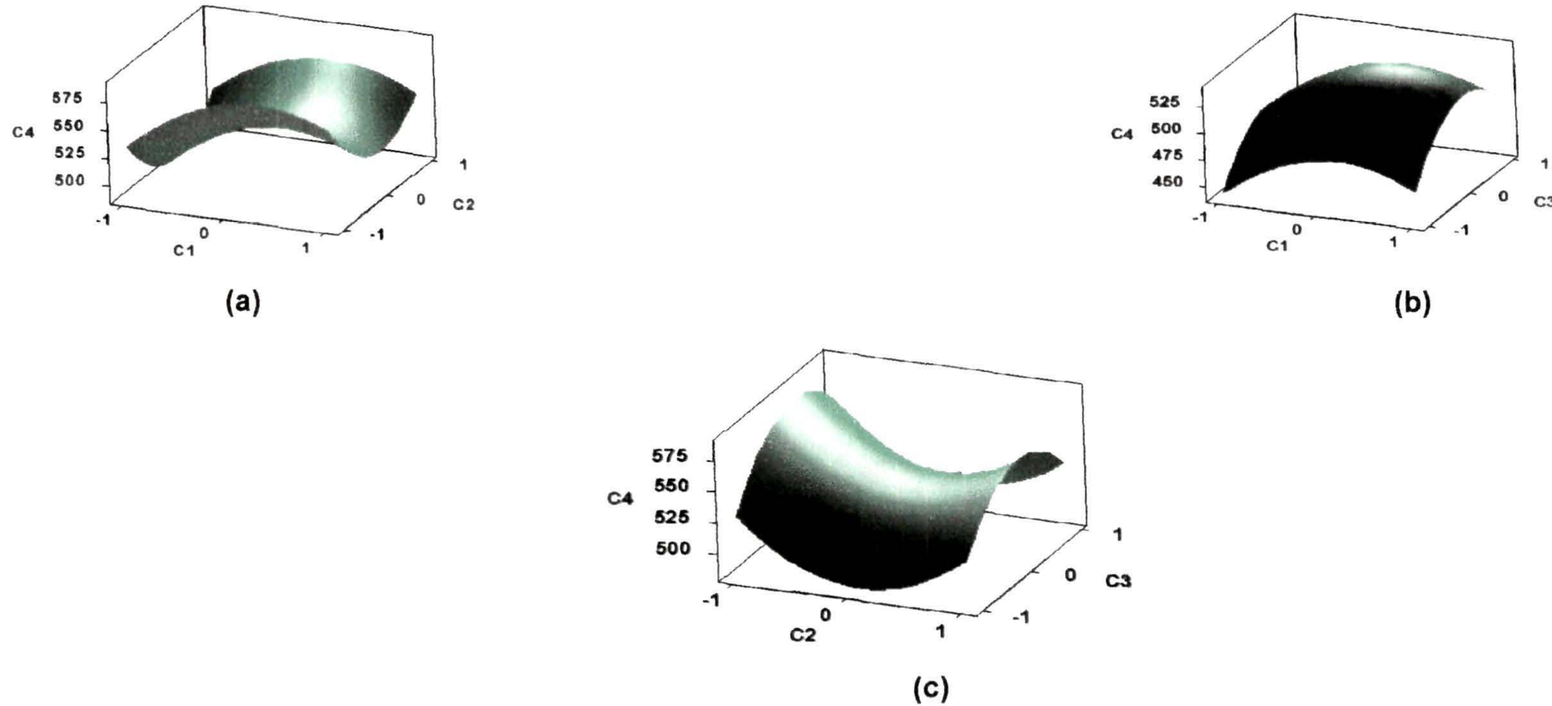


Fig.6.15 Response surface plots for alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. The interaction between (a) pH of the medium (C_1) and concentration (%, w/v) of casein level (C_2), hold value C_3= 0 (b) pH of the medium (C_1) vs concentration (%, w/v) of ammonium sulphate level (C_3), hold value C_2= 0 and (c) concentration (%, w/v) of casein (C_2) vs concentration of ammonium sulphate level (C_3) (% w/v) hold value C_1 = 0.

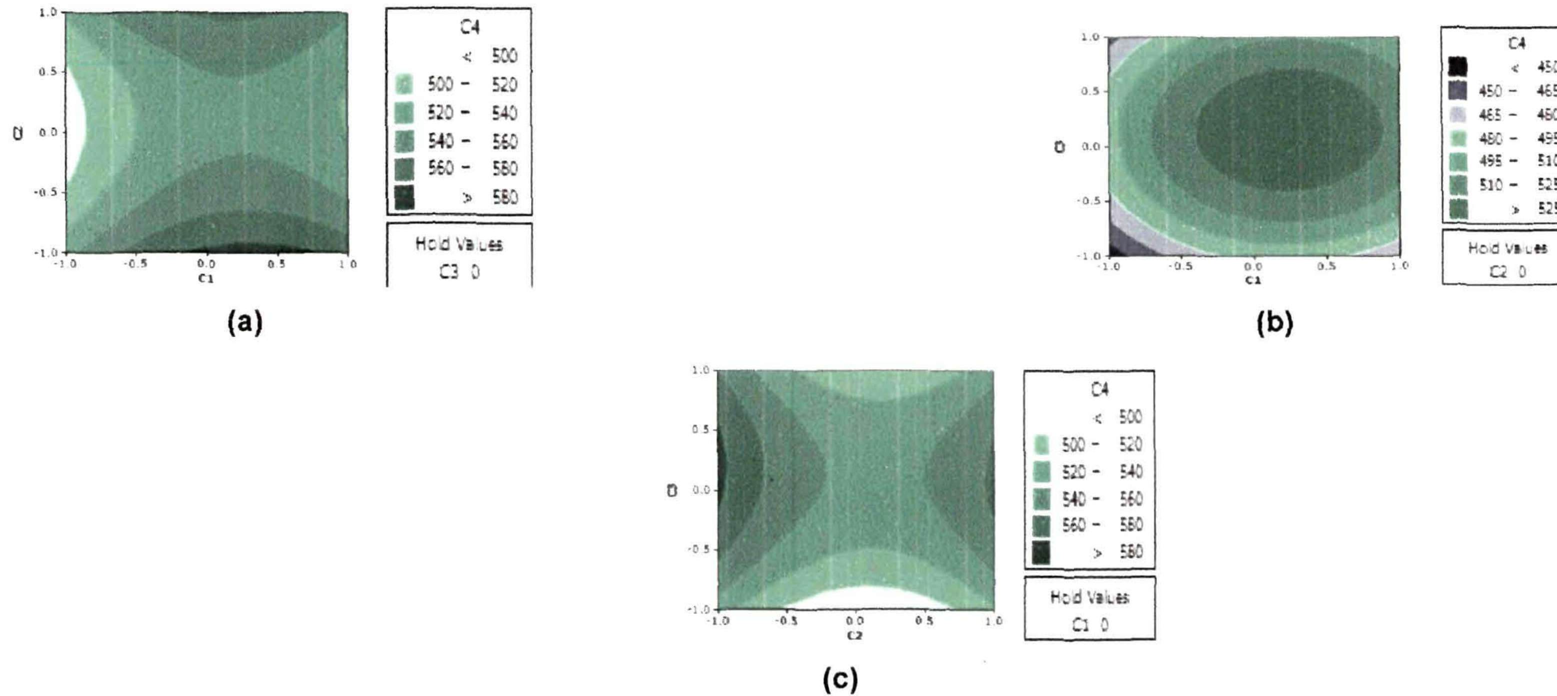


Fig.6.16 2D Countor surface plots for alkaline protease production by *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in SmF. The interaction between (a) pH of the medium (C_1) and concentration (%, w/v) of casein level (C_2), hold value C_3= 0 (b) pH of the medium (C_1) vs concentration (%, w/v) of ammonium sulphate level (C_3), hold value C_2= 0 and (c) concentration (%, w/v) of casein (C_2) vs concentration of ammonium sulphate level (C_3) (% w/v) hold value C_1 = 0.

6.3.1. Response surface plots

Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels (zero, for instance), are more helpful in understanding both the main and the interaction effects of these two factors. These plots can easily be obtained by calculating from the model the values taken by one factor where the second varies with constraint of a given Y value. The yield values for different concentrations of the variables can also be predicated from the respective response surface plots (Fig. 6.15a-c). The maximum predicted yield is indicated by the surface confined in the response surface diagram.Fig. 6.15a shows the response surface plot obtained as a function of pH of the medium (C_1) vs. casein concentration (C_2), while all other variables are maintained at central level. An increase in alkaline protease yield with increase in pH of the medium, versus casein concentration was observed. Fig.6.15b shows interaction of pH of the medium (C_1) vs ammonium sulphate concentration (C_2), while other variables are maintained at zero level. An increased alkaline protease yield with increase in pH of the medium vs. ammonium sulphate concentration was observed. Fig.6.15c demonstrates significant interaction of casein (C_2) vs ammonium sulphate concentrations. An initial increase in casein and ammonium sulphate concentration linearly enhanced alkaline protease production. However, further increase may lead to decrease in protease production (Fig.6.15c).

6.3.2. Countor plots

2D Contour plots are shown in Figs. 6.16a–c which depicts the interaction between two variables by keeping the other variables at their middle levels for protease production. The maximum predicted value is indicated by the

surface confined in the smallest ellipse in the contour diagram. The response surfaces having circular contour plot indicates no interaction where as, an elliptical or saddle nature of the contour plot indicates significant interaction between the corresponding variables. The Fig. 6.16a and b showing the countor plots indicated significant interaction of pH of the medium with casein for protease production. It could be seen that the protease production increased upon increasing the initial pH from 7.0 to 9.0, but any further increase in its values resulted in decreased protease production. Therefore, the optimal initial pH was around 7.0. The Fig. 2b shows interaction of pH (C_1) with ammonium sulphate level (C_2) increase in protease production was observed by the formation of elliptical; increase of pH of the medium vs ammonium sulphate level leads to maximum protease production. Based on regression equation model the interaction of casein with ammonium sulphate displayed increase in protease production (Fig.6.16c)

6.4 Batch fermentation using the optimal medium

The validation of the statistically optimized condition for the production of alkaline protease by *P. tezpurensis* sp.nov. strain AS-S24-II was confirmed by carrying out batch fermentation in a 5-l fermentor. Protease yield, accompanied by the increase of viable cell numbers (1 x 10^8 cell /ml), reached its maximum of 598.0 x 10^{-3} U / l at 48 h post-incubation and remained constant thereafter, suggesting that alkaline protease production by *P. tezpurensis* sp.nov. strain AS-S24-II was at least partially associated with cell growth.

6.5 Isolation and purification of detergent-stable alkaline protease

The 80% acetone-precipitated fraction demonstrated the maximum laundry detergent stability, and separation of this fraction on a gel filtration column resolved it into six protease peaks, designated as GF-I to GF-VI (Fig.6.17). The protease(s) present in GF-II fraction demonstrated appreciable detergent stability and was re-fractionated on an RP-HPLC C18 Nova-Pak column (Fig. 6.18). The peak with the retention time of 14.07 min (HP-III) showed highest alkaline protease activity and was found to be homogenous by 15% SDS-PAGE. About 8.0 µg of protein, both under reducing and non-reducing condition, yielded a single Coomassie-stained band of apparent molecular weight of 43 kDa in SDS-PAGE showing monomeric nature of the protease (Fig.6.19). A summary of purification of this enzyme is displayed in Table 6.7.

Table 6.7 Summary of purification of a detergent stable alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Data represent a typical experiment.

Purification step	Total protein (mg)	Total activity (Units)	Yield of protein (%)	Specific activity (units/mg)	Purification (fold)
Cell-free supernatant	64.0	160.0 X 10^3	100	2.5 X 10^3	1.0

Continued....

Table 6.7 Continued....

80% acetone precipitation	0.260	754.0	0.4	2.9×10^3	1.2
GF-II	0.186	595.0	0.3	3.2×10^3	1.3
HP-III	0.140	588.0	0.2	4.2×10^3	1.7

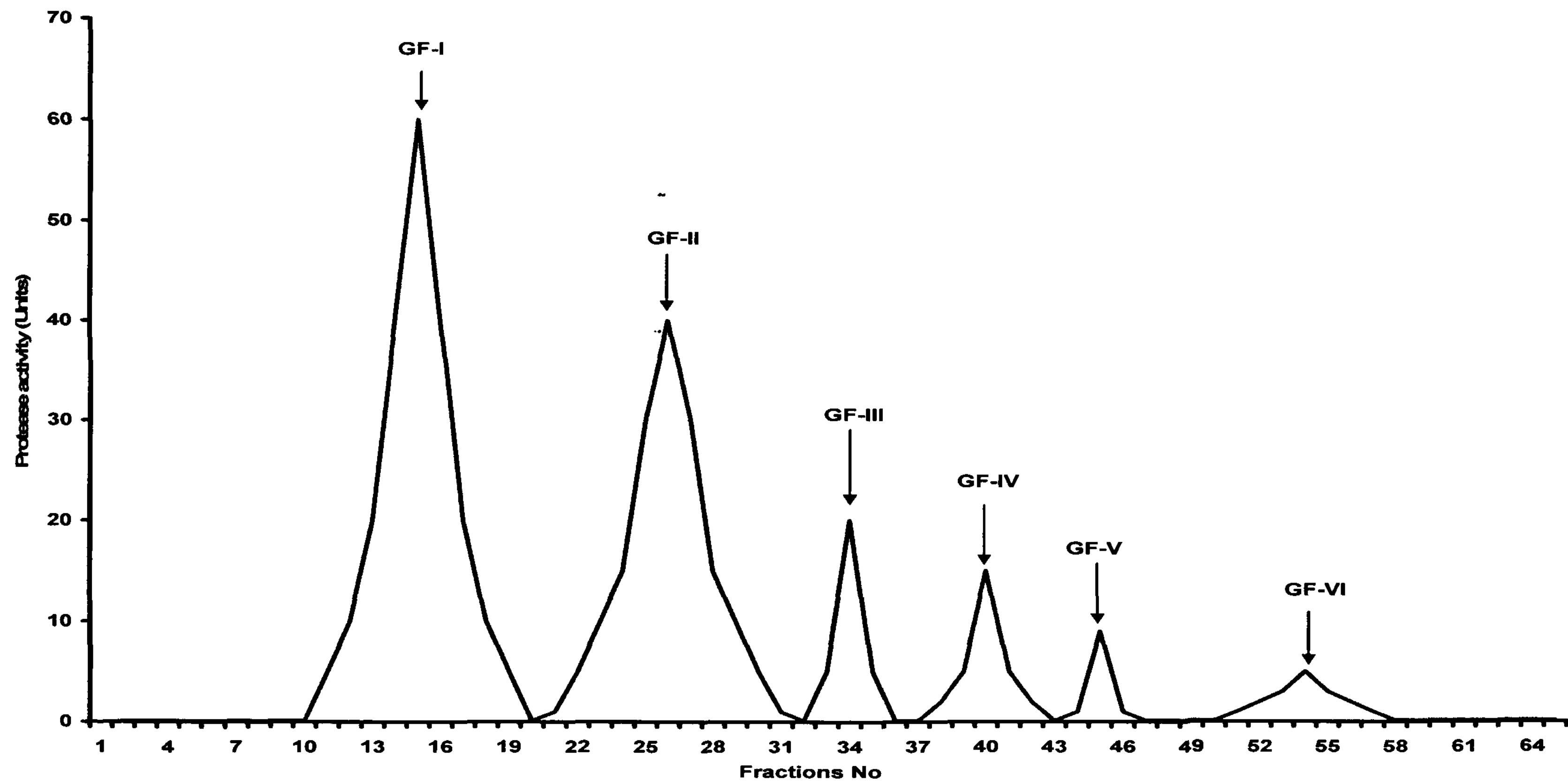


Fig.6.17 Gel filtration of 80% acetone fraction of alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II on Sephacyrl S-200 column. Data represent a typical experiment.

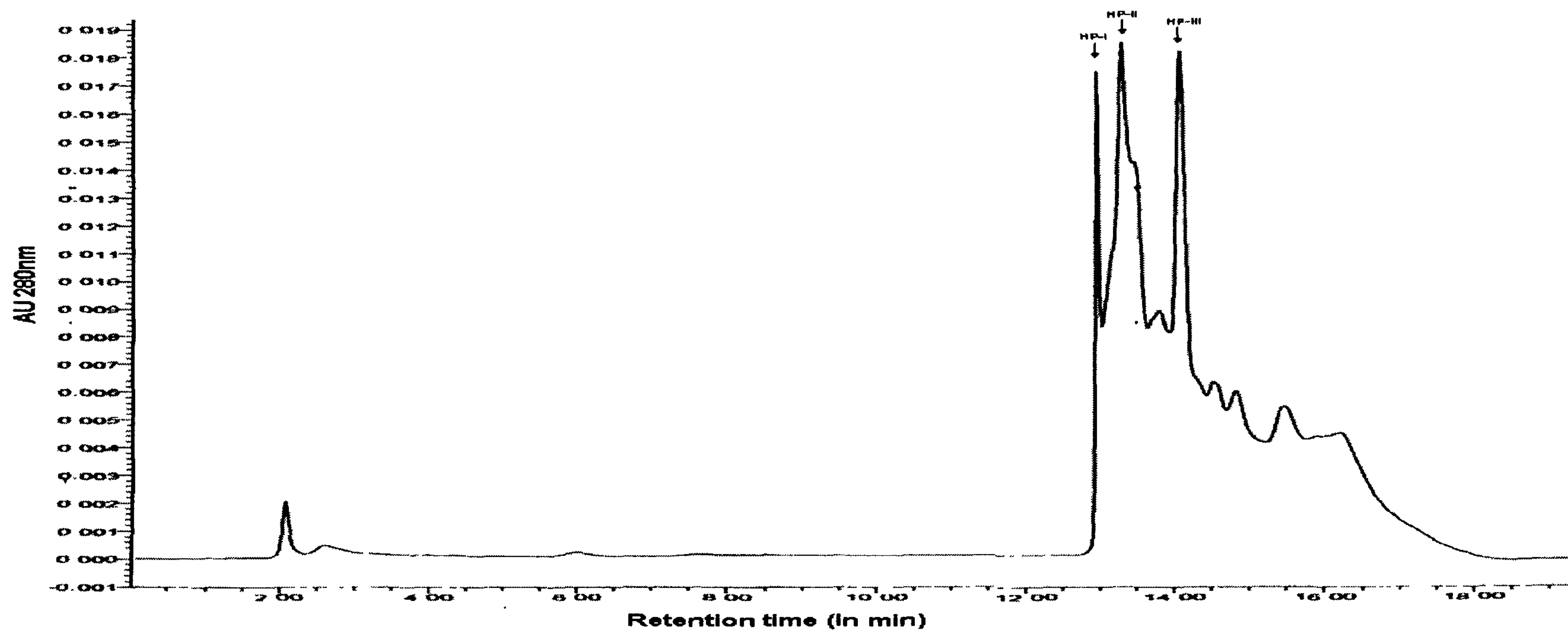


Fig.6.18 RP-HPLC of GF-II protease fraction of alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Initial peak with a retention time of 2.0 min represents the solvent peak.

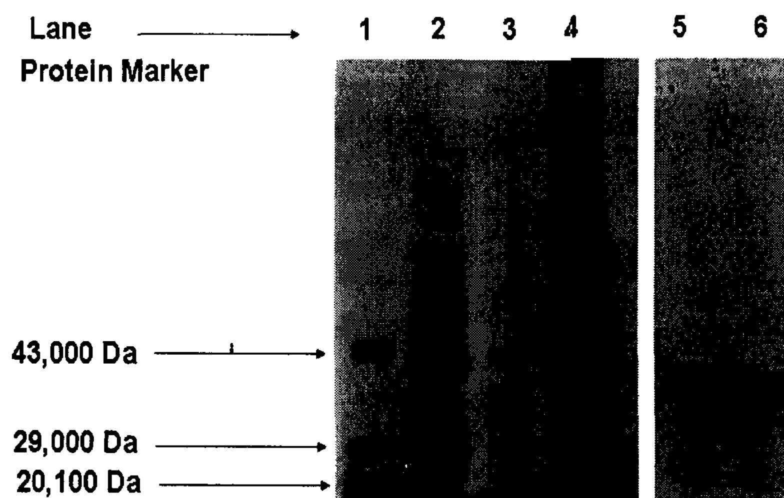


Fig.6.19 SDS-polyacrylamide gel (15%) electrophoresis of purified protease (HP-III). Lane 1, protein molecular weight standards: ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (20.1 kDa); lane 2, reduced crude protease (30 µg); lane 3, 60% acetone precipitated fraction (30 µg), lane 4, 80% acetone precipitated fraction (30 µg), lane 5, reduced alkaline protease (8 µg), lane 6, non-reduced alkaline protease (8 µg)

6.6 Biochemical characterization

6.6.1 Effect of pH and incubation temperature on detergent stable alkaline protease

The purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II remained active over a broad range of pH and temperature; however, the optimum activity of enzyme was shown at pH 9.5 and at 45–50°C temperature range (Table 6.8).

Table 6.8 Biochemical properties and substrate specificity of alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Values are mean ± S.D. of triplicate determinations.

Biochemical property	**Value**
Molecular mass (kDa)	
(a) SDS-PAGE	43.0
(b) Gel-filtration	42.9
Optimum pH	9.0- 9.5
Optimum temperature	45-50°C
Substrate specificity (unit / mg protein) (mean ± S.D., n=3)	**Purified protease**
(a) Casein	4200.0 ± 210.0
(b) Chicken-feather keratin	1300.0 ± 65.0
(c) Bovine serum fibrinogen	1050.0 ± 21.0
(d) Bovine serum albumin	768.0 ± 16.0
(e) Fibrin	562.6 ±11.3
(f) Bovine serum globulin	230.0 ± 11.5
(g) Human-hair keratin	0
(h) Collagen	0

6.6.2 Thermo-stability study of purified protease

The protease showed remarkable thermo stability as was evident from the heating experiment. The enzyme retained 68.0% of its original activity post-heating at 60°C for 120 min at pH 9.5, and it was found that Ca^{2+} had no role in influencing the thermo stability of the enzyme (Fig.6.20).

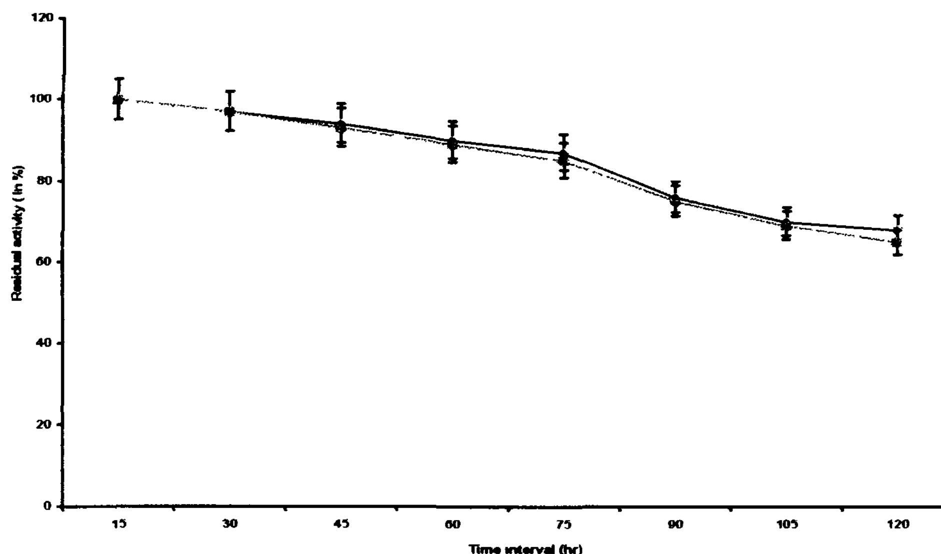


Fig.6.20 Thermo-stability study of detergent-stable protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II in presence of $CaCl_2$ (◆) and in absence of $CaCl_2$ (■). Values are mean ± S.D. of triplicate determinations.

6.6.3 Substrate specificity study

The crude protease (cell-free culture supernatant) from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II demonstrated a broad spectrum of substrate specificity. It was shown to hydrolyse both soluble (casein, bovine *serum albumin, fibrinogen, haemoglobin) and insoluble* (keratin from chicken-feather and fibrin) protein substrates to a considerable extent (Table 6.8). The purified protease showed a preference for the hydrolysis of casein compared to other protein substrates (Table 6.8).

6.6.4 Kinetic study of purified protease

The Km and Vmax values for purified protease towards casein were determined as 0.227 mg / ml and 25.0 µmol / mg/ min, respectively, documenting the excellent substrate (casein) specificity of the enzyme (Fig.6.21).

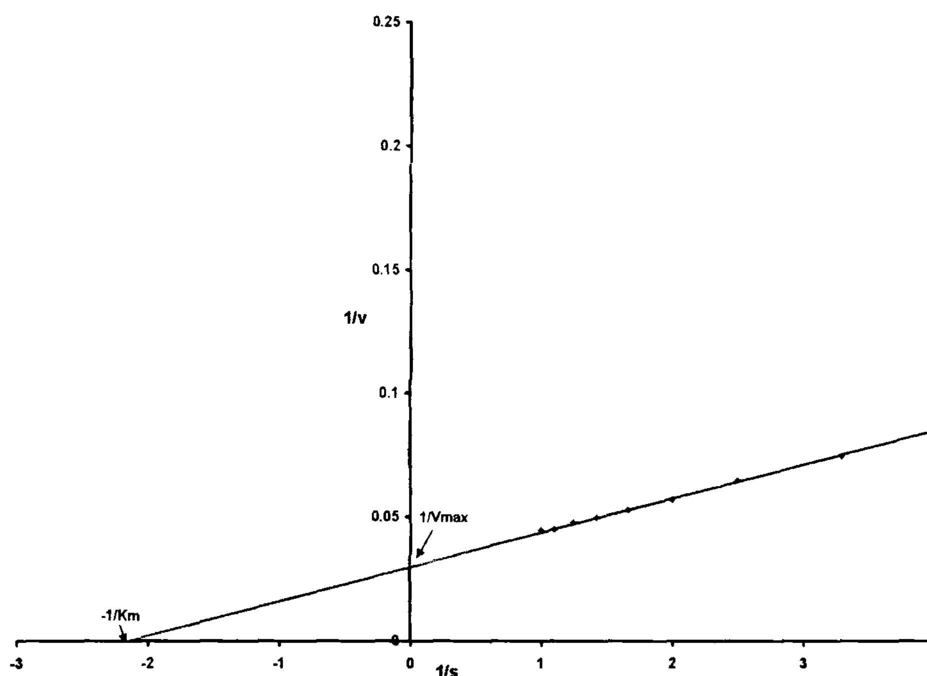


Fig.6.21 Lineweaver-Burk plot to determine the K_m and V_{max} values of detergent stable alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. The substrate used was casein at pH 9.5 and incubation temperature was 45°C.

6.6.5 Effect of polyols on stability of protease against heat-denaturation

The influence of various polyols (5%) such as glycerol, mannitol, sorbitol and xylitol, on the thermostability of alkaline protease was examined at 60°C. *The data presented in Fig. 6.22 show that glycerol and mannitol* stabilized the protease against heat-denaturation.

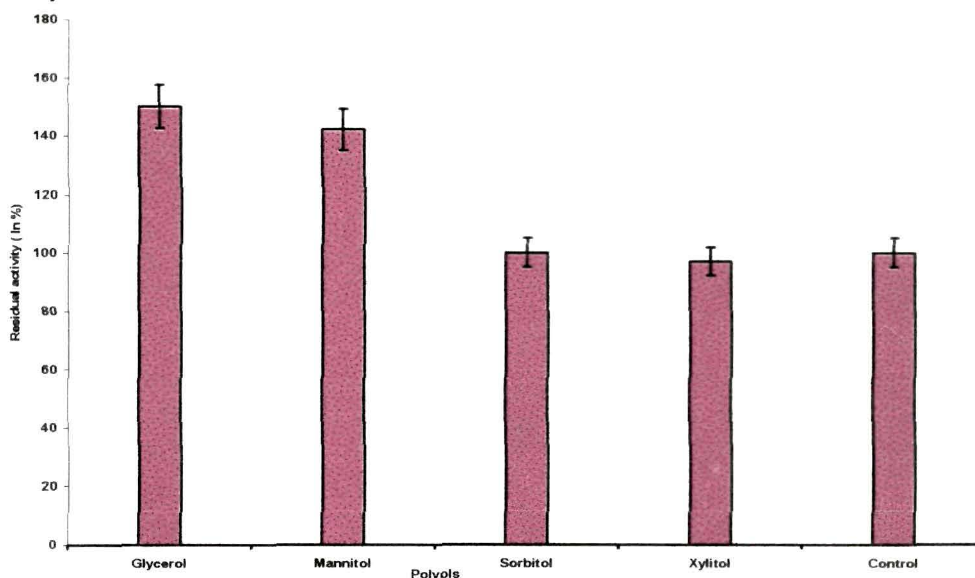


Fig.6.22 Effect of polyols on thermostability of protease under heated condition from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. The enzyme without polyols served as control (100% residual activity). Values are mean ± S.D. of three experiments.

6.6.6 Effect of metal ions on purified alkaline protease

Study on the influence of metal ions on alkaline protease activity of *P.tezpurensis* sp.nov. strain *AS-S24-II* showed that all the tested cations, viz. Ni^{2+} ; Cd^{2+} ; Co^{2+} ;Mg^{2+}; Fe^{2+}; Hg^{2+}; Cu^{2+} ;Mn^{2+}; Ca^{2+} and Zn^{2+}, inhibited the enzyme activity. Maximum inhibition of protease activity was observed in the presence of Co^{2+} (90 % inhibition) whereas Hg^{2+} exerted minimum inhibition (48%) of protease activity compared to control (Fig.6.23).

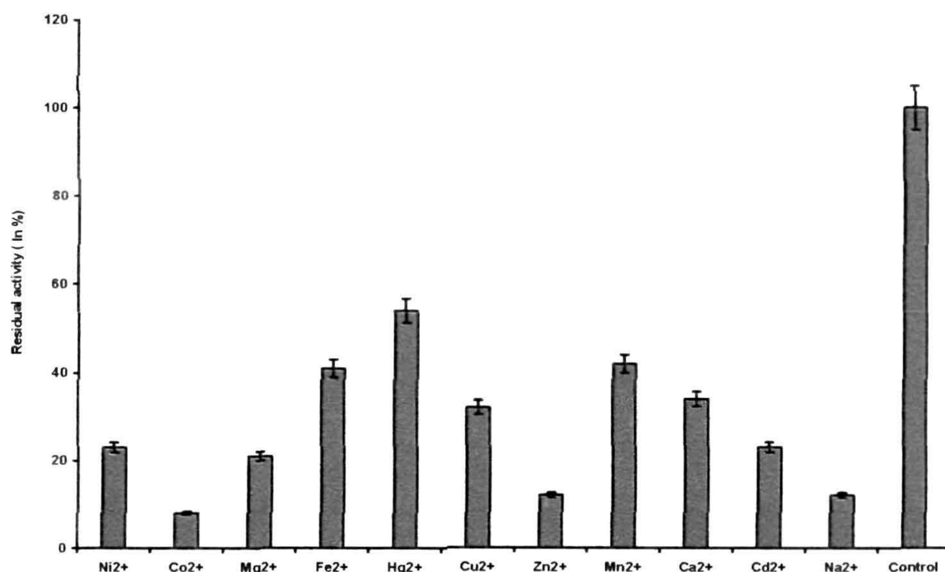


Fig.6.23 Effect of metal ions on protease activity of detergent stable alkaline protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II Values are mean ± S.D. of three experiments.

6.6.7 Effect of denaturing agents, oxidizing and bleaching agents, inhibitors, chelators, and surfactants on protease activity

A comparison of effects of different inhibitors, metal chelator, surfactants, denaturing agents, oxidizing and bleaching agents and DTT on purified alkaline protease is shown in Table 6.9. The enzyme activity was significantly affected by PMSF, IAA, 4-bromophenacyl bromide and TPCK, thus suggesting a role for serine, cysteine and histidine residues in the catalysis process (Table 6.9). Further, a significant inhibition of protease activity in the presence of DTT was also observed. The anionic surfactant SDS dose-dependently inhibited the protease activity whereas the protease activity was enhanced in presence of oxidizing agent (H_2O_2) and bleaching agent (*sodium perborate*).

Table 6.9. Effect of different inhibitors, surfactant, denaturing agents, oxidizing and bleaching agents and metal chelators on purified protease. Values are mean ± S.D. of triplicate determinations.

Inhibitors/surfactants	**Residual activity (%)**
Control	100
Inhibitors/chelator (1 mM)	
PMSF	0
4-BPB	7.6 ± 0.4
IAA	0
TLCK	41.5 ± 2.1
TPCK	2.7 ± 0.1
EDTA	100
Anionic surfactant (SDS)	
20 mM	102.0 ± 5.1
40 mM	92.0 ± 4.6
60 mM	76.0 ± 3.8
80 mM	55.0 ± 2.8
Non-ionic surfactants (1% v/v)	
Trition-X-100	72.3 ± 3.6
Tween 20	80.2 ± 4.0
Oxidizing agent	
H_2O_2 (% v/v)	
5.0	130.0 ± 2.6
10.0	124.0 ± 2.5
Bleaching agent	
Sodium perborate (% v/v)	
0.5	119.0 ± 2.4

Inhibitors/surfactants	Residual activity (%)
1.0	108.0 ± 2.2
DTT (1 mM)	13.7 ± 0.7

6.6.8 Effect of organic –solvent on purified protease

The organic-solvent (at a final concentration of 20% v/v) stability of purified alkaline protease is shown in Fig. 6.24. It was observed that xylene and ethanol slightly enhanced the protease activity, whereas the n-hexane, 2-propanol, methanol, and acetonitrile did not affect on enzyme stability. However, in presence of benzene, the enzyme was destabilized and lost about 35% of its original activity (Fig.6.24).

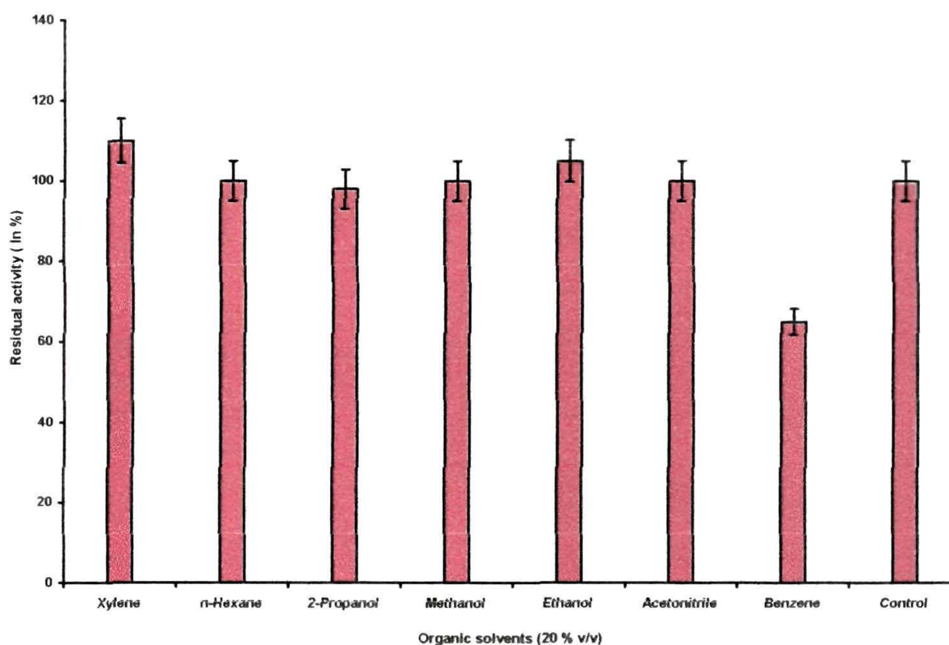


Fig.6.24 Organic solvent stability of alkaline protease from *Paenibacillus tezpurensis* sp.nov.strain AS-S24-II. Enzyme activity in the absence of solvent was considered as 100% activity and other values were compared with that. Each value represents mean ± S.D. of three experiments.

6.6.9 Pharmacological properties of protease

The pharmacological property of purified protease is summarized in Table 6.10. The protease at a concentration of 15 µg / ml showed 3.0 % hemolysis of the washed human erythrocytes, and it did not have any undesirable haemorragic effect on goat liver, heart, lungs and kidney tissues (Table 6.10). Further, as shown in Table 6.10, purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II did not interfere with the normal clotting time of goat platelet- poor plasma (PPP).

Table 6.10 Pharmacological properties of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II. Values are mean ± S. D. of three determinations.

Pharmacological properties	Values
Hemolysis (% Hb released / 15.0 µg of alkaline protease 90 min post incubation at 37 °C)	
Control (without alkaline protease)	1.4 ± 0.1
Treatment	3.0 ± 0.5
Ca-Clotting time (s)	
Control (without alkaline protease)	129.0 ± 6.5
Treated (with 15.0 µg / ml of alkaline protease)	132.0 ± 7.0
In vitro tissue damaging activity (% hemoglobin release by 15.0 µg / ml of alkaline protease 5 h post incubation at 37 °C)	
a) ***Heart***	
Control (without alkaline protease)	0.09 ± 0.01
Treatment	0.27 ± 0.01
b) ***Lung***	
Control (without alkaline protease)	0.13 ± 0.01
Treatment	0.36 ± 0.02

Pharmacological properties	Values
c) ***Liver***	
Control (without alkaline protease)	0.16 ± 0.08
Treatment	0.28 ± 0.01
d) ***Kidney***	
Control (without alkaline protease)	0.12 ± 0.05
Treatment	0.27 ± 0.01

6.7 Industrial application of purified protease

6.7.1 Detergent compatibility study of protease

As shown in Fig. 6.25, the stability of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II towards tested laundry detergents demonstrated a promising result. At a purified protease concentration below 3.0 μg / ml, the enzyme activity in the presence of detergents (7 mg/ml) could not be detected; however, at a concentration of 3.0 μg / ml, the enzyme retained 90.0% to 105% of its original activity in the presence of detergents at the tested temperature ranges (Fig.6.25). Based on screening results, maximum stability of purified protease was observed with "Sunlight" detergent; dose dependent study of purified protease with sunlight detergent demonstrated that optimum concentration for wash performance activity was 8.0 μg / ml and below this dose no washing activity was observed (Fig. 6.26). With 8.0 μg / ml dose of purified protease maximum blood stain was removed from cotton fabrics when supplemented to laundry detergents (Fig.6.27).

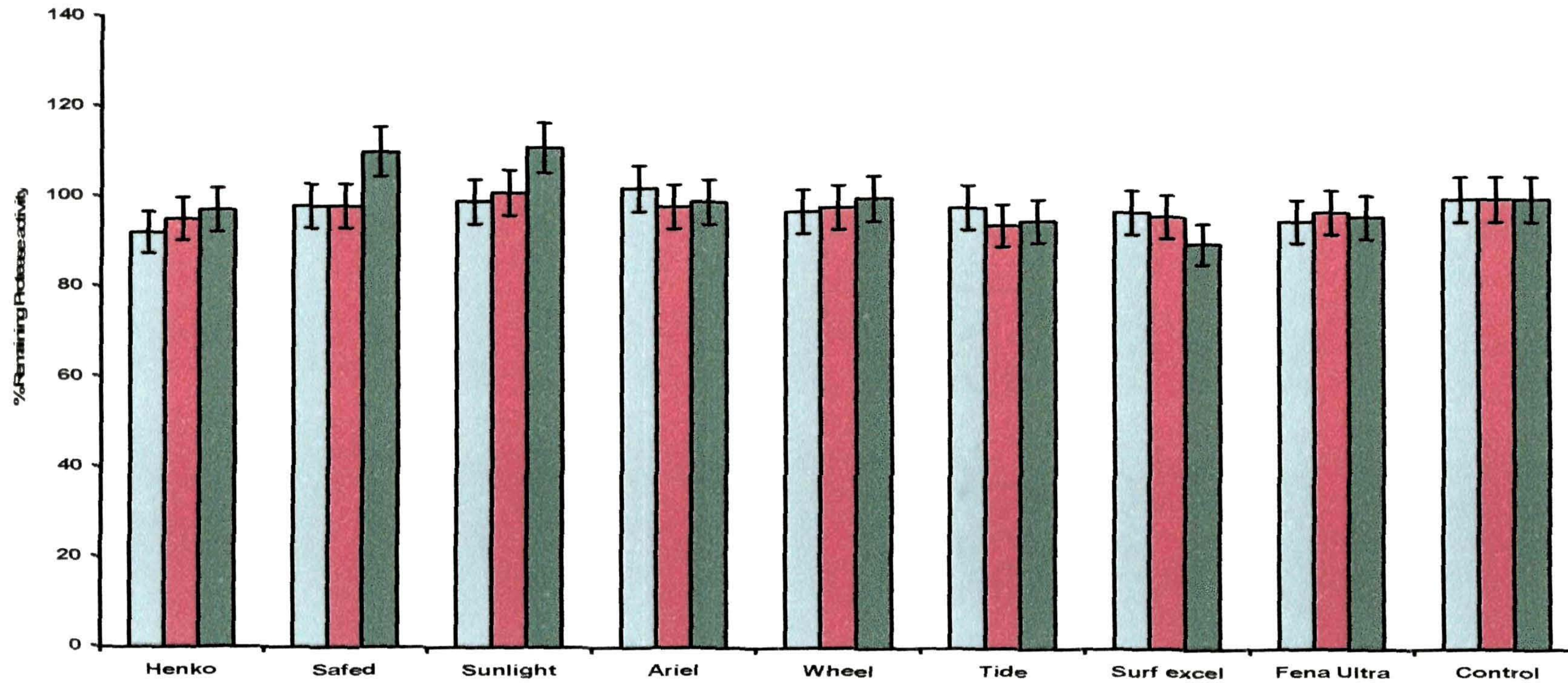


Fig.6.25 Detergent compatibility pattern of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II at the tested temperature ranges 25 ( ), 37 ( ) and 45°C ( ). Values are mean ± SD of triplicate determinations.

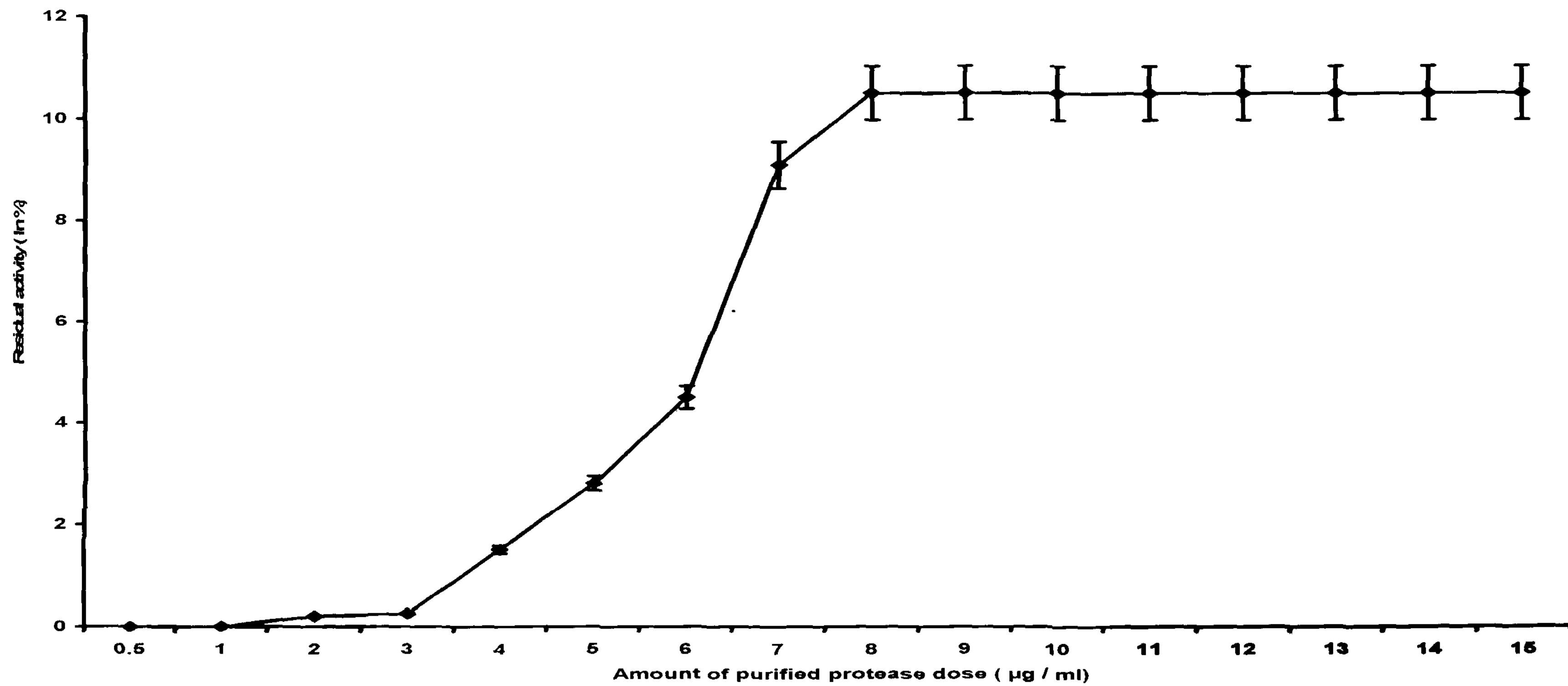


Fig.6.26 Dose-dependent wash performance study of purified protease in presence of sunlight® detergent (7.0 mg /ml) .

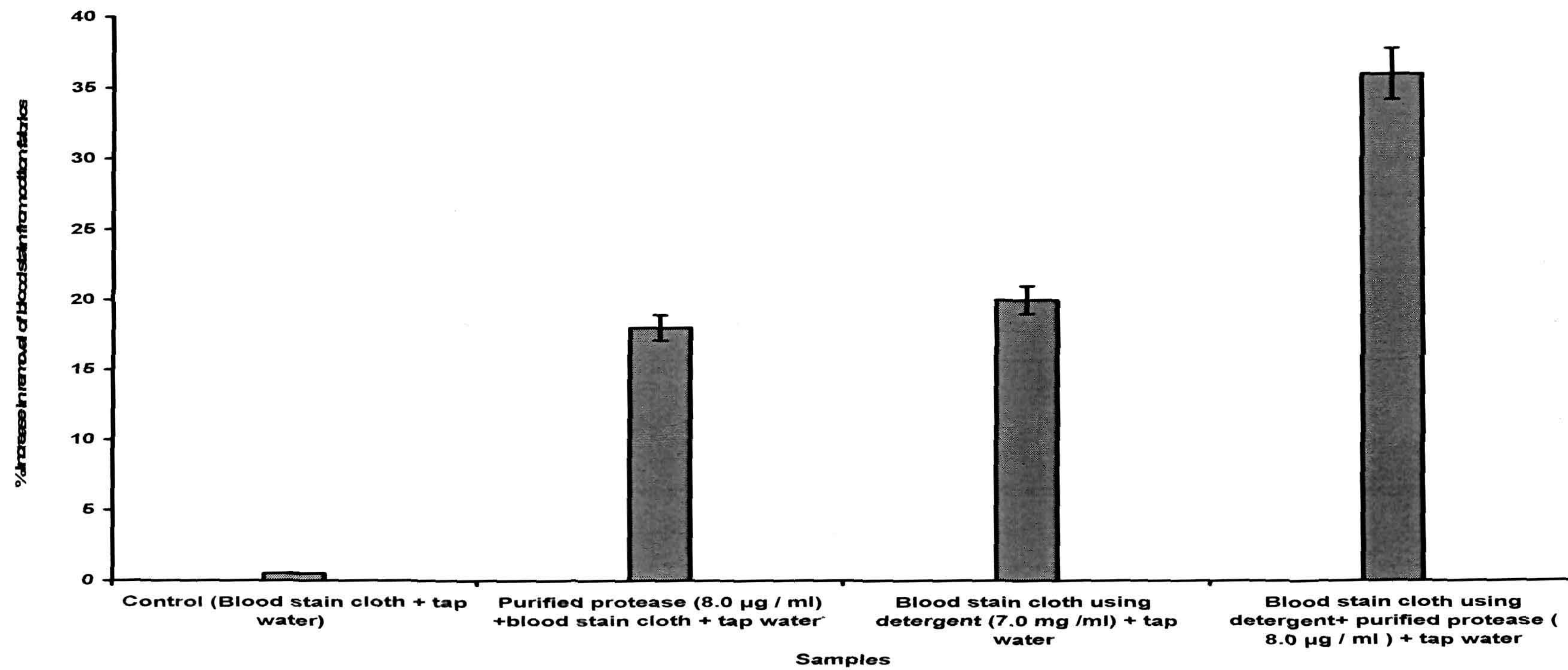


Fig.6.27 Wash performance study of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II at 37°C. Values are mean ± SD of triplicate determinations.

6.7.3 Dehairing activity of purified protease

In the present study, purified alkaline protease displayed a promising dehairing activity, as was evident from the fact removal of hair from raw skin incubated with purified protease (50U / ml) at 37°C for 12 h, was much easier as compared with control (Fig.6.28) suggesting application of protease leather industry as a dehairing agent.

(a) **(b)**

Fig. 6.28 Dehairing activity of purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with purified protease from *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II (50 U / ml) for 12 h at 37°C.

CHAPTER VII

RESULTS

Sudhir K Rai

PhD Thesis, Tezpur University, 2010

Chapter 7

Process optimization, isolation, purification, biochemical characterization and industrial applications of an alkaline β-keratinase (protease) isolated from *Bacillus subtilis* strain RM-01

7.1 Production of alkaline β-keratinase under solid-state fermentation

Pre-screening of fermentation parameters were done for optimization of alkaline β-keratinase production under solid-state fermentation system.

7.1.1 Time-course of keratinase production

In the SSF system, when chicken feather was used as a substrate for β-keratinase production by *Bacillus subtilis* strain RM-01, the β-keratinase production was observed to increase linearly with time. Maximum keratinase yield of 8.9 U/gds was achieved post 96 hours of incubation at 50°C under un-optimized condition (Fig.7.1). Thereafter, the β-keratinase production declined. Henceforth, 96 hours was considered as the optimized period for β-keratinase production in the subsequent experiments.

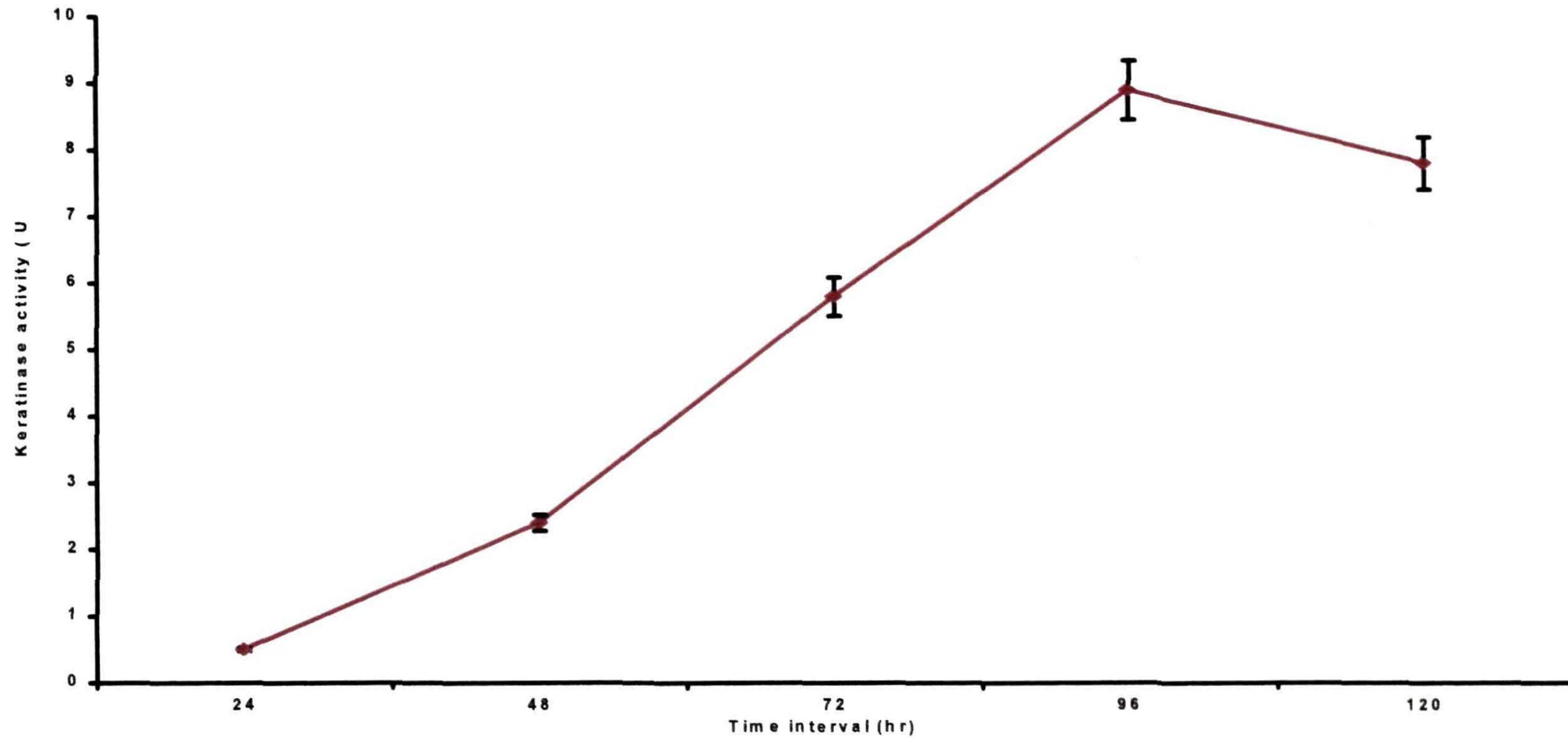


Fig.7.1 Alkaline β-keratinase production from *Bacillus subtilis* strain RM-01 under solid state fermentation. Values are mean ± S.D. of three experiments.

7.1.2 Effect of initial moisture content of the substrate and moistening agent on β-keratinase production

With an increase in the moisture content of the substrate from 50% to 100%, β-keratinase production was concomitantly enhanced, and further increase in the moisture content of substrate resulted in a steady decline in the β-keratinase yield (Fig.7.2).

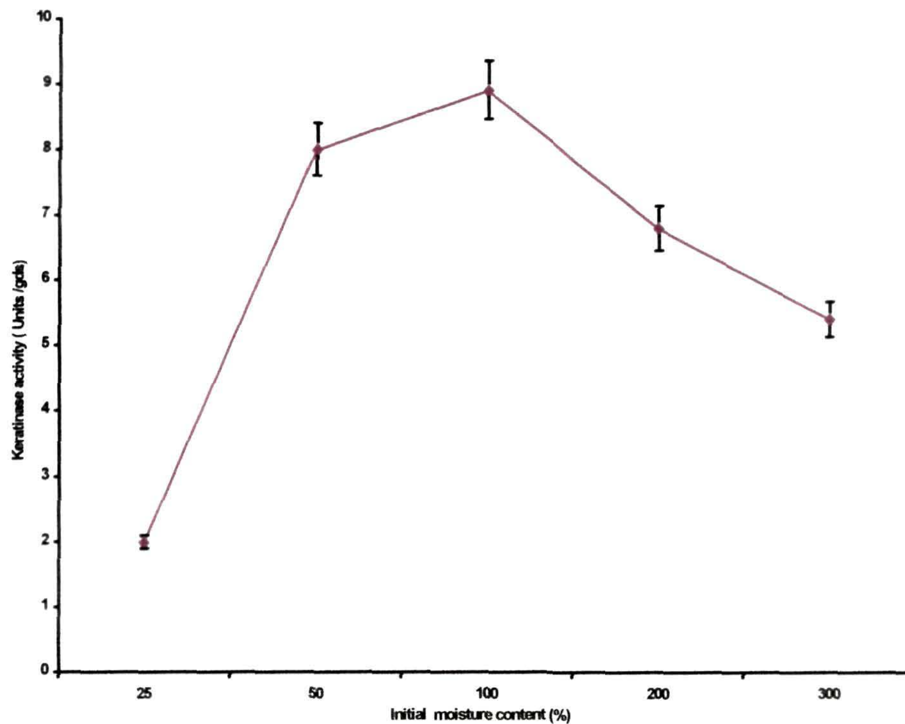


Fig. 7.2 Influence of initial moisture content on alkaline β-keratinase production from *B.subtilis* strain RM01. Values are mean ± S.D. of three determinations post 96 incubation at 50°C temperature. Moistening agent was distilled water, pH adjusted to 8.0.

7.1.3 Influence of pH of the moistening agent on β-keratinase production

It was very important to note that amongst the different moistening agents used, distilled water adjusted to pH 8.0 was found to be superior compared to K-phosphate or Tris-HCl buffer, pH 8.0 in supporting the maximal β-keratinase production (Fig.7.3).

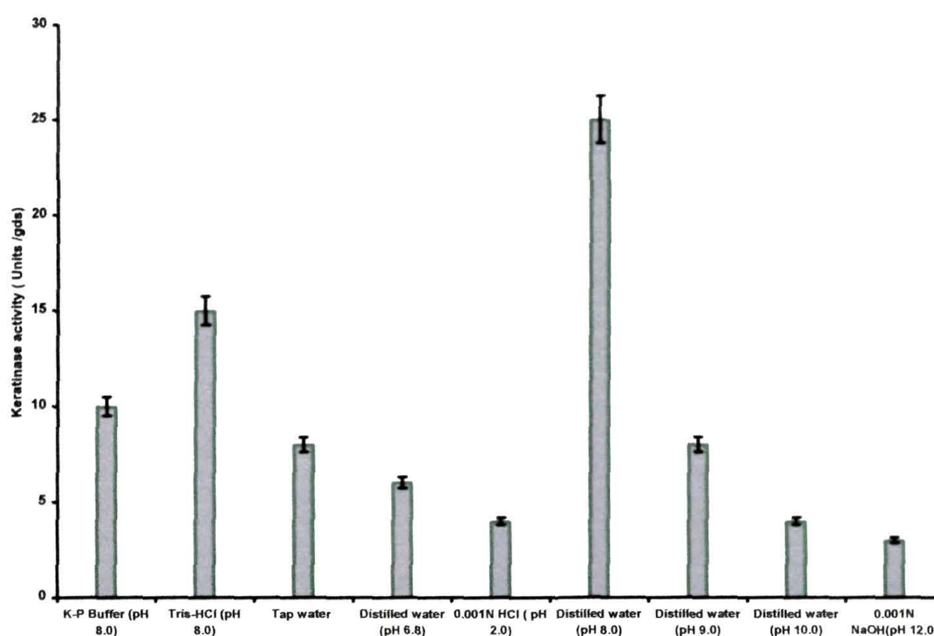


Fig.7.3 Influence of moistening agent on alkaline β-keratinase production by *B.subtilis* strain RM-01 using chicken feather as a substrate. Values represent mean ± S.D. of three experiments post 96 h of incubation at 50°C.

7.1.4 Effect of inoculum's size on protease production

As shown in Fig.7.4, with an increase in inoculum's size from 0.5 ml to 2.0 ml (5.0 g substrate), there was a concomitant increase in alkaline β-keratinase production by *B. subtilis* strain RM-01. Increasing the inoculum's

size beyond 2.0 ml. Resulted in a steady decline in β-keratinase production by *B. subtilis* strain RM-01 (Fig.7.4).

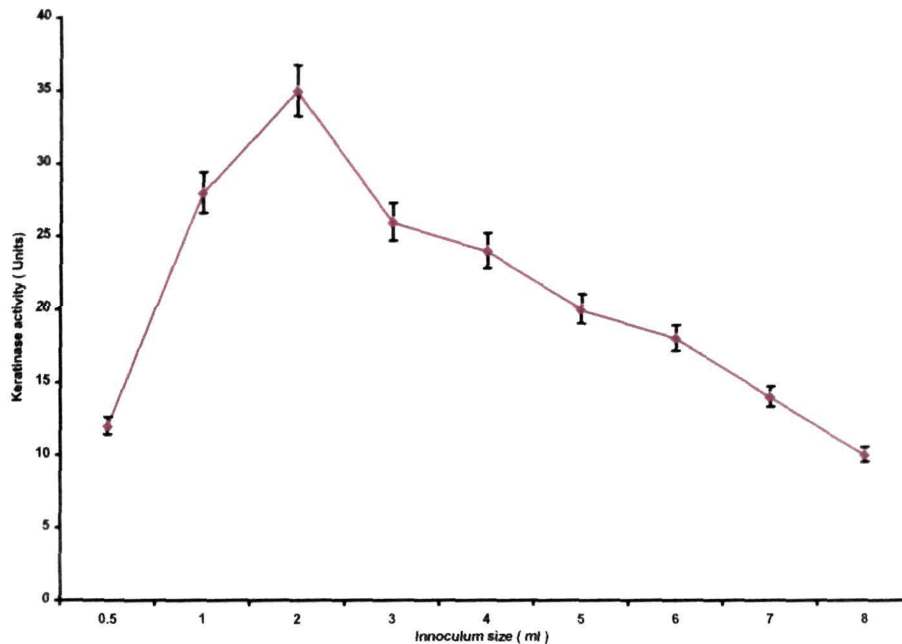


Fig.7.4 Influence of inoculum size on alkaline β-keratinase production by *B.subtilis* strain RM-01 on raw chicken-feather under SSF. Values are mean ± S.D. of three experiments.

7.1.5. Effect of supplementation of co-carbon and co-nitrogen sources on alkaline β-keratinase production

Among tested carbon sources, maltose demonstrated about 5.6 fold increase in β-keratinase production followed by casein (5.0 fold) compared with control (Fig.7.5). In a sharp contrast, galactose, lactose, fructose, sucrose, and starch inhibited the β-keratinase production by *B. subtilis* strain RM-01. As shown in fig. 7.6, amongst the tested co-nitrogen sources, $NaNO_3$ was most effective in enhancing keratinase yield (69.3 U/gds) followed by yeast extract (34.8 U/gds).

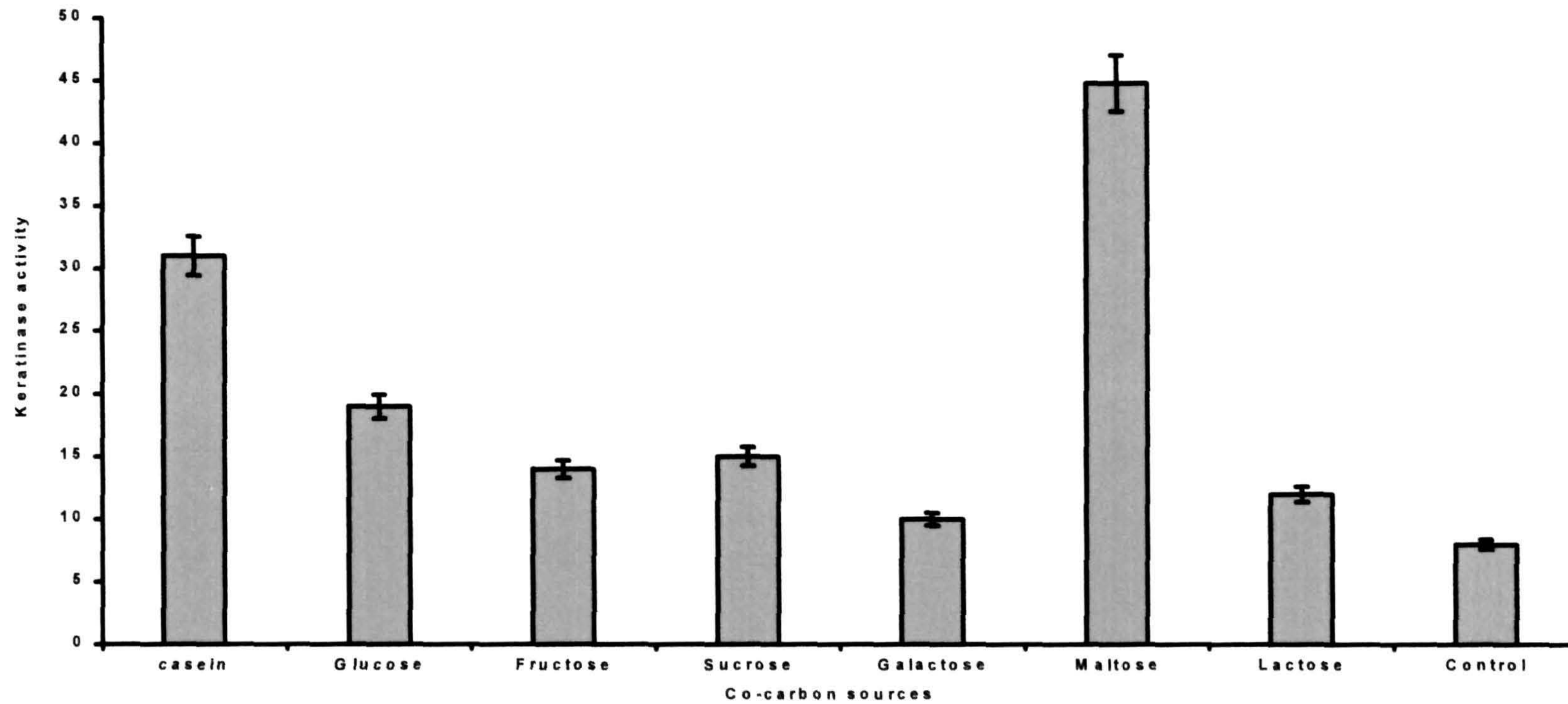


Fig.7.5 Effect of supplementation of co-carbon sources (10%, w/w) to chicken feather (90%, w/w) on β-keratinase production by *B. subtilis* strain RM-01. Values are mean ± S.D. of three experiments.

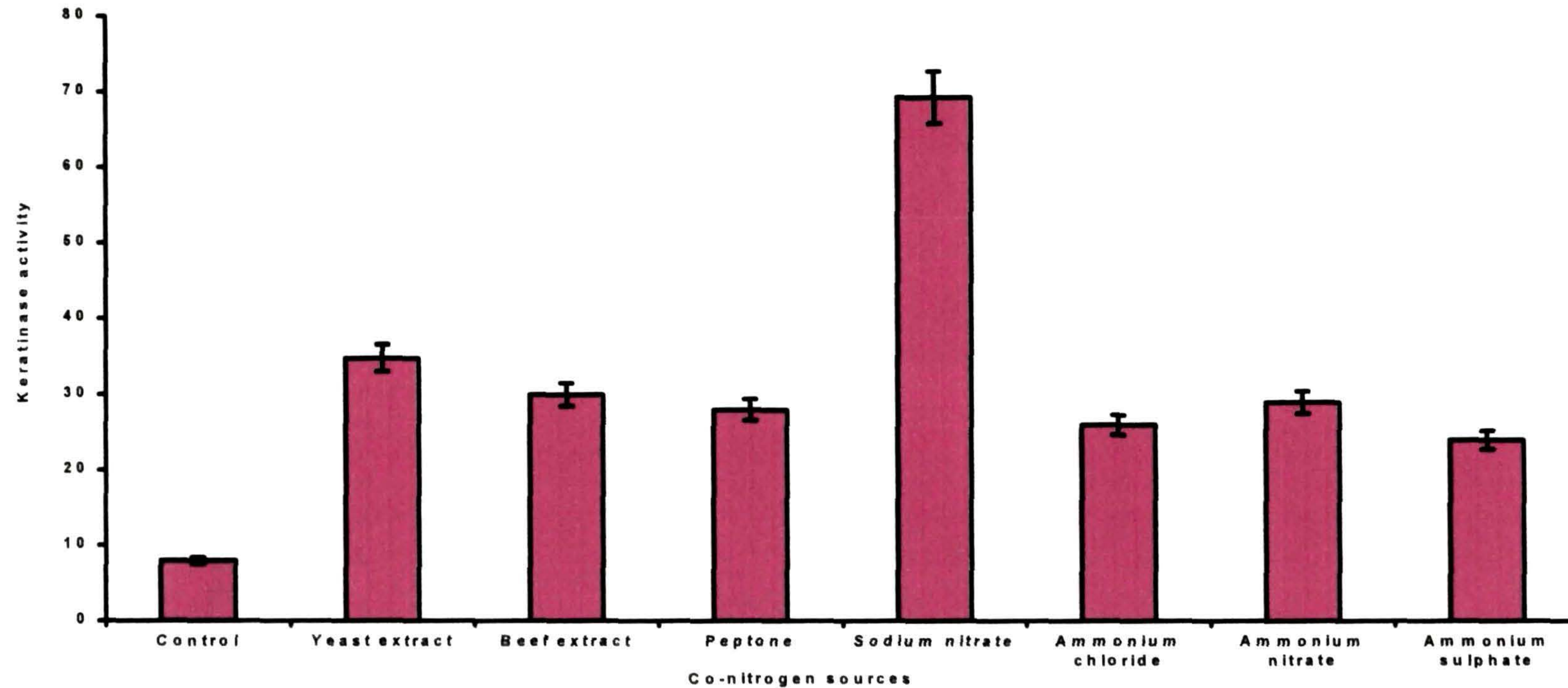


Fig.7.6 Effect of supplementation of co-nitrogen sources (1.0%, w/w) to chicken feather (substrate) for β-keratinase production by *B. subtilis* strain RM-01. Values are mean ± S.D. of three experiments.

7.1.6. Extraction of alkaline β-keratinase from fermented matter

Among the different extractions medium used for the recovery of protease from the fermented matter, distilled water containing 0.1%(v/v) triton X-100, pH 8.0 served as best extraction medium (Fig.7.7).

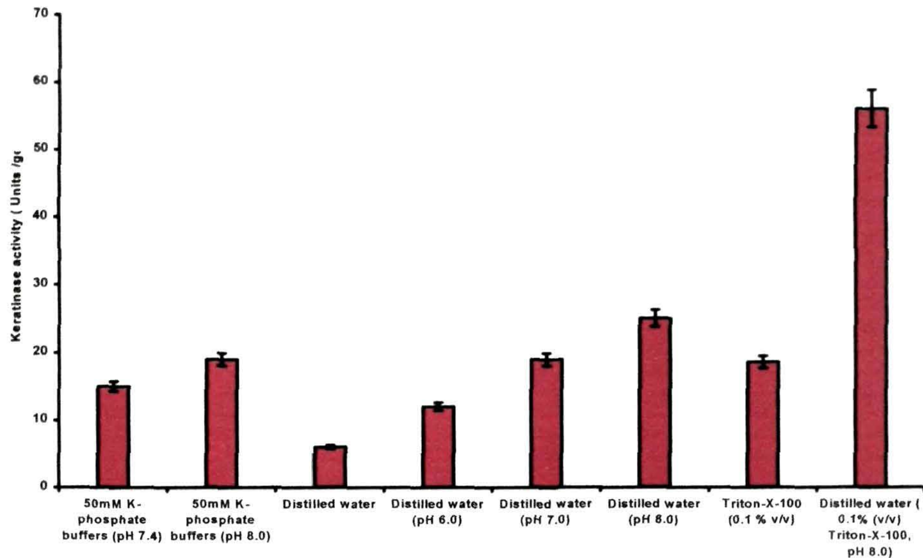


Fig.7.7 Screening of best extraction medium for maximum alkaline β-keratinase extraction from fermented material. Values are mean ± S.D. of three experiments.

7.1.7. Batch fermentation

Maximum β-keratinase production (95 U/gds) under statistically optimized condition was observed post-72 h of incubation in contrast to 96 h during the initial screening process (non-optimized condition). The β-keratinase production under optimized condition remained almost constant up to 96 h of bacterial growth and thereafter, a decline in β-keratinase production was observed.

7.2 Production of alkaline β-keratinase production under submerged fermentation (SmF)

7.2.1. Effect of carbon source on alkaline β-keratinase production

Amongst the different carbon sources screened for alkaline β-keratinase production under submerged fermentation, maximum alkaline β-keratinase production was observed in presence of 1% (w/v) maltose followed by casein Fig.7.8.

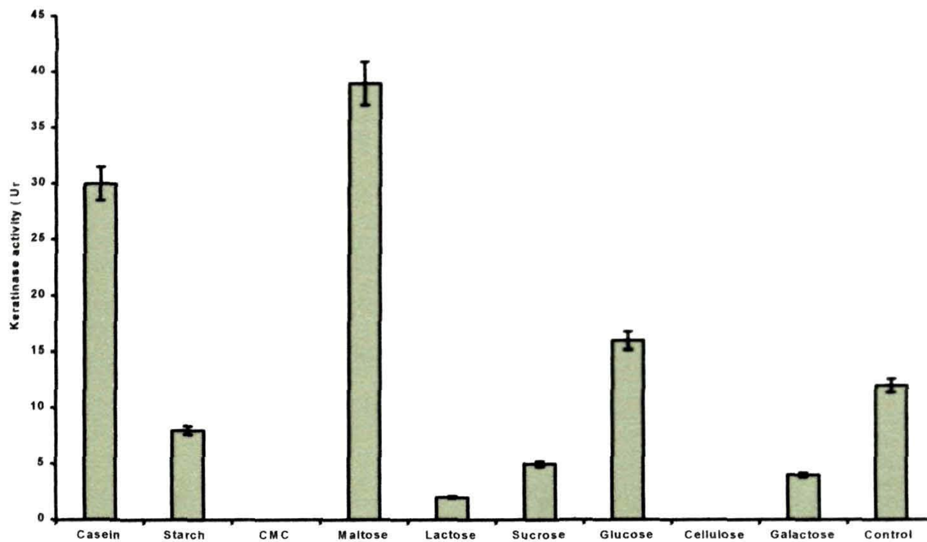


Fig.7.8 Effect of different carbon sources on alkaline β-keratinase production from *B.subtilis* strain RM-01 under SSF. Values are mean ± S.D. of three experiments.

7.2.2. Effect of nitrogen source on alkaline β-keratinase production

Various nitrogen sources were tested for alkaline β-keratinase production and maximum production was observed in presence of 0.1% (w/v) sodium nitrate (Fig.7.9).

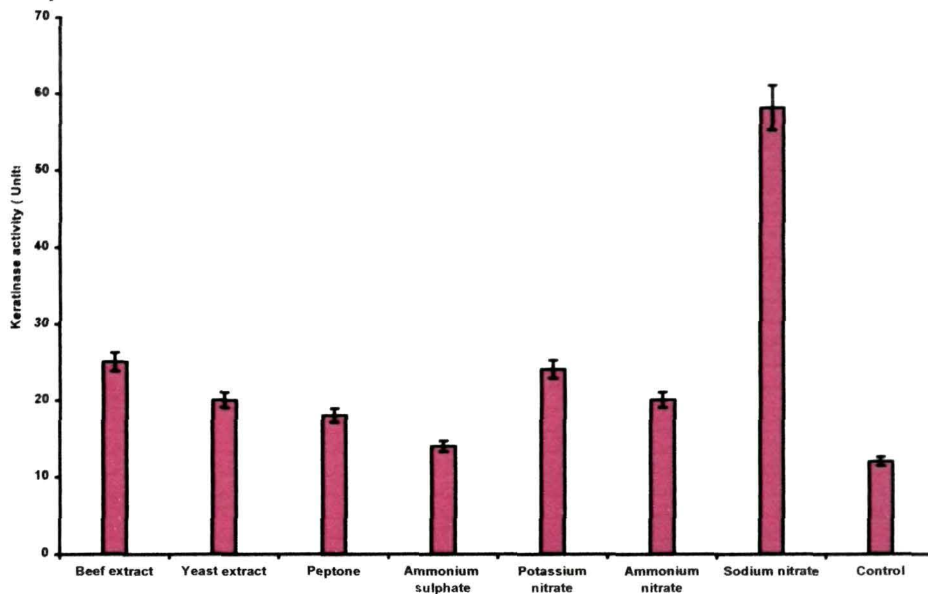


Fig.7.9 Effect of various nitrogen sources on alkaline β-keratinase production from *B.subtilis* strain RM-01 under SSF. Values are mean ± S.D. of three experiments.

7.2.3 Effect of pH on alkaline β-keratinase production

The effect of pH on alkaline β-keratinase production by *B.subtilis* strain RM-01 was determined by adjusting the medium pH and assaying the enzyme activity. It was observed that with an increase in the pH of the medium from 6.0 to 8.0, β-keratinase production enhanced concomitantly. However, a further increase in pH resulted in decline in β-keratinase yield (Fig.7.10).

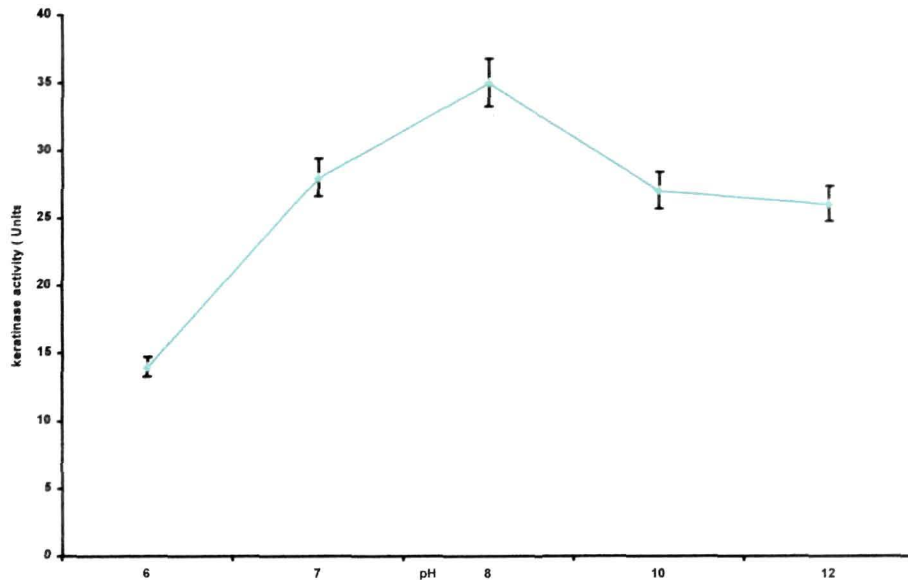


Fig.7.10 Effect of pH on alkaline β-keratinase production from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.

7.2.4. Effect of temperature on alkaline β-keratinase production

In the tested range of incubation temperature on alkaline β-keratinase production from *B.subtilis* strain RM-01, it was observed that β-keratinase production linearly increased from 25 to 50°C and beyond this temperature protease production was slightly declined (Fig.7.11). Therefore, 50°C was considered as optimum temperature for β-keratinase production by *B.subtilis* strain RM-01.

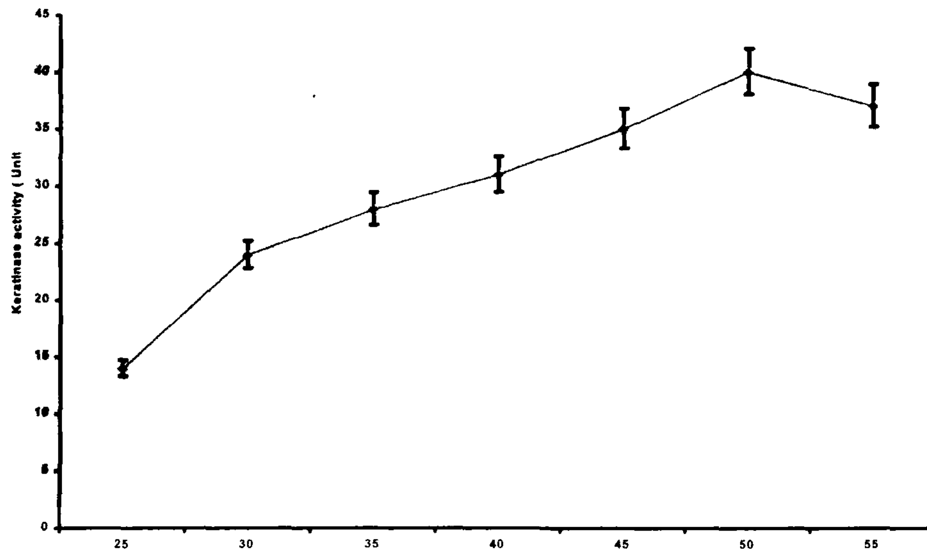


Fig.7.11 Effect of incubation temperature on alkaline β-keratinase production from *B.subtilis* strain RM-01 in SmF. Values are mean ± S.D. of three experiments.

7.2.4. Kinetics of alkaline β-keratinase production

Study of kinetics for alkaline β-keratinase production from *B.subtilis* strain RM-01 demonstrated maximum keratinase production by *B.subtilis* strain RM-01 attained post 96h of incubation (Fig. 7.12). Thereafter, a sharp fall in alkaline β-keratinase production was observed. The bacterial dry biomass and protein content of culture supernatant also displayed the similar trend.

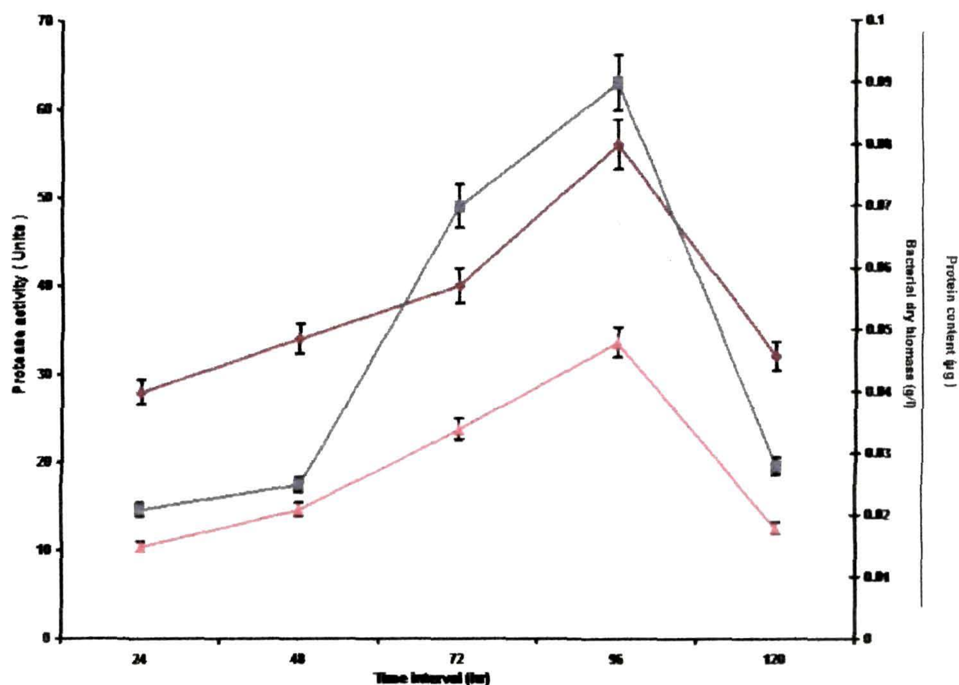


Fig.7.12 Effect of incubation time on alkaline β-keratinase production in SmF (■), bacterial dry biomass (●), and protein content (▲) from *B.subtilis* strain RM-01 under SSF. Values are mean± S.D. of three experiments.

7.2.5. Effect of agitation rate on alkaline β-keratinase production

Influence of agitation rate on the alkaline β-keratinase production displayed a significant increase in β-keratinase production (40U) by *B.subtilis* strain RM-01 at 45°C with a constant shaking at 200 rpm (Fig. 7.13).

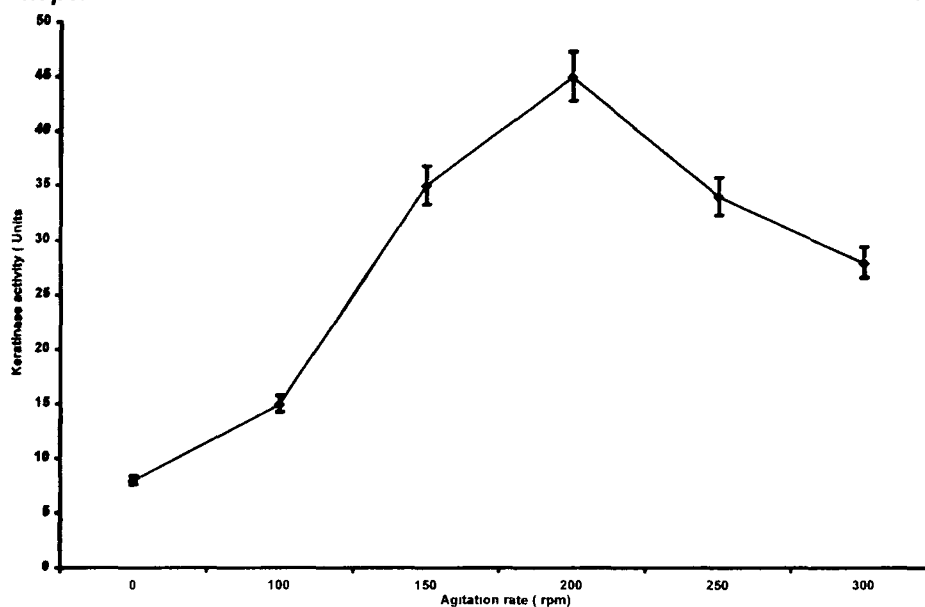


Fig.7.13 Effect of agitation rate on alkaline β-keratinase production from *B.subtilis* strain RM-01 under SmF. Values are mean ± S.D. of three experiments.

7.3. Statistical optimization of alkaline β-keratinase production under solid- state fermentation

7.3.1 Screening of effective key factors for alkaline β-keratinase production by Plackett-Burman design

Screening results displayed the significance of selected factors on the β-keratinase production (Table 7.1). The analysis of regression coefficients and t-value of six parameters showed that maltose, sodium nitrate, and pH of the medium displayed a positive effect for β-keratinase production whereas, innoculum level, incubation temperature and incubation time had a negative effect on enzyme production (Table 7.2). The model equation for β-keratinase production was derived (eq 7.1) neglecting the variables which were insignificant.

$$Y = 15.0 + 27.4X_1 + 7.9X_2 + 138.3X_3 \text{------} (7.1)$$

Where, Y = β-keratinase activity (U /gds), X_1 = pH of the medium, X_2 = maltose level, and X_3 = sodium nitrate level.

On the basis of calculated t- and p-values (Table 7.2), casein, potassium nitrate, and pH of the medium were chosen for further optimization. The innoculum level (% v/v) and incubation time (h) were kept at middle level.

Table 7.1 Plackett–Burman store design showing six variables with coded values along with the observed results for β-keratinase production by *B.subtilis* strain RM-01.

Maltose (% w/v)	Sodium nitrate level (% w/v)	Incubation time (h)	Incubation temperature (In °C)	pH of the medium	Innoculum size (% v/v)	β-keratinase yield (Units)
1 (15.0)	-1(1.20)	1 (96)	-1 (40)	-1 (7.0)	-1 (1.5)	38.0
1 (15.0)	1 (1.30)	-1 (48)	1 (50)	-1 (7.0)	-1 (1.5)	34.0
-1 (5.0)	1 (1.30)	1 (96)	- 1(40)	1 (9.0)	-1 (1.5)	25.0
1 (2.5)	-1 (1.20)	1 (96)	1 (50)	-1 (7.0)	1 (5.0)	54.0
1 (2.5)	1 (1.30)	-1 (48)	1 (50)	1 (9.0)	-1 (1.5)	67.0
1 (2.5)	1 (1.30)	1 (96)	-1 (40)	1 (9.0)	1 (5.0)	25.0
-1 (1.5)	1 (1.30)	1 (96)	1 (50)	-1 (7.0)	1 (5.0)	29.0
-1 (1.5)	-1 (1.20)	1 (96)	1 (50)	1 (9.0)	-1 (1.5)	64.0
-1 (1.5)	-1 (1.20)	-1 (48)	1 (50)	1 (9.0)	1 (5.0)	58.0
1 (2.5)	-1 (1.20)	-1 (48)	-1 (40)	1 (9.0)	1 (5.0)	68.0
-1 (1.5)	1 (1.30)	-1 (48)	-1 (40)	-1 (7.0)	1 (5.0)	67.0
-1 (1.5)	-1 (1.20)	-1 (48)	- 1 (40)	-1 (7.0)	-1 (1.5)	57.0
0 (2.0)	0 (1.25)	0 (72)	0 (45)	0 (8.0)	0 (2.5)	15.0
0 (2.0)	0 (1.25)	0 (72)	0 (45)	0 (8.0)	0 (2.5)	15.0
0 (2.0)	0 (1.25)	0 (72)	0 (45)	0 (8.0)	0 (2.5)	15.0

Table 7.2 Statistical analysis of Plackett–Burman design showing coefficient values, t- and P-value for each variable for keratinase activity (p-value <0.05).

Variables	**Keratinase yield (Units)**			
	Co-efficient	**SE Coef**	**t-Stat**	**p-value**
Intercept	15.0	0.5774	25.98	0.001
Incubation time (h)	-38.9	0.7217	-53.87	0.000
Innoculum level (%v/v)	-45.8	0.7638	-59.90	0.000
pH of the medium	27.4	0.7638	35.84	0.001
Agitation rate (rpm)	-133.6	2.6497	-50.43	0.000
Sodium nitrate (% w/v)	7.9	0.4330	18.19	0.003
Maltose level (% w/v)	138.3	2.6615	51.95	0.000

7.3.2 Optimization by response surface methodolgy

The results of the observed and predicted β-keratinase activity (response) as function of pH, co carbon level and co-nitrogen level with reference to the experiments performed according to the CCD are shown in Table 7.3. Maximum β-keratinase activity (95.3 U/gds) was obtained with the data set of pH 8, maltose level of 10%, and sodium nitrate of 1.25 %. The parameters of Eq. (3.2) were determined by multiple regression analysis by the application of RSM. The overall second-order polynomial regression equation showing the empirical relationship between keratinase activity (Y) and three test variables in coded units is represented by Eq. 7.2:

$$Y = 74.124 - 16.8722C_1 + 0.5889C_2 + 11.1889C_3 - 23.2994C_1^2 - 1.8661C_2^2 + 9.9339C_3^3 + 0.0250C_1C_2 + 0.0250C_1C_3 + 0.0292C_2C_3$$

------------- (7.2)

The coefficients of the model including the significance of each coefficient as determined by t test and p values are shown in Table 7.4. Results showed that all the effects of C_1, C_2 and C_3, except quadratic effects of C_3 (C_1C_3 and C_2C_3) are significant ($p<0.0001$). The ANOVA of the quadratic regression model demonstrated that the computed F value (Table 7.5) for linear regression was much greater than the tabulated (P)>F value (Table 7.5). The coefficient (R^2) and adjusted R^2 values were calculated as 100%.

Table 7.3 Observed and predicted values of alkaline β-keratinase production by *B.subtilis* strain RM-01 post 96 h of incubation at 50°C. The observed values are average of triplicate determinations ± S.D. Boundaries of the experimental domain and spacing of levels are expressed in coded (within parenthesis) and natural units C_1, pH of the medium ; C_2 , maltose level (% w/v); C_3, sodium nitrate (% w/v).

Independent Variables			Y Response (Keratinase activity in U/gds)	
C_1	C_2	C_3	Observed value	Predicted value
1(9.0)	-1(7.5)	-1(0.75)	64.25	64.068
-1(7.0)	-1(7.5)	0(1.0)	65.2	65.269
-1(7.0)	-1(7.5)	1(1.25)	86.3	86.338
-1(7.0)	0(10.0)	-1(0.75)	66.4	66.469
-1(7.0)	0(10.0)	0(1.0)	67.7	67.699
-1(7.0)	0(10.0)	1(1.25)	88.8	88.797
-1(7.0)	1(12.5)	-1(0.75)	65.1	65.138

Continued....

Table 7.3 Continued....

-1(7.0)	1(12.5)	0(1.0)	66.4	66.397
-1(7.0)	1(12.5)	1(1.25)	87.55	87.524
0(8.0)	-1(7.5)	-1(0.75)	70.35	70.446
0(8.0)	-1(7.5)	0(1.0)	71.65	71.671
0(8.0)	-1(7.5)	1(1.25)	92.8	92.765
0(8.0)	0(10.0)	-1(0.75)	72.85	72.871
0(8.0)	0(10.0)	1(1.25)	95.3	95.249
0(8.0)	1(12.5)	-1(0.75)	71.6	71.565
0(8.0)	1(12.5)	0(1.0)	72.9	72.849
0(8.0)	1(12.5)	1(1.25)	94	94.001
1(9.0)	-1(7.5)	-1(0.75)	30.2	30.224
1(9.0)	-1(7.5)	0(1.0)	31.5	31.475
1(9.0)	-1(7.5)	1(1.25)	52.6	52.593
1(9.0)	0(10.0)	-1(0.75)	32.7	32.675
1(9.0)	0(10.0)	0(1.0)	34	33.955
1(9.0)	0(10.0)	1(1.25)	55.1	55.103
1(9.0)	1(12.5)	-1(0.75)	31.4	31.393
1(9.0)	1(12.5)	0(1.0)	32.7	32.703
1(9.0)	1(12.5)	1(1.25)	53.8	53.880
0(8.0)	0(10.0)	0(1.0)	73.93	74.126
0(8.0)	0(10.0)	0(1.0)	74.45	74.126
0(8.0)	0(10.0)	0(1.0)	73.77	74.126
0(8.0)	0(10.0)	0(1.0)	74.18	74.126
0(8.0)	0(10.0)	0(1.0)	74.27	74.126

Table 7.4 Model coefficients estimated by multiple regression (model adequacy determination)

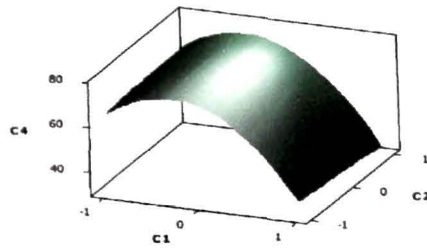
Factor	Coefficient	t-value	p-value
Constant	74.1264	1569.805	0.000<0.05
C_1	-16.8722	-540.816	0.000<0.05
C_2	0.5889	18.876	0.000<0.05
C_3	11.1889	358.644	0.000<0.05
$C_1 C_1$	-23.2994	-466.437	0.000<0.05
$C_2 C_2$	-1.8661	-37.357	0.000<0.05
$C_3 C_3$	9.9339	198.870	0.000<0.05
$C_1 C_2$	0.0250	0.654	0.520>0.05
$C_1 C_3$	0.0250	0.654	0.520>0.05
$C_2 C_3$	0.0292	0.763	0.454>0.05

R^2 = 100.00%, R^2 (pred) = 99.99%, R^2 (adj) = 100.00%.

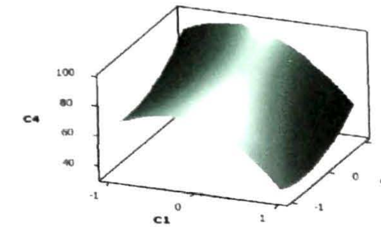
Table 7.5 Analysis of Variance (ANOVA) of alkaline β-keratinase produced by *B.subtilis* strain RM-01

Source	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
Regression	9	10842.7	10842.7	1204.75	32.82	0.000
Linear	3	6829.8	6593.3	2197.76	59.87	0.000
C_1	1	5231.5	4118.8	4118.78	112.21	0.000
C_2	1	31.4	10.4	10.38	0.28	0.600
C_3	1	1567.0	1627.9	1627.88	44.35	0.000
Square	3	3872.9	3914.3	1304.77	35.55	0.000
$C_1 * C_1$	1	2988.7	3455.4	3455.40	94.14	0.000
$C_2 * C_2$	1	11.0	5.5	5.52	0.15	0.702

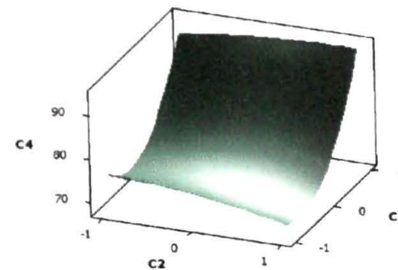
Source	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
$C_3 * C_3$	1	873.2	824.7	824.73	22.47	0.000
Interaction	3	140.0	140.0	46.66	1.27	0.310
$C_1 * C_2$	1	42.8	30.5	30.47	0.83	0.373
$C_1 * C_3$	1	49.5	30.5	30.47	0.83	0.373
$C_2 * C_3$	1	47.6	47.6	47.65	1.30	0.267
Residual error	21	770.8	770.8	36.71		
Lack-of-Fit	16	190.8	190.8	11.93	0.10	1.000
Pure error	5	580.0	580.0	116.00		
Total	30	11613.6				



(a)



(b)



(c)

Fig.7.14 Response surface plots for alkaline β-keratinase production by *B.subtilis* strain RM-01. The interaction between (a) pH of the medium and concentration (%, w/v) of maltose, hold value C_3=0 (b) pH of the medium vs concentration (%, w/v) of sodium nitrate, hold value C_2=0 and (c) concentration (%, w/v) of maltose vs concentration of sodium nitrate (% w/v) hold value C_1=0. Values are mean ± S.D. of three experiments.

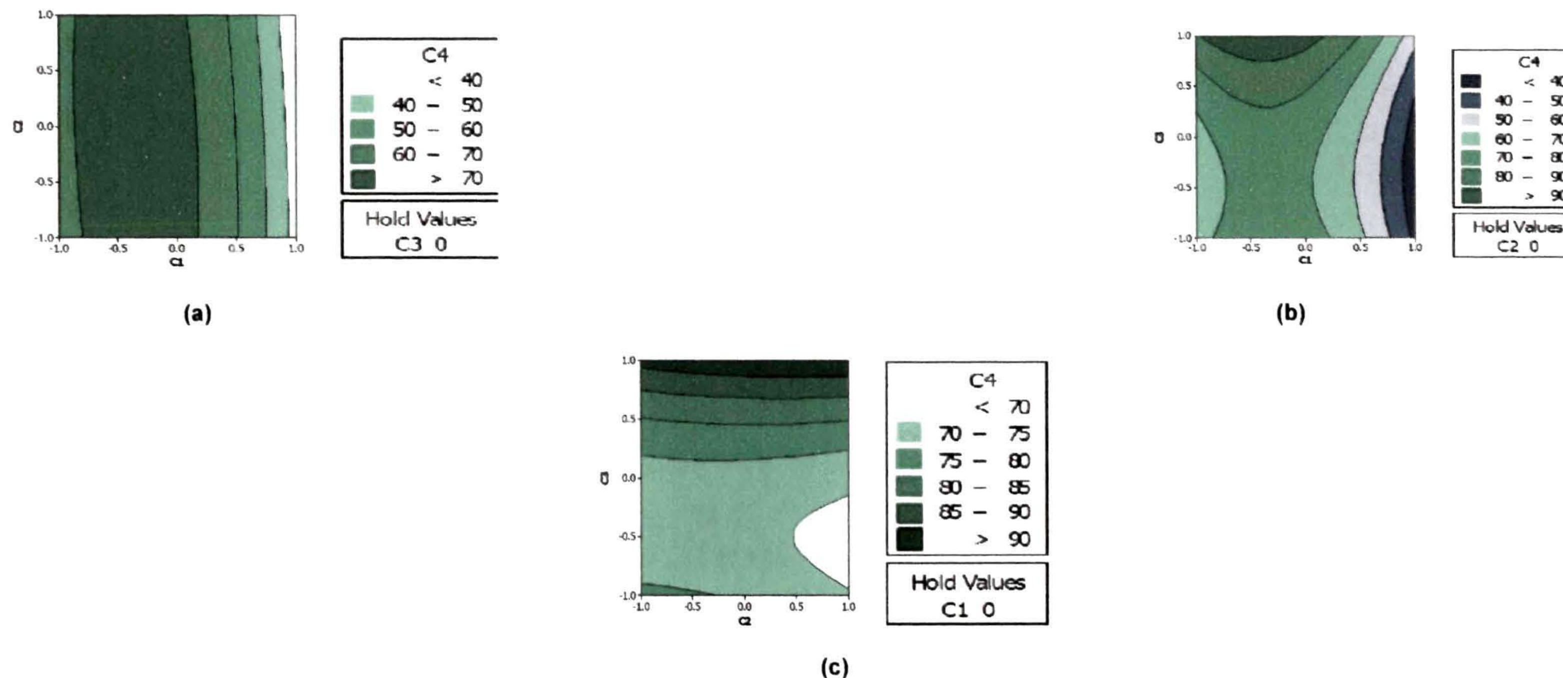


Fig.7.15 Countor plots for alkaline β-keratinase production by *B.subtilis* strain RM-01. The interaction between (a) pH of the medium and concentration (%, w/v) of maltose, hold value C_3=0 (b) pH of the medium vs concentration (%, w/v) of sodium nitrate, hold value C_2=0 and (c) concentration (%, w/v) of maltose vs concentration of sodium nitrate (% w/v) hold value C_1=0. Values are mean ± S.D. of three experiments.

7.3.1 Response surface plots

The three-dimensional response surface plots as a function of two factors at a time, maintaining the third factor at the minimum (zero) level, are depicted in Figs. 7.14a–c. A small trough in response surface plot (Fig. 7.14a) indicated an initial decrease in alkaline β-keratinase production with the initial increase of maltose level. Figure 7.14b shows that alkaline β-keratinase production was increased with an initial increase in sodium nitrate concentration. Interaction of maltose and sodium nitrate suggests, increase in maltose level resulted in a decrease β-keratinase yield, whereas an increase in sodium nitrate level significant enhanced the β-keratinase production (Fig.7.14c).

7.3. 2. Countor plots

The maximum predicted value is indicated by the smallest ellipse in the contour diagram Figs.7.15a–c shows the 2D contour plots of protease production for each pair of variables by keeping the other three variables constant at its middle level. It could be seen that the protease production increased upon increasing the initial pH from 7.0 to 9.0, but any further increase in its values resulted in decreased alkaline β-keratinase production. Therefore, the optimal initial pH was at around 8.0. Similarly, the alkaline β-keratinase production increased with increasing the sodium nitrate from 0.5 to1.5 % (w/v), and any further increase in its values resulted in decreased alkaline β-keratinase production also the optimal maltose concentration was around 10.0 % (w/v).

7.4. Batch fermentation under optimized condition

Maximum β-keratinase production under statistically optimized condition was observed post 72 h of incubation (Fig. 7.16) in contrast to 96 h during the initial screening process (see section 7.1.1). The β-keratinase production under optimized condition remained almost constant up to 96 h

of bacterial growth, demonstrating that the accumulated β-keratinase was stable over time (Fig.7.16).

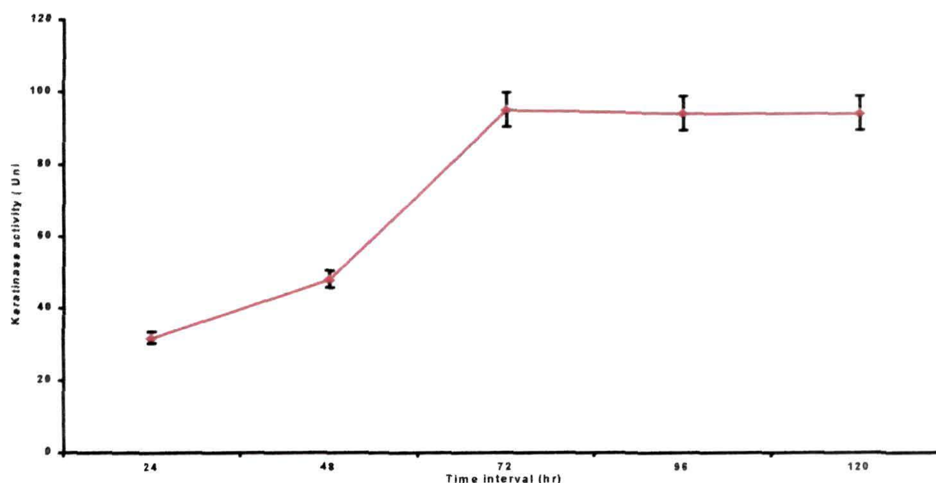


Fig.7.16 Batch fermentation for alkaline β-keratinase production from *B.subtilis strain RM-01 under statistically optimized conditions in SSF.* Values are mean ± S.D. of three experiments.

7.5 Isolation and purification of β-keratinase from *B.subtilis* strain RM-01

Fractionation of CM-cellulose unbound β-keratinase (peak CM-I as shown in Fig.7.17) on a gel filtration column resulted in its separation into five peaks GF-I to GF-V (Fig.7.18). The peak GF-III containing proteins in the molecular weight range of 15–22 kDa showed maximum β -keratinase activity and was separated into six major peaks (HP-I to HP-VI) on a reverse phase C_{18} HPLC column (Fig.7.19). The peak with the retention time of 12.57min (HP-IV) showing β -keratinase activity was found to be homogenous by SDS-PAGE. About 15 µg of protein (HP-IV), both under reducing and non-reducing condition, yielded a single Coomassie stained band of apparent molecular weight 20.1 (Fig.7.20). A summary of purification of this β-keratinase is displayed in Table 7.6.

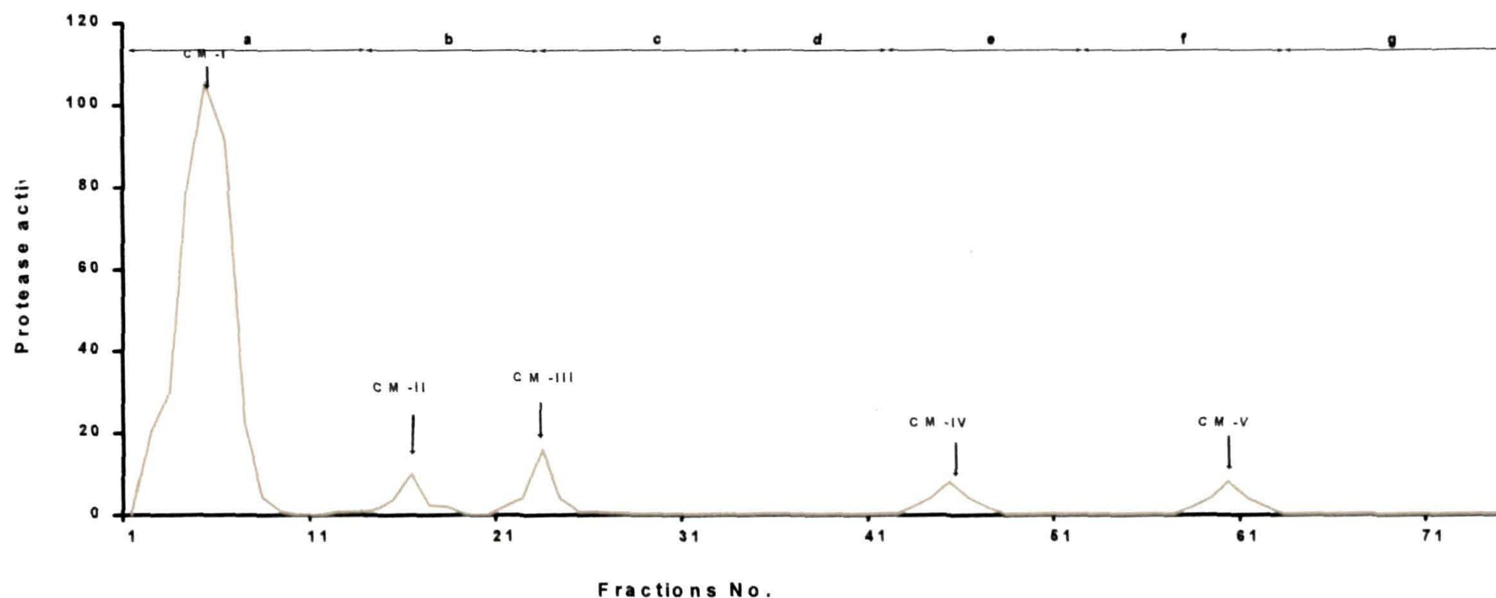


Fig.7.17 CM-Cellulose profile of cationic keratinase secreted from *B.subtilis* strain RM-01. Buffers used for step elution were: (a) 100 mM K-phosphate buffer, pH 7.5 ; (b) 100 mM K-phosphate buffer, pH8.0 ; (c) 150 mM K-phosphate buffer,pH8.0 ; (c) 200 mM K-phosphate buffer, pH 8.0; (d) 200 mM K-phosphate buffer, pH 8.0 ; (e) 300 mM K-phosphate buffer, pH 8.0 ; (f) 300 mM K-phosphate buffer, pH 8.5. Data represent a typical experiment.

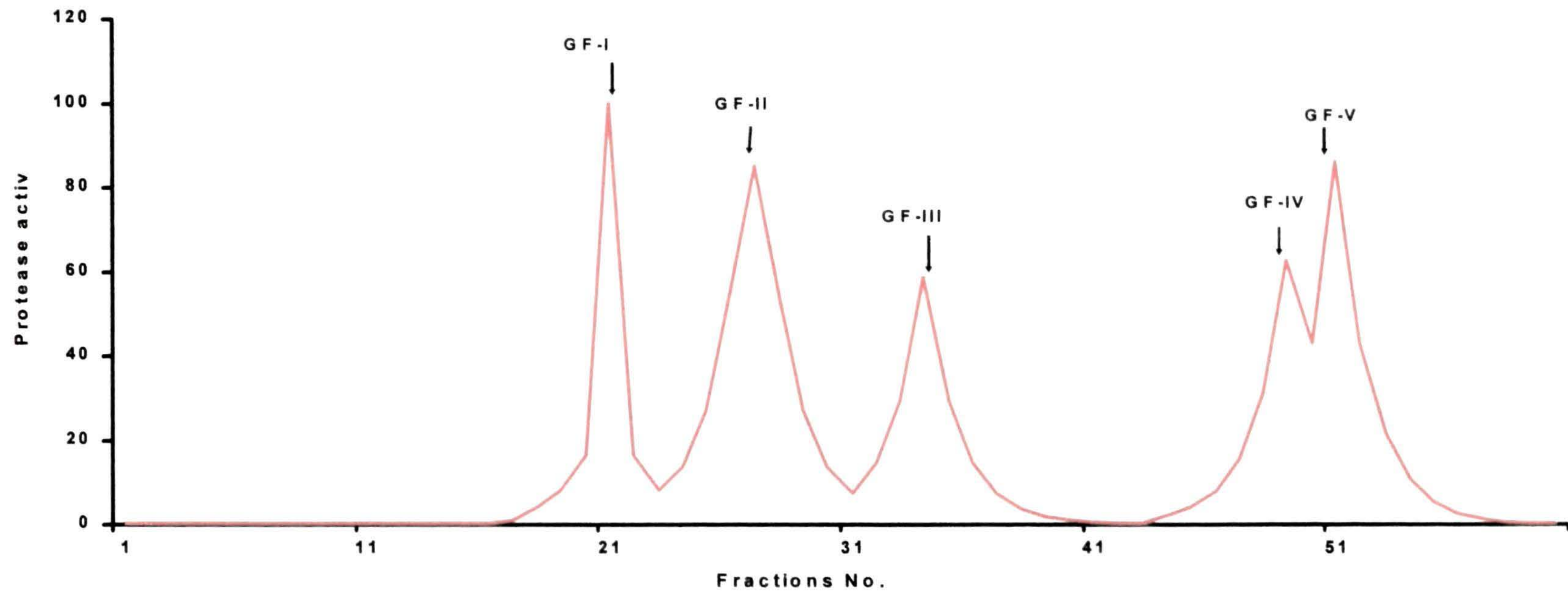


Fig.7.18 Gel-filtration profile of CM-1 (non-retained by CM-cellulose) fraction from *B.subtilis* RM-01 bacterial strain on Sephacryl S-200 column. Data represent a typical experiment.

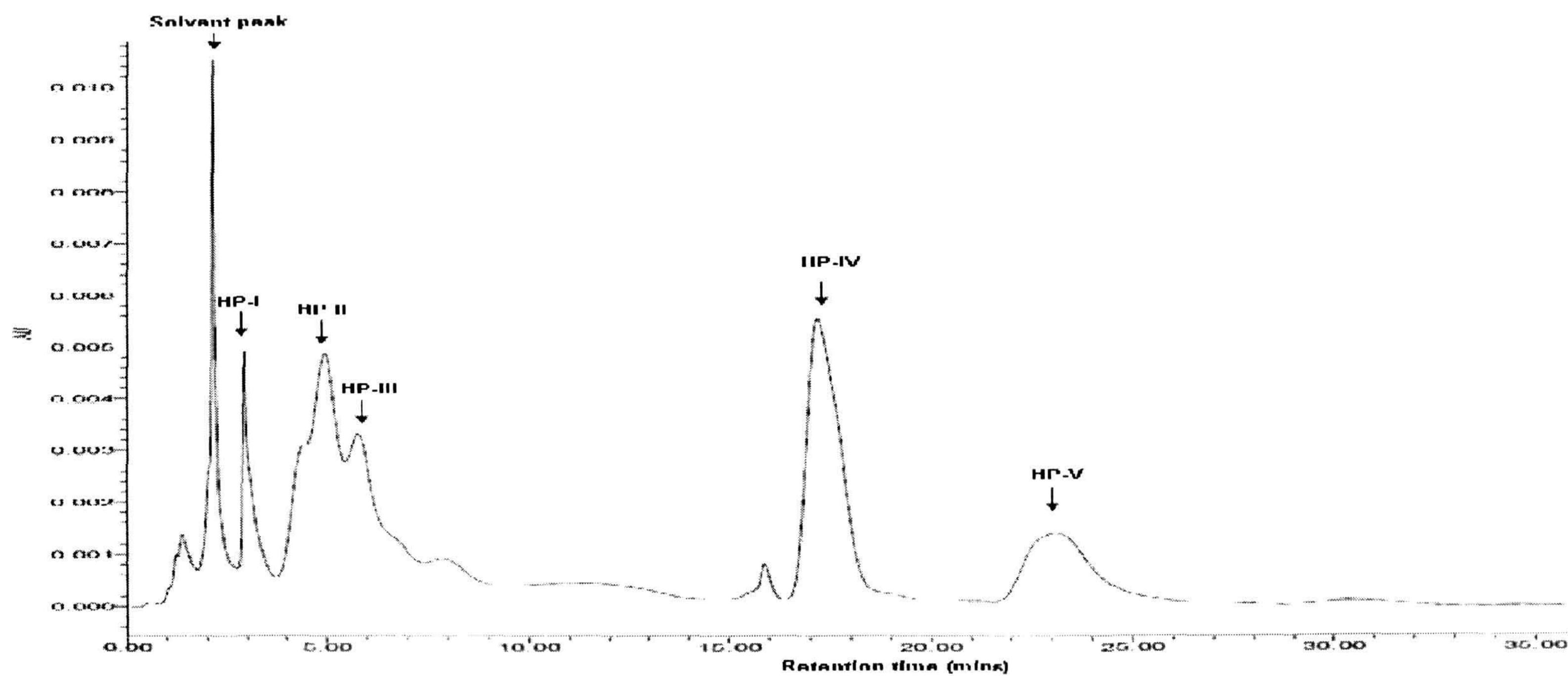


Fig.7.19 RP-HPLC profile of fraction GF-III. The HPLC experiment condition is described in the text. Data represent a typical experiment.

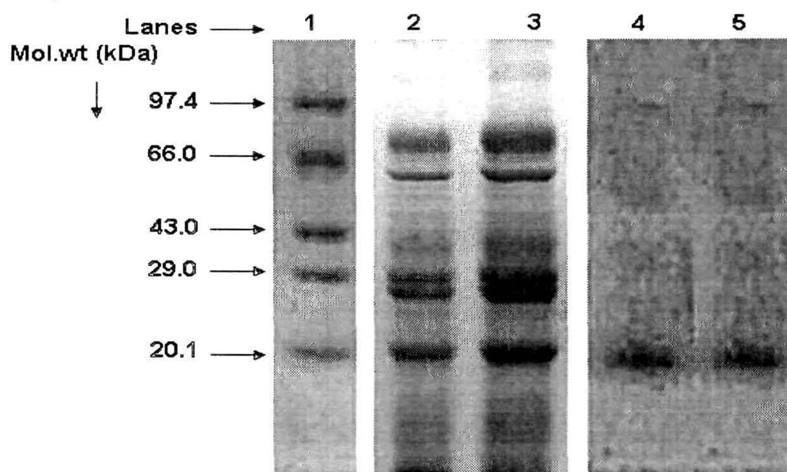


Fig.7.20 SDS-polyacrylamide gel (12.5%) electrophoresis of purified β-keratinase. Lane 1, Protein molecular weight standards: phosphorylase b (97.4 kDa), BSA (66.0 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and soybean trypsin inhibitor (20.1 kDa); lane 2 reduced crude β-keratinase (30 µg); lane 3 non-reduced crude β-keratinase (30 µg); lane 4 reduced β-keratinase (15 µg); lane 5 non-reduced β-keratinase (15 µg).

Table 7.6 Summary of purification of β-keratinase from *B.subtilis* strain RM-01. Results represent a typical experiment.

Purification step	Total protein (mg)	Total activity (Units)	Yield of protein (%)	Specific activity (units/mg)	Purification (fold)
Cell-free supernatant	180.0	134694.0	100	748.3	I
CM-I	4.0	3658.0	2.2	915.0	1.2
GF-III	1.2	3412.0	0.6	2843.0	3.8
HP-IV	0.5	3400.0	0.3	6800.0	9.1

7.6 Biochemical characterization of alkaline β-keratinase from *B.subtilis* strain RM-01

7.6.1 Effect of pH and temperature on alkaline β-keratinase activity

The enzyme showed activity at pH range of 7.0–11.0 and at 25–55°C temperature range; however, optimum activity was observed at pH 8.0 and at a temperature of 45°C (Fig.7.21 and Fig.7.22, respectively).

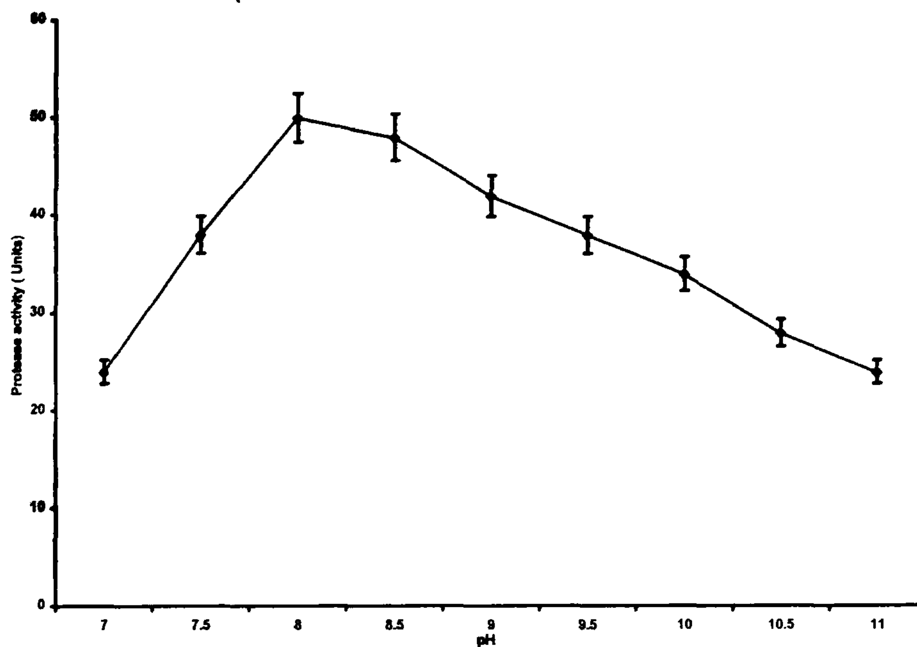


Fig.7.21 Optimum pH requirement for alkaline β-keratinase from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.

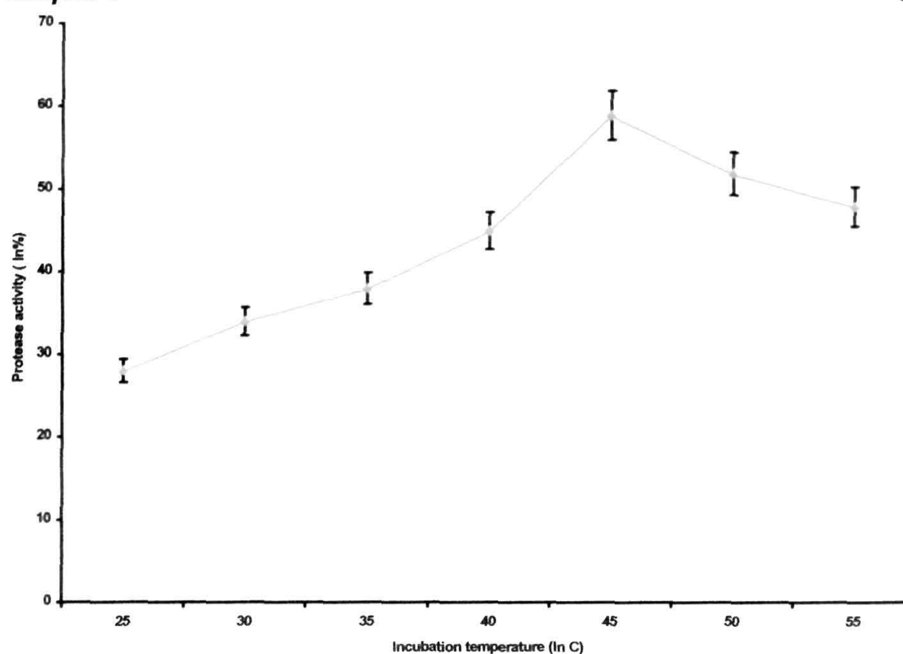


Fig.7.22 Effect of incubation temperature on alkaline β-keratinase activity from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.

7.6.2 Substrate specificity study

Amongst the tested substrates, chicken-feather keratin was found to be the most preferred substrate for this enzyme followed by casein; human hair keratin was least hydrolyzed by this enzyme (Fig.7.23).

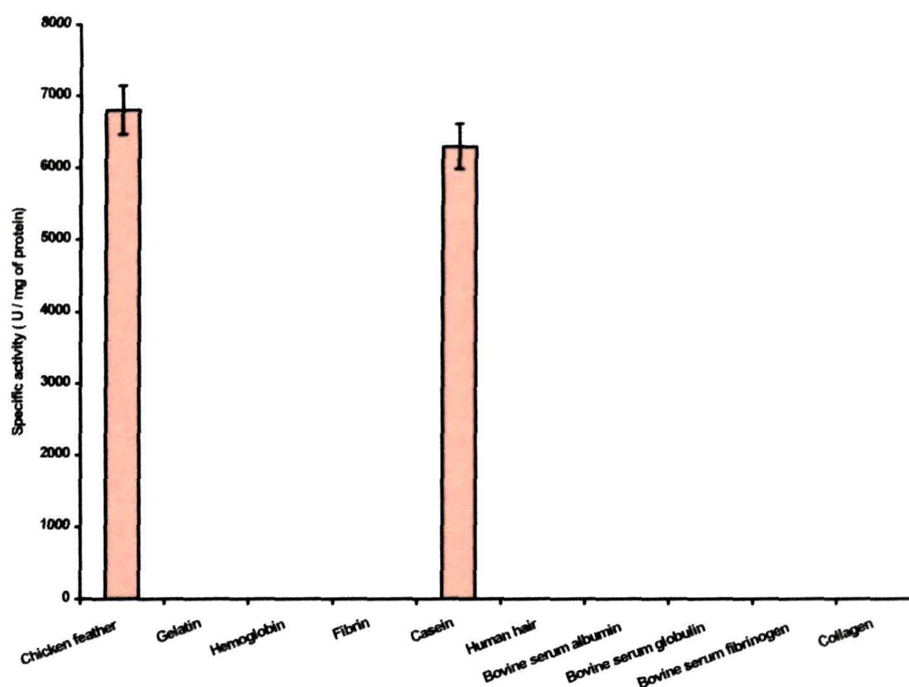


Fig.7.23 Substrate specificity study of purified alkaline β-keratinase from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.

7.6.3 Thermo-stability study of purified alkaline β-keratinase

The β-keratinase purified from *B. subtilis* strain RM-01 retained 100% stability of its original activity post-heating at 60°C for 30 min and thereafter there was a steady decline of upto 60% the enzyme activity (Fig.7.24).

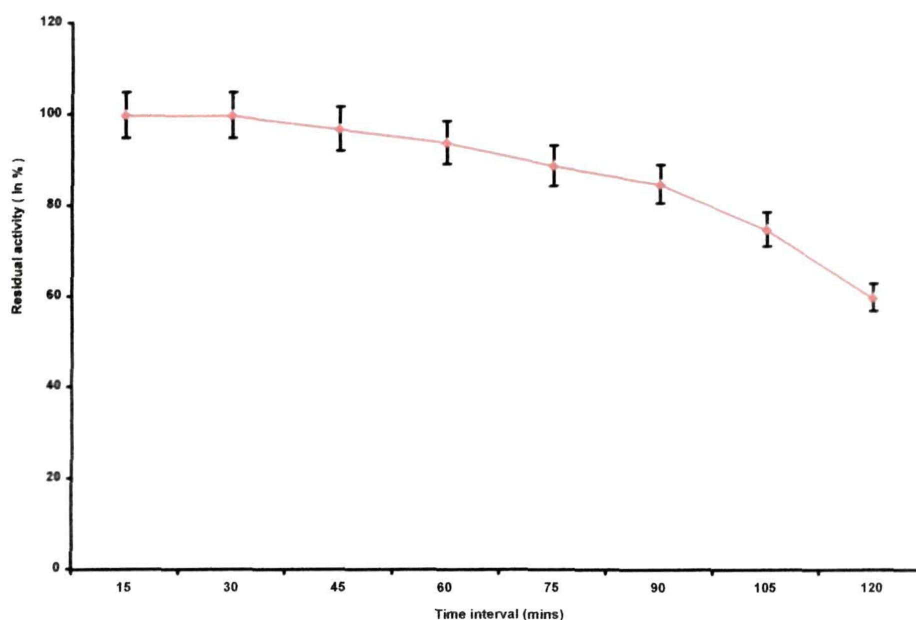


Fig.7.24 Thermo-stability of alkaline β-keratinase from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments. Using chicken-feather keratin as a substrate.

7.6.4 K_m and V_{max} values of purified alkaline β-keratinase

The K_m and V_{max} values of purified β-keratinase towards soluble keratin were determined as 5.0 mg /ml and 1.25 µmol /min / mg, respectively (Fig.7.25).

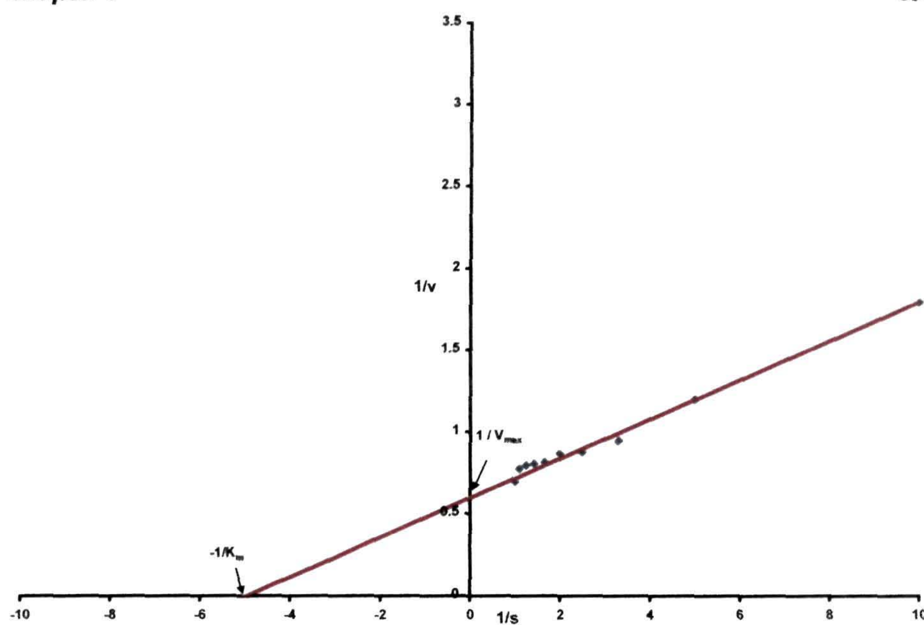


Fig. 7.25 Lineweaver Burk plot to determine the K_m and V_{max} values of purified alkaline β-keratinase from *Bacillus subtilis* strain RM-01.

7.6.5 Effect of metal ions on alkaline β-keratinase activity

All the tested metal ions, except Fe^{2+}, were shown to inhibit the enzyme activity. About 150% (1.5 fold) enhancement of β-keratinase activity was observed in presence of Fe^{2+} (Fig.7.26).

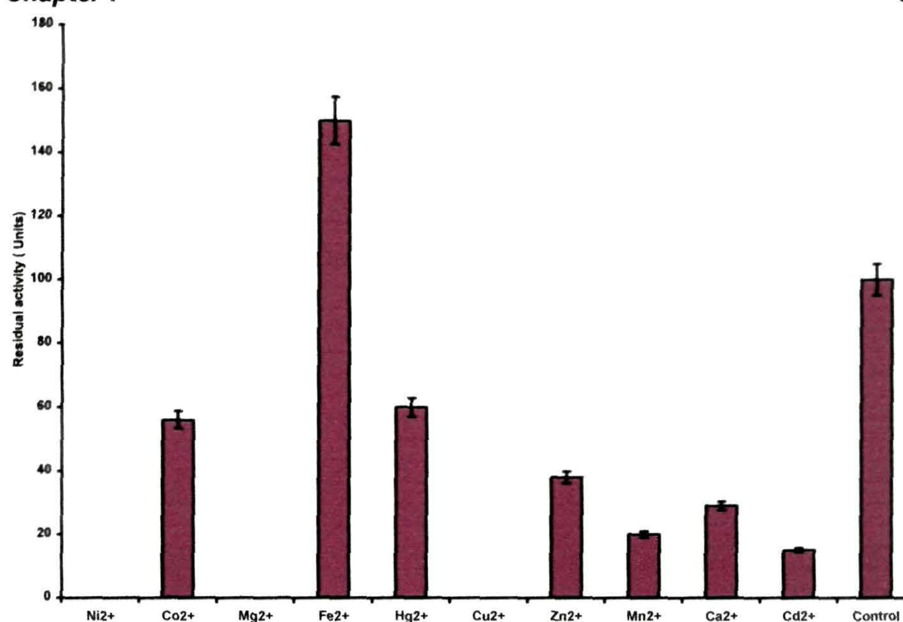


Fig. 7.26 Effect of metal ions on alkaline β-keratinase activity from *B.subtilis* strain RM-01. Values are mean ± S.D. of three experiments.

7.6.6 Effect of denaturing agents, oxidizing and bleaching agents, inhibitors, chelators and surfactants on alkaline β-keratinase activity

The β- keratinase from *B. subtilis* strain RM-01 retained more than 80% of its original activity post-treatment with EDTA, surfactants, oxidizing and bleaching agents (Table 7.8), whereas the enzyme activity was significantly effected by PMSF, IAA and 4-bromophenacyl bromide thus suggesting a role for serine, cysteine, and histidine residues in the catalysis process (Table 7.7).

Table 7.7 Effect of denaturing agent, oxidizing and bleaching agents, inhibitors, chelators, and surfactants on purified β-keratinase from *Bacillus subtilis* strain RM-01 at pH 8.0 and 45°C temperature. Values are mean ± S.D. of three experiments.

Parameters	Values
Control	100
Inhibitors (1 mM)	
PMSF	10.5 ± 1.0
4-Bromophenacyl bromide	18.3 ± 1.8
IAA	2.8 ± 0.2
Chelator (1 mM)- EDTA	84.3 ± 3.0
Anionic surfactant (SDS)	
40mM	100.0
60mM	94.0 ± 2.0
Non-ionic surfactants (1% v/v)	
Trition-X-100	87.4 ± 8.0
Tween 20	82.9 ± 4.0
Tween 80	100.0
Denaturing agent	
Urea (8 M)	91.6 ± 8.0
Oxidizing agent	
H_2O_2 (% v/v)	
5.0	110.0 ± 2.2
10.0	100.0
Bleaching agent	
Sodium perborate (% v/v)	
0.5	108.0 ± 2.2
1.0	99.0 ±1.9

7.6.7 The stabilizing effect of polyols against heat –denaturation of alkaline β-keratinase

Thermo-stability of enzyme could also be enhanced by modification of the environment, such as addition of various polyols or simple sugars. Effect of polyols on the thermo-stability property of alkaline β-keratinase from *B.subtilis* strain RM-01 is shown in (Fig.7.27). Thermo-stability of alkaline β-keratinase at 60°C was significantly improved post addition of polyols into the reaction media. The highest improvement in stability (relative activity of 138.0%) was obtained with glycerol which was 1.3 fold higher as compared with control. On the other hand, xylitol did not play any role in improving the thermostability of the β-keratinase.

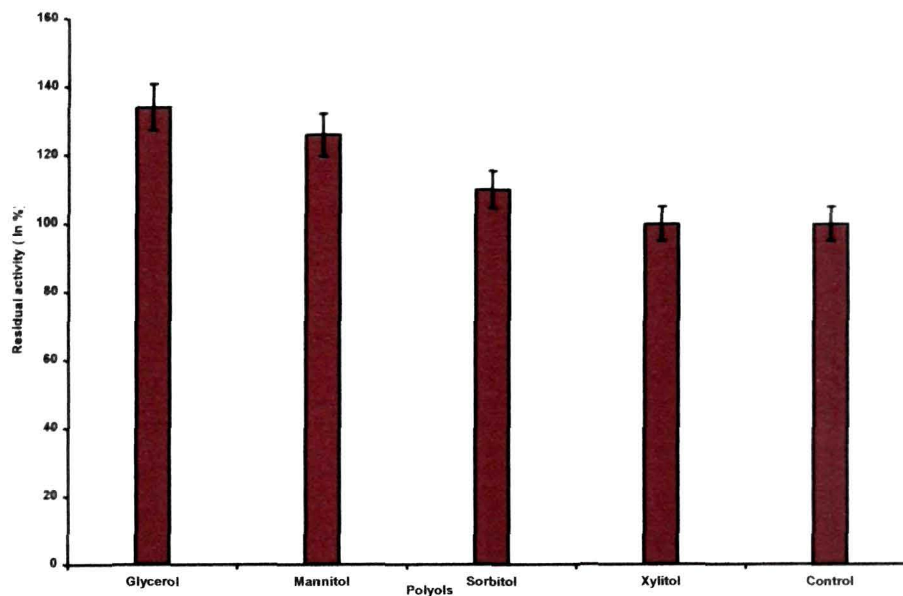


Fig.7.27 Effect of polyols on alkaline β-keratinase stability post heating at 60°C for 30 min. The enzyme activity without polyols served as control (100%) activity. Values are mean ± S.D. of three experiments.

7.7.8 Effect of organic solvent on alkaline β-keratinase activity

The enzyme was stable to all the organic solvents tested after 90 min incubation (Fig. 7.28). In presence of organic solvents such as xylene , methanol, n-hexane, 2-propanol, acetonitrile, ethanol and benzene (20%, v/v) the activity of alkaline β-keratinase was observed as 130%, 109%, 102%, 99%, 90% and 75%, respectively when compared to the control. Therefore, β-keratinase in the present study showed different tolerancy profile for different organic solvents.

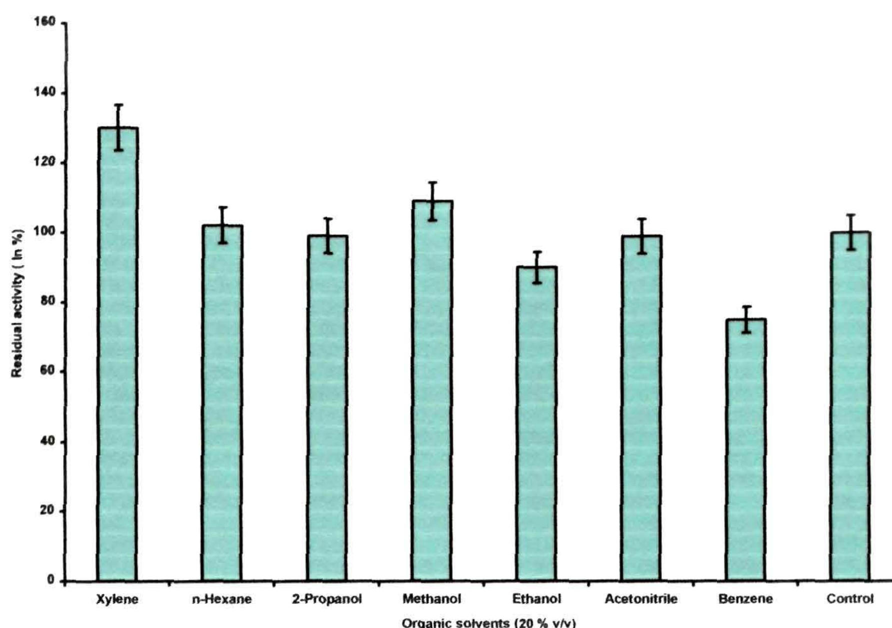


Fig.7.28 Organic solvent stability of alkaline β-keratinase from *B.subtilis* strain RM-01. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean ± S.D. of three experiments.

7.7 Pharmacological properties of alkaline β-keratinase

Purified β-keratinase from *Bacillus subtilis* strain RM-01 at a dose of 7.0 µg/ml caused 3.0% hemolysis of the washed human erythrocytes. Moreover, this enzyme did not display any tissue-damaging effect on goat liver, heart, lungs and kidney tissues (Table 7.8). Further, as shown in Table 7.8, the normal clotting time of goat platelet- poor plasma (PPP) was insignificantly ($p>0.05$) effected by the purified β-keratinase from *B.subtilis* strain RM-01.

Table 7.8 Pharmacological properties of purified β-keratinase from *Bacillus subtilis* strain RM-01. Values are mean ± S. D. of three determinations.

Pharmacological properties	Values
Hemolysis (% Hb released / 15.0 µg of β-keratinase 90 min post incubation at 37 °C)	
Control (without β-keratinase)	1.4 ± 0.1
Treatment	3.0 ± 0.5
Ca-Clotting time (s)	
Control (without β-keratinase)	129.0 ± 6.5
Treated (with 15.0 µg / ml of β-keratinase)	132.0 ± 7.0
In vitro tissue damaging activity (% hemoglobin release by 15.0 µg / ml of β-keratinase 5 h post incubation at 37 °C)	
a) ***Heart***	
Control (without β-keratinase)	0.09 ± 0.01
Treatment	0.27 ± 0.01
b) ***Lung***	

Pharmacological properties	Values
Control (without β-keratinase)	0.13 ± 0.01
Treatment	0.36 ± 0.02
c) ***Liver***	
Control (without β-keratinase)	0.16 ± 0.08
Treatment	0.28 ± 0.01
d) ***Kidney***	
Control (without β-keratinase)	0.12 ± 0.05
Treatment	0.27 ± 0.01

7.8 Binding efficiency and specific activity of β-keratinase

The cyanamide treatment of iron-oxide MNPs (method II) prior to enzyme coupling was found to be a better method as compared to aminofunctionalization of MNP (method I) when we considered the (i) binding efficiency of β-keratinase onto MNPs, and (ii) increase in the specific activity of the enzyme post immobilization onto iron-oxide MNPs (Table 7.9).

Table 7.9 A comparison of binding efficiency (under non-optimized condition) of β-keratinase onto iron-oxide MNP and increase in specific activity of the enzyme post binding to MNP by two different methods. Values are mean ± S.D. of three different experiments.

Immobilization method	Enzyme binding (%)	Specific activity (U/mg protein)	Increase in specific activity* (%)
Method I#	69 ± 4.8	27200 ± 29	4
Method II+	81 ± 6.6	55080 ± 22	8.1

* Compared to specific activity of free β-keratinase

Treatment of MNP with #3-(amino propyl) triethoxy silane and +cyanamide prior to enzyme coupling.

7.9 Statistical optimization of iron-oxide MNP β-keratinase binding

Table 7.10 shows the observed and predicted values of Y response (specific activity). The maximum Y response was observed when 25 mg MNP was coupled with β-keratinase at pH 8.0. The response surface and contour plots (Fig.7.29a and 7.29b) have shown that both the tested factors (amount of MNP and pH of 20 mM Tris-HCl buffer) independently exerted their influence in both linear and quadratic manner onto the specific activity of the iron-oxide MNP-coupled β-keratinase. However, their (C_1 and C_2) interaction effect was not significant ($p > 0.05$) (Table 7.11). The overall second-order polynomial equation for keratinase activity may be represented as

$$Y = 60097.5 - 14099.0 C_1 - 5008.98 C_1^2 + 6454.98 C_2 - 17785.1 C_2^2 \quad \text{...... (7.3)}$$

Table 7.10 Observed and predicted values of Y response by RSM. The observed values are mean of triplicate determinations. Data within bracket indicates the coded value.

Run no.	Independent Variables		Y Response (Keratinase activity U/mg)		Residual value
	C_1	C_2	Observed	Predicted	
1	25 (-1)	7.5 (-1)	45184.7	44947.510	237.190
2	25 (-1)	8.0 (0)	68713.2	69187.614	-474.414
3	25 (-1)	8.5 (1)	58094.7	57857.476	237.224
4	50 (0)	7.5 (-1)	35383.0	35857.381	-474.381

...Continued

Table 7.10 Continued......

5	50 (0)	8.5 (1)	48292.9	48767.348	-474.448
6	50 (0)	8.0 (0)	61130.6	60097.486	1033.114
7	50 (0)	8.0 (0)	60013.2	60097.486	-84.286
8	75 (1)	7.5 (-1)	16986.7	16749.510	237.190
9	75 (1)	8.0 (0)	40515.2	40989.614	-474.414
10	75 (1)	8.5 (1)	29896.7	29659.476	237.224

The sample coefficient of determination (R^2) measures the closeness of fit of the sample regression equation to the observed value of Y. As shown in Table 7.11, the value of R^2 indicated that more than 99% of sample variations for specific activity of the iron-oxide MNP-coupled β-keratinase were explained by the two independent variable chosen. The analysis of variance (ANOVA) by Fisher's statistical test was conducted for the second-order response surface model and the larger magnitude of the t value and smallest *p* value vouched for the significance of the corresponding coefficient of the model (Table 7.11).

Table 7.11 Model coefficients estimation by multiple regression analysis (model adequacy checking).

Factor	Coefficient	SE coefficient	Computed t-value	p-value
Constant	60097.5	443.2	135.605	0.0
C_1	-14099.0	302.7	-46.570	0.0
C_2	6454.98	302.7	21.321	0.0
C_1^2	-5008.87	485.5	-10.317	0.0
C_2^2	-17785.1	485.5	-36.634	0.0
$C_1.C_2$	-0.0	370.8	-0.000	1.0

R^2 =99.9 R^2 (pred) = 99.4, R^2 (adj) = 99.7

(a) **(b)**

Fig.7.29 Legend shows response surface plot (a) and contour plot (b) of specific activity of iron-oxide MNP bound β-keratinase vs pH of buffer (Tris-HCl) system used. C_1, amount of MNP (in coded value), C_2, pH of Tris-HCl (in coded value), and C_3 (specific activity in U/ mg).

7.9.1. Response surface plots

In order to determine the optimal levels of each variable for iron-oxide MNP bound β-keratinase and pH of buffer (Tris-HCl), three-dimensional response surface plots were constructed by plotting the response (protease production) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels. As is shown in Fig.7.29a, a linear increase in iron-oxide MNP was lead to decrease in specific activity of bound β-keratinase. In contrast, significant increase in pH of the Tris-HCl buffer system enhanced the specific activity (Fig.7.29a).

7.9.2. Countor surface plots

The 2D countor surface plots are generally the graphical representations of the regression equation. The 2D response surface plots are presented in Figure 7.29b from which the values of β-keratinase activity for different concentrations of the variables could be predicted. Each contour curve represented an infinite number of combinations of two test variables with the other two maintained at their respective zero level. The maximum predicted value was indicated by the surface confined in the smallest ellipse in the contour diagram. The contour plots in Figure 7.29b showed that there were ellipsis near the centre point, which meant there were well defined optimum iron-oxide MNP bound β-keratinase and pH of buffer (Tris-HCl) system. However, the ellipsis was a small enough, as the surfaces were rather symmetric and a little flat near the optimum.

7.10 Characterization of bare and β-keratinase bound iron-oxide MNPs

The SEM images showed that the bare iron-oxide MNPs exist as discrete particles with a size of about 50-70 nm (Fig. 7.30a). On the other hand, the enzyme coupled MNP demonstrated the size ranged from 120-400 nm

suggesting agglomeration in association with the binding process (Fig. 7.30b).

(a) **(b)**

Fig.7.30 Scanning electron microscopic images of (a) bare bare Fe_3O_4 MNP, and (b) Fe_3O_4 MNP bound β-keratinase.

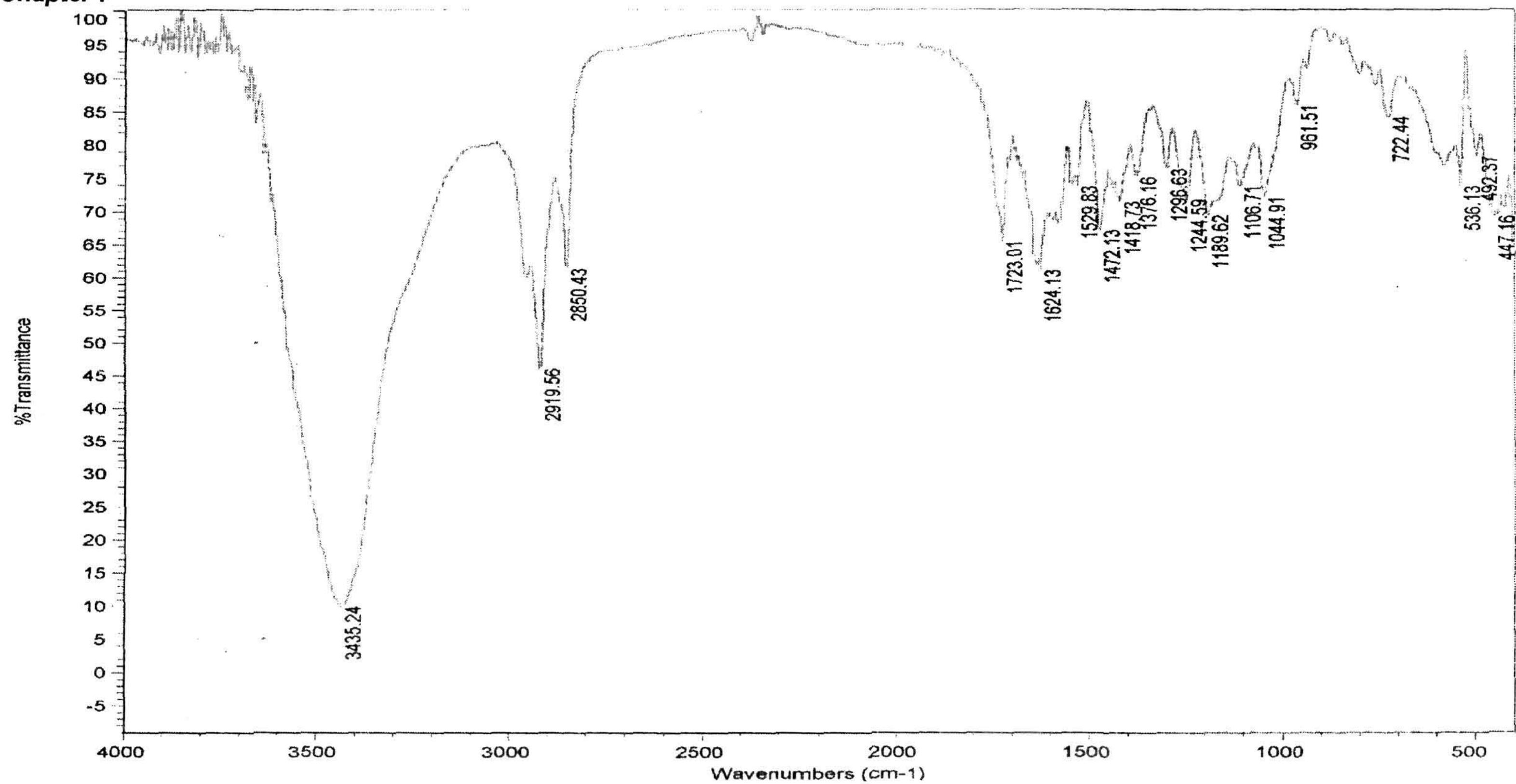


Fig.7.31 (a) FTIR spectra of iron oxide magnetic nanoparticle.

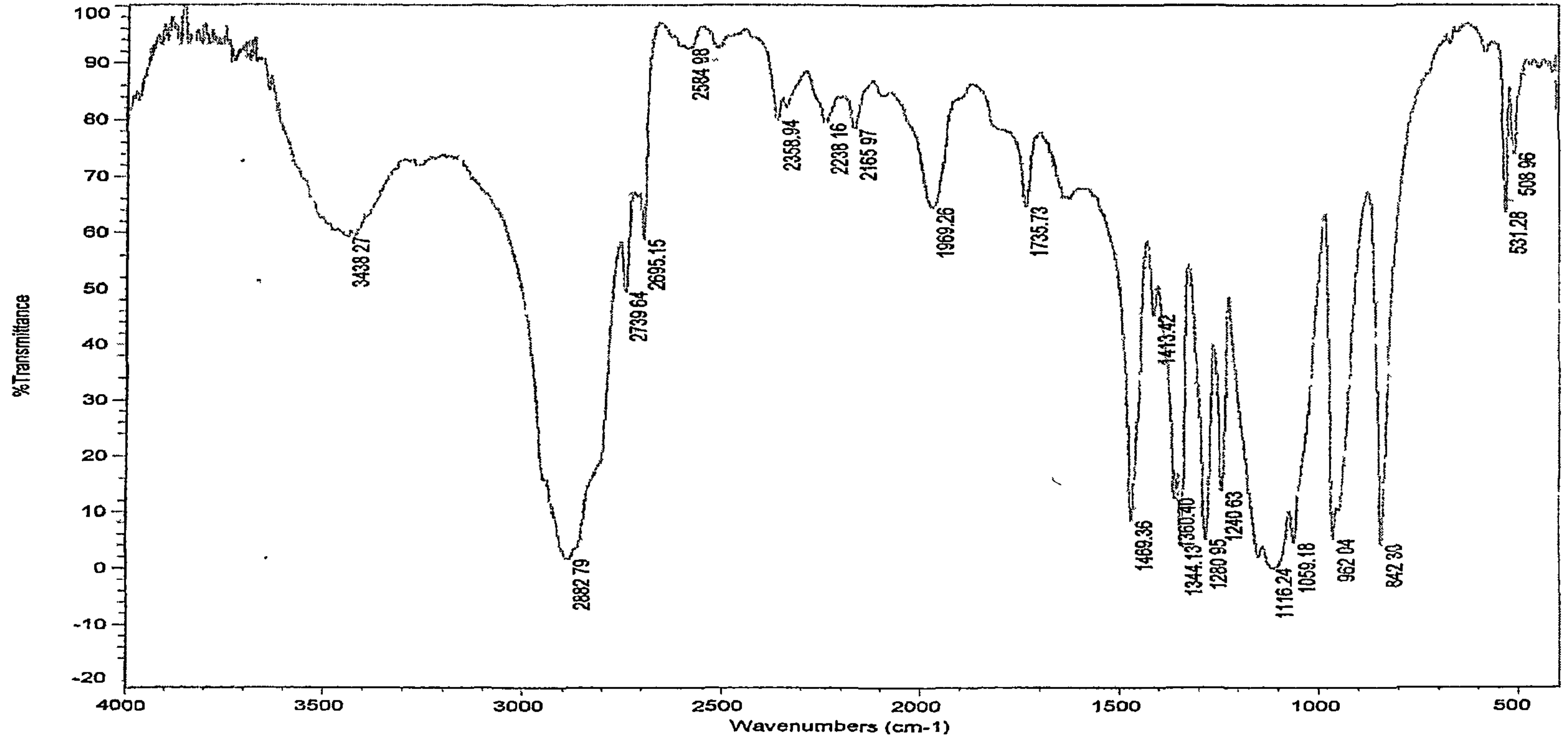
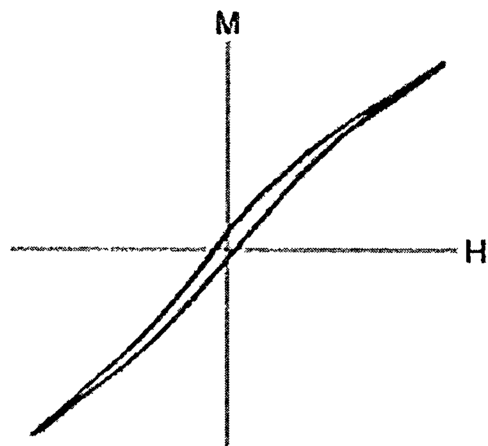
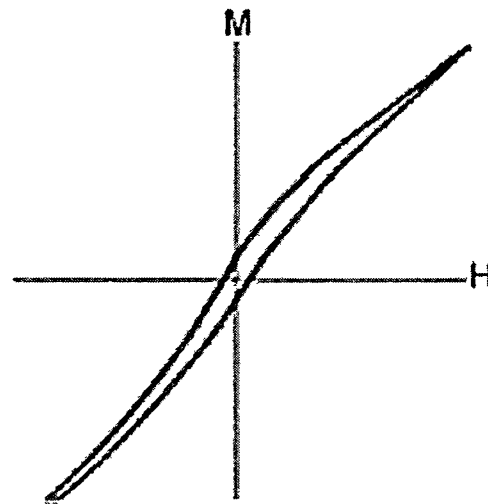


Fig.7.31 (b) FTIR spectra of MNP-coupled β-keratinase.



(a)



(b)

Fig. 7.32 Magnetization curve of iron oxide magnetic nanoparticle (a) Before (b) after enzyme immobilization.

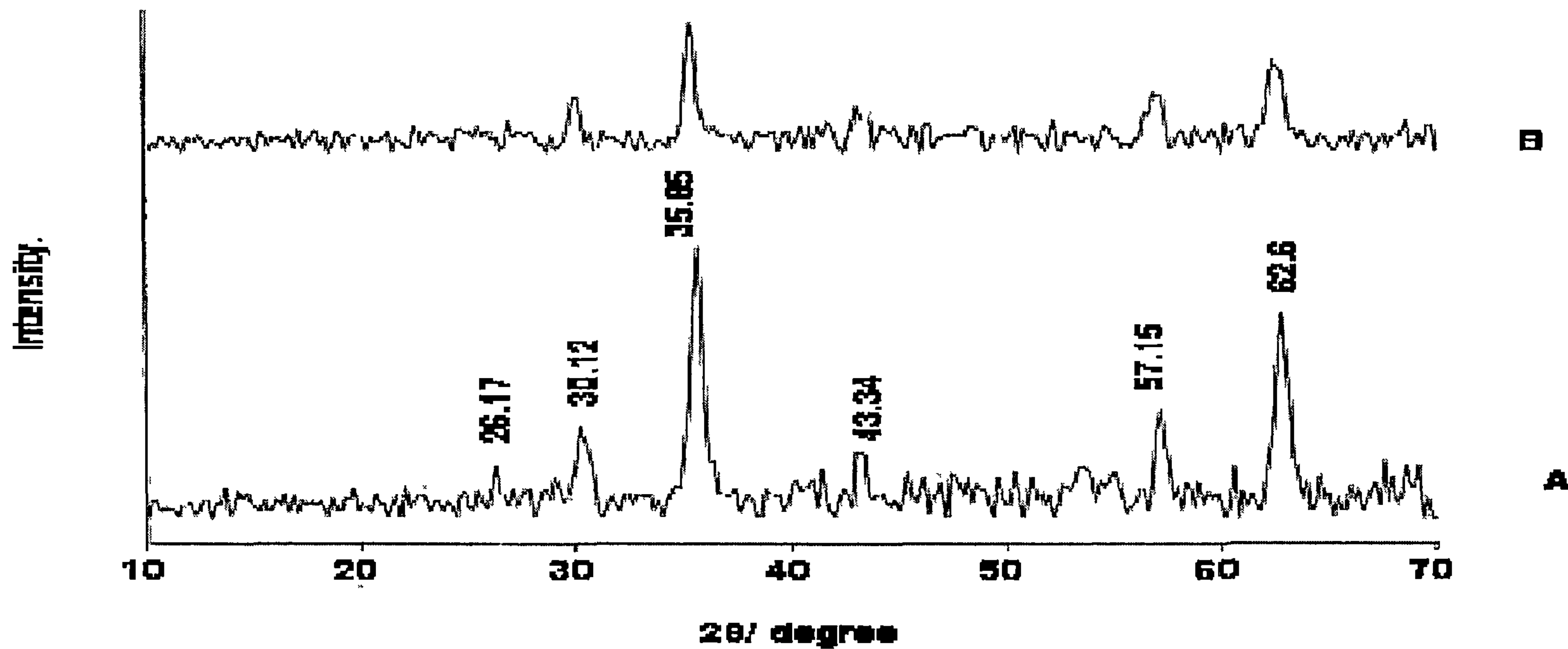


Fig.7.33 X-ray diffraction pattern of (A) bare Fe_3O_4 MNP (B) Fe_3O_4 MNP bound β-keratinase.

FTIR results also confirmed the binding of the purified β-keratinase onto magnetic Fe_3O_4 nanoparticles. The FTIR spectra of bare Fe_3O_4 MNP showed the characteristic band of metal oxygen interaction at round 572.79 cm^{-1}, typical for the synthesized iron-oxide MNP (Fig.7.31a). However, the change to 564.56 cm^{-1} indicated possible modulation of interaction due to activation with carbodiimide and the enzyme binding. Band at 1517.69 cm^{-1} was indicative of the carbodiimide functionalization of the MNP (Fig.7.31b). The C-N stretching bands were observed in the FTIR spectra of enzyme-bound carbodiimide activated iron-oxide MNP at 1465.12, 1517.69 and 1629 cm^{-1} which were absent in the FTIR spectra for bare iron-oxide MNP(Fig.7.31b). Besides, in the FTIR spectra for enzyme coupled iron-oxide MNP, the characteristics band of 1632 cm^{-1} shown by bare Fe_3O_4 MNP could not be observed. Instead a high intensity band of 1629.92 cm^{-1} appeared (Fig.7.31b). Similarly, the characteristics band at 3424.54 cm^{-1} of bare iron-oxide MNP disappeared post binding with the enzyme and a new high intensity band of 3452.15 cm^{-1} was observed in the FTIR spectra of iron-oxide MNP-β-keratinase conjugate(Fig.7.31b). This result proves the binding of β-keratinase onto iron-oxide MNPs since the washing of iron-oxide MNP β-keratinase conjugate ruled out the possibility of non-specific binding of protein(s) to MNPs(Fig.7.31b).

The low field (178 G) hysteresis loop indicates the presence of proportionately large density single domain super-paramagnetic nanoparticles (Fig.7.32). The magnetization at this field remained the same for iron-oxide nanoparticles with and without enzyme immobilization. The hysteresis loop did not show saturation up to this field indicating that the specimen could withstand to relatively larger field strength. The saturation magnetization (μ_s), remanent magnetization (μ_r) and coercivity (H_c) values for free iron-oxide MNP were determined to be 4.648 emu/cm^3, 2.703 emu/cm^3 and 9.28 G, respectively and for enzyme-conjugated iron-oxide

MNP these values were determined as 4.680 emu/cm^3, 2.703 emu/cm^3, and 9.7 G, respectively (Fig.7.32). The magnetic susceptibility of the iron oxide nanoparticles, (λ = 0.366) remained the same both before and post enzyme immobilization indicating that β-keratinase immobilization had not altered the magnetic behavior of the iron-oxide nanoparticles to a statistically significant extent.

The XRD pattern of the bare and β-keratinase bound Fe_3O_4 MNP (Figs 7.33a and 7.33b) revealed the characteristic peaks (26.1°, 30.1°, 35.8°, 43.1°, 57.1° and 62.6°) for pure Fe_3O_4. however, the only difference was observed in the intensity of X-ray diffraction from iron-oxide MNP post binding with enzyme (Fig. 7.33b). Using the software X-PERT, the nanocrystallite size for the bare MNP was found to be about 38.2 nm, and the β-keratinase bound MNP also showed almost the same crystallite size of 38.35 nm.

7.11 Biochemical and kinetic properties of free and MNP bound β-keratinase

7.11.1 Effect of pH on free and MNP bound β-keratinase

This was evident from the fact that the keratinolytic activity of free β-keratinase was restricted to pH range of 7.0 to 10.0 whereas the MNP-bound β-keratinase showed activity in the pH range of 6.0 to 12.0 (Fig.7.34).

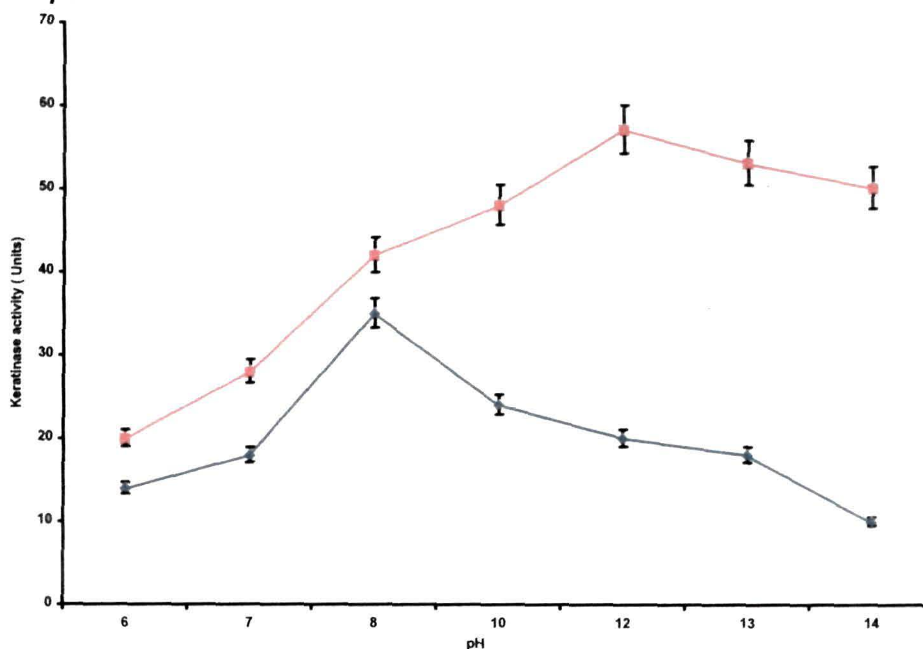


Fig.7.34 Effect of pH on activity of free β-keratinase (◆) and MNP coupled β-keratinase (■).Values are mean ± S.D. of three experiments.

7.11.2 K_m and V_{max} free and MNP bound β-keratinase

The kinetic studies showed that K_m and V_{max} values for free β-keratinase towards keratin were 5.0 mg/ml and 1.25 μmol/ min/ mg, respectively; whereas the same values for Fe_3O_4 MNP-bound β-keratinase were determined as 2.5 mg/ ml and 20 μmol/ min/ mg , respectively. Identically, the K_m and V_{max} values for free β-keratinase towards casein were determined as 4.2 mg/ ml and 1.42 μmol/min/mg, respectively. The specific activity of free and MNP-bound β-keratinase towards keratin was determined as 6800 U/mg and 61713 U/mg, respectively indicating that the catalytic efficiency of MNP bound β-keratinase compared to free β-keratinase was enhanced more than 9 folds.

7.12 Thermal and storage stabilities

A comparison of the thermal stabilities of the free and Fe_3O_4 MNP-bound β-keratinase post incubation for an identical time period showed that the latter was more stable compared to the former (Fig.7.35). The storage stability study demonstrated that the free enzyme lost 90% of its original activity post storage for 4 weeks at 4°C, whereas the iron-oxide MNP-bound enzyme retained nearly 70% of its original activity under identical storage condition (Fig.7.36).

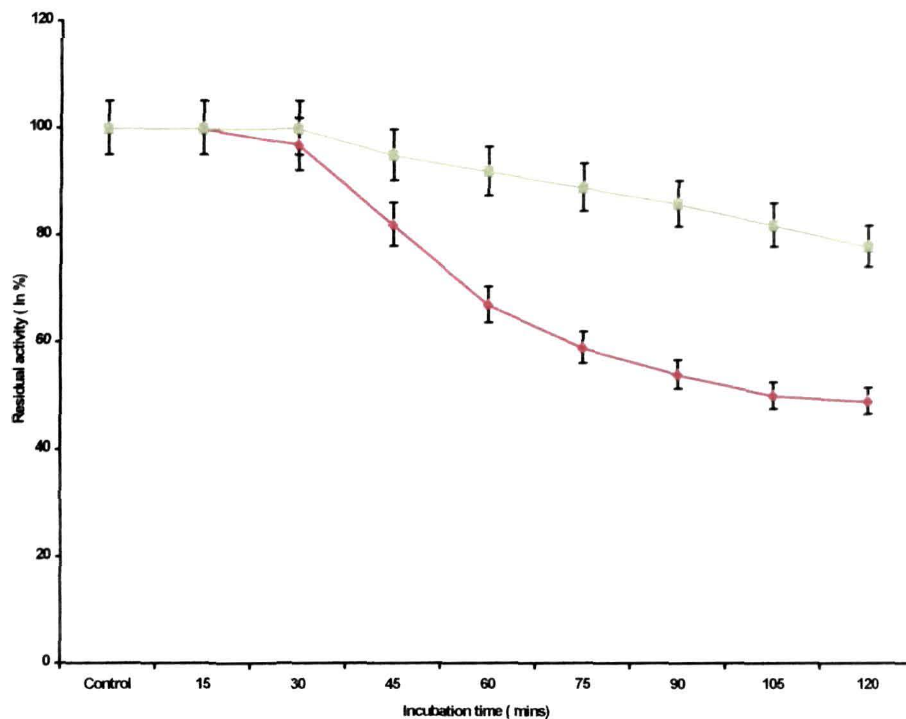


Fig.7.35 Thermostability study of free β-keratinase and MNP –coupled β-keratinase at 60°C for 120 min. Values are mean ± S.D. of three experiments.

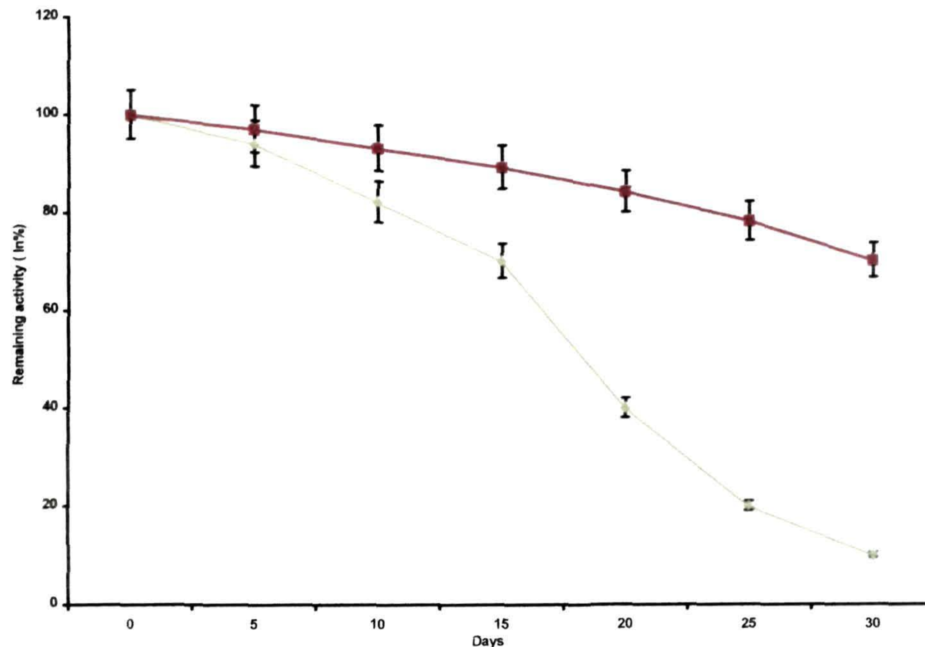


Fig. 7.36 Storage stability study of free β-keratinase and MNP –coupled β-keratinase for 30 days at 4°C. Values are mean ± S.D. of three experiments.

7.14 Industrial applications of alkaline β-keratinase

7.14.1 Detergent compatibility study

The alkaline β-keratinase demonstrated significant stability and compatibility with all the tested commercial laundry detergents at 37°C (Fig. 7.37). This excellent laundry detergent stability of alkaline β-keratinase prompted us to evaluate its stain removal potency for application in commercial laundry detergent formulations. It was observed that alkaline β-keratinase at a concentration of 0.5 mg /ml could remove 32.0 ± 2.1% (mean ± S.D., n = 3) of blood stain from cotton fabrics as shown in Fig 7.38.

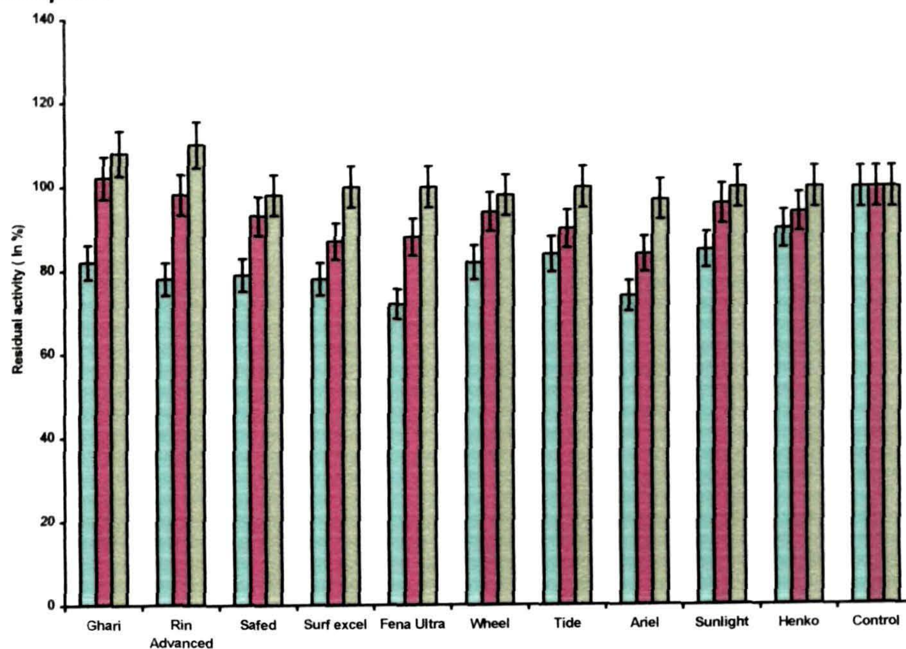


Fig.7.37 Detergent stability and compatibility of alkaline β-keratinase from *B.subtilis* strain RM-01 at 25 ( ), 37 ( ) and 45 ( ) °C. Enzyme activity in the absence of detergent was considered as 100% activity and other values were compared with that. Values represent mean ± S.D of three experiments.

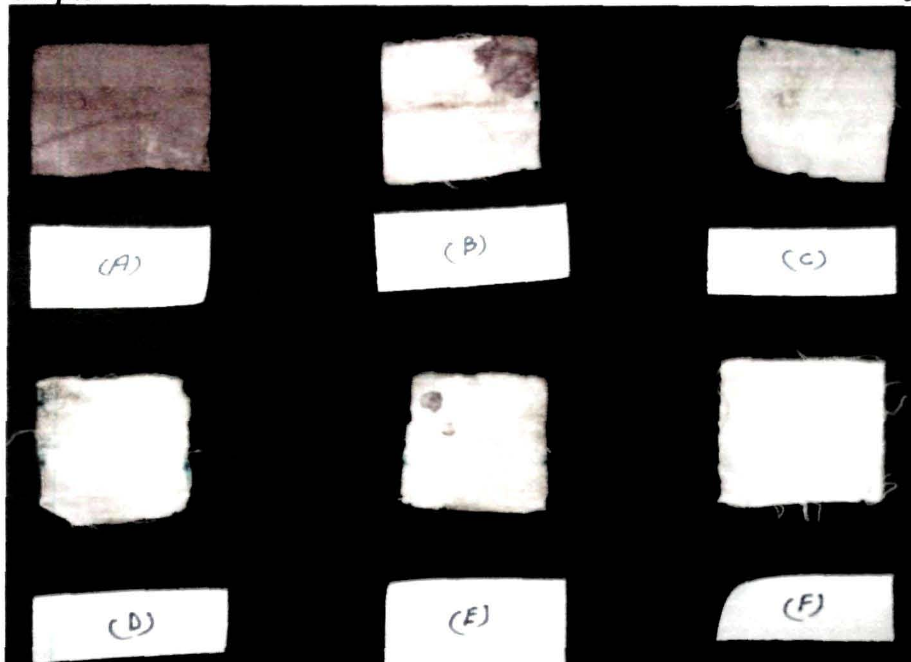
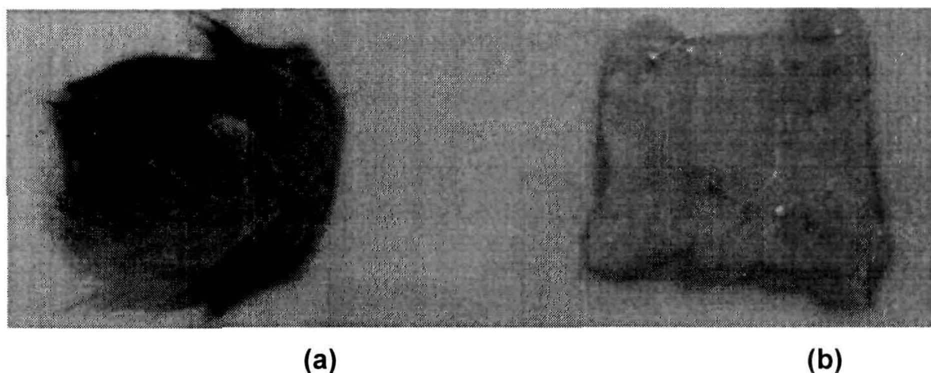


Fig.7.38 Wash performance study of alkaline β-keratinase from *B.subtilis* strain RM-01 at 37°C. From left, A: control (Tap water with blood stain cloth), B: stain cloth washed with alkaline β-keratinase (0.5 mg / ml), C: blood stain cloth washed with unheated detergent solution, D: heated detergent with blood stain cloth, E: unheated detergent with purified alkaline β-keratinase (0.5 mg / ml) with blood stain cloth, F: blood stained cloth washed with heated detergent containing purified alkaline β-keratinase (0.5 mg / ml).

7.14.2 Dehairing activity

The enzyme dehaired the goat skin within 6 h at pH 8.0 and ambient temperature 37°C, with 50 U / ml enzyme used for a 2.5 cm x 2.5 cm piece of goat skin (Fig. 7.39). The enzyme used in dehairing process possibly is non-collagenolytic in nature, as such the hide matrix remains intact. In general, the dehairing process requires the enzyme to be active

under alkaline condition; this criterion was satisfied by alkaline β-keratinase from *B.subtilis* strain RM-01 and is thus a suitable candidate for application in leather industry as a dehairing agent.



(a) **(b)**

Fig. 7.39 Dehairing activity of alkaline β-keratinase (a) goat skin incubated in 100 mM Tris- buffer, pH 8.0 for 6 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with alkaline β-keratinase (50U / ml) for 6 h at 37°C.

7.14.3 Kinetics of chicken-feather hydrolysis by MNP-coupled β-keratinase

A comparison of kinetics of chicken-feather keratin hydrolysis by free and iron-oxide MNP-bound β-keratinase showed that the latter was more efficient than the former in digesting the chicken-feather protein (keratin) as it was evident from the HPLC analysis of the released free amino acids from the feathers. About three fold increase in the rate of chicken-feather hydrolysis was observed by iron-oxide MNP-bound β-keratinase compared to free β-keratinase post 40 h of incubation under identical condition (Fig.7.40). After 40 h of incubation, the amino acids were recovered from the feather-hydrolisate in the following order: cysteine >valine >threonine >lysine >isoleucine >phenylalanine ≈methionine.

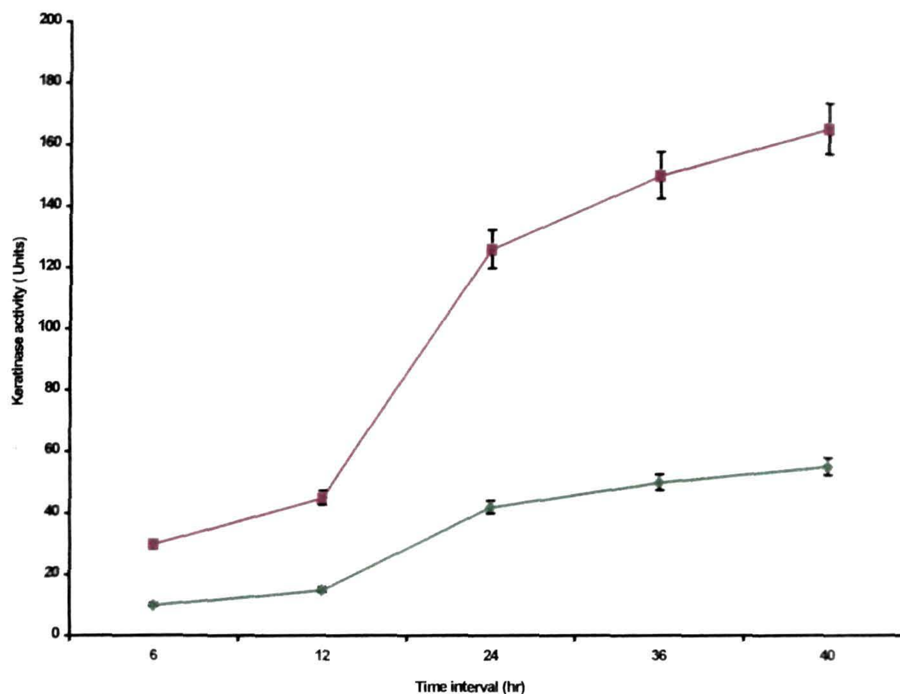


Fig.7.40 Kinetics of chicken-feather hydrolysis by free β-keratinase (◆) and MNP-coupled β-keratinase (■). Values represent mean ± S.D of three experiments.

7.14.4 SEM Study of feather-degradation

The SEM study confirmed that β-keratinase gradually degraded the native-chicken feather (keratin) and subsequently modified the structure of the feather. A comparison of feather-degradation by iron-oxide MNP bound and free β-keratinase revealed that disintegration of barbules started post 2 h and 4 h of treatment of feathers with MNP bound and free β-keratinase, respectively. After 12 h of incubation with β-keratinase-iron-oxide MNP conjugate, the surface of feather keratin was affected severely. At 18 h,

feather keratin began to break down, followed by hole formation in rachis at 24 h (Fig. 7.41c). It was no longer possible to see the entire structure of keratin after 40 h of incubation with β-keratinase-iron-oxide MNP conjugate (Fig. 7.41d). The SEM study suggested the superiority of iron-oxide MNP-bound β-keratinase compared to free β-keratinase in degrading the chicken-feather under identical experimental condition.

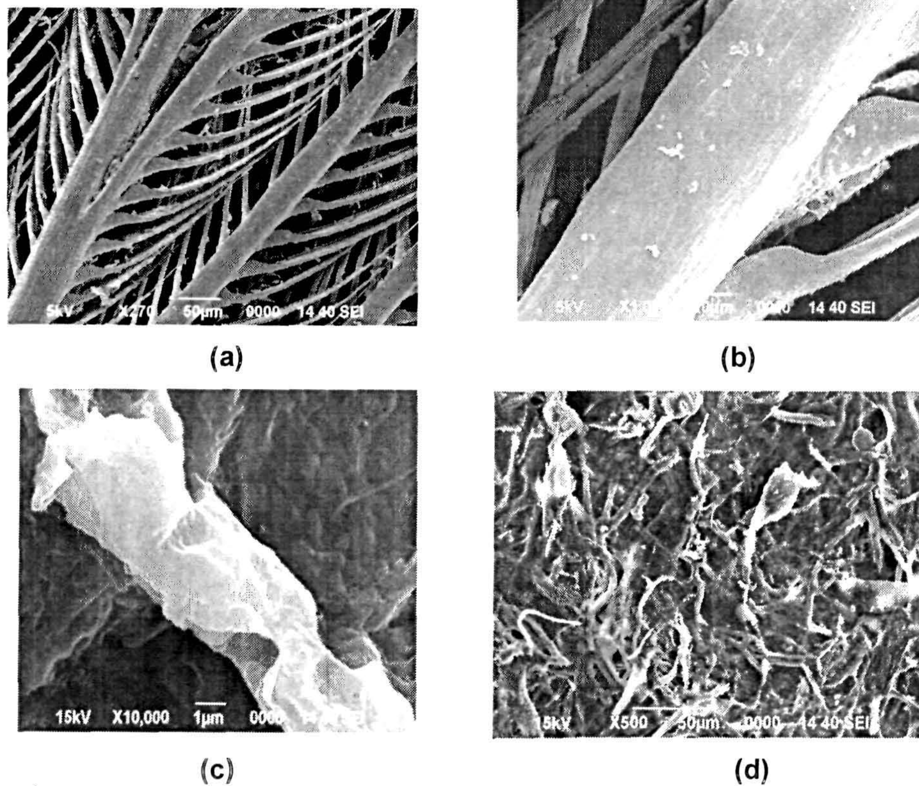


Fig.7.41 Scanning electron microscopic images of (a) native chicken-feather (X 270) (b) native chicken-feather barb (X 1000) (c) rachis of chicken-feather post 24 h treatment with Fe_3O_4 MNP bound β-keratinase (X 10,000) and (d) chicken-feather post 40 h treatment with Fe_3O_4MNP bound β-keratinase (X 500).

7.14.5 Analysis of liberated free fatty acids from chicken-feather by β-keratinase

GC analysis of the hydrolysed products from keratin fibres yields at least 7 volatile products as shown in Figs.7.42 and 7.43. Examination of the corresponding MS indicated that, most products are derived from lipids (Fig.7.44). The molecular ion peaks at m/z 190, 276, 466,171,466,466 and at RT 19.430, 19.488, 25.936, 28.363, 30.078, 31.405, and 32.615respectively were found to increase sharply post treatment with MNP-coupled β-keratinase than free β-keratinase

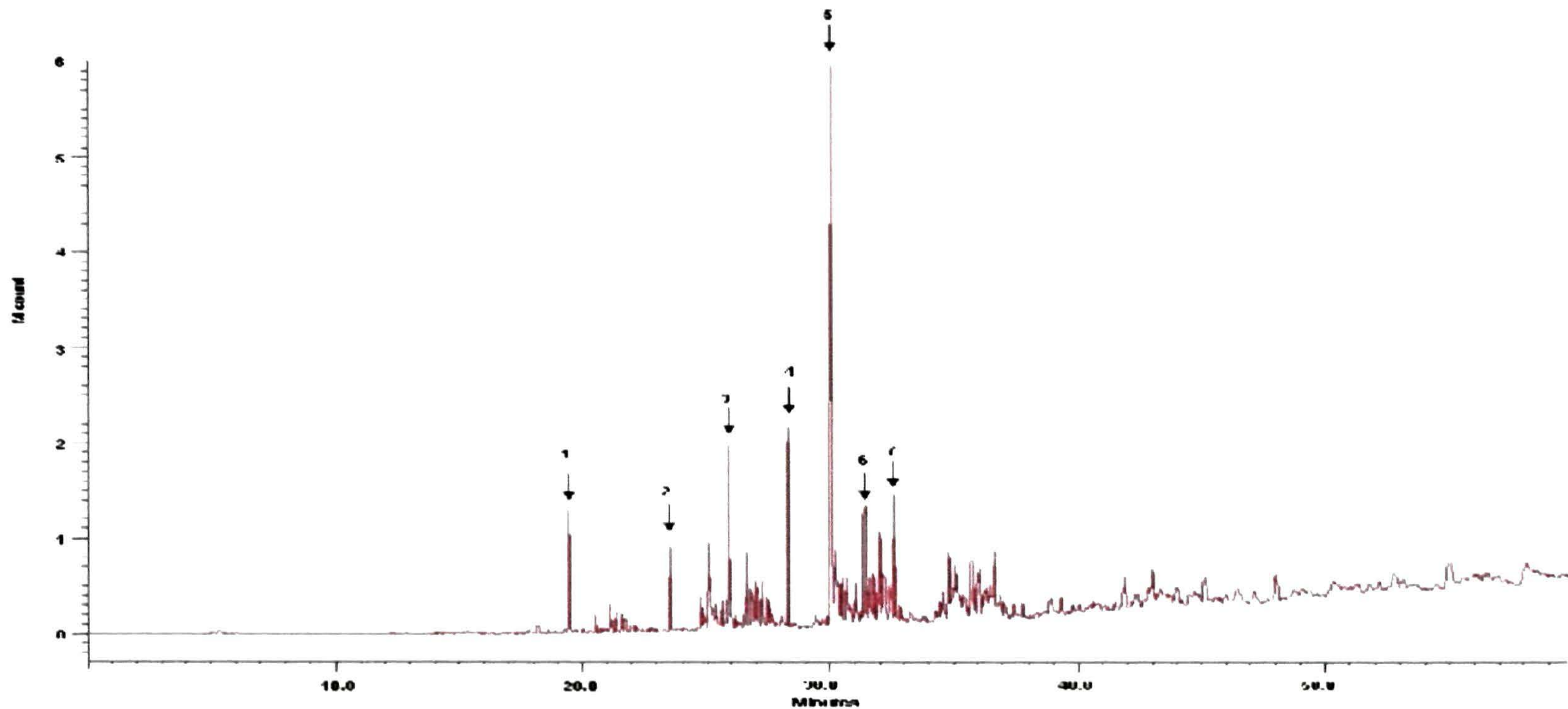


Fig.7.42 Gas chromatographic profile of free β-keratinase treated chicken feather fermented products.

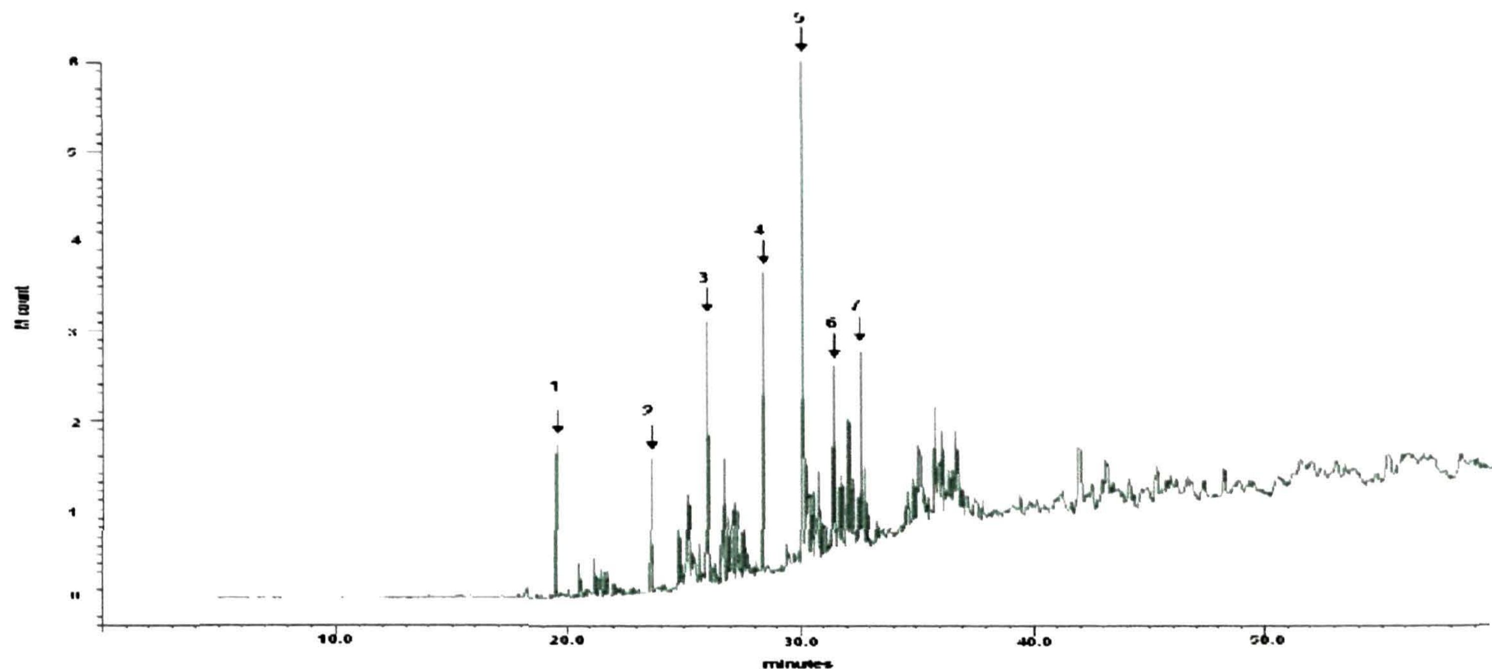
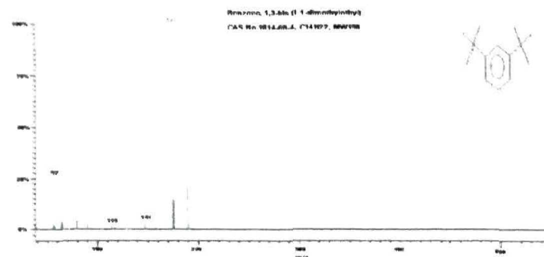
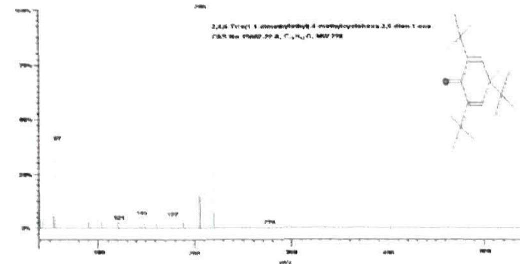


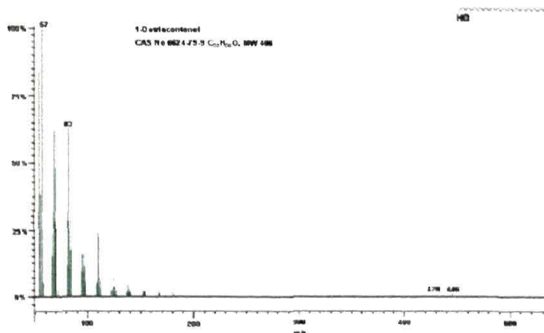
Fig.7.43 Gas chromatographic profile of MNP coupled β-keratinase treated chicken feather fermented products.



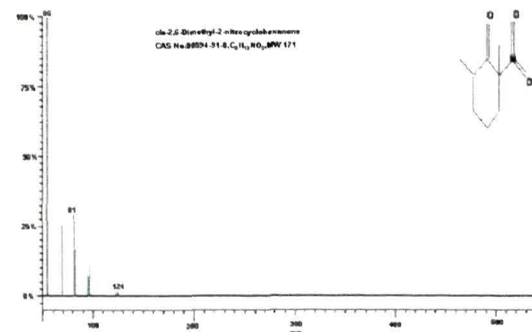
(a)



(b)



(c)



(d)

Fig. 7.44 Mass spectrum (MS) of volatile product released from hydrolyzed chicken feather post treated with MNP-coupled β-keratinase. Legends show ion chromatogram of each peak: (a) retention time 19.430; (b) retention time 25.936; (c) retention time 28.363, 31.405, and 32.615 have similar products; (d) retention time 30.078.

CHAPTER VIII

RESULTS

Sudhir K Rai

PhD Thesis, Tezpur University, 2010

Chapter 8

Process optimization, partial purification, biochemical characterization and industrial application of an alkaline protease isolated from *Bacillus* sp. AS-S20-I

8.1 Alkaline protease production under solid-state fermentation

8.1.1 Screening of different agro-industrial and waste materials for alkaline protease production

The types of substrate used are the key factor influencing the protease synthesis by bacteria in solid-state fermentation (SSF) system. Different substrates such as mustard oil cake (MOC), wheat bran (WB), rice bran (RB), *Imperata cylindrica* (IC) grass, waste potato peel (PP), banana leaf (BL), and used tea leaves (TL) were evaluated for protease synthesis by *Bacillus* sp. AS-S20-I in SSF (Fig. 8.1). Maximum enzyme production was observed in presence of *Imperata cylindrica* grass (IC) (191.8 U/gds) and potato peel (PP) (311.81 U/gds) post 24 h of incubation, while minimum protease production (2.5 U/gds) was shown by waste TL as substrate/ support material. The protein content of the culture supernatants as well as the bacterial dry biomass also followed the same trend like protease production by bacterium on different substrates in SSF (Fig.8.2).

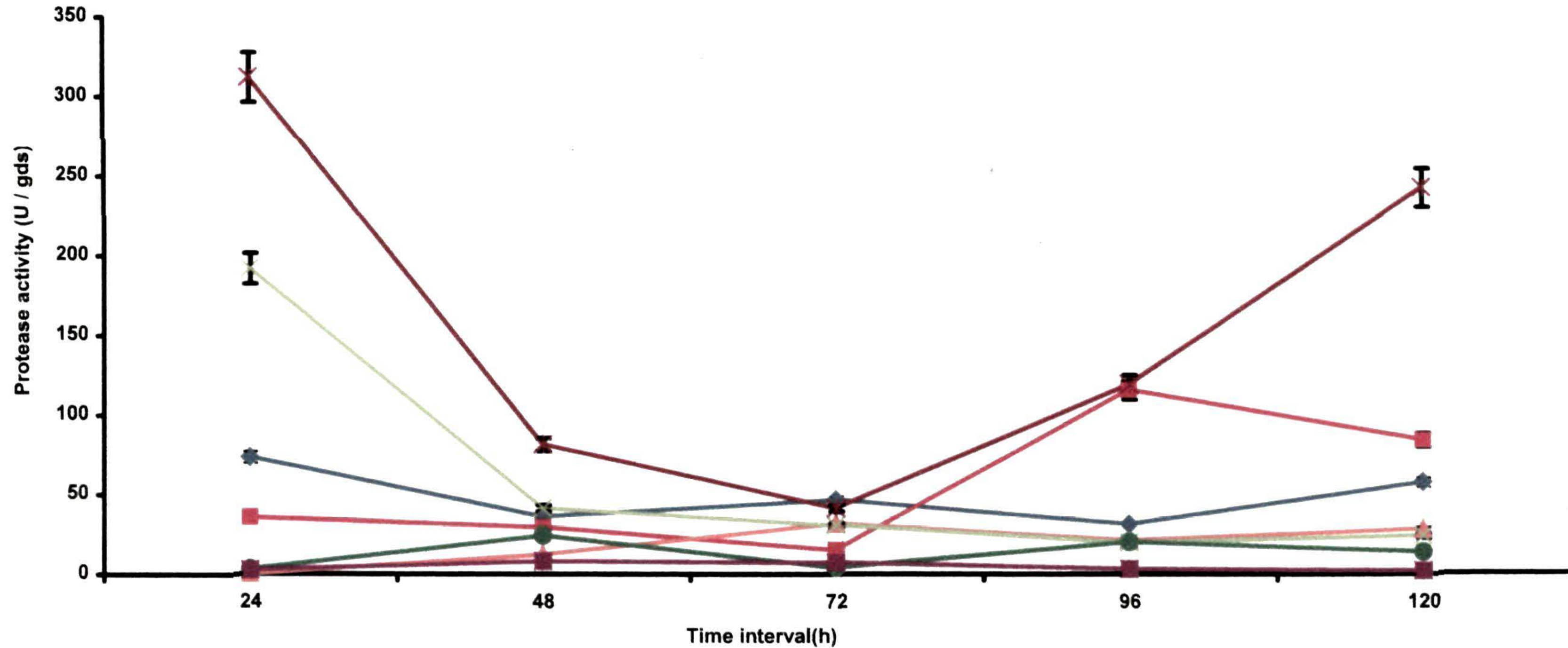


Fig.8.1 Screening of different agro-industrial waste residues such as MOC (◆), WB (■), RB (▲), IC(×), PP(☒), BL(●), and TL(■) for the production of alkaline protease by *Bacillus* sp.strain AS-S20-I at different time intervals. Values are mean ± S.D. of three experiments.

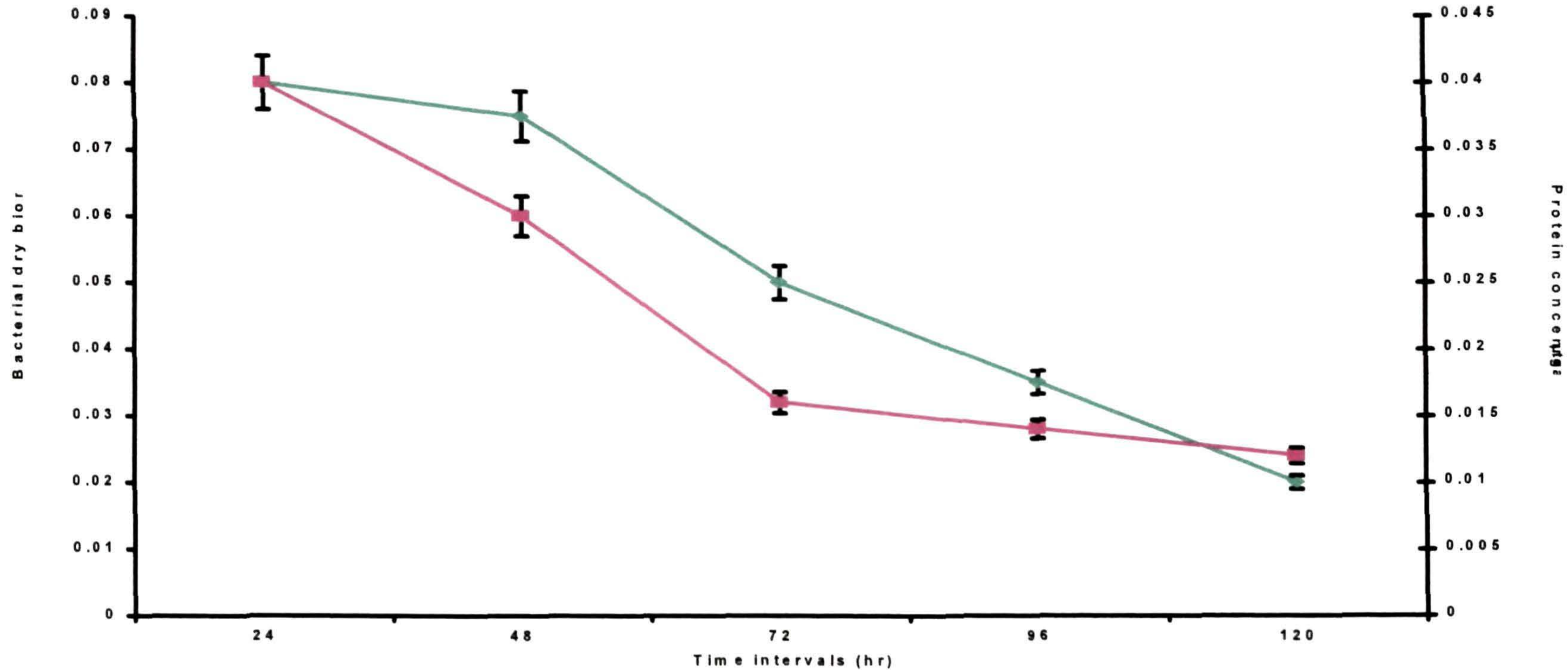


Fig.8.2 Kinetics of alkaline protease production by *Bacillus* sp. strain AS-S20-I in SSF. Legends show bacterial dry biomass (◆) and protein concentration (■).Values are mean ± S.D. of three experiments.

8.1.2. Effect of initial moisture content of the substrate and moistening agent on protease production

The moisture content is a critical factor in solid-state fermentation. Its importance for microbial growth and thereby enzyme production has been well established. To check the influence of moisture on protease production during SSF, IC or PP was moistened with different amounts of distilled water (25-300%) prior to fermentation. Results show that 50% of moisture content of solid substrate was optimal for production of protease in IC and PP (290 and 245 U/gds) of substrate, respectively (Fig.8.3). Distilled H_2O adjusted to pH 11.0 was most efficient moistening agent for protease production compared to other moistening agents (Fig. 8.4).

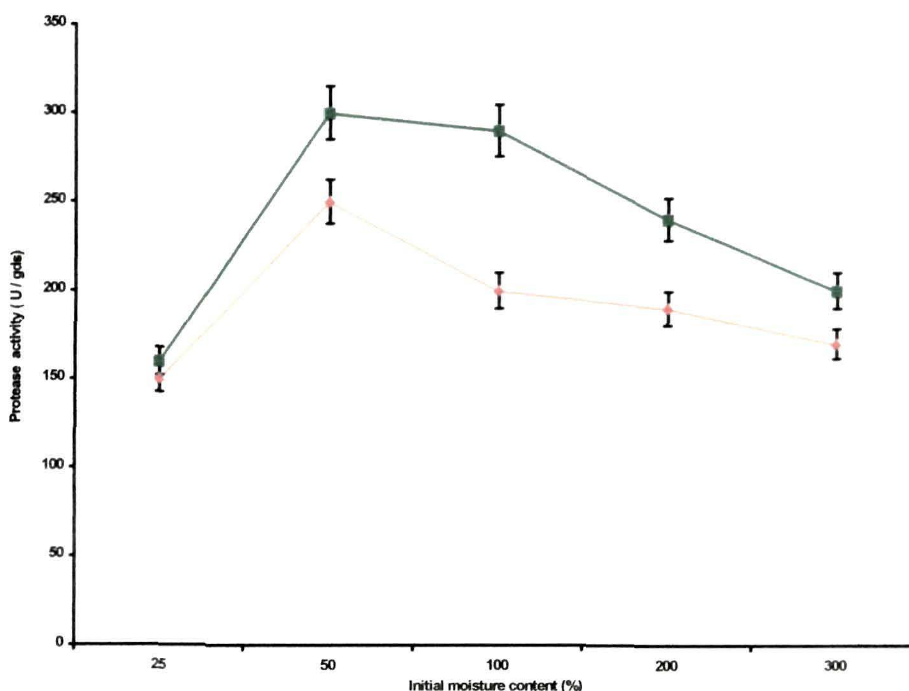


Fig.8.3 Influence of initial moisture content of the substrates [PP (◆) or IC (■)] on protease production. Values are mean ± S.D. of three experiments post 24h incubation at 50°C. Moistening agent was distilled water, pH adjusted to 11.0.

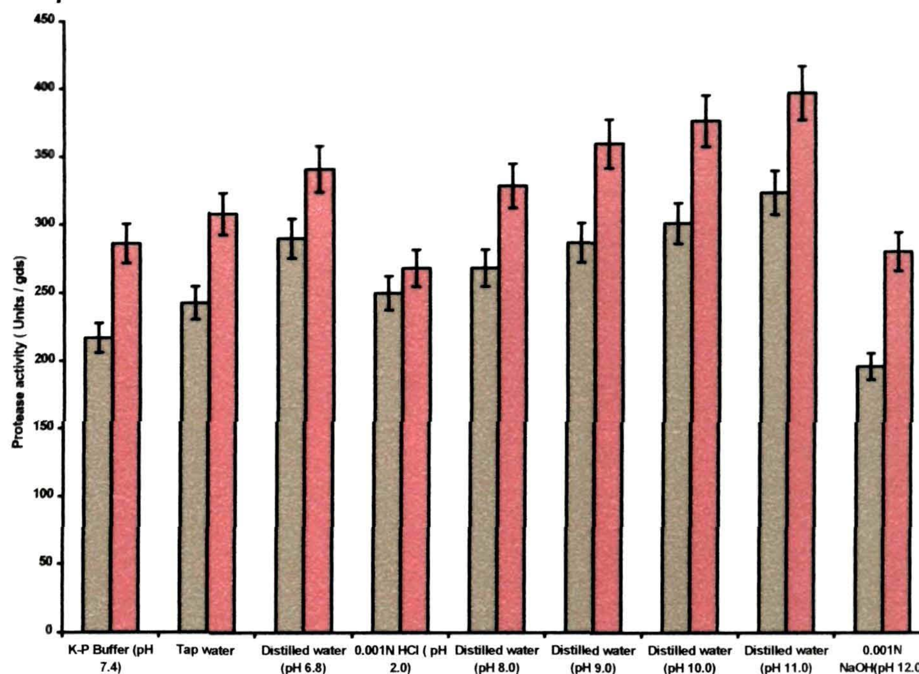


Fig.8.4 Influence of moistening agent on protease production using either IC (■) or PP (■) as a substrate. Values are mean ± S.D. of three experiments post 24h of incubation of *Bacillus* sp. strain AS-S20-I at 50°C.

8.1.3. Effect of inoculum's size on protease production

As shown in Fig.8.5, with an increase in inoculum's size from 0.5 ml to 1.5 ml (5.0 g substrate), protease production by *Bacillus* sp. strain AS-S20-I was enhanced linearly, while increasing the inoculum's size from 1.5 ml to 4.5 ml did not have a significant impact in enhancing the protease yield ($p>0.05$). However, an increase in inoculum's size beyond 4.5 ml resulted in a steady decline in protease production in SSF by *Bacillus* sp. strain AS-S20-I (Fig.8.5).

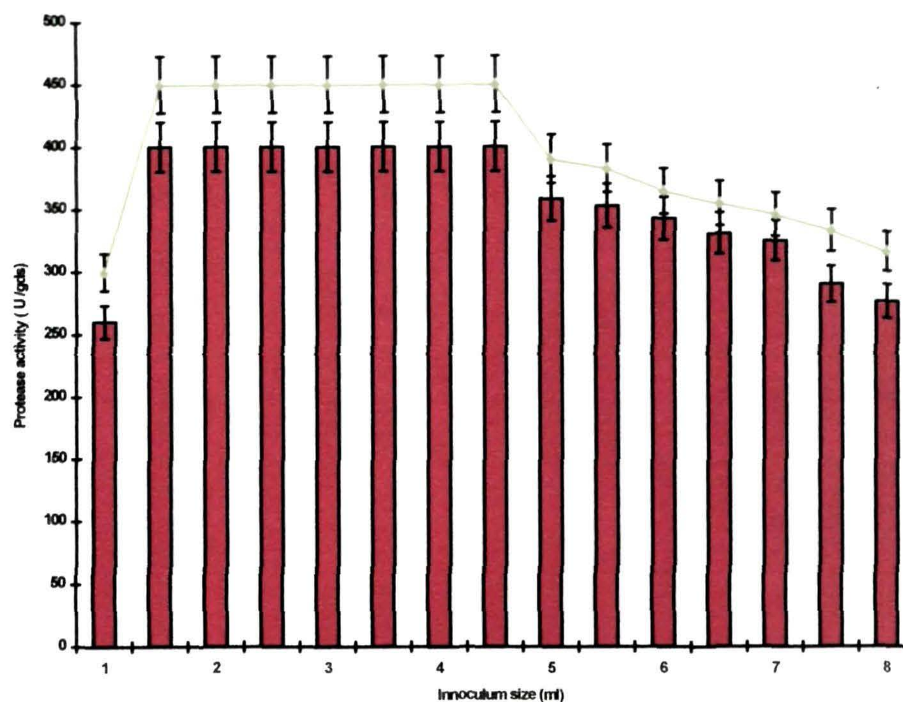


Fig.8.5 Influence of inoculum size on protease production by *Bacillus* sp. strain AS-S20-I strain using PP (◆) or IC (■) as substrate under SSF. Values are mean ± S.D. of three experiments.

8.1.4. Effect of supplementation of co-carbon and co-nitrogen sources on protease production

In this study, various carbon sources, viz. glucose, casein, galactose, maltose, lactose, fructose, and sucrose were used to investigate their influence on protease production (Fig.8.6). It was found that potato peel displayed a significant increase in protease production followed by casein as the best co-carbon source. And then by maltose for increasing the protease production (Fig. 8.6). About 3.6 fold increase in protease production was observed in presence of casein as compare with control (medium without casein).

Among the tested nitrogen compounds, 0.1 % (w/w) ammonium sulphate followed by potassium nitrate served as the best co-nitrogen sources for protease production by *Bacillus* sp. strain AS-S20-I on IC (Fig.8.7). Ammonium sulphate increased the protease production to 4.6 times higher as compare with the control. Thus, casein and ammonium sulphate were found to be the best carbon and nitrogen sources supporting microbial growth as well as enzyme production in SSF. When supplemented to IC as a solid substrate.

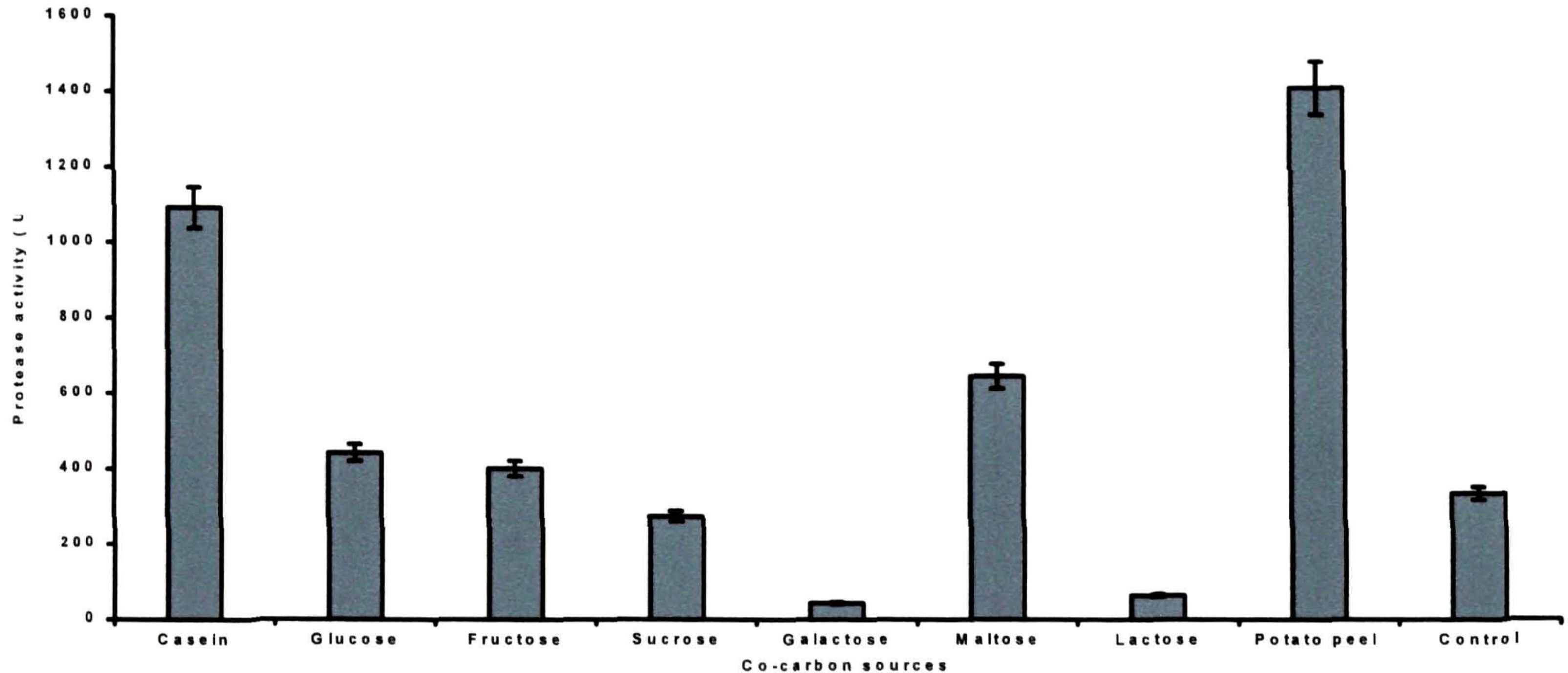


Fig. 8.6 Effect of supplementation of co-carbon sources (10 % w/w) to IC (90% w/w) (substrate) on protease production by *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments.

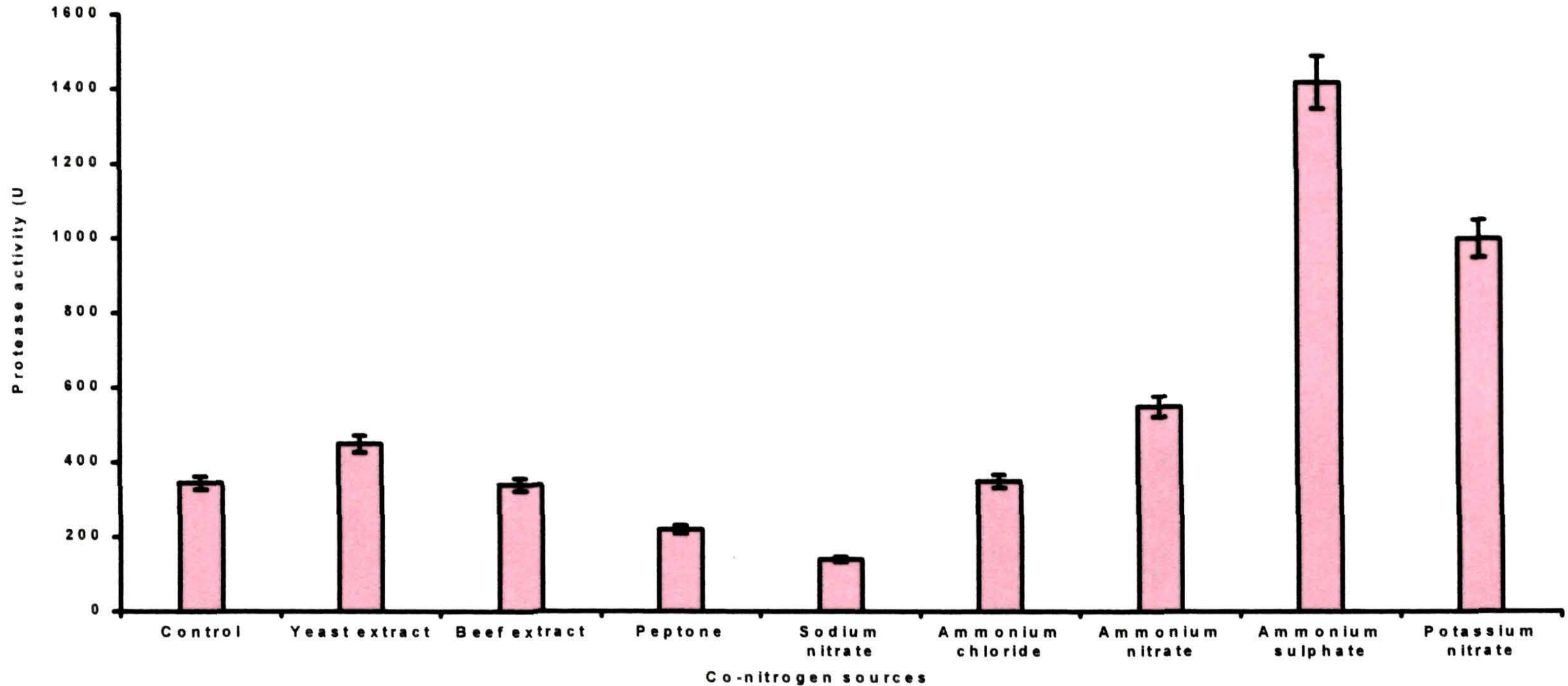


Fig.8.7 Effect of supplementation of co-nitrogen sources (1.0% w/w) to IC (99.0% w/w) (substrate) on protease production by *Bacillus* sp.strain AS-S20-I. Values are mean ± S.D. of three experiments.

8.1.5 Protease extraction from fermented matter

Tested extractions medium used for the recovery of protease from the fermented matter, distilled water containing 0.1%(v/v) triton X-100, pH 11.0 served as best extraction medium (Fig.8.8).

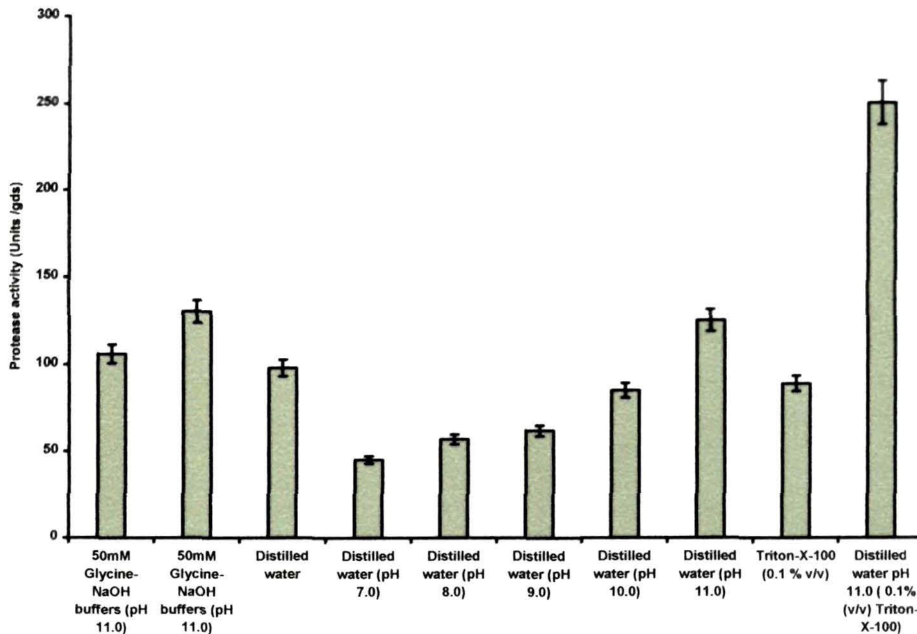


Fig.8.8 Screening of best extraction medium for optimum protease recovery from fermented matter. Values are mean ± S.D. of three experiments.

8.1.6 Batch fermentation for alkaline protease production

Batch fermentation for protease production from *Bacillus* sp. strain AS-S20-I was studied using the various combinations of IC and PP with or without supplementation of best co-carbon and co-nitrogen sources. Results showed that when IC and PP were mixed in a ratio of 1:1 without any co-carbon and nitrogen sources, a significant increase in protease yield ($p < 0.05$) could be achieved (Table 8.1), thus demonstrating no requirement of

additional co-carbon and co-nitrogen sources when PP was supplemented to IC.

Table 8.1 Influence of different combinations of co-substrates on alkaline protease production by *Bacillus* sp.strain AS-S20-I in solid-state fermentation. Values are mean ± S.D. of three experiments.

Substrate(s)	Protease activity (U/gds)
I.cylindrica (100%)	191.8 ± 9.59
Potato peel (100%)	311.81 ± 15.5
I.cylindrica (89%w/w)+ casein (10 % w/w) + ammonium sulphate (1% w/w)	1300.00 ± 65.0
I.cylindrica (90%w/w) + Potato peel (10%)	1409.0 ± 70.45
I.cylindrica (80%w/w) + Potato peel (20 %w/w)	1736.0 ± 86.8
I.cylindrica (50%w/w) + Potato peel (50%w/w)	2082.0 ± 104.1
Potato peel (89% w/w) + casein (10 % w/w) + ammonium sulphate (1% w/w)	846.0± 42.3

8.2 Alkaline protease production under submerged fermentation

8.2.1 Effect of carbon source on protease production

Carbon source such as glucose, fructose, galactose, lactose, sucrose, starch, carboxylmethyl cellulose, casein, and mannitol were incorporated at 10% level in the medium. It was observed that cellulose and

carboxylmethyl cellulose were least effecting carbon source for protease production. The best carbon source supporting protease production by *Bacillus* sp. strain AS-S20-I in SmF (Fig. 8.9).

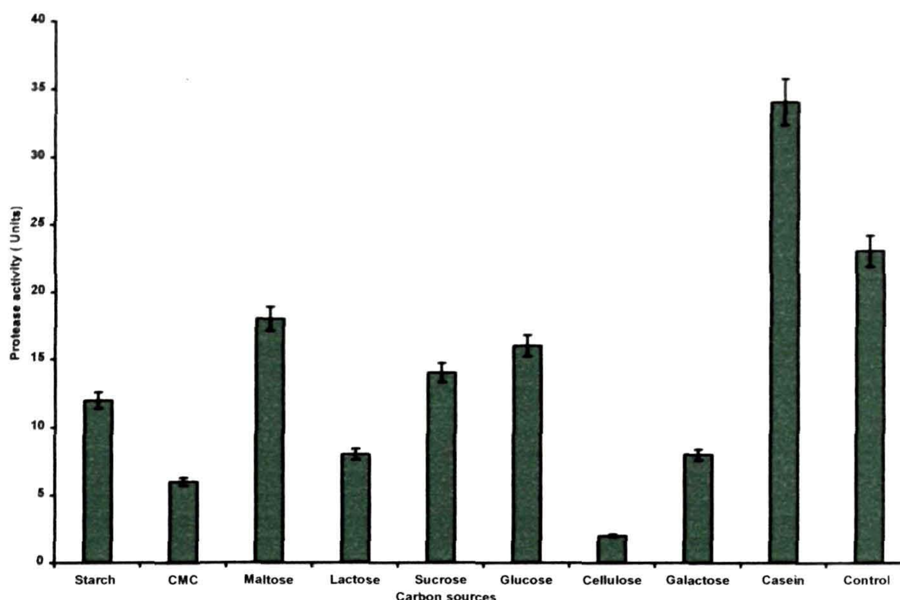


Fig.8.9 Effect of different carbon sources on alkaline protease production by *Bacillus* sp.strain AS-S20-I under submerged fermentation. Values are mean ± S.D. of three experiments.

8.2.2 Effect of nitrogen source on protease production

Various nitrogen sources were tested for alkaline protease production and maximum alkaline production was observed in presence of 0.1% (w/v) ammonium sulphate (Fig.8.10).

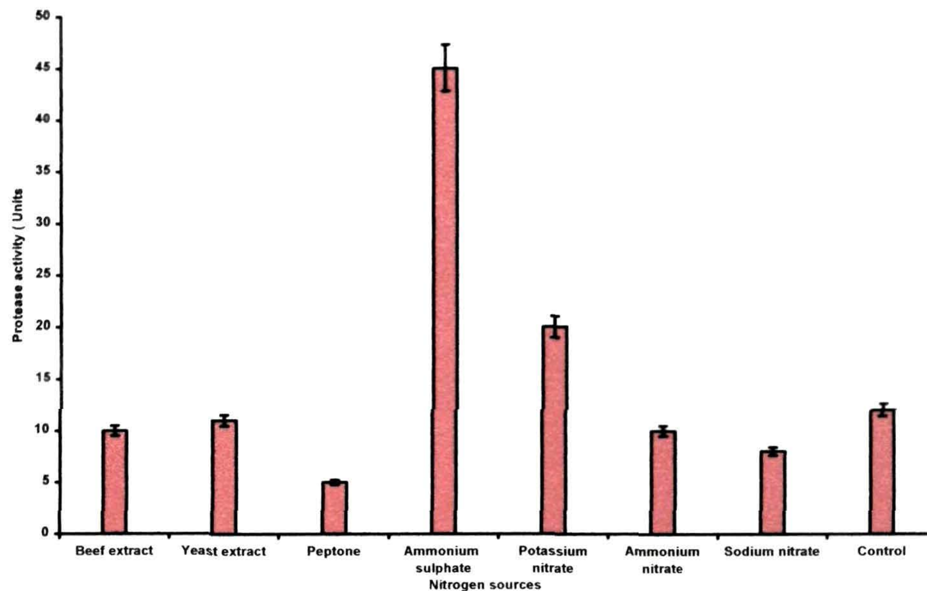


Fig.8.10 Effect of various nitrogen sources on alkaline protease production from *Bacillus* sp. strain AS-S20-I under SmF. Values are mean ± S.D. of three experiments.

8.2.3 Effect of pH on protease production

The effect of pH on alkaline protease production from *Bacillus* sp. strain AS-S20-I was determined by adjusting the medium pH and than producing protease at that pH. Result showed that with an increase in the pH of the medium from 6 to 9.5, the protease production was enhanced significantly ($p<0.05$). The protease production remained constant from pH 9.5-11.0; however, a further increase in pH of the medium resulted in a decline in protease yield (Fig.8.11).

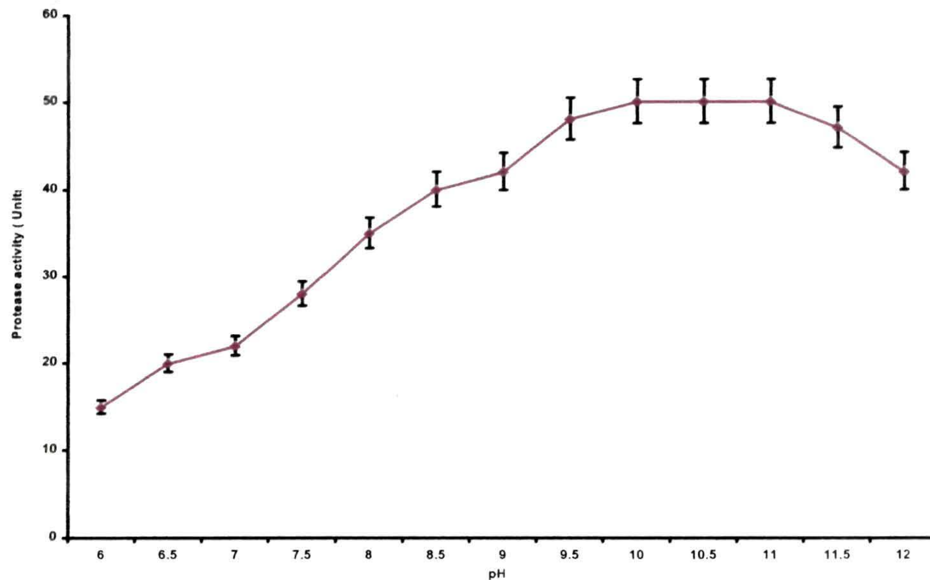


Fig.8.11 Effect of medium pH on alkaline protease production by *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments.

8.2.4. Effect of temperature on protease production

The effect of temperature on alkaline protease production from *Bacillus* sp.strain AS-S20-I showed that protease production was linearly increased from 25 to 50°C and beyond this temperature protease production was decreased suggesting 50°C is the optimum temperature for protease production by *Bacillus* sp. strain AS-S20-I (Fig.8.12).

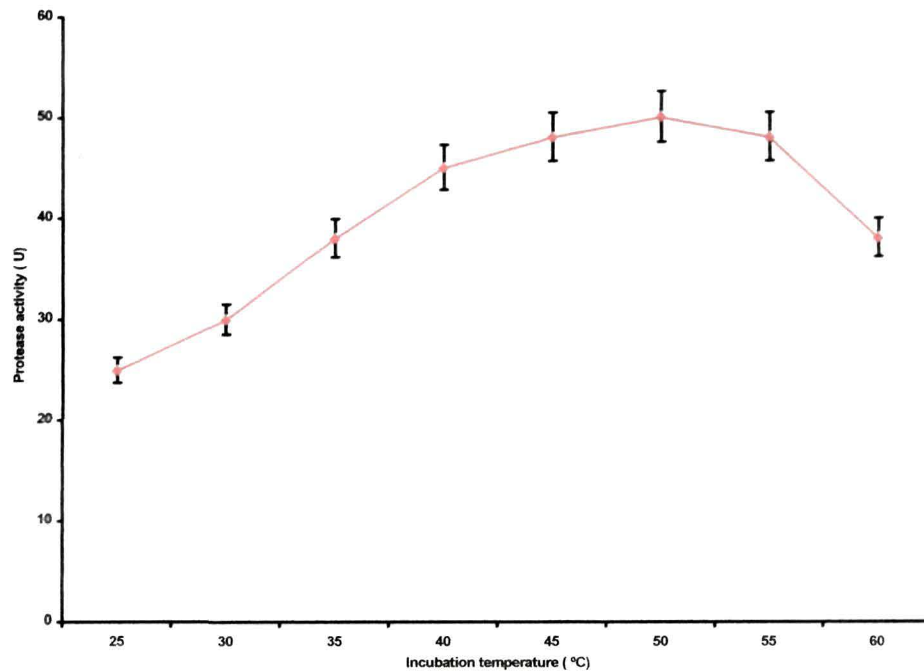


Fig.8.12 Influence of incubation temperature on alkaline protease production by *Bacillus* sp.strain AS-S20-I in SmF. The pH of the medium was adjusted to 9.5. Values are mean ± S.D. of three experiments.

8.2.5 Kinetics of protease production

Result showed that maximum alkaline protease production was achieved post 48h of incubation (Fig. 8.13). The protease production was slightly declined upto 72h of incubation and beyond this period, a sharp fall in protease production was recorded (Fig. 8.13).

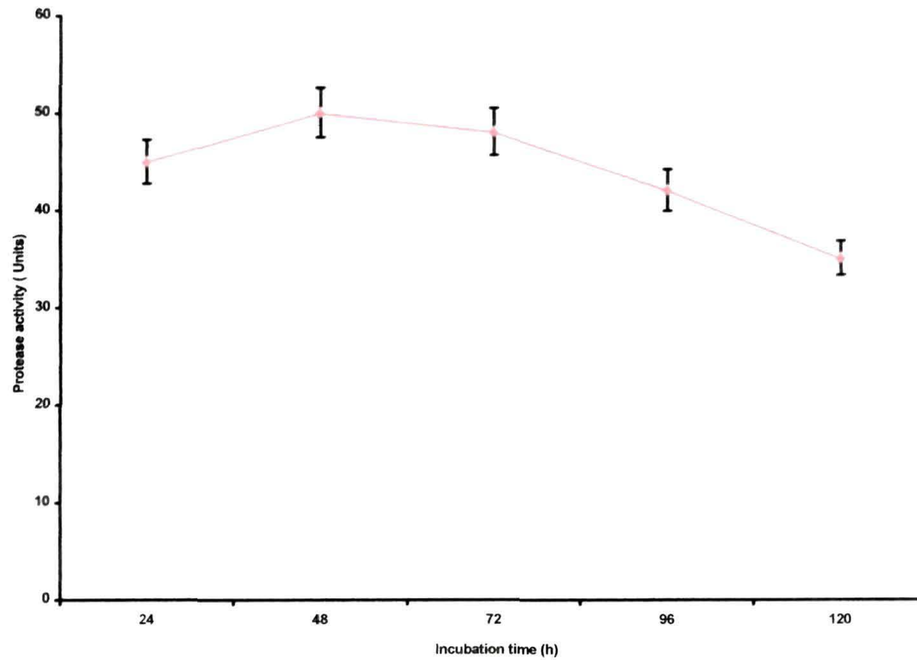


Fig. 8.13 Effect of incubation time on alkaline protease production by *Bacillus* sp. strain AS-S20-I in SmF . Values are mean± S.D. of three experiments.

8.2.6 Effect of agitation rate on protease production

The effect of the agitation speed on the protease production showed that optima alkaline protease production was observed at 200 rpm (Fig. 8.14), further increase in agitation rate displayed a sharp fall in protease yield.

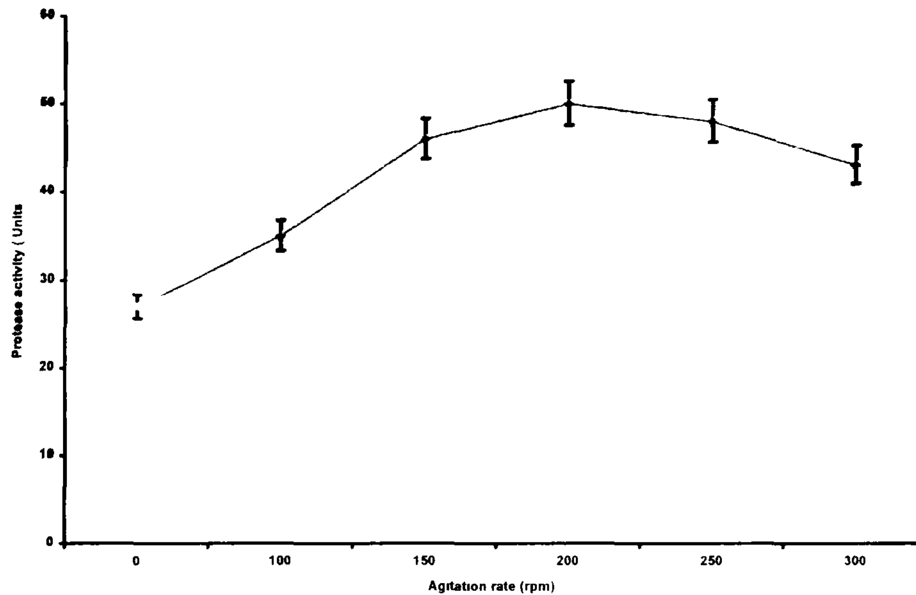


Fig.8.14 Effect of agitation rate on alkaline protease production from *Bacillus* sp. strain AS-S20-I under SmF. Values are mean ± S.D. of three experiments.

8.3 Statistical optimization of alkaline protease production under submerged fermentation

8.3.1 Evaluation of factors effecting production using Plackett-Burman design

In the present study, maximum protease activity was detected in the 48 h post-inoculated cell-free extract of *Bacillus* sp. strain AS-S20-I. Screening result demonstrated a wide variation of protease activity from 59.0 Units to 145.8 Units in the fifteen trials (Table 8.2). The analysis of regression coefficients and t-value of six parameters showed that amongst the tested parameters casein, ammonium sulphate, and pH of the medium stimulated the protease production whereas, innoculum level and incubation time had

a negative effect on protease production (Table 8.2). Neglecting the variables which were insignificant, the model equation for protease production can be written as

$$Y = 101.680 + 4.870 X_1 + 12.377 X_2 + 13.885 X_3$$
----(8.1)

Where X_1 = pH of the medium, X_2 = casein level, and X_3 = ammonium sulphate level.
On the basis of calculated t-values (Table 8.3), casein, ammonium sulphate, and pH of the medium were chosen for further optimization by RSM. Innoculum level (% v/v) and incubation time (h) were kept at middle level.

Table 8.2 Plackett–Burman design showing six variables with coded values along with the observed results for protease production by *Bacillus* sp. strain AS-S20-I .

Casein (% w/v)	Ammonium sulphate (% w/v)	Incubation time (h)	Agitation rate (rpm)	pH	Innoculum size (% v/v)	Protease yield (U)
1 (1.5)	-1(0.1)	1 (96)	-1 (150)	-1 (9.0)	-1 (1.5)	59.6
1 (1.5)	1 (0.25)	-1 (48)	1 (250)	-1 (9.0)	-1 (1.5)	82.8
-1 (0.5)	1 (0.25)	1 (96)	- (150)	1 (11.0)	-1 (1.5)	105.3
1 (1.5)	-1 (0.1)	1 (96)	1 (250)	-1 (9.0)	1 (5.0)	94.1
1 (1.5)	1 (0.25)	-1 (48)	1 (250)	1 (11.0)	-1 (1.5)	111.0
1 (1.5)	1 (0.25)	1 (96)	-1 (150)	1 (11.0)	1 (5.0)	116.0
-1 (0.5)	1 (0.25)	1 (96)	1 (250)	-1 (9.0)	1 (5.0)	112.1
-1 (0.5)	-1 (0.1)	1 (96)	1 (250)	1 (11.0)	-1 (1.5)	111.4
-1 (0.5)	-1 (0.1)	-1 (48)	1 (250)	1 (11.0)	1 (5.0)	146.0
1 (1.5)	-1 (0.1)	-1 (48)	-1 (150)	1 (11.0)	1 (5.0)	122.0
-1 (0.5)	1 (0.25)	-1 (48)	-1 (150)	-1 (9.0)	1 (5.0)	112.1
-1 (0.5)	-1 (0.1)	-1 (48)	- 1 (150)	-1 (9.0)	-1 (1.5)	84.0

Casein (% w/v)	Ammonium sulphate (% w/v)	Incubation time (h)	Agitation rate (rpm)	pH	Innoculum size (% v/v)	Protease yield (U)
0 (1.0)	0 (0.2)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	90.0
0 (1.0)	0 (0.2)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	90.0
0 (1.0)	0 (0.2)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	90.0

Table 8.3 Statistical analysis of Plackett–Burman design showing coefficient values, t- and p-value for each variable for protease activity (p-value <0.05).

Variables	Protease yield (Units)			
	Co-efficient	SE Coef	t-Stat	p-value
Intercept	101.680	2.065	49.24	0.000
pH of the medium	4.870	2.309	2.11	0.015
Innoculum level (%v/v)	1.870	2.309	0.81	0.441
Incubation time (h)	-4.870	2.309	-2.11	0.068
Agitation rate (rpm)	-7.127	2.309	-3.09	0.068
Casein level (% w/v)	12.377	2.309	5.36	0.001
Ammonium sulphate (% w/v)	13.885	2.309	6.01	0.000

Based on the preliminary screening result on the influence of process parameters on protease production, the three main parameters viz pH of the medium (C_1), concentration of carbon source (% w/v) (C_2), and concentration of nitrogen source (% w/v) (C_3) were considered as most

significant factors influencing protease production. Requirements of these were further optimized by statistical analysis.

8.3.2 Statistical optimization of protease production in SmF

RSM was employed to characterize the individual and interactive effects of pH, casein and ammonium sulphate concentration on protease production (Table 8.4). The overall second-order polynomial regression equation showing the empirical relationship between protease activity (Y) and three test variables in coded units can be represented by equation 8.2.

$$Y = 562.090 - 57.643C_1 + 211.277C_2 + 2.523C_3 - 53.226C_1^2 - 75.226C_2^2 + 12.774C_3^2 + 45.729C_1C_2 - 13.729C_1C_3 - 3.379C_2C_3 \quad \text{------------------------ (8.2)}$$

The coefficients of the model including the significance of each coefficient as determined by t test and p-values showed that all the effects of C_1, C_2 and C_3, were significant ($p < 0.0001$) (Table 8.5). The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the computed F value for linear regression was much greater than the tabulated (P) > F value (Table 8.5). The goodness-of-fit of the model was checked by determining the coefficient of determination (R^2) and adjusted R^2. The observed values of R^2 explain that the fitted model could explain 99.87% of the total variation and therefore, vouches for adequacy of the model (Table 8.6). The results showed that C_1 (pH of the medium), C_2 (casein level) and C_3 (ammonium sulphate level) had significant effect ($p<0.001$) on protease production by bacterium under study (Table 8.6).

Table 8.4 Observed responses and predicted values of alkaline protease production by *Bacillus* sp. strains AS-S20-I in SmF. Values are mean± S.D. of three experiments.

Run no.	Independent Variables			Y Response (Protease yield in U)		Residual value
	C_1	C_2	C_3	Observed value	Predicted value	
1	-1(7.0)	-1(0.50)	-1(0.15)	319.000	318.877	0.123
2	1(9.0)	-1(0.50)	-1(0.25)	140.000	139.591	0.409
3	-1(7.0)	1(1.50)	-1(0.15)	656.600	656.731	-0.131
4	1(9.0)	1(1.50)	-1(0.15)	660.300	660.360	-0.060
5	-1(7.0)	-1(0.50)	1(0.25)	358.130	358.138	-0.008
6	1(9.0)	-1(0.50)	1(0.25)	124.000	123.937	0.063
7	-1(7.0)	1(1.50)	1(0.25)	682.000	682.477	-0.477
8	1(9.0)	1(1.50)	1(0.25)	631.000	631.191	-0.191
9	0(8.0)	0(1.0)	-1(0.15)	567.000	566.507	0.493
10	0(8.0)	0 (1.0)	1 (0.25)	451.000	451.221	-0.221
11	-1(7.0)	0(1.0)	0(0.20)	275.000	275.587	-0.587
12	1(9.0)	0(1.0)	0(0.20)	699.000	698.141	0.859
13	0(8.0)	-1(0.50)	0(0.20)	572.000	572.341	-0.341
14	0(8.0)	1(1.50)	0(0.20)	578.000	577.387	0.613
15	0(8.0)	0(1.0)	0(0.20)	562.000	562.090	-0.090
16	0(8.0)	0(1.0)	0(0.20)	562.000	562.090	-0.090
17	0(8.0)	0(1.0)	0(0.20)	562.000	562.090	-0.090
18	0(8.0)	0(1.0)	0(0.20)	562.000	562.090	-0.090
19	0(8.0)	0(1.0)	0(0.20)	562.000	562.090	-0.090
20	0(8.0)	0(1.0)	0(0.20)	562.000	562.090	-0.090

R^2 = 100.00%, R^2 (pred) = 100.00%, R^2 (adj) = 100.00%

Table 8.5 Analysis of Variance (ANOVA) of alkaline protease produced by *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments.

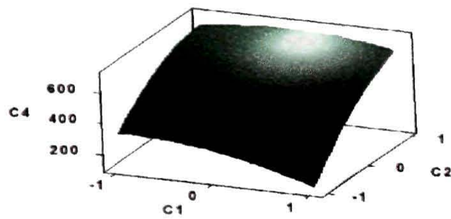
Source	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
Regression	9	555453	555453	61717	258685.17	0.000
Linear	3	479671	479671	159890	670175.59	0.000
Square	3	57454	57454	19151	80272.65	0.000
Interaction	3	18328	18328	6109	25607.26	0.000
Residual error	10	2	2	0		
Lack-of-Fit	5	2	2	0		
Pure error	5	0	0	0		
Total	19	555455				

Table 8.6 Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for alkaline protease production by *Bacillus* sp. strain AS-S20-I in SmF under shake-flask study. Values are mean ± S.D. of three experiments.

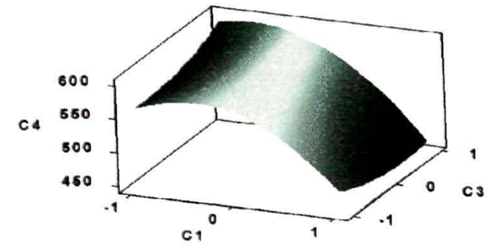
Factor	Coefficient	SE coefficient	Computed t-value	p-value
Constant	562.090	0.1679	3347.451	0.000
C_1	-57.643	0.1545	-373.190	0.000
C_2	211.277	0.1545	1367.841	0.000
C_3	2.523	0.1545	16.334	0.000
C_1^2	-53.226	0.2945	-180.706	0.000
C_2^2	-75.226	0.2945	-255.398	0.000
C_3^2	12.774	0.2945	43.369	0.000
$C_1 C_2$	45.729	0.1727	264.800	0.000
$C_1 C_3$	-13.729	0.1727	-79.499	0.000
$C_2 C_3$	-3.379	0.1727	-19.565	0.000

8.3.1 Response surface plots

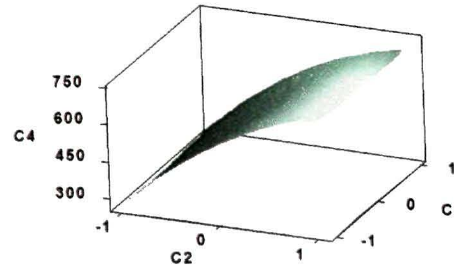
The three-dimensional (3-D) response surfaces were plotted on the basis of the model equation to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum protease production by *B.* sp. strain AS-S20-I (Figs.8.16a-c). Protease production varied significantly upon changing the initial concentrations of casein and ammonium sulphate. The three-dimensional plots depicted that with an increase in either pH of the medium, or concentration of ammonium sulphate resulted in protease production to optimum value; whereas, further increase in concentration beyond the optimum value decreased the protease yield. These results reinforced that the response equation provided a suitable model for the CCD experiment.



(a)



(b)

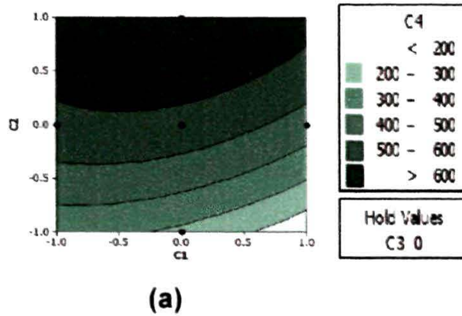


(c)

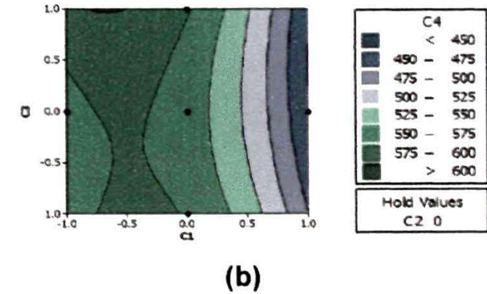
Fig.8.15 Response surface plots for alkaline protease production by *Bacillus* sp. strain AS-S20-I. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0 (coded value) (b) pH of the medium vs concentration (%, w/v) of ammonium sulphate, hold value C_2 = 0(coded value), and (c) concentration (%, w/v) of casein vs concentration of ammonium sulphate (% w/v) hold value C_1 = 0(coded value). Values are mean ± S.D. of three experiments.

8.3.2 Countor plots

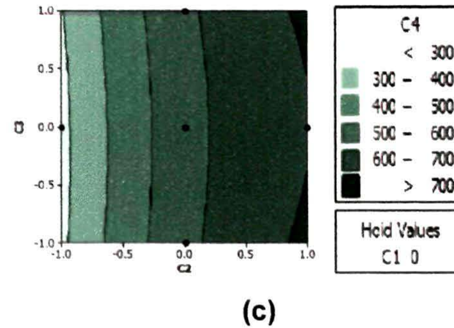
The countor plots were plotted on the basis of the model equation to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum protease production by *Bacillus* sp. strain AS-S20-I (Figs.8.17a-c). Protease production varied significantly upon changing the initial concentration of casein and the concentration of ammonium sulphate. The countor plots showed that either with an increase in pH of the medium, or concentration of ammonium sulphate resulted in protease production to optimum value, whereas, further increase in concentration beyond the optimum value decreased the protease yield. These results reinforced that the response equation provided a suitable model for the CCD experiment.



(a)



(b)



(c)

Fig.8.16 Countor plots for alkaline protease production by *Bacillus* sp. strain AS-S20-I. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0 (coded value) (b) pH of the medium vs concentration (%, w/v) of ammonium sulphate, hold value C_2 = 0 (coded value) and (c) concentration (%, w/v) of casein vs concentration of ammonium sulphate (% w/v) hold value C_1 = 0 (coded value). Values are mean ± S.D. of three experiments.

8.4 Batch fermentation using the optimal medium

On the basis of regression equation so obtained, the optimum level of test variables for maximum protease yield was predicted by using the Minitab 16 Statistical Software® program as 3.0 % (w/v) of casein, 0.12 % (w/v) of ammonium sulphate and at pH 10.9 of the medium. The optimal protease production in a process-controlled fermenter was found to be significantly higher (749.0 x 10^3 U /l) than that observed under the shake-flask study (699.0 x 10^3 U /l).

8.4 Partial purification of alkaline protease

The electrophoresis of the crude protease and acetone fraction are presented in Fig 8.17. Protein profiling of *Bacillus* sp. strain AS-S20-I suggested that this bacterium secretes a major protein with molecular mass of 66.0 kDa, and two minor protein of molecular weights43.0 and 37.0 kDa, respectively.

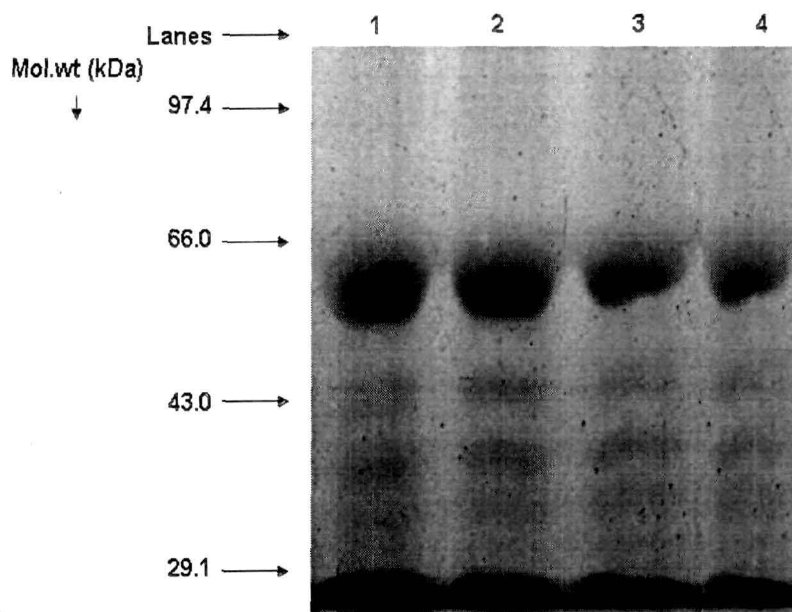


Fig 8.17 12.5 % SDS-polyacrylamide gel electrophoresis of crude and acetone precipitated proteases. Molecular weight markers are phosphorylase b (97,400 Da), BSA (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da); lane 1, crude protease in reduced condition (40.0 µg); lane 2, crude protease in non-reduced condition (40.0 µg); lane 3, acetone precipitate fraction under reduced condition (15.0 µg); lane 4, acetone precipitate fraction under non-reduced condition (15.0 µg).

8.5 Biochemical characterization of partially purified protease

8.5.1 Effect of pH, incubation time and temperature on partially purified alkaline protease

The partially purified protease displayed maximum caseinolytic activity within 15 min of post -incubation at 45°C incubation temperature with casein as a substrate.

8.5.2 Effect of pH on partially purified alkaline protease

Although the enzyme remained active over a broad range of pH (5.0–12.0) the enzyme showed characteristics pH optima at 10.0 (Fig. 8.18).

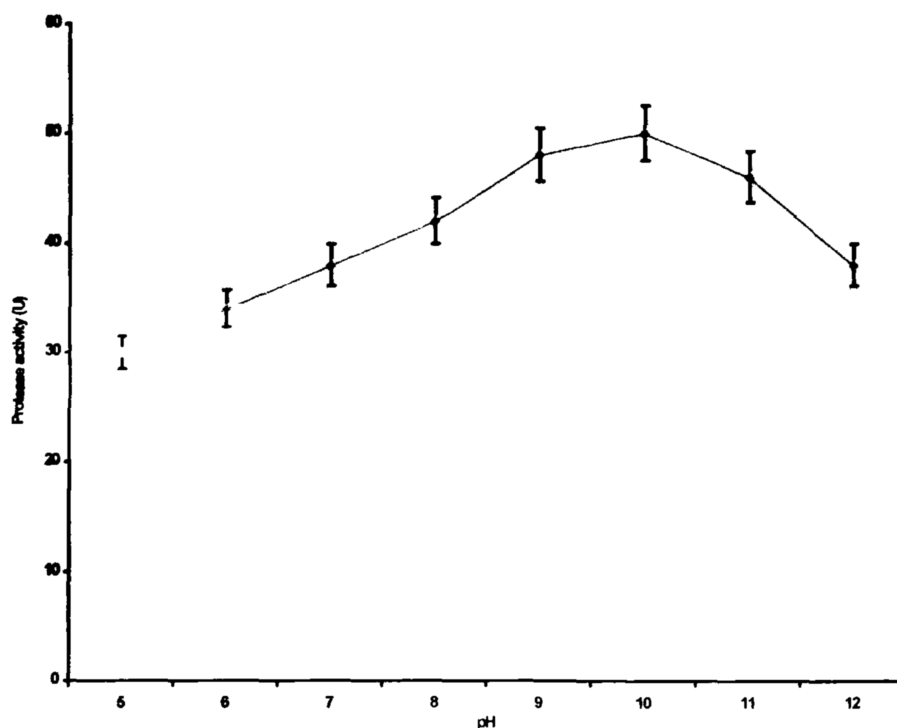


Fig. 8.18 Influence of pH on alkaline protease activity from *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments.

8.5.3 Thermo-stability study of partially purified alkaline protease

The protease from *B.* sp. strain AS-S20-I retained 100% of its original activity after heating at 60°C for 15 min and thereafter, a steady decline in the protease activity was observed (Fig.8.19).

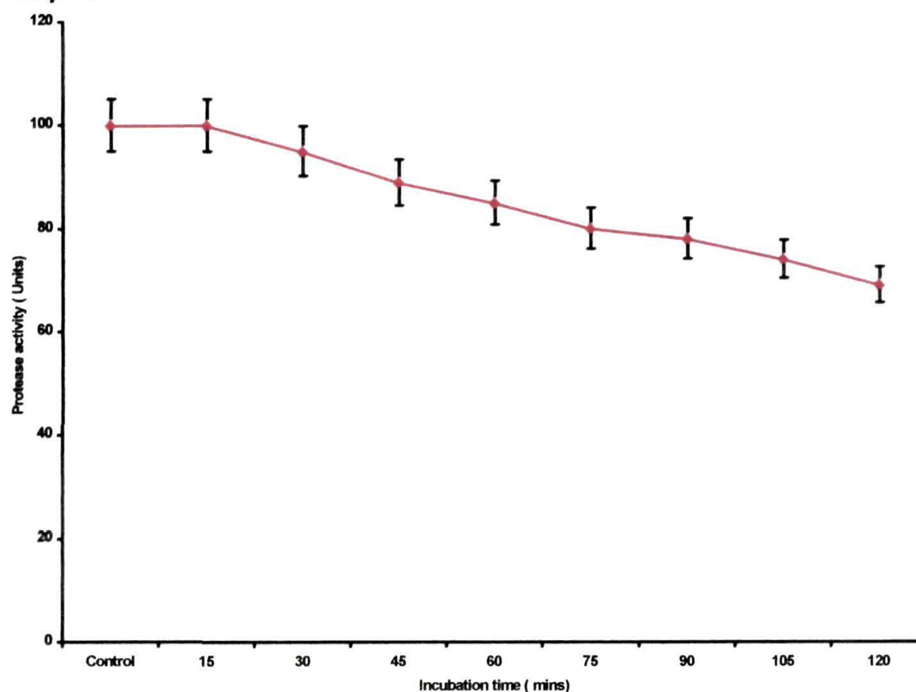


Fig.8.19 Thermo-stability study of partially purified protease from *Bacillus* sp. strain AS-S20-I heated at 60°C for various time interval. Values are mean ± S.D. of three experiments.

8.5.4 Substrate specificity of partially purified alkaline protease

Among the tested protein substrates, casein served the most preferred substrate, followed by chicken-feather keratin, bovine serum fibrinogen, albumin, globulin, fibrin, hemoglobin, and gelatin (Table 8.7). In contrast, human hair and collagen could not be hydrolyzed by this enzyme.

Table 8.7 Substrate specificity of partially purified alkaline protease from *Bacillus* sp.strain AS-S20-I. Values are mean ± S.D. of three experiments.

Substrate specificity	Specific activity (U / mg of protein)
Fibrin	7100.0 ± 355.0
Casein	3830.0 ± 192.0
Raw chicken feather	3408.0 ± 170.0
Bovine serum albumin	2144.0 ± 107.0
Bovine serum fibrinogen	1532.0 ± 77.0
Bovine serum globulin	957.0 ± 48.0
Hemoglobin	766.0 ± 38.0
Gelatin	574.0 ± 23.0
Human hair	0.0
Collagen	0.0

8.5.5 Thermo-stability study of partially purified alkaline protease in presence or absence of polyols

The residual protease activity post heating of partially purified alkaline protease at 60°C for 120 min in presence and absence of polyols is displayed in Fig.8.20. It was observed that glycerol stabilized the enzyme to the maximum extent against heat-denaturation.

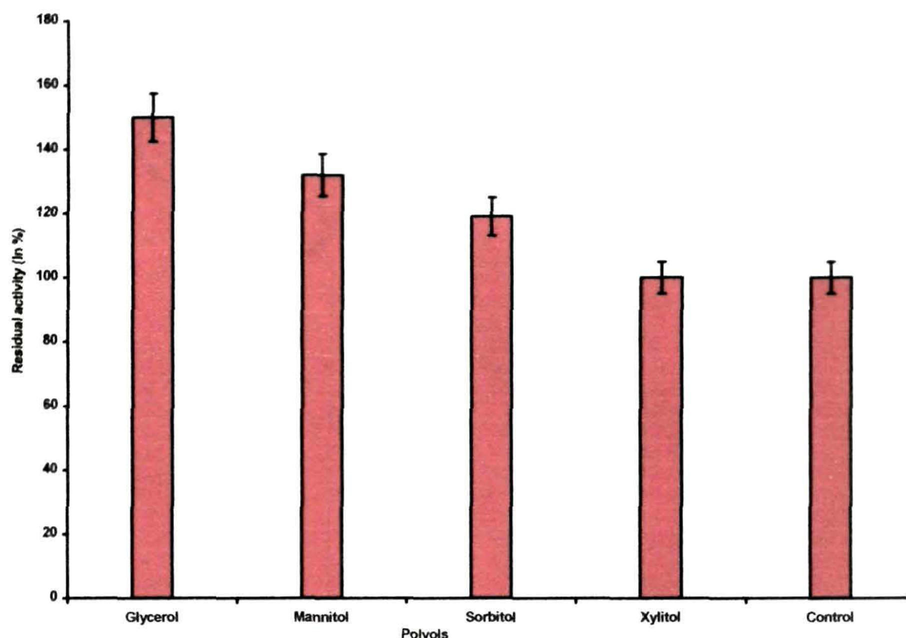


Fig.8.20 Effect of polyols against heat-denaturation of partially purified alkaline protease from *Bacillus* sp. strain AS-S20-I. The protease was heated at 60°C for 120 min either in presence or absence of polyols. The enzyme activity without polyols was considered as control (100%) and values were compared with that. Values are mean ± S.D. of three experiments.

8.5.6 K_m and V_{max} values

The K_m and V_{max} values of partially purified protease towards fibrin were determined as 0.16 mg /ml and 20.0 µmol /min / mg respectively. (Fig.8.21)

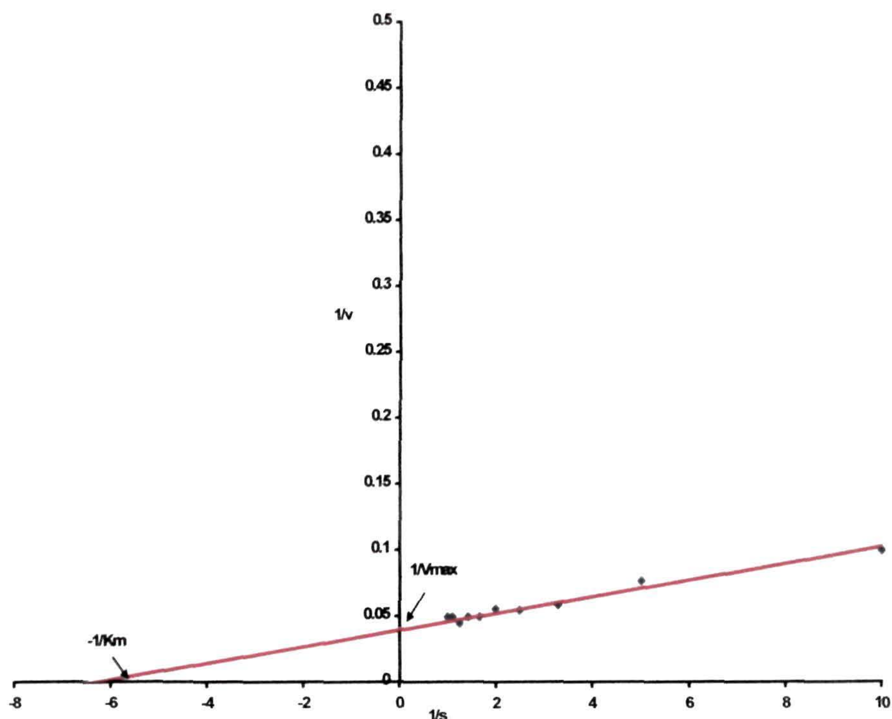


Fig.8.21 Lineweaver-Burk plot to determine the K_m and V_{max} values of partially purified protease using casein as a substrate. Values are mean ± S.D. of three experiments.

8.5.7 Effect of metal ions on partially purified protease

Study on the influence of different metal ions on alkaline protease activity of strain AS-S20-I showed that all the tested cations, viz. Ni^{2+}, Cd^{2+}, Co^{2+},Mg^{2+}, Fe^{2+}, Hg^{2+}, Cu^{2+},Mn^{2+}, Ca^{2+} and Zn^{2+}, inhibited the enzyme activity. Maximum inhibition of protease activity was observed in the presence of Cu^{2+} (78% inhibition) whereas Cd^{2+} exerted minimum inhibition (12.77 %) of protease activity compared to control (Fig.8.22).

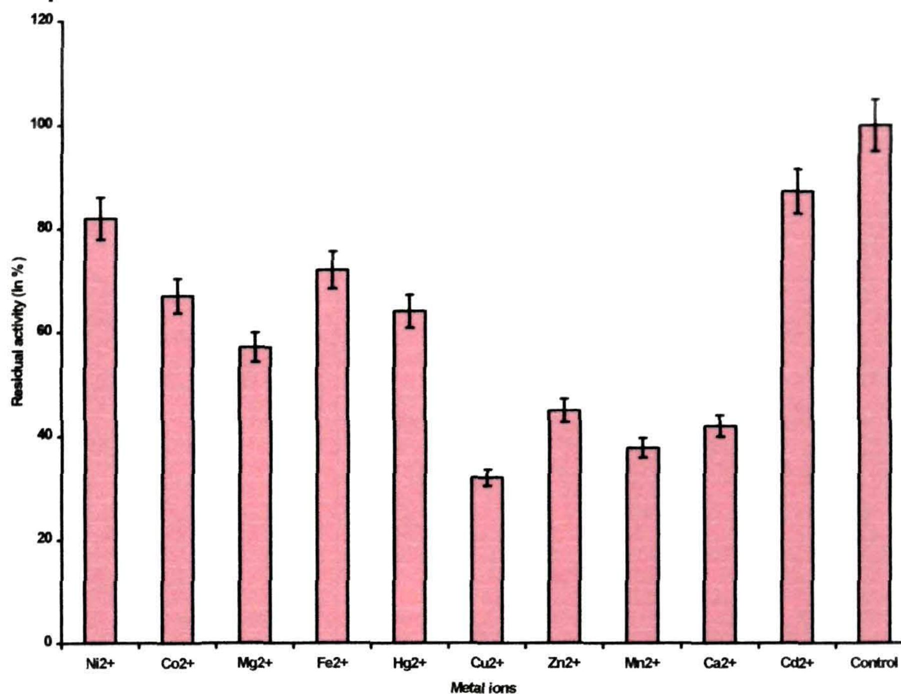


Fig.8.22 Effect of metal ion on partially purified protease from *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D. of three experiments.

8.5.8 Effect of inhibitors, chelators, denaturing agent, oxidizing and bleaching agent, and surfactants on partially purified protease

An effect of different inhibitors, metal chelator, surfactants and DTT on partially purified protease is shown in Table 8.8. The partially purified protease retained 100% to 80% of its original activity post-treatment with different concentrations of EDTA, SDS, Tween-20, Tween-80, Triton-X-100, and urea (Table 8.8). The oxidizing agent like H_2O_2 and bleaching agent (sodium perborate) enhanced the enzyme activity (Table 8.8). However, the enzyme activity was significantly affected by PMSF, IAA, 4-bromophenacyl bromide and TPCK, thus suggesting a role for serine, cysteine and histidine residues in the catalysis process (Table 8.8). Further, a significant inhibition of protease activity in the presence of DTT was also observed. For example, in presence of 4.0mM DTT the protease

lost more than 90.0% of the original activity of protease was abolished (Table 8.8).

Table 8.8 Effect of chemical/chelators/ inhibitors/ denaturing/ oxidizing/ bleaching agents on catalytic activity of partially purified protease from *Bacillus* sp. strain AS-S20-I. Values are mean ± S.D of triplicate determinations.

Reagents / Effectors	Percent residual activity
Control	100
Inhibitors/ surfactants	
PMSF (2 mM)	25.0 ± 0.5
(4 mM)	2.4 ± 0.05
4-BpB (2 mM)	73.0 ± 1.5
(4 mM)	12.0 ± 0.2
IAA (2 mM)	28.0 ± 0.5
(4 mM)	10.0 ± 0.2
EDTA (2 mM)	100.2 ± 5.01
(4 mM)	85.4 ± 4.27
TLCK (2 mM)	110.1 ± 5.51
(4 mM)	91.4 ± 4.57
TPCK (2 mM)	45.2 ± 2.26
(4 mM)	12.4 ± 0.62
SDS (40 mM)	98.4 ± 4.92
Trition-X-100 (1% v/v)	100.4 ± 5.02
Tween 20 (1% v/v)	99.3 ± 4.97
Tween 80 (1% v/v)	95.2 ± 4.76
Denaturing agent	
Urea (6 M)	93.2 ± 4.66
Oxidizing agent	
H_2O_2 (% v/v)	

Reagents / Effectors	Percent residual activity
5.0	140.0 ± 2.8
10.0	125.0 ± 2.5
Bleaching agent	
Sodium perborate (% v/v)	
0.5	129.0 ± 2.6
1.0	114.0 ± 2.3
DTT(2 mM)	45.2 ± 2.26
(4 mM)	8.90 ± 0.45

8.5.9 Effect of organic –solvents on partially purified protease

The organic-solvent (at a final concentration of 20% v/v) stability of partially purified protease is shown in Fig. 8.23. All the organic solvent destabilized the protease resulting in loss of protease activity in presence of organic solvents. The maximum inhibition was produced by methanol followed by benzene.

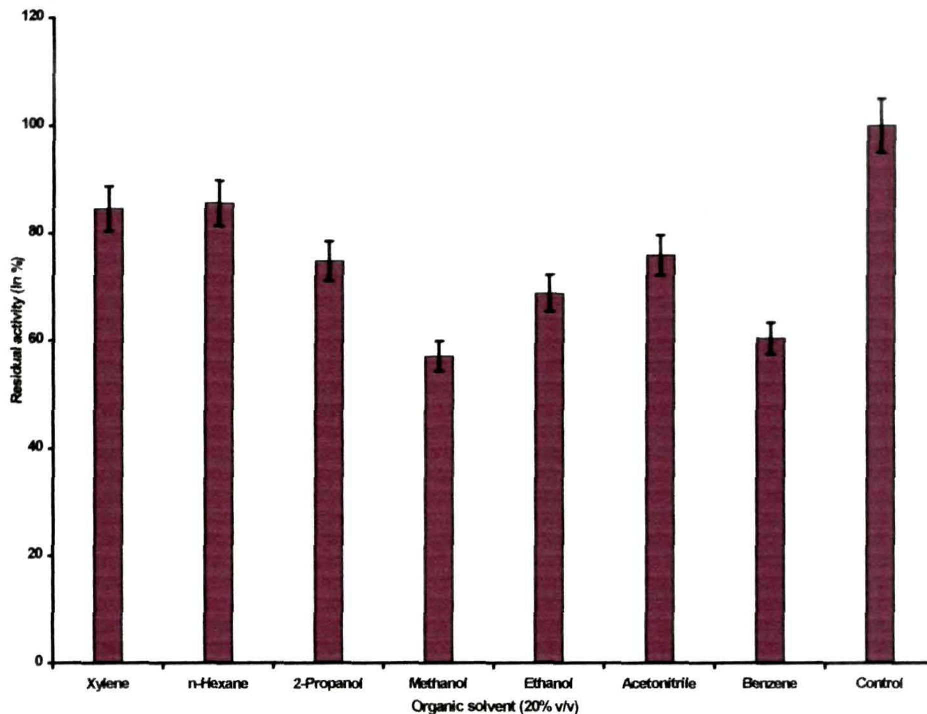


Fig. 8.23 Organic-solvent stability of partially purified protease from *Bacillus* sp. strain AS-S20-I. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean ± S.D. of three experiments.

8.5.10 Fibrin(ogen)lytic study

The specific activity of protease from *B.* sp. strain AS-S20-I was determined as 1689.0 U / mg of protein against fibrin, The fibrin degradation pattern demonstrated that the degradation of α-chain of fibrin was faster compared to the rate of degradation of β-, and γ-chains (Fig. 8.24). In contrast, the crude enzyme preferentially degraded the β-chain compared to α-chain of fibrinogen. However, the γ-chain of fibrinogen could not be degraded within 12 h of incubation (Fig.8.25).

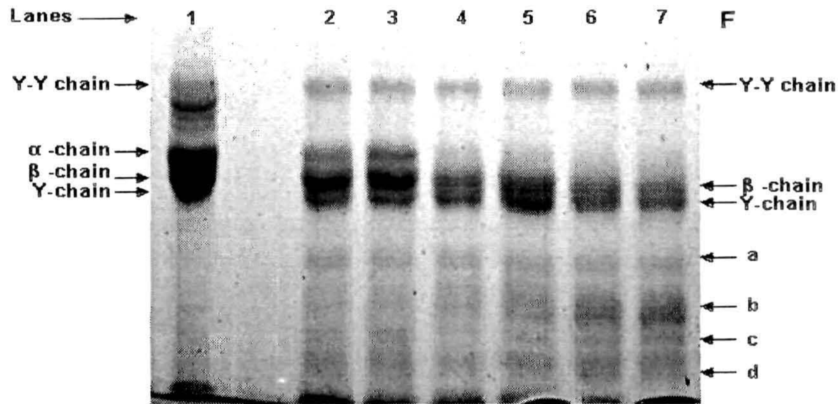


Fig. 8.24 Degradation of fibrin by partially purified alkaline protease from *B.* sp. *strain* AS-S20-I. The fibrin degradation products were separated by 12.5% SDS-PAGE. Lanes 1: control; Lane 2-7: fibrin degradation pattern by 5.0 µg of partially purified alkaline protease post 30, 60, 120, 240, 360, 480, and 720 min of incubation, respectively at 37°C.

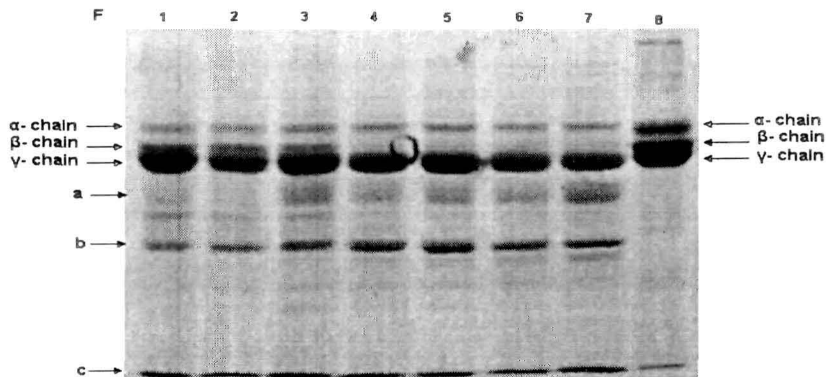


Fig.8.25 Degradation pattern of fibrinogen by partially purified alkaline protease from *B.* sp. strain AS-S20-I. The fibrinogen degradation products were separated by 12.5% SDS-PAGE. Lanes 1-7: fibrin degradation pattern by 5.0 µg of partially purified alkaline protease post 30, 60, 120, 240, 360, 480, and 720 min of incubation, respectively at 37°C; Lane 8: control.

8.5.11 Pharmacological properties of partially purified protease from *Bacillus* sp. strain AS-S20-I

Partially purified protease from *Bacillus* sp. strain AS-S20-I at a dose of 15 µg/ml induced 2.4 % hemolysis of the washed human erythrocytes. At the same dose, it did not exert any haemorrage to goat liver, heart, lungs and kidney tissues (Table 8.9). Further, as shown in Table 8.9, this partially purified protease did not interfere with the normal clotting time of goat platelet- poor plasma (PPP).

Table 8.9 Pharmacological properties of partially purified protease from *Bacillus* sp. strain AS-S20-I. Values are mean± S.D. of three experiments.

Pharmacological properties	Values
Hemolysis (% Hb released / 15.0 µg of partially purified protease 90 min post incubation at 37 °C)	
Control (without partially purified protease)	1.4 ± 0.1
Treatment	2.4 ± 0.05
Ca-Clotting time (s)	
Control (without partially purified protease)	129.0 ± 6.5
Treated (with 15.0 µg / ml of partially purified protease)	132.0 ± 7.0
In vitro tissue damaging activity (% hemoglobin release by 15.0 µg / ml of partially purified protease 5 h post incubation at 37 °C)	
a) ***Heart***	
Control (without partially purified protease)	0.09 ± 0.01
Treatment	0.27 ± 0.01
b) ***Lung***	
Control (without partially purified protease)	0.13 ± 0.01
Treatment	0.36 ± 0.02

Pharmacological properties	Values
c) ***Liver***	
Control (without partially purified protease)	0.16 ± 0.08
Treatment	0.28 ± 0.01
d) ***Kidney***	
Control (without partially purified protease)	0.12 ± 0.05
Treatment	0.27 ± 0.01

8.6 Industrial applications of partially purified protease

8.6.1 Blood clot lytic activity of partially purified protease

The in vitro clot lytic activity showed that incubation of protease at a dose of 0.78 mg /ml with 300.0 ± 10.0 mg of natural or synthetic blood clot (induced by thrombin) for 16h can completely lyse the clot (Fig.8.26).

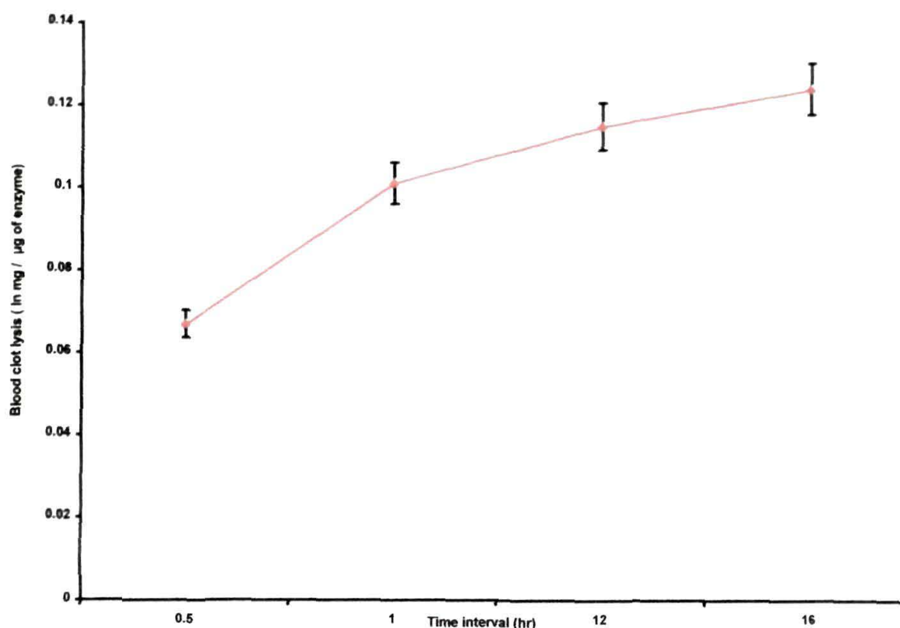


Fig.8.26 Thrombolytic activity of partially purified protease from *Bacillus* sp. strain AS-S20-I. Values represent mean ± S.D of three experiments.

8.6.2 Detergent compatibility and stain removal potency of protease

The partially purified protease at a dose of 6.0 µg / ml retained 60- 100% of its original activity when pre-incubated at 37°C in presence of commercial laundry detergents in the following order: Surf excel® = Sunlight® = Safed® > Wheel® > Henko® > Ariel® > Fena Ultra® > Tide® (Fig.8.27). The blood stain removal from cotton fabrics shown by a 1:1 (v/v) mixture of detergent: partially purified protease solution was significantly higher ($p < 0.05$) compared to the detergents solutions or partially purified protease solution alone in the tested temperature. For example, about 35% and 22% increase in removal of blood stain was observed post washing the stained cotton fabrics with detergent supplemented with partially purified protease, and Surf excel® detergents, respectively reinforcing application of the enzyme under study in detergent formulations for improving the wash performance of the detergents (Fig. 8.28).

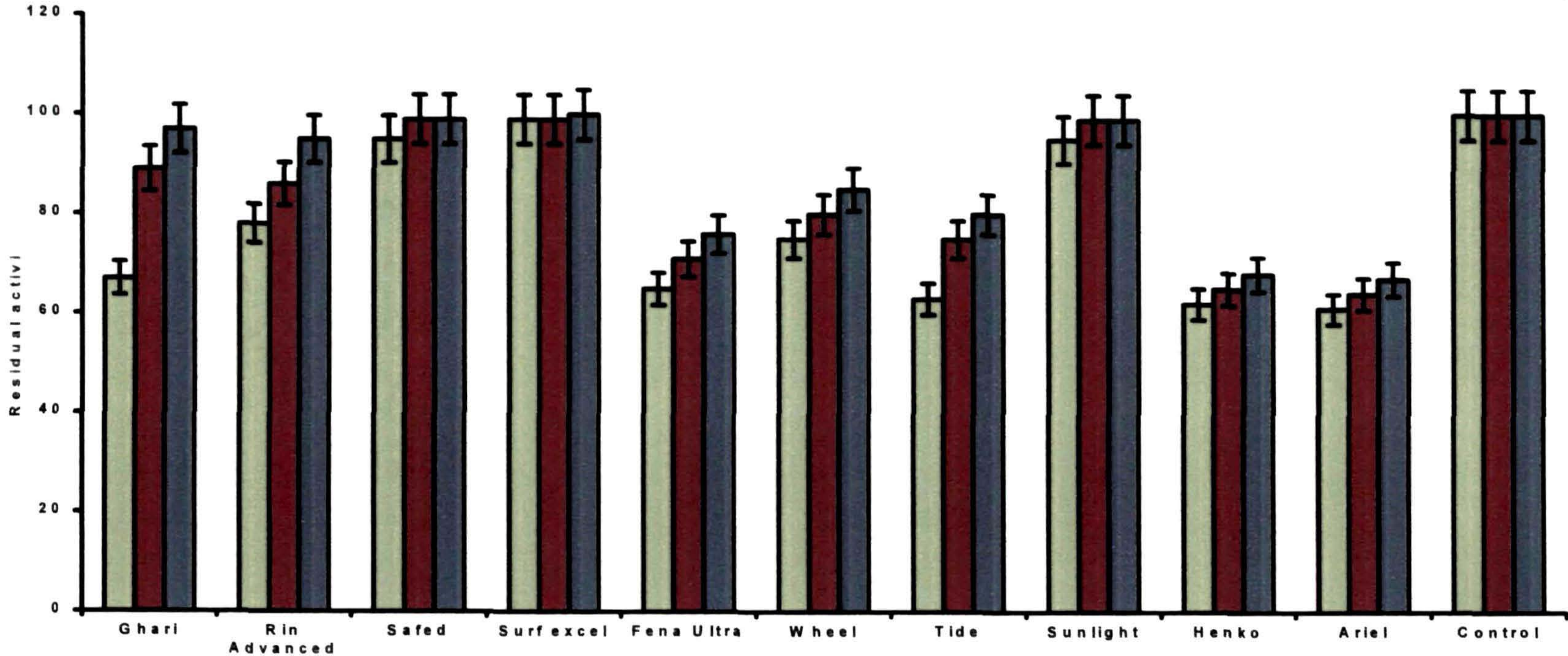


Fig.8.27 Detergent stability and compatibility of partially purified protease (6.0 µg / ml) from *Bacillus* sp. strain AS-S20-I at the tested temperature ranges 25 (■), 37 (■) and 45°C (■). Values are mean ± SD of triplicate determinations.

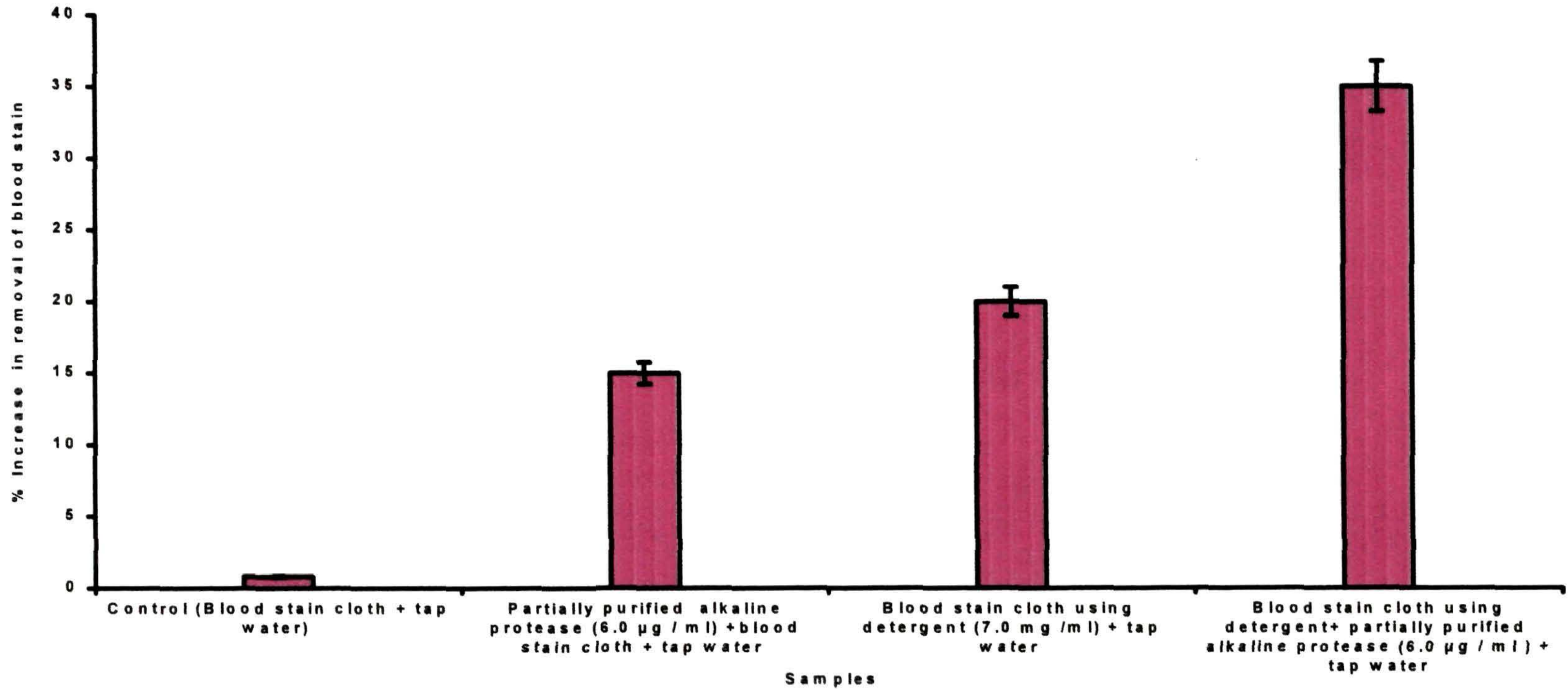
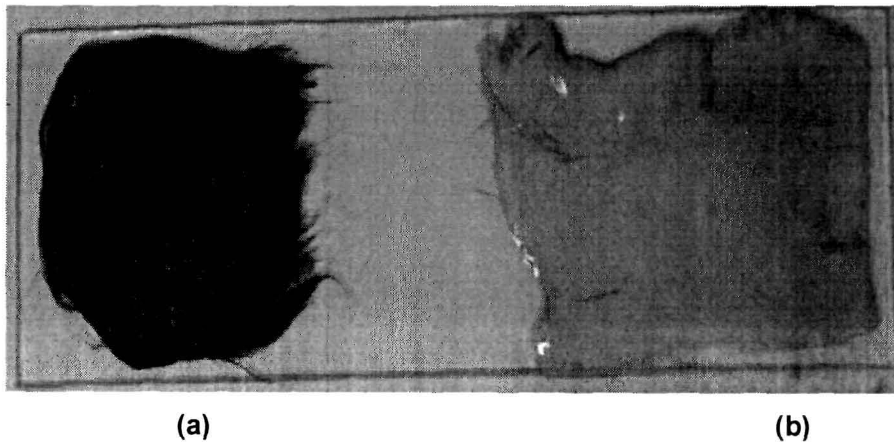


Fig.8.28 Wash performance test of partially purified protease (6.0 µg /ml) from *Bacillus* sp. strain AS-S20-I at 37°C. The values represent mean ± S.D. of three determinations.

8.6.3 Dehairing activity

The dehairing effect of partially purified protease from *Bacillus* sp. strain AS-S20-I showed a promising result (Fig. 8.29). When the raw skin was incubated with the partially purified protease at 37°C for 12 h, the hairs could be pulled out easily., which was not possible when the skin was incubated with buffer only.



(a) (b)

Fig. 8.29 Dehairing activity of partially purified protease from *Bacillus* sp.strain AS-S20-I (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with partially purified protease (50 U / ml) for 12 h at 37°C.

CHAPTER IX

RESULTS

Sudhir K Rai

PhD Thesis, Tezpur University, 2010

Chapter 9

Process optimization, purification, biochemical characterization, and industrial application of an alkaline protease isolated from *Bacillus licheniformis* AS-S24-I

9.1 Alkaline protease production under solid-state fermentation

9.1.1 Screening of different agro-industrial and waste materials for alkaline protease production

Various agro-industrial and kitchen waste substrates were screened for their ability to produce alkaline protease in this study. In SSF, all the tested substrates except used tea-leaves supported protease production. The study on the potential of agro-industrial residues as substrates for alkaline protease production by *Bacillus licheniformis* strain AS-S24-I showed IC and PP were the most effective substrates for this purpose (Fig.9.1). Maximum protease production was observed with PP (340.0 U/gds) followed by IC (219.0 U/gds) as substrate post 60 h of incubation, while minimum protease production (1.7 U/gds) was shown by waste TL as substrate/ support material (Fig.9.1). The protein content of the culture supernatants as well as the bacterial dry biomass followed the same trend as protease production on different substrates (Fig.9.2).

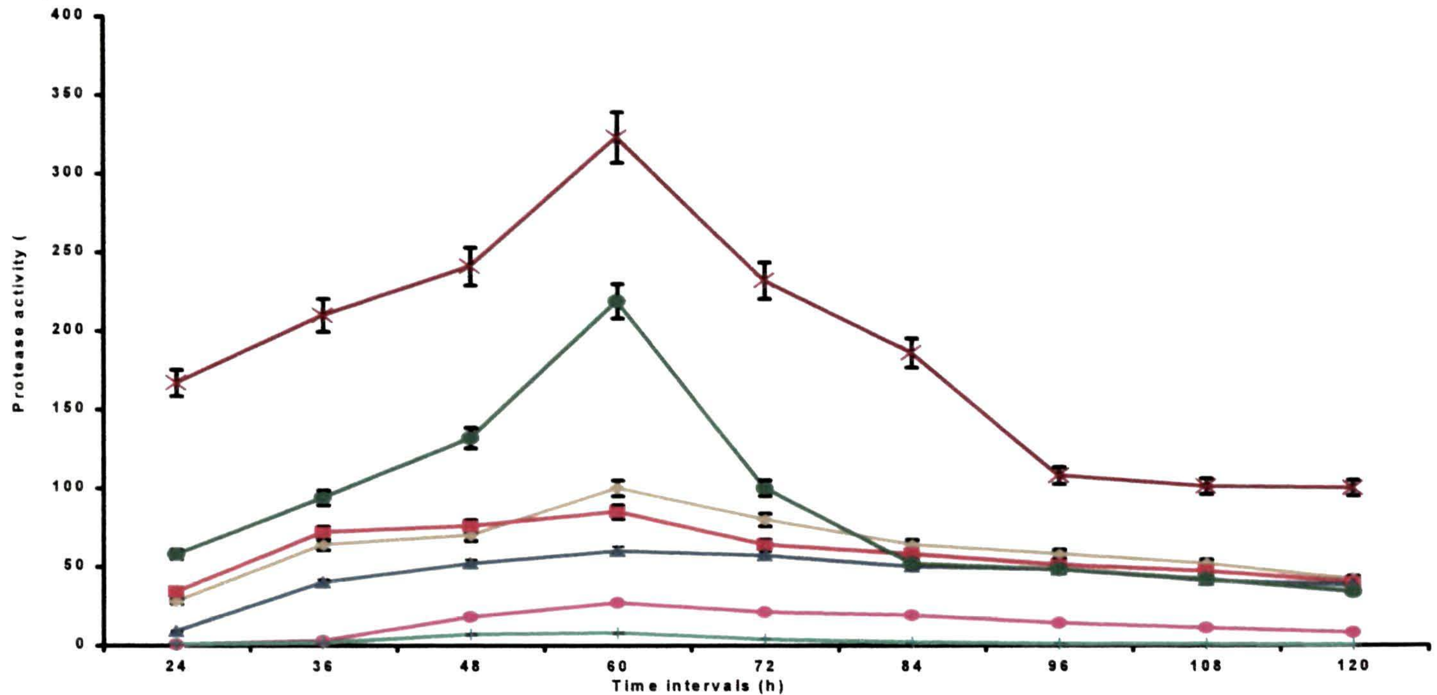


Fig.9.1 Screening of different waste residues such as MOC (◆), WB (■), RB (▲), IC(●), PP(☒), BL(●), and TL(+) for the production of alkaline protease by *B. licheniformis* strain AS-S24-I at different time intervals. Values are mean ± S.D. of three experiments.

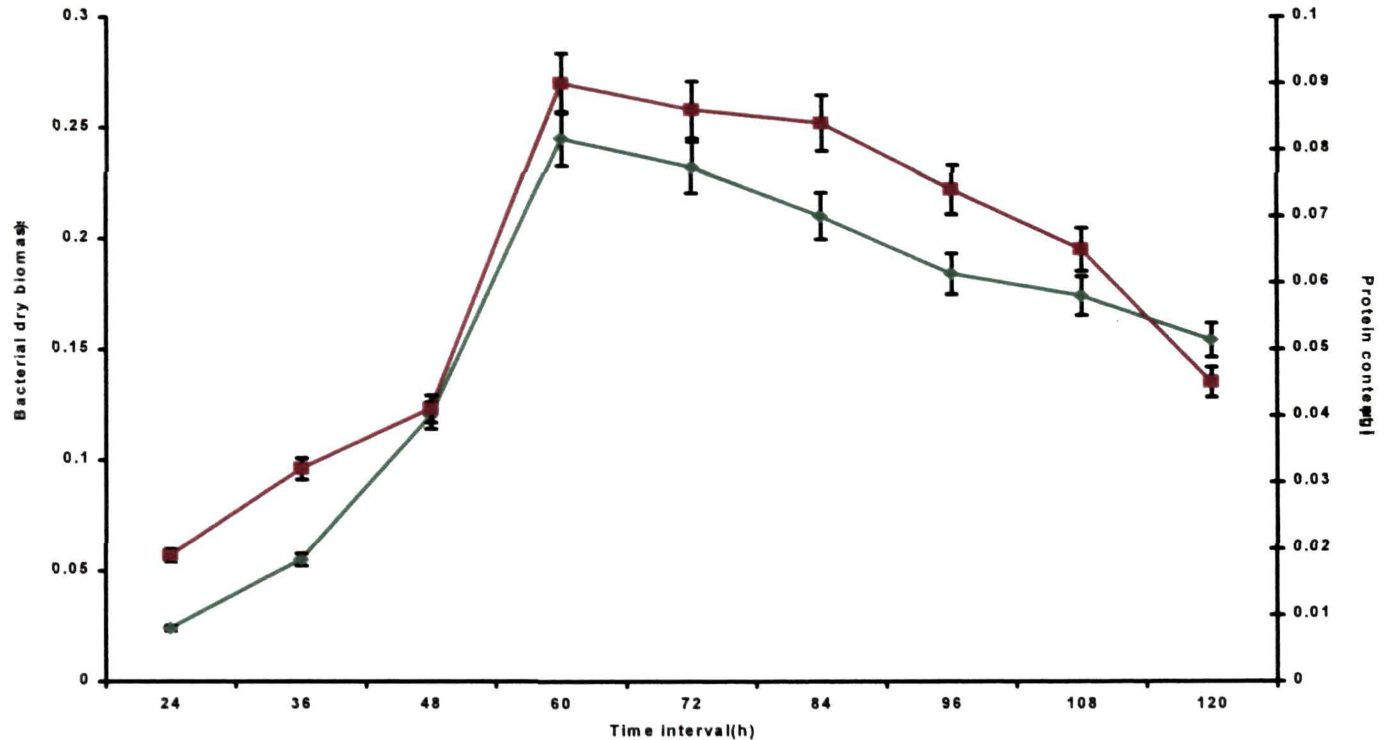


Fig.9.2 Kinetics of alkaline protease production from *B. licheniformis* strain AS-S24-I. Legends show bacterial dry biomass (◆) and protein concentration (■).Values are mean ± S.D. of three experiments.

9.1.2 Effect of initial moisture content of the substrate and moistening agent on protease production

Among the several factor that are important for bacterial growth and protease production under solid-state fermentation by *B.licheniformis* strain AS-S24-I using a particular substrate, moisture level (content)/ water activity is one of the most critical factors. In present study, maximum protease production was observed with 40% moisture content with an enzyme activity of 260 U/gds (Fig. 9.3). Distilled H_2O adjusted to pH 10.0 was most efficient moistening agent for protease production compared to other moistening agents (Fig. 9.4).

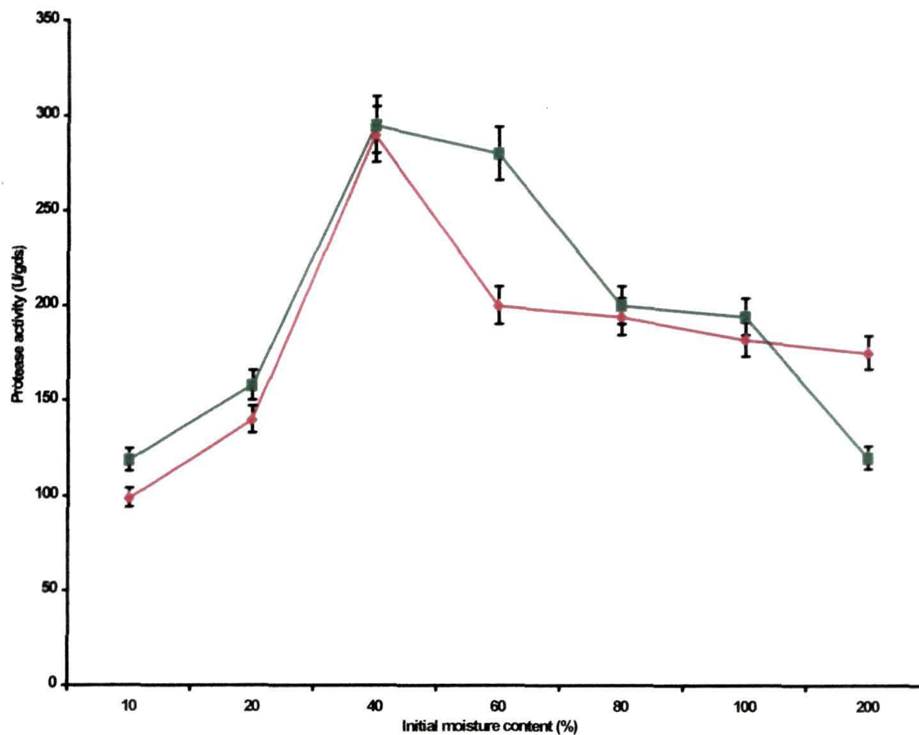


Fig.9.3 Influence of initial moisture content of the substrates [PP (■) or IC (♦)] on protease production. Values are mean ± S.D. of three experiments post 60h incubation at 45°C. Moistening agent was distilled water, adjusted to pH 10.0 with 0.01N NaOH.

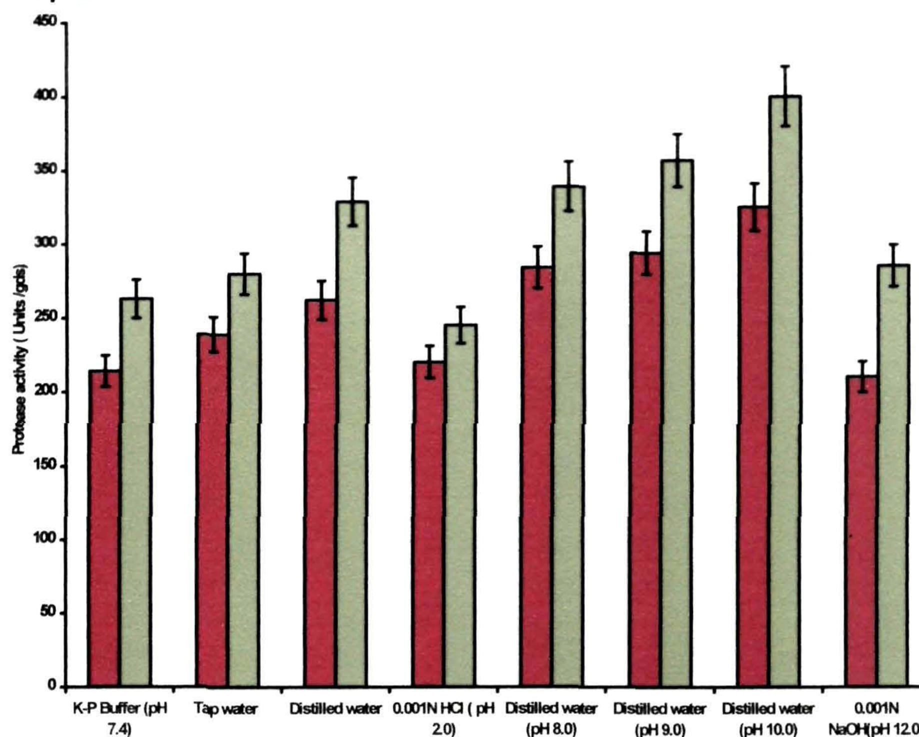


Fig.9.4 Influence of moistening agents on alkaline protease production by *B.licheniformis* strain AS-S24-I in SSF using either IC (■) or PP (■) as a solid substrate. Values are mean ± S.D. of three experiments post 60h of incubation 45°C.

9.1.3 Effect of inoculum's size on protease production

Size of inoculum is an important biological factor, which determines biomass as well as enzyme production during fermentation. The results presented in Fig. 9.5 showed that there was a significant increase in alkaline protease production with an increase in inoculum size up to an optimal level of 0.5-4.0ml after that enzyme yield was constant upto the range of 6.0ml of culture volume. A decrease in protease yield was observed with an increase in inoculum volume beyond 6.0 ml. Maximum enzyme production (440 U/g) was obtained when SSF medium was inoculated with 4.0 ml of inoculum.

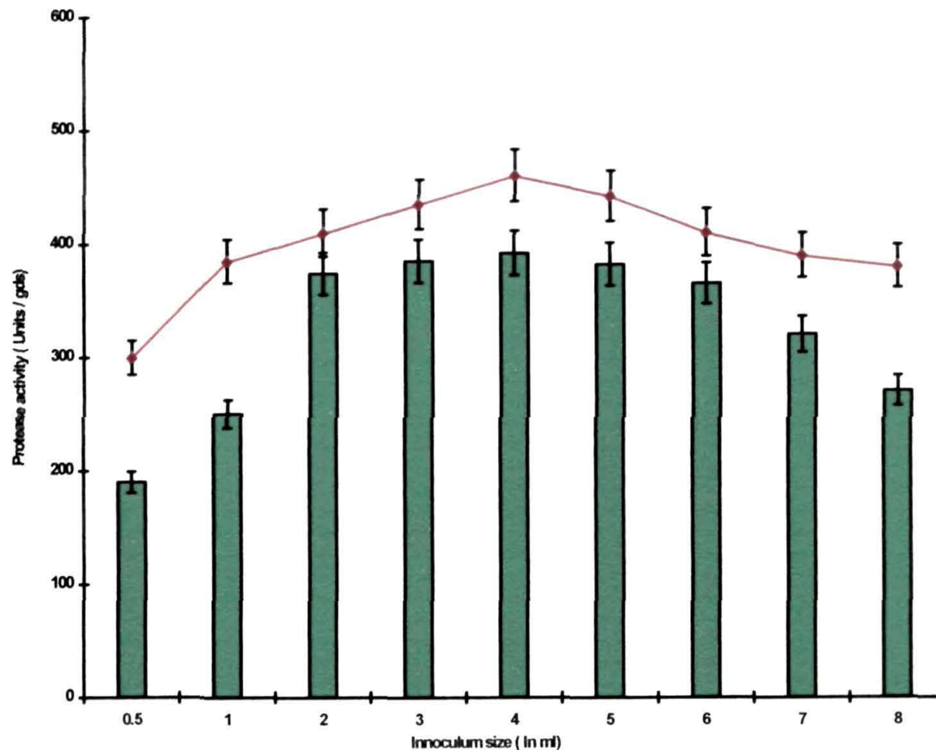


Fig.9.5 Influence of inoculum size on protease production by *B. licheniformis* strain AS-S24-I using PP (♦) or IC (■) as substrate under SSF. Values are mean ± S.D. of three experiments.

9.1.4 Effect of supplementation of co-carbon and co-nitrogen sources on protease production

In order to study the effect of carbon sources on protease production, cultivations were performed with 1.0 % (w/w) carbon (casein, glucose, fructose, galactose, maltose, sucrose and lactose). Maximum yield of protease was observed when the substrates was supplemented with glucose (1200 U/ gds) as co-carbon sources followed by galactose (850 U /gds) and lactose (794.0 U / gds) along with IC as main substrate (Fig.9.6), whereas the other supplemented carbon sources did not accelerate the bacterial growth and protease production by *B.licheniformis* strain AS-S24-I under SSF system.

Among the tested nitrogen compounds, maximum extracellular alkaline protease production was obtained in presence of 0.1 % (w/w) beef extract (890.0 U / gds) followed by yeast extract (875.0 U / gds) which served as the best co-nitrogen sources for protease production by *B. licheniformis* AS-S24-I strain on IC (Fig.9.7). The protease production in presence of other organic and inorganic nitrogen sources was as followed: peptone (389.0 U / gds), sodium nitrate (495.0 U / gds), ammonium nitrate (285.0 U / gds), ammonium chloride (195.0 U / gds), and ammonium sulphate (176.0 U / gds). These results are displayed in Fig 9.7.

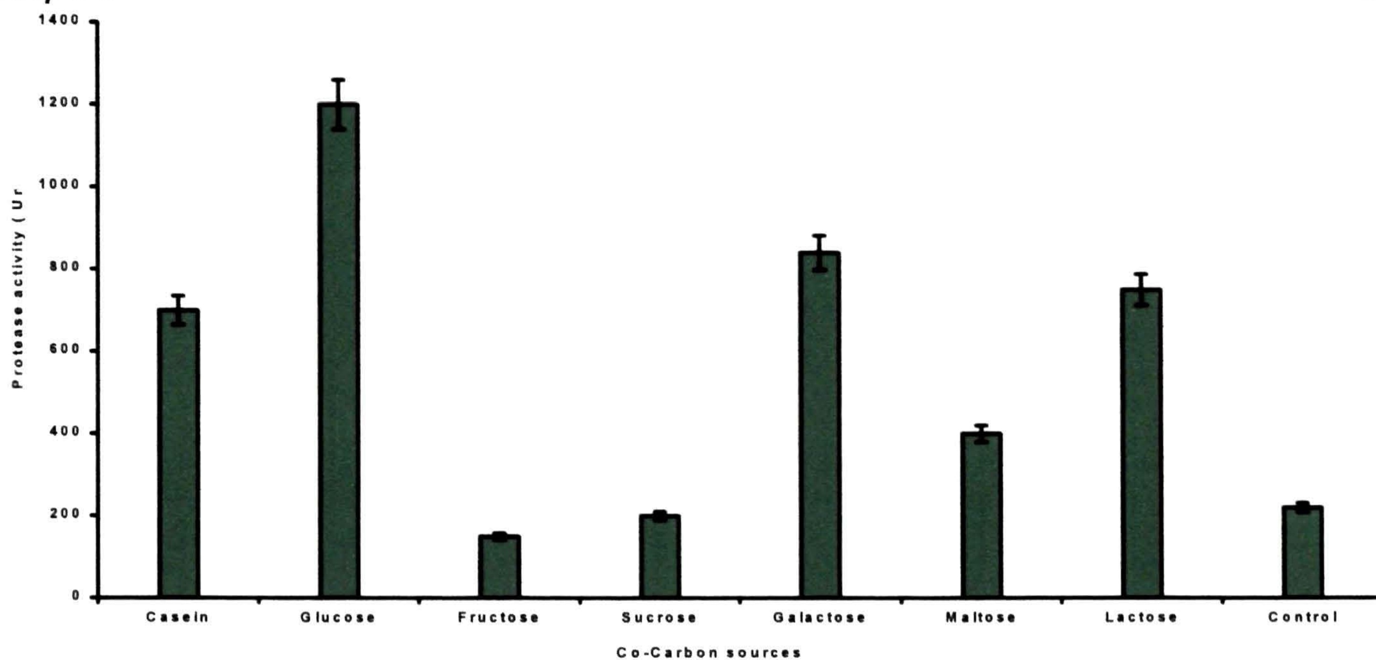


Fig. 9.6 Effect of supplemented co-carbon sources (10 % w/w) to IC (90 % w/w) (substrate) on alkaline protease production by *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments.

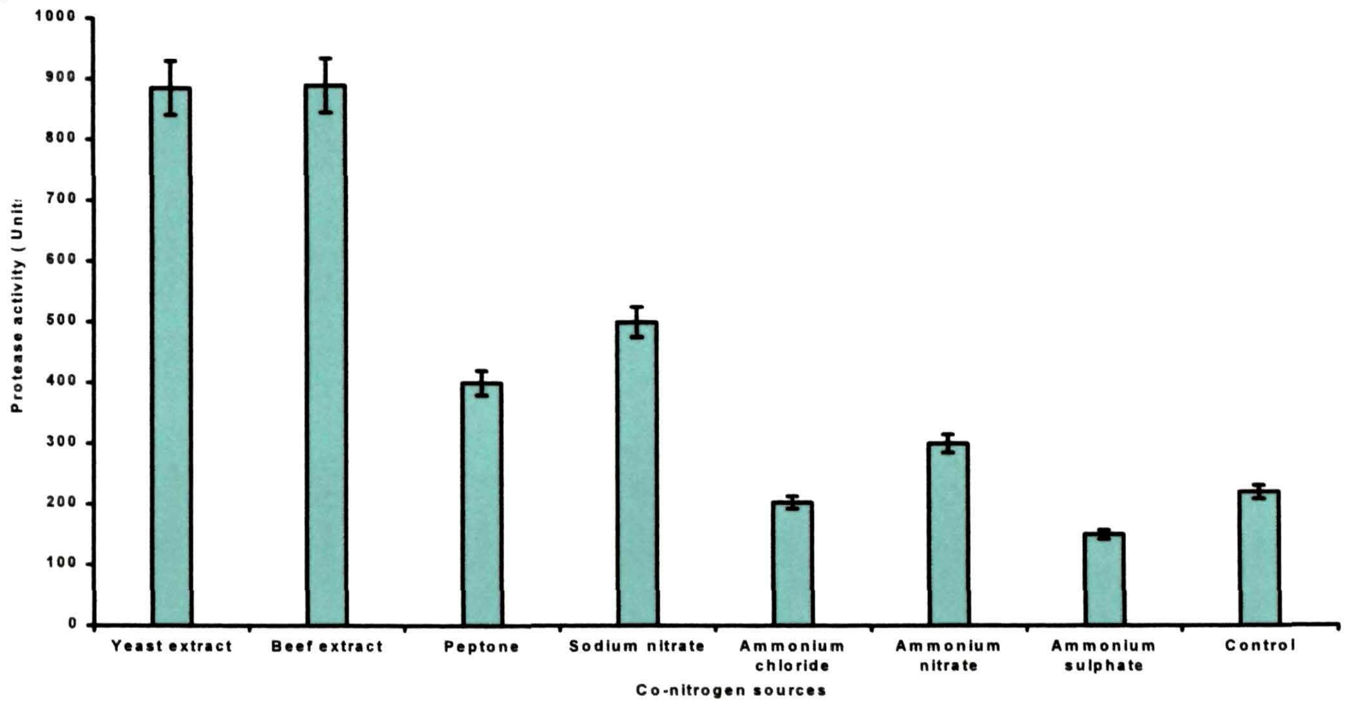


Fig.9.7 Effect of supplemented co-nitrogen sources (1.0% w/w) to IC (99.0 % w/w) (substrate) for protease production from *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments.

9.1.5 Protease extraction from fermented matter

Among the different extractions medium used for the recovery of protease from the fermented matter (IC), distilled water containing 0.1%(v/v) triton X-100, pH 10.0 served as best extraction medium (Fig.9.8).

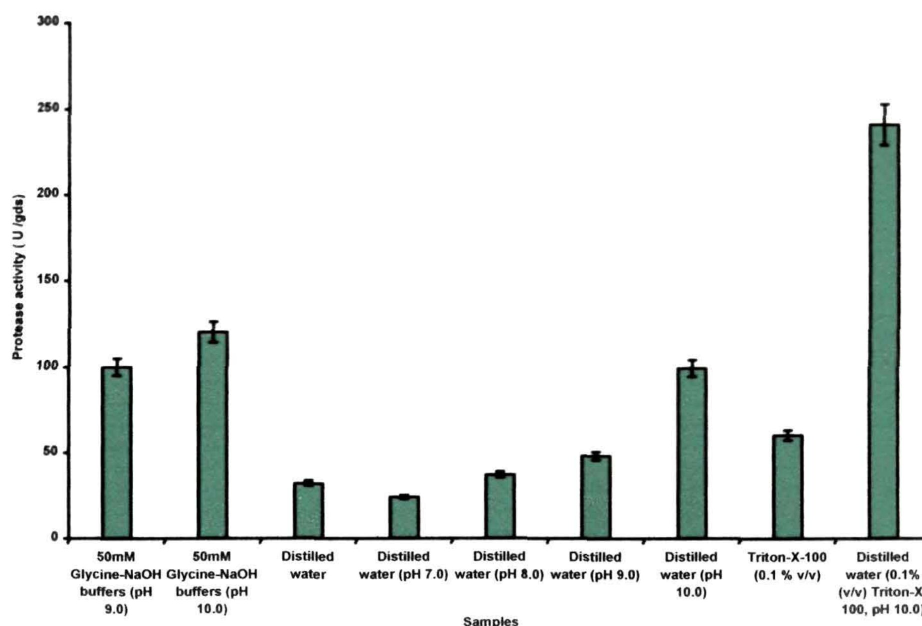


Fig.9.8 Screening of best medium for alkaline protease extraction from the fermentation mesh. Values are mean ± S.D. of three experiments.

9.1.6 Batch fermentation

A fermenter provides a suitable environment for growth and enzyme production, where a consistency in conditions like moisture level, innoculum level, incubation temperature, regular nutrition supply can be maintained in a better manner. When *B.licheniformis* strain AS-S24-I was cultivated in a rectangular trays fermentor, a 1.9-fold increase in enzyme yield was recorded when IC and PP powders were mixed in a ratio of 1:1 without any additional co- carbon and nitrogen sources (Table 9.1).

Table 9.1 Influence of different combinations of co-substrates on alkaline protease production by *B. licheniformis* strain AS-S24-I under solid-state fermentation Values are mean ± S.D. of three experiments.

Substrate(s)	Protease activity (U/gds)
l.cylindrica (100%)	219.0±10.95
Potato peel (100%)	340.0 ± 17.0
l.cylindrica (89%w/w)+ glucose (10 % w/w) + Beef extract (1% w/w)	1200.00 ± 60.0
l.cylindrica (90%w/w) + Potato peel (10%)	1500.0 ± 75.0
l.cylindrica (80%w/w) + Potato peel (20 %w/w)	1836.0± 91.8
l.cylindrica (50%w/w) + Potato peel (50%w/w)	2300.0 ± 115.0
Potato peel (89% w/w) + glucose (10%) + Beef extract (1%)	1000.0 ± 50.0

9.2 Alkaline protease production under submerged fermentation

9.2.1 Effect of different carbon source on protease production

Among different types of different carbon sources tested, 1.0 % (w/v) glucose (50.0 U) supported a high level protease production, followed by galactose (41.0 U), and production was least in presence of carboxylmethyl cellulose is a substrate (Fig.9.9).

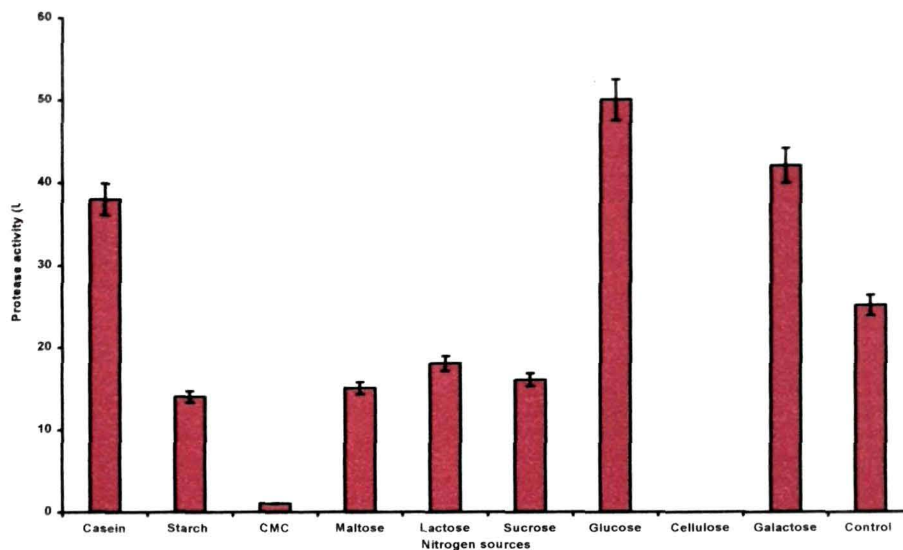


Fig.9.9 Effect of different carbon sources on alkaline protease production from *Bacillus licheniformis* strain AS-S24-I under SmF. Values are mean ± S.D. of three experiments.

9.2.2 Effect of nitrogen source on protease production

A wide range of organic and inorganic nitrogen sources were tested for successive protease production by *B.licheniformis* strain AS-S24-I in SSF. Maximum alkaline production was observed in presence of 0.1% (w/v) yeast extract followed by beef extract with accelerating bacterial growth and protease production (Fig. 9.10). Inorganic nitrogen sources were found to be less effective as compared to the complex organic sources of nitrogen in supporting the bacterial growth and protease production by soil borne *B.licheniformis* strain AS-S24-I.Sodium nitrate (inorganic nitrogen source) and tryptone (organic nitrogen source) suppressed the protease production by the strain under study.

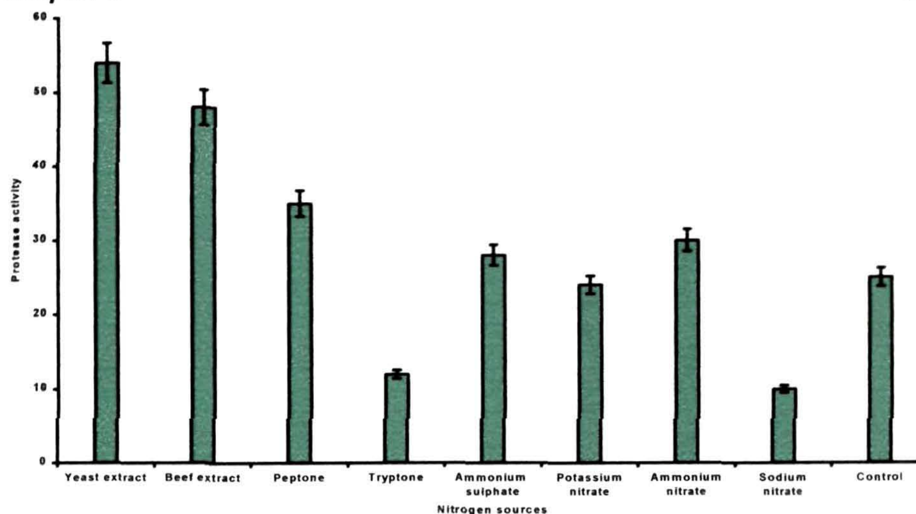


Fig.9.10 Effect of various nitrogen sources on alkaline protease production from *B. licheniformis* strain AS-S24-I in SmF. Values are mean ± S.D. of three experiments.

9.2.3 Effect of pH on protease production

Metabolic activities of the microorganisms are very sensitive to the pH change. Protease production by *B.licheniformis* strain AS-S24-I was found to be affected if pH level is higher or lower compared to the optimum value (pH 10.0). Results demonstrated that with an increase in the pH of the medium from 7.0 to 12.0, optimum protease production was observed at pH 10.0. Increase in medium pH beyond 10.0 resulted in a steady decline in protease yield (Fig.9.11).

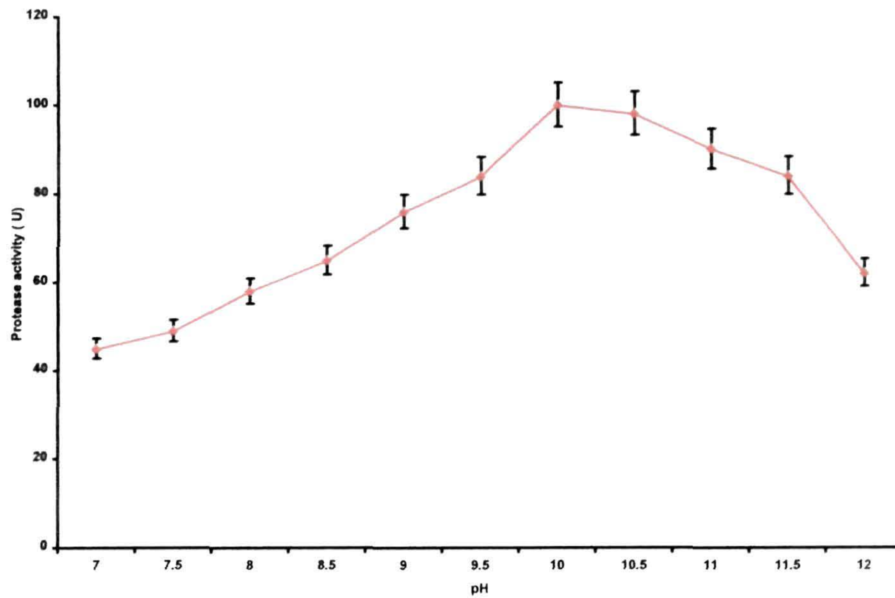


Fig.9.11 Effect of pH on alkaline protease production by *B. licheniformis* strain AS-S24-I in SmF. Values are mean ± S.D. of three experiments.

9.2.4 Effect of temperature on protease production

A linear increase in enzyme production was observed when bacteria were incubated at different temperature from 25- 50°C. Maximum bacterial growth and enzyme production was observed at 45-50°C range (Fig.9.12) A decrease in enzyme production was observed beyond 50°C.

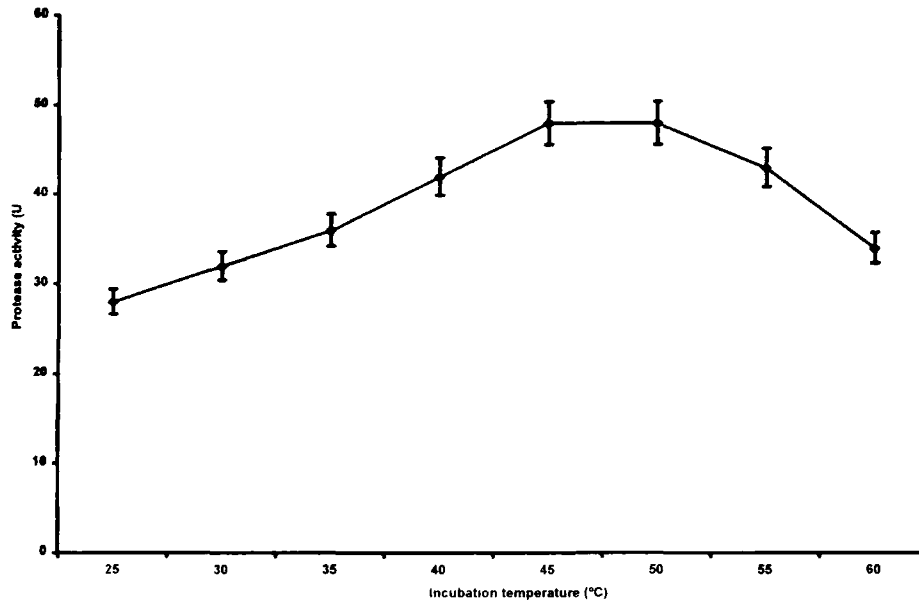


Fig.9.12 Effect of incubation temperature on alkaline protease production by *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments.

9.2.5 Kinetics of protease production

Result showed that optimum alkaline protease production was achieved post 72h of incubation (Fig. 9.13). Thereafter, a sharp fall in this enzyme yield was recorded.

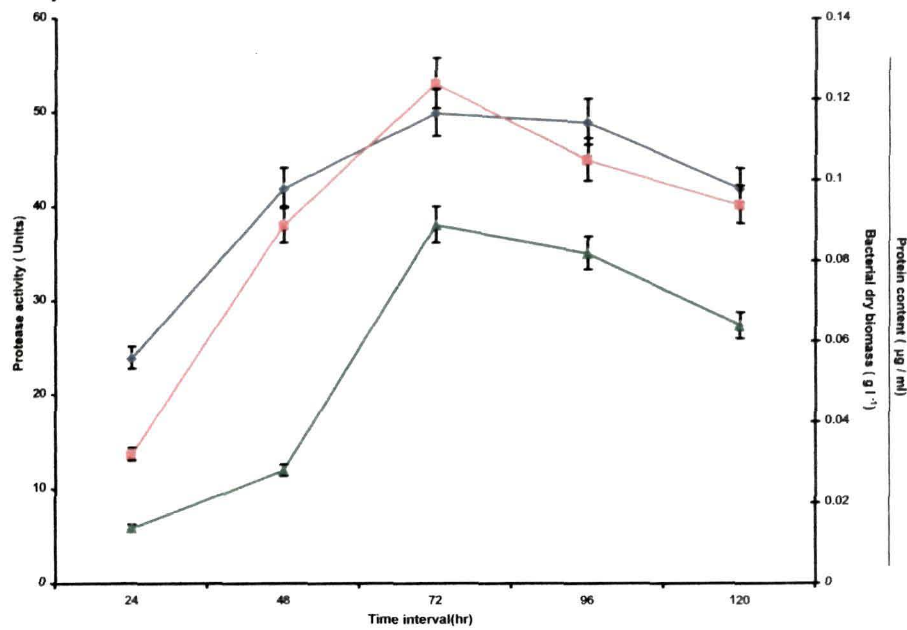


Fig. 9.13 Effect of incubation time on alkaline protease production (♦), bacterial dry biomass (■), and protein content (▲) from *B. licheniformis* strain AS-S24-I under SmF condition. Values are mean ± S.D. of three experiments.

9.2.6 Effect of agitation rate on protease production

Agitation rates effected protease production as the yield were very low in static conditions, but there was a gradual rise in enzyme production with increasing agitation rates and were stable from 150-200 rev/min (Fig.9.14), due to improved availability of oxygen and nutrients.

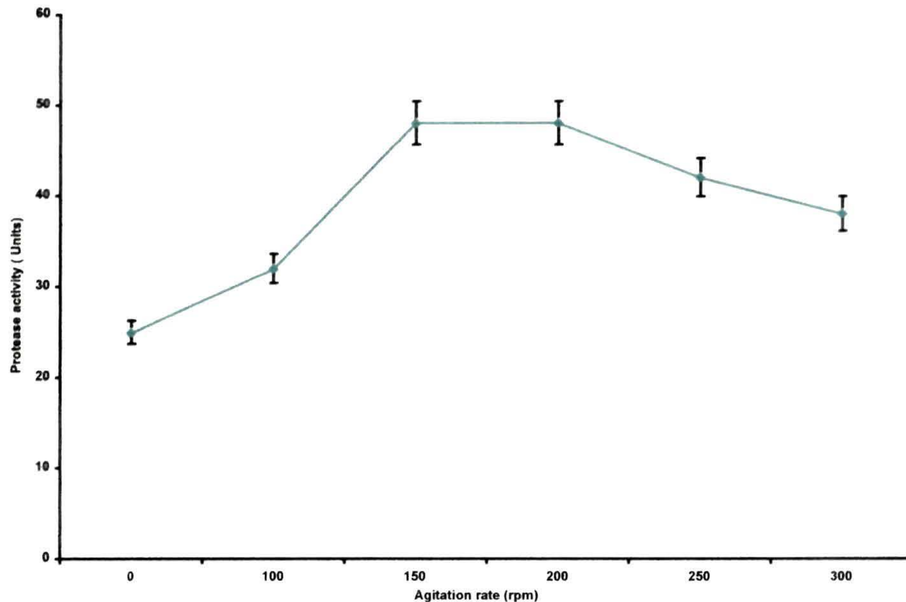


Fig.9.14 Effect of agitation rate on alkaline protease production from *B. licheniformis* strain AS-S24-I under SmF. Values are mean ± S.D. of three experiments.

9.3 Statistical optimization of alkaline protease production under submerged fermentation

9.3.1 Screening of essential key factors effecting protease (keratinase) production using Plackett-Burman design

In the present study the bacterium was found to secrete a high titer of alkaline keratinase, and maximum keratinase activity was estimated in the 72 h post-inoculated cell-free extract of *B. licheniformis* strain AS-S24-I. Screening result (Table 9.2) demonstrated a wide variation of protease (keratinase) activity from 33.0 Units to 81.0 Units in the fifteen trials. This variation reflected the significance of factors on the β-keratinase activity. The analysis of regression coefficients and t-value of six parameters showed that glucose, yeast extract, and pH of the medium displayed a positive effect for keratinase production where as, innoculum level and

incubation time had a negative effect on enzyme production (Table 9.3). Neglecting the variables which were insignificant, the model equation for keratinase production can be written as 9.1.

$$Y = 23.25 + 0.417 X_1 + 3.083 X_2 + 2.750 X_3$$

--------(9.1)

Where X_1 = pH of the medium, X_2 = yeast extract level, and X_3 = glucose level.

On the basis of calculated t-values (Table 9.3), glucose, yeast extract, and pH of the medium were chosen for further optimization. Innoculum level (% v/v) and incubation time (h) were kept at middle level.

Table 9.2 Plackett–Burman store design showing six variables with coded values along with the observed results for protease (β-keratinase) production by *B.licheniformis* strain AS-S24-I.

Glucose level (% w/v)	Yeast extract level (% w/v)	Incubation time (h)	Agitation rate (rpm)	pH	Innoculum size (% v/v)	Protease (Keratinase) yield (Units)
1 (1.5)	-1(0.05)	1 (96)	-1 (150)	-1 (9.0)	-1 (1.5)	33.125
1 (1.5)	1 (0.15)	-1 (48)	1 (250)	-1 (9.0)	-1 (1.5)	46.025
-1 (0.5)	1 (0.15)	1 (96)	- (150)	1 (11.0)	-1 (1.5)	58.525
1 (1.5)	-1 (0.05)	1 (96)	1 (250)	-1 (9.0)	1 (5.0)	52.275
1 (1.5)	1 (0.15)	-1 (48)	1 (250)	1 (11.0)	-1 (1.5)	61.450
1 (1.5)	1 (0.15)	1 (96)	-1 (150)	1 (11.0)	1 (5.0)	64.375
-1 (0.5)	1 (0.15)	1 (96)	1 (250)	-1 (9.0)	1 (5.0)	62.275
-1 (0.5)	-1 (0.05)	1 (96)	1 (250)	1 (11.0)	-1 (1.5)	61.875
-1 (0.5)	-1 (0.05)	-1 (48)	1 (250)	1 (11.0)	1 (5.0)	81.025
1 (1.5)	-1 (0.05)	-1 (48)	-1 (150)	1 (11.0)	1 (5.0)	67.70

Glucose level (% w/v)	Yeast extract level (% w/v)	Incubation time (h)	Agitation rate (rpm)	pH	Innoculum size (% v/v)	Protease (Keratinase) yield (Units)
-1 (0.5)	1 (0.15)	-1 (48)	-1 (150)	-1 (9.0)	1 (5.0)	62.275
-1 (0.5)	-1 (0.05)	-1 (48)	- 1 (150)	-1 (9.0)	-1 (1.5)	46.45
0 (1.0)	0 (1.0)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	50.00
0 (1.0)	0 (1.0)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	50.00
0 (1.0)	0 (1.0)	0 (72)	0 (200)	0 (10.0)	0 (2.5)	50.00

Table 9.3 Statistical analysis of Plackett–Burman design showing coefficient values, t- and P-value for each variable for protease (keratinase) activity (p-value <0.05).

Variable	Protease (keratinase) yield (Units)				
	Effect	Co-efficient	SE Coef	t-Stat	p-value
Intercept		23.25	0.5678	40.95	0.000
pH of the medium	-3.167	0.417	0.5678	0.73	0.027
Innoculum level (%v/v)	0.833	-1.583	0.5678	-2.79	0.487
Incubation time (h)	-2.167	-1.083	0.5678	-1.91	0.098
Agitation rate (rpm)	2.167	1.083	0.5678	1.91	0.098
Yeast extract (% w/v)	6.167	3.083	0.5678	5.43	0.001
Glucose level (% w/v)	5.500	2.750	0.5678	4.84	0.002

9.3.2 Statistical optimization of keratinase production using RSM

The predicted response for protease (β-keratinase) production using RSM was found as 780.1 X 10^3 U/ l, while the actual experimental value was observed as 776.0 x 10^3 U/ l by shake-flask study, and this difference in observed and predicted values is non-significant ($p > 0.05$) thus proving the validity of the model (Table 9.4). The overall second-order polynomial regression equation showing the empirical relationship between protease (keratinase) activity (Y) and three test variables in coded units can be represented by equation 9.2.

$$Y = 729.060 + 9.749 C_1 + 4.849 C_2 + 21.880 C_3 - 5.520 C_1^2 - 4.459 C_2^2 + 2.789 C_3^3 - 8.875 C_1 C_2 + 4.875 C_1 C_2 - 0.125 C_2 C_3 \quad \text{------------------------------------} \ (9.2)$$

The coefficients of the model including the significance of each coefficient as determined by t test and p-values showed that C_1 (pH of the medium), C_2 (glucose level), and C_3 (yeast extract level) had significant effect ($p<0.001$) on alkaline protease production by bacterium under study (Table 9.5).The positive co- efficient observed for the C_1, C_2 and C_3 variables indicated a linear effect of these parameters for the increase in β-keratinase production. Further, all the effects of C_1, C_2 and C_3, except quadratic effect of C_3C_2 were significant ($p < 0.05$). The goodness-of-fit of the model was analyzed by determining the coefficient of determination (R^2) and adjusted R^2. The observed values of R^2 suggest that the fitted model could explain 99.64% of the total variation and therefore, vouches for adequacy of the model (Table 9.5).On the basis of analysis of variance (ANOVA) of the quadratic regression model, it could be inferred that the computed F value for linear regression was much greater than the tabulated (P) > F value (Table 9.6).

Table 9.4. Observed responses and predicted values of β-keratinase activity of culture supernatants from *B.licheniformis* strain AS-S24-I. The observed values are average of triplicate determinations.

Run no.	Independent Variables			Y Response Protease (Keratinase) yield (in U)		Residual value
	C_1	C_2	C_3	Observed	Predicted value	
1	-1 (8.0)	-1(0.5)	-1(0.10)	679.0	681.266	-2.266
2	1(10.0)	-1(0.5)	-1(0.10)	710.0	708.763	1.237
3	-1(8.0)	1(1.5)	-1(0.10)	710.0	708.965	1.035
4	1(10.0)	1(1.5)	-1(0.10)	700.0	700.962	-0.962
5	-1(8.0)	-1(0.5)	1(0.20)	715.0	715.527	-0.527
6	1(10.0)	-1(0.5)	1(0.20)	760.0	762.524	-2.524
7	-1(8.0)	1(1.5)	1(0.20)	740.0	742.725	-2.725
8	1(10.0)	1(1.5)	1(0.20)	755.0	754.223	0.777
9	-2(7.0)	0(1.0)	0(0.15)	699.0	697.052	1.948
10	2(11.0)	0(1.0)	0(0.15)	730.0	729.842	0.158
11	0(9.0)	-2(0.25)	0(0.15)	710.0	708.292	1.708
12	0(9.0)	2(2.0)	0(0.15)	725.0	724.603	0.397
13	0(9.0)	0(1.0)	-2(0.05)	700.0	700.149	-0.149
14	0(9.0)	0(1.0)	2(0.25)	776.0	780.100	-4.1
15	0(9.0)	0(1.0)	0(0.15)	729.0	729.060	-0.060
16	0(9.0)	0(1.0)	0(0.15)	729.0	729.060	-0.060
17	0(9.0)	0(1.0)	0(0.15)	729.0	729.060	-0.060
18	0(9.0)	0(1.0)	0(0.15)	729.0	729.060	-0.060
19	0(9.0)	0(1.0)	0(0.15)	729.0	729.060	-0.060
20	0(9.0)	0(1.0)	0(0.15)	729.0	729.060	-0.060

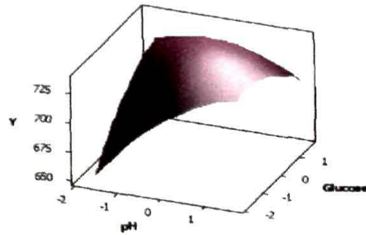
Table 9.5 Analysis of variance (ANOVA) of alkaline β-keratinase activity produced by *B.licheniformis* strain AS-S24-I strain. Values are mean ± S.D. of three experiments.

Source	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
Regression	9	9839.19	9839.19	1093.24	309.14	0.000
Linear	3	8157.24	8157.24	2719.08	768.89	0.000
pH	1	1297.89	1297.89	1297.89	367.01	0.000
Glucose	1	321.16	321.16	321.16	90.82	0.000
Yeast extract	1	6538.19	6538.19	6538.19	1848.84	0.000
Square	3	861.57	861.57	287.19	81.21	0.000
pH*pH	1	423.02	439.12	439.12	124.17	0.000
Glucose*Glucose	1	326.49	286.58	286.58	81.04	0.000
Yeast extract*Yeast extract	1	112.06	112.06	112.06	31.69	0.000
Interaction	3	820.38	820.38	273.46	77.33	0.000
pH*Glucose	1	630.13	630.12	630.12	178.18	0.000
pH*Yeast extract	1	190.13	190.13	190.13	53.76	0.000
Glucose*Yeast extract	1	0.12	0.12	0.12	0.04	0.855
Residual error	10	35.36	35.36	3.54		
Lack-of-Fit	5	35.36	35.36	7.07		
Pure error	5	0.00	0.00	0.00		
Total	19	9874.55				

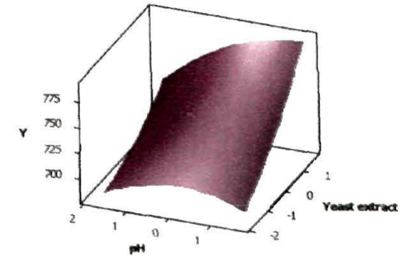
Table 9.6 Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for protease (β-keratinase) production by *B.licheniformis* strain AS-S24-I in SmF under shake-flask study ($p<0.05$).

Factor	Coefficient	SE coefficient	Computed t-value	p-value
Constant	729.060	0.7670	950.574	0.000
pH	9.749	0.5089	19.158	0.000
Glucose	4.849	0.5089	9.530	0.000
Yeast extract	21.880	0.5089	42.998	0.000
pH * pH	-5.520	0.4954	-11.143	0.000
Glucose * Glucose	-4.459	0.4954	-9.002	0.000
Yeast extract * Yeast extract	2.789	0.4954	5.629	0.000
pH * Glucose	-8.875	0.6649	-13.349	0.000
pH * Yeast extract	4.875	0.6649	7.332	0.000
Glucose * Yeast extract	-0.125	0.6649	-0.188	0.855

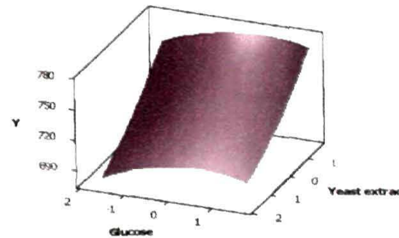
R^2 = 99.64%, R^2 (pred) = 97.04%, R^2 (adj) = 99.32%



(a)

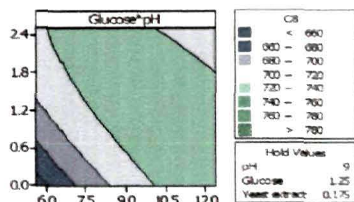


(b)

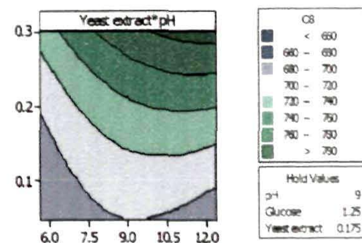


(c)

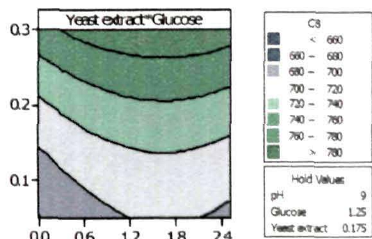
Fig. 9.15 Response surface plots for alkaline protease production by *B.licheniformis* strain AS-S24-I. The interaction between (a) pH of the medium and concentration (%, w/v) of glucose, hold value C_3 = 0 (b) pH of the medium vs concentration (%, w/v) of yeast extract, hold value C_2 = 0 and (c) concentration (%, w/v) of glucose vs concentration of yeast extract (% w/v), hold value C_1=0. Values are mean ± S.D. of three experiments.



(a)



(b)



(c)

Fig.9.16 Countor plots for alkaline protease production by *B.licheniformis* strain AS-S24-I. The interaction between (a) pH of the medium and concentration (%, w/v) of glucose, hold value C_3 = 0 (b) pH of the medium vs concentration (%, w/v) of yeast extract, hold value C_2 = 0 and (c) concentration (%, w/v) of glucose vs concentration of yeast extract (% w/v), hold value C_1=0. Values are mean ± S.D. of three experiments.

9.3.1 Three dimensional surface plots

The three-dimensional (3-D) response surfaces were plotted on the basis of the model equation to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum keratinase production by *B. licheniformis* strain AS-S24-I. The response surfaces shown in Fig. 9.15a-c were based on the final model, holding one variable constant at its optimum level, while the other two within their experimental range. It was observed that with an initial increase in the pH of the medium and concentration of the glucose resulted in an increase in keratinase yield (Fig. 9.15a). The response surface plot based on interaction of pH of the medium and concentration of yeast extract (nitrogen source) demonstrated enhancement of keratinase production (Fig. 9.15b). However, the interaction of glucose (C_2) and yeast extract (C_3) was insignificant in higher concentration ($p>0.05$) resulting decrease in keratinase yield (Fig. 9.15c). These results reinforced that the response equation provided a suitable model for the CCD experiment.

9.3.2 Two dimensional countor plots

Figure 9.16a clearly illustrated that an initial increase in pH of medium and concentration of glucose increases protease yield particularly above pH level 10, with a glucose concentration 1.8 % w/v as elliptical formation occurred at this concentration. Fig. 9.16b shows that interaction of pH of medium and concentration of glucose increases protease yield. However, further increase in the glucose level results in decrease in protease yield. The Fig.9.16c shows interaction of (C_2) glucose and (C_3) yeast extract level was significant in increasing protease yield to optimal value. These results reinforced the premises that the response equation provided a suitable model for the CCD experiment.

9.4 Batch fermentation

On the basis of regression equation so obtained, the optimum levels of test variables for maximum keratinase yield were predicted by using the Minitab 16 Statistical Software® program as 0.4 % (w/v) of glucose, 0.30 % (w/v) of yeast-extract and pH 10.0 of the medium. The optimal keratinase production in a process-controlled fermenter was found to be significantly higher (796.0 x 10^3 U/ l post 60 h of incubation) than that observed under the shake-flask study (780.0 x 10^3 U/ l post 72 h of incubation).

9.5 Isolation and purification of alkaline protease

Maximum protease (β-keratinase activity) was determined that 80% acetone-precipitated fraction. Separation of this fraction on a gel filtration column resolved it into seven protease peaks, designated as GF-I to GF-VII (Fig.9.17). The GF-VI fraction (containing proteins in the molecule weight range of 40-45 kDa) demonstrated maximum β-keratinase activity. When GF-VI peak was fractionated by RP-HPLC, the peak HP-III (Alkarnase) with retention time of 8.50 min showed β-keratinase activity (Fig. 9.18). This peak was found to be homogenous by 15% SDS-PAGE analysis, both under reducing and non-reducing conditions, yielded a single Coomassie-stained band of apparent molecular weight of 43.0 kDa (Fig. 9.19). This enzyme was name as Alkarnase. A summary of purification of Alkarnase is displayed in Table 9.7.

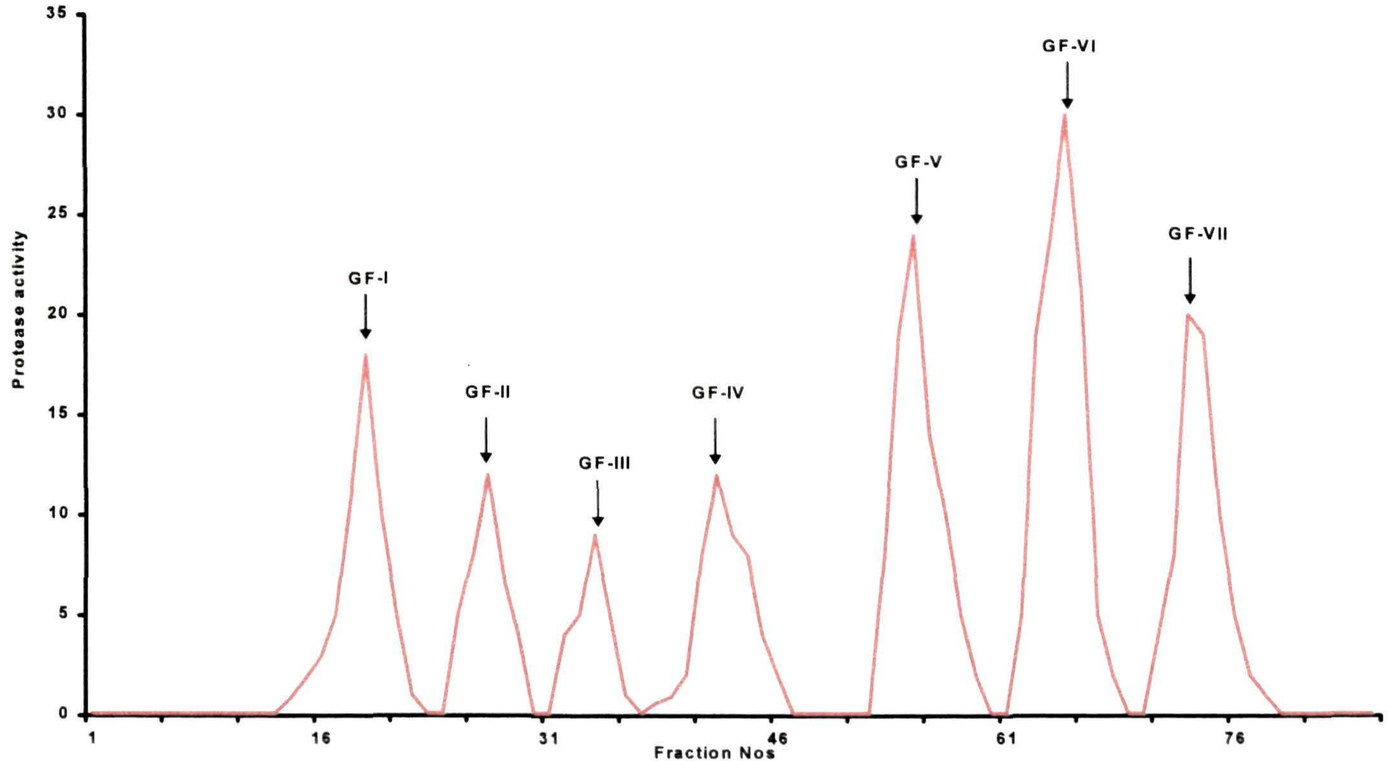


Fig. 9.17 Sephacryl S-200 gel filtration profile of 80 % (v/v) acetone precipitated fraction. Data represent a typical experiment.

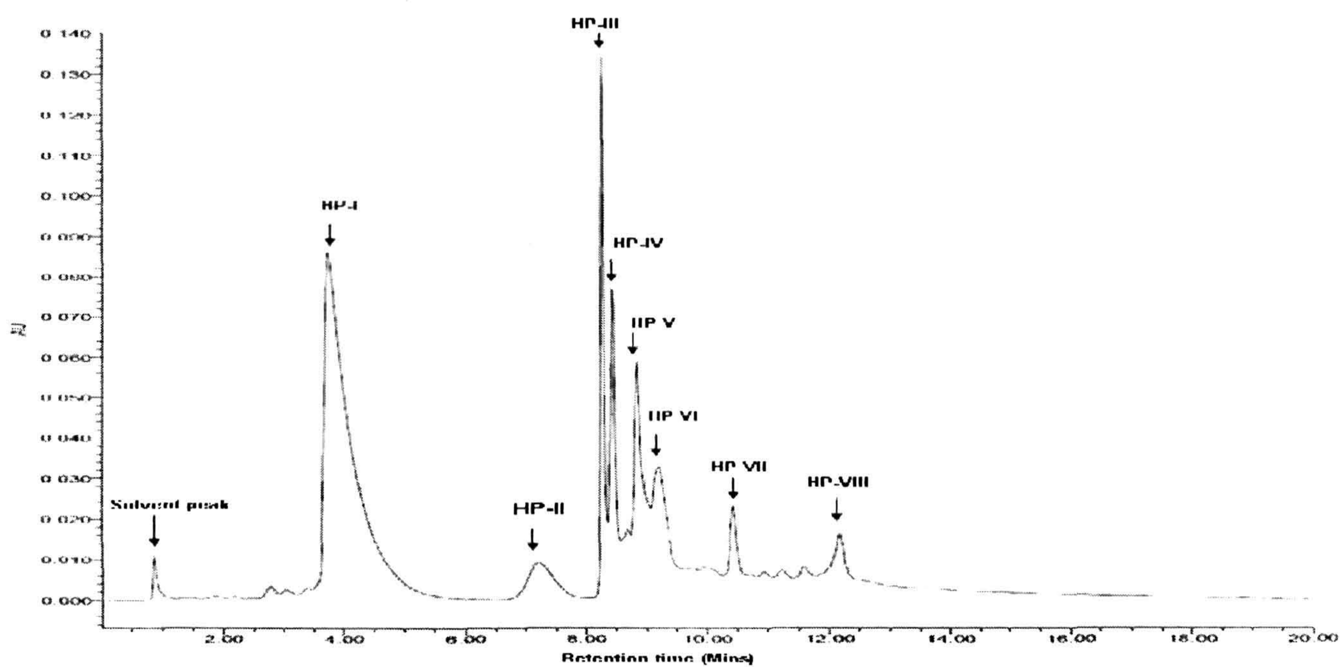


Fig.9.18 RP-HPLC profile of fraction GF-VI from *B.licheniformis* strain AS-S24-I.

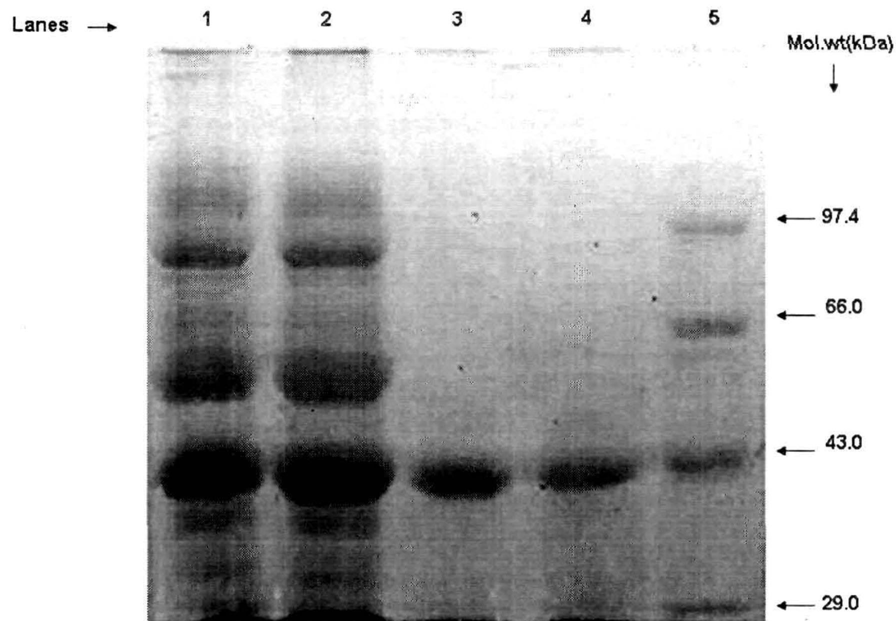


Fig. 9.19 SDS-PAGE (12.5%) of HP-III fraction (Alkarnase) from *B. licheniformis* strain AS-S24-I. Lane 1 –crude keratinase (50 µg); Lane 2- acetone precipitated keratinase (50 µg), Lane 3- reduced Alkarnase (10.0 µg); Lane 4- non-reduced Alkarnase (10.0 µg); Lane 5- Protein molecular weight marker (kDa) :phosphorylase b (97.4), BSA (66.0),ovalbumin (43.0), carbonic anhydrase (29.0).

Table 9.7 Summary of purification of Alkarnase (an alkaline β-keratinase) from *B.licheniformis* strain AS-S24-I. Data represent a typical experiment.

Purification step	Total protein (mg)	Total activity (Units)	Enzyme yield (%)	Specific activity (units/mg)	Purification (fold)
Cell-free supernatant	127.0	796.0	100	6.3×10^3	1
80% acetone precipitation	21.0	670.0	84.0	32.0×10^3	5.0
GF-VI	13.9	605.0	76.0	44.0×10^3	7.0
HP-III(Alkarnase)	3.0	408.0	51.0	136.0×10^3	22.0

9.6 Biochemical characterization of Alkarnase

The various biochemical properties and characteristics of the isolated β-keratinase (Alkarnase) were characterized in view of its potential application in the field of emerging industrial biotechnology.

9.6.1 Effect of pH and temperature on activity of Alkarnase

The Alkarnase remained active over a broad range of pH (6-13) and temperature (25- 60°C); however, the optimum activity was observed at pH 9.0-10 (Fig.9.20) and at 45-50°C (Fig. 9.21).Further increase in pH and temperature leads to a decrease in the protease activity.

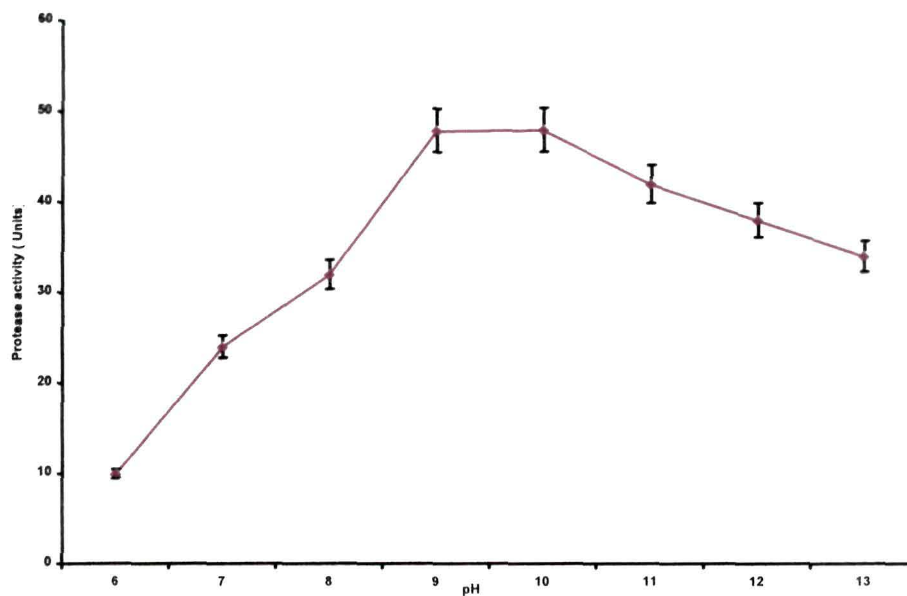


Fig. 9.20 Effect of pH on protease activity. Values are mean ± S.D. of three experiments.

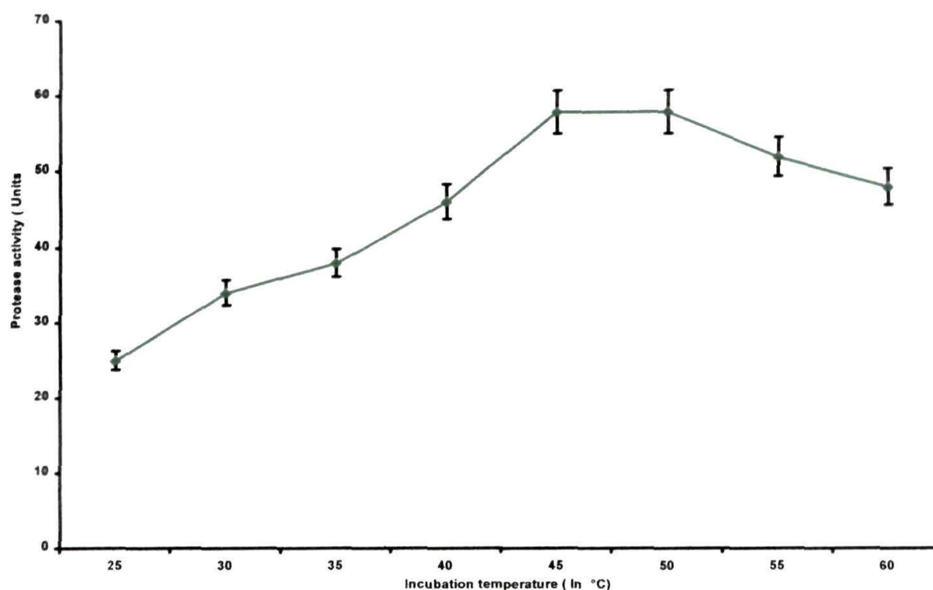


Fig.9.21 Effect of incubation temperature on protease activity of Alkarnase. Values are mean ± S.D. of three experiments.

9.6.2 Substrate specificity study of alkarnase

The specificity (U/mg of protein) of the purified protease towards different substrates was observed in the following order: raw chicken-feather > casein > bovine serum globulin > bovine serum albumin > bovine serum fibrinogen > hemoglobulin > gelatin. Human hair (α-keratin) and collagen could not be degraded by this protease. Based on its substrate specificity, this protease was considered as an alkaline β-keratinase and was named as Alkarnase. (Table 9.8).

Table 9.8 Substrate specificity of Alkarnase from *B. licheniformis* strain AS-S24-I. Values are mean ± S.D. of three experiments.

Substrate specificity	Specific activity (U / mg of protein)
Raw chicken-feather	13,600.00 ± 680.0
casein	5081.0 ± 254.0
bovine serum globulin	500.9 ± 25.0
bovine serum albumin	350.8 ±18.0
bovine serum fibrinogen	330.5 ±17.0
hemoglobulin	150.5 ± 8.0
gelatin	100.3 ± 5.0
Human hair	0
Collagen	0

9.6.3 K_m and V_{max}

The K_m and V_{max} values for Alkarnase towards soluble keratin were determined as 0. 1 mg / ml and 100.0 μmol / min / mg, respectively. (Fig. 9.22)

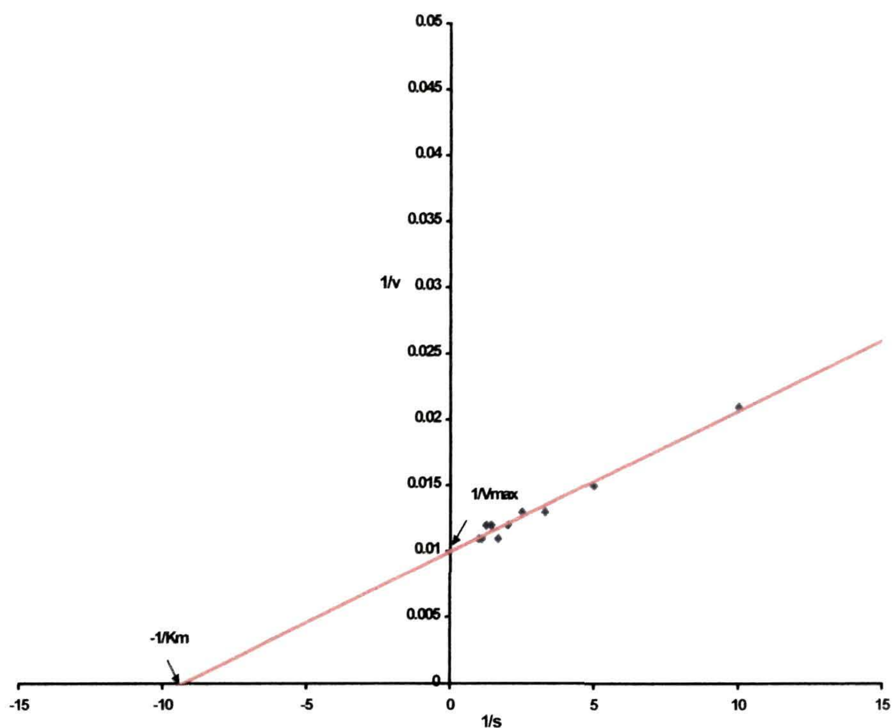


Fig.9.22 Lineweaver–Burk plot for determination of K_m and V_{max} values of Alkarnase. Values are mean ± S.D. of three experiments.

9.6.4 Effect of metal ions on Alkarnase

All the tested metal ions inhibited the keratinolytic activity of Alkarnase however, maximum (100%) inhibition was produce by Ni^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+} and Ca^{2+} ions (Fig. 9.23)

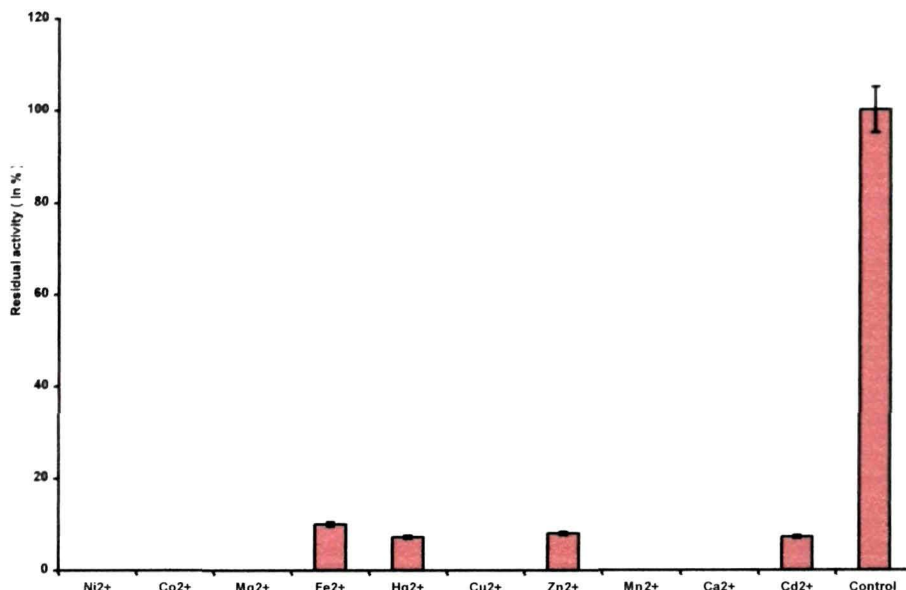


Fig.9.23 Effect of metal ions on activity of Alkarnase. Values are mean ± S.D. of three experiments.

9.6.5 Effect of chemicals /inhibitors/chelators/ surfactants/ denaturing/oxidizing/bleaching agents on Alkarnase

The serine protease inhibitor, PMSF is known to strongly inhibit the serine residue at the active site causing a significant inhibition of enzyme activity. The activity of Alkarnase was inhibited to 91.4% and 98.8 % , in presence of 2.0 and 4.0mM PMSF, whereas iodoacetic acid (IAA) displayed 100% inhibition at 4.0mM concentration. The 4-pBPB also displayed maximum inhibition of about 95.0% of Alkarnase activity. High stability was observed for alkarnase toward various surfactants (Table 9.9). Upon incubation with 1% of Tween-20, -40, and Triton X-100 at room temperature (24°C) for 30.0 min, Alkarnase exhibited enhanced residual activities between 99.0-125 %. Alkarnase retained 98.0 and 90.0 % of original activity after incubation with 2 and 4mM EDTA at room temperature 24°C for 30.0 min. Alkarnase still retained 110.0,100.0 and 95.0% of its original activity, respectively, even after incubation with 20.0mM,-40mM and 60mM SDS.

Alkarnase demonstrated 112% residual activity in presence of 5% (v/v) H_2O_2 and 0.5 % (w/v) sodium perborate at room temperature 24°C for 30.0 min (Table 9.9). About, 88.0 % decrease in protease activity was observed post addition of DTT with Alkarnase which suggests the presence of intra-molecular disulphide bonds in Alkarnase molecules (Table 9.9).

Table 9.9 Effect of protease inhibitors, chelator, surfactants, urea, oxidizing and bleaching agents on catalytic activity of Alkarnase from *B.licheniformis* strain AS-S24-I strain. Experiment was done as described in the text. Values represent mean ± S.D of three determinations.

Metal ions/ reagents	Relative activity (%)
Control	100
Inhibitors	
PMSF (2mM)	8.6 ± 0.4
(4mM)	1.2 ± 0.2
4-pBPB (2mM)	14.3 ± 0.8
(4mM)	5.2 ± 0.5
IAA (2mM)	2.4 ± 0.1
(4mM)	0.0
Metal-ion chelator	
EDTA (2mM)	98.0 ± 1.0
(4mM)	90.0 ± 0.9
Anionic surfactant (SDS)	
20 mM	110.0 ± 5.5
40 mM	100.0 ± 2.1
60 mM	95.0 ± 0.9
Non-ionic surfactants (1% v/v)	
Trition-X-100	100.0 ± 5.0
Tween 20	125.0 ± 6.3

Metal ions/ reagents	Relative activity (%)
Tween 40	99 ± 4.6
Tween 60	95 ± 5.0
Urea (8 M)	80.0 ± 4.0
Oxidizing agents	
H_2O_2 (% v/v)	
5.0	112.0± 7.1
10.0	100.0 ± 5.0
Sodium perborate (% w/v)	
0.5	112.0± 6.6
1.0	103.0 ± 6.4
DTT	
4mM	12.0 ± 0.6

9.6.7 Thermo-stability study of Alkarnase

Thermostability study demonstrated that the Alkarnase was stable post heating at 60°C for 15 min. However, about 20 % of original activity of Alkarnase was lost post heating it at 60°C for 120 min (Fig.9.24).

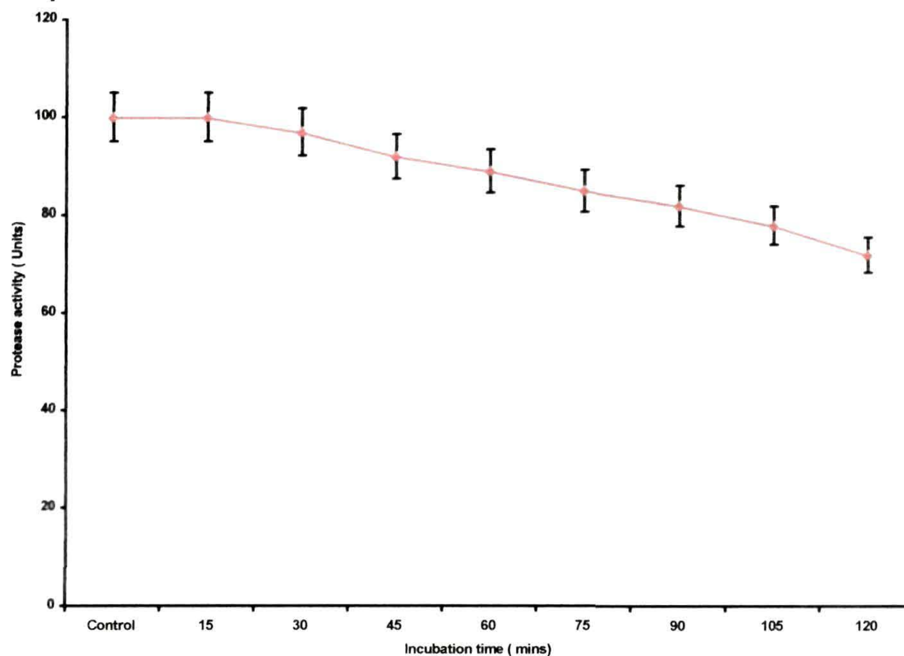


Fig.9.24 Thermo-stability study of Alkarnase from *B. licheniformis* strain AS-S24-I post heating at 60°C for 120 min. Values are mean ± S.D. of three experiments.

9.6.8 Effect of polyols on stability Alkarnase against heat denaturation

The influence of various polyols (5.0%) such as glycerol, mannitol, sorbitol and xylitol on the thermostability of Alkarnase was examined. Data presented in Fig.9.25 show that glycerol and mannitol stabilized this keratinase to the maximum extent against heat –denaturation, whereas xylitol exhibited least activity in stabilizing the enzyme against heat denaturation.

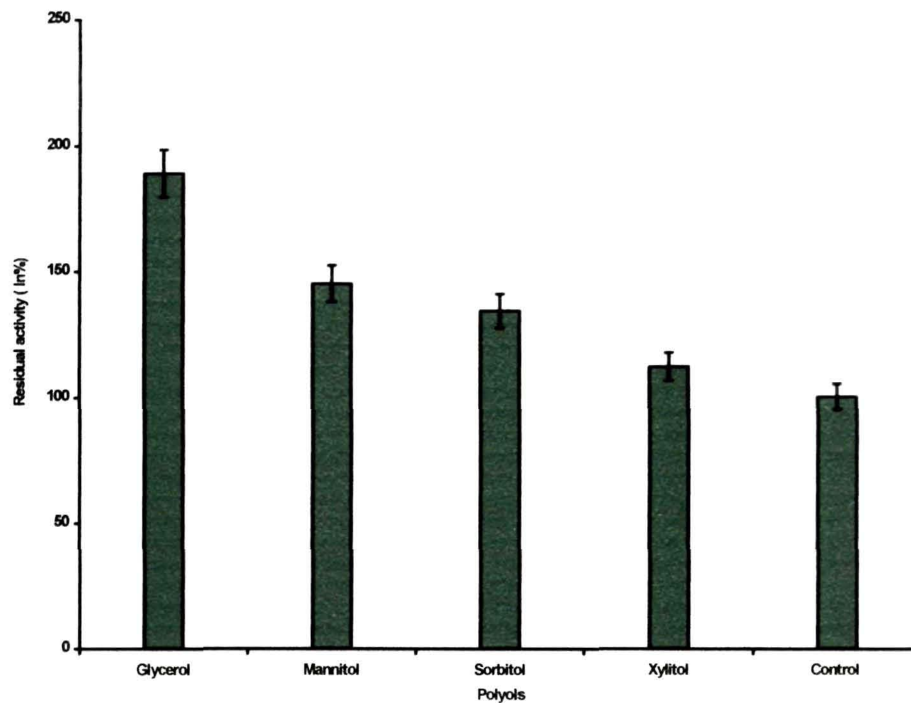


Fig. 9.25 Effect of polyols on thermostability of Alkarnase from *B.licheniformis* strain AS-S24-I post heating at 60°C for 120 min. The Alkarnase activity without polyols served as control (100% activity). Values are mean ± S.D. of three experiments.

9.6.9 Effect of organic solvents on Alkarnase activity

Present study demonstrates the effects of various organic solvents with different Log P (20%, v/v) on the stability of the Alkarnase. Residual activity of Alkarnase is displayed in Fig.9.26. The stability of the enzyme was highly enhanced by n-hexane and xylene, and no loss of activity was observed in these solvents post 90 mins of incubation at room temperature (Fig.9.26). The enzyme was highly unstable in presence of benzene followed by ethanol.

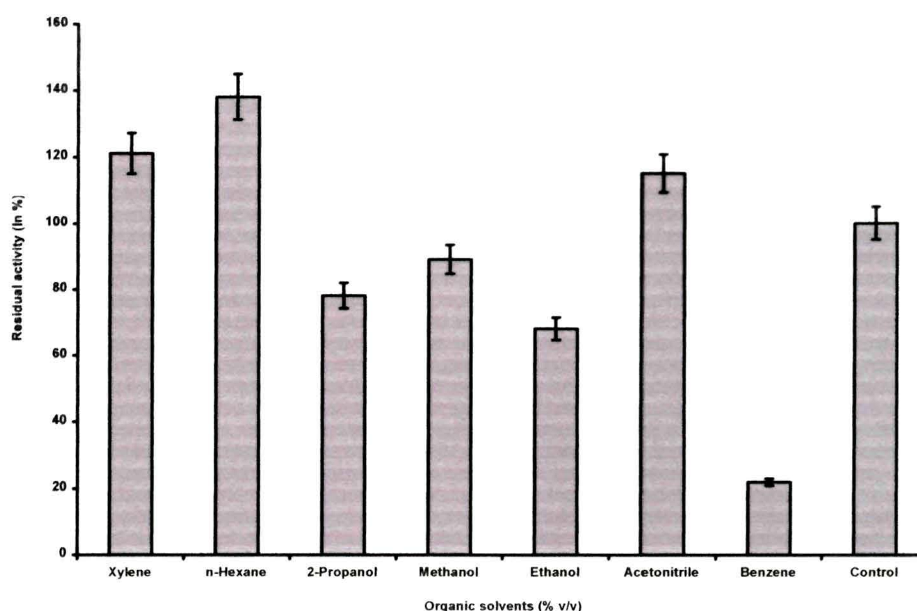


Fig. 9.26 Organic solvent stability of Alkarnase from *B. licheniformis* strain AS-S24-I. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean ± S.D. of three experiments.

9.5.10 Pharmacological properties of Alkarnase from *B. licheniformis* strain AS-S24-I

In order to develop safer laundry detergents for household and industrial applications, it is essential to explore the pharmacological properties and toxicity level of purified enzymes; however, only limited attempts have been made in this direction. Alkarnase from *B. licheniformis* strain AS-S24-I induced only 2.4% hemolysis of the washed human erythrocytes at a dose of 15 µg/ml. Moreover, it did not display any detrimental effect on goat liver, heart, lungs and kidney tissues (Table 9.10). Further, as shown in Table 9.10, Alkarnase had no affect on the normal clotting time of goat platelet-poor plasma (PPP).

Table 9.10 Pharmacological properties of Alkarnase from *B. licheniformis* strain AS-S24-I. Values are mean± S.D. of three experiments.

Pharmacological properties	Values
Hemolysis [a] (%)	
Control (without Alkarnase)	1.2 ± 0.1
Treatment with Alkarnase (15.0 µg /ml) .	2.4 ± 0.1
Ca-Clotting time (s)[b]	
Control (without Alkarnase)	125.0 ± 6.3
Treated (with 15.0 µg/ml of Alkarnase)	133.0 ± 6.7
In vitro tissue damaging activity [c]	
a) ***Heart***	
Control (without Alkarnase)	0.12 ± 0.01
Treatment with Alkarnase	0.28 ± 0.01
b) ***Lung***	

Pharmacological properties	Values
Control (without Alkarnase)	0.13 ± 0.01
Treatment with Alkarnase	0.36 ± 0.02
c) ***Liver***	
Control (without Alkarnase)	0.12 ± 0.01
Treatment with Alkarnase	0.29 ± 0.02
d) ***Kidney***	
Control (without Alkarnase)	0.13 ± 0.01
Treatment with Alkarnase	0.32 ± 0.02

[a]Percent of Hb released by 15 μg/ml of Alkarnase post 90 min incubation at 37°C. RBC treated with 1% (v/v) Triton-X-100 was considered as 100 % activity.
[b]Ca^{2+} clotting time of platelet poor goat plasma by 15 μg/ml of Alkarnase.
[c]Percent of Hb released from damaged tissues by 15 μg/ml of Alkarnase post 5 h incubation at 37°C. Hemoglobin released from the tissues treated with 1% (v/v) Triton X-100 was considered as 100 % activity.

9.6 Industrial application of Alkarnase

9.6.1 Detergent compatibility and stain removal potency of Alkarnase
Proteolytic activity of Alkarnase was monitored in the presence of different commercial detergents at a concentration of 7.0 mg /ml at pH 10.0; pre-incubated at 25.0, 37.0 and 45.0°C temperatures for a period of 60 min. The proteolytic activity was assayed at every 10 min intervals. Alkarnase at a dose of 7.0 μg /ml demonstrated protease activity in presence of detergents at all the tested temperature. The most promising results was observed at 45°C in presence of Henko (120.0%) followed by Sunlight = Surf excel = Fena Ultra = Wheel = Rin advanced = Safed = Ariel (100%) and Ghari (97.0%) detergents (Fig. 9.27).

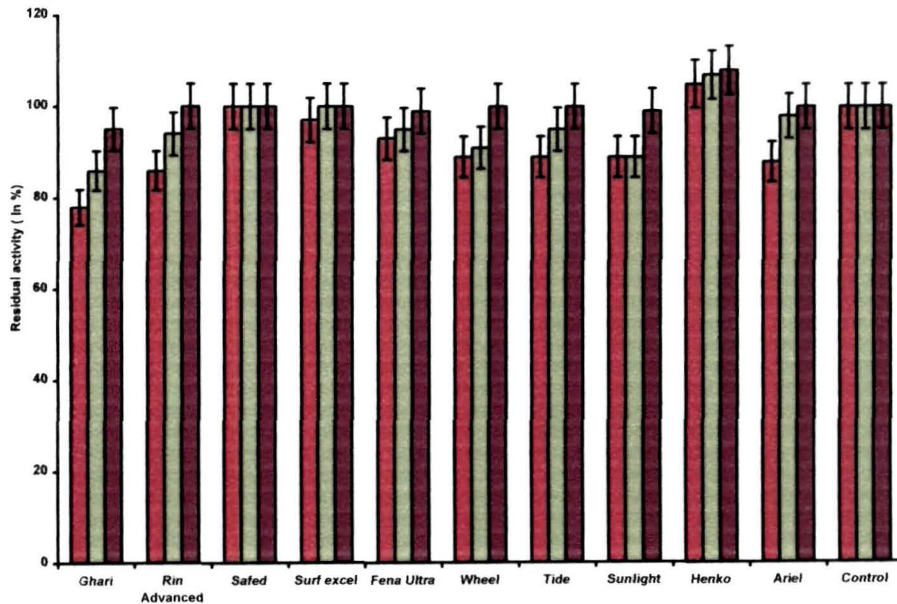


Fig.9.27 Detergent stability and compatibility of Alkarnase (7.0 µg / ml) from *B. licheniformis* strain AS-S24-I at the tested temperature ranges 25(■), 37 (■) and 45 °C (■). Values are mean ± SD of triplicate determinations.

Wash performance analysis of the Alkarnase on small square pieces (7cm ×7 cm) of a cotton fabric was performed at room temperatures. The enzyme and detergent combinations were found to be superior for efficient removal of blood stain, when compared to the use of detergent alone (Fig.9.28). It was observed that Henko® detergent supplemented with Alkarnase (dissolved in tap water) at a concentration of 7.0 µg / ml could remove 36.0 ± 3.0 % (mean ± S.D., n=3) of blood stain from cotton fabrics, where as the control tap water removed 3.0 ± 0.2% (mean ± S.D., n=3) of blood stain from cotton fabrics under identical experimental condition.

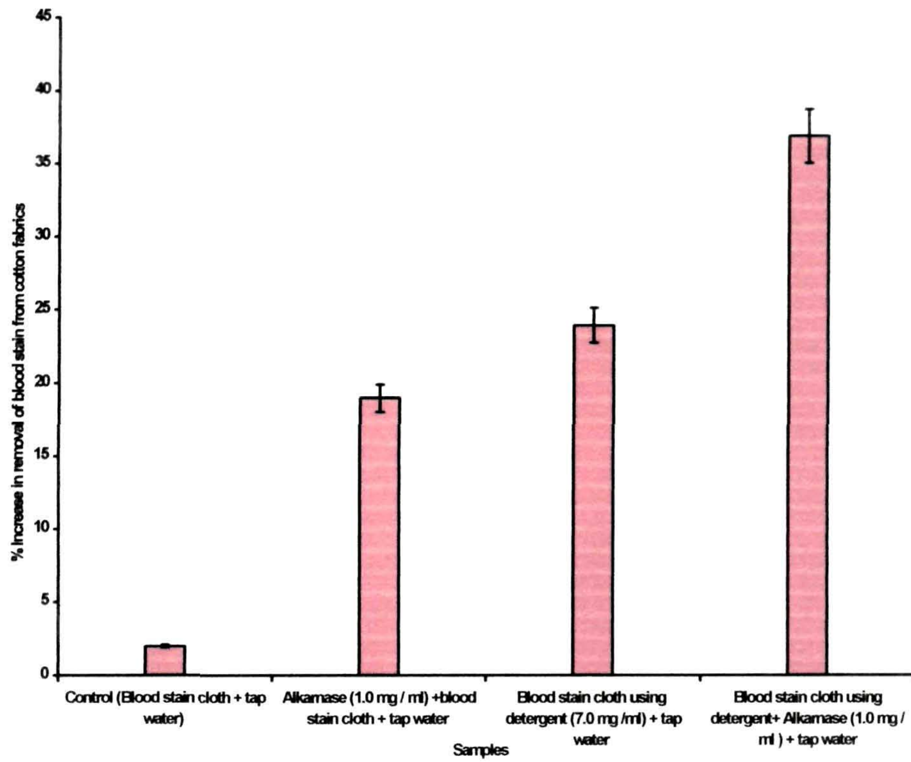


Fig.9.28 Wash performance test of Alkarnase from *B. licheniformis* strain AS-S24-I at 37°C. The values represent mean ± S.D. of three determinations.

9.6.2 Dehairing activity of Alkarnase

In the present study, dehairing of skin was observed post treatment with Alkarnase (50.0 U / ml) at 37°C for 6 h; on the other hand, treatment of skin with the buffer (control) could not lead to its dehairing (Fig.9.29). Absence of collagenase activity and lack of toxicity in the protease preparation (Alkarnase) from *B. licheniformis* strain AS-S24-I may be considered as promising properties for its future application as a green chemical in leather industry for dehairing purpose.

(a) **(b)**

Fig. 9.29 Dehairing activity of Alkarnase (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 10.0 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with Alkarnase (50U / ml) for 12 h at 37°C.

CHAPTER X

RESULTS

Chapter 10

Process optimization, purification, biochemical characterization, and industrial application of an alkaline protease isolated from *Brevibacillus* sp. strain AS-S10-II

10.1 Alkaline protease production under solid-state fermentation

10.1.1 Screening of different agro-industrial and waste materials for alkaline protease production

Brevibacillus sp. strain AS-S10-II was employed in the present study for bacterial growth and protease production with cheaper agro-industrial and kitchen waste materials. Several solid substrates obtained from a local market and industrial by-products have been tested for protease production. As shown in Fig. 10.1, IC followed by PP mixture (1:1) were found to be most effective substrates in SSF system for protease production. Alkaline protease production was observed to be maximum in presence of IC (224.0 U /gds) and PP (346.0 U/gds) post 48h of incubation at 50°C; followed by wheat bran (WB) and mustard oil cake (MOC) (Fig. 10.1). Least production was achieved in presence of banana leaves and tea leaves (Fig.10.1). Among different substrates, IC and PP demonstrated an appropriate supporting sustained bacterial growth and protease production in SSF systems. These substrates IC and PP significantly enhances the protein content of the culture supernatants as well as the bacterial dry biomass post 48h of incubation at 45°C temperature as compared with other substrates (Fig.10.2).

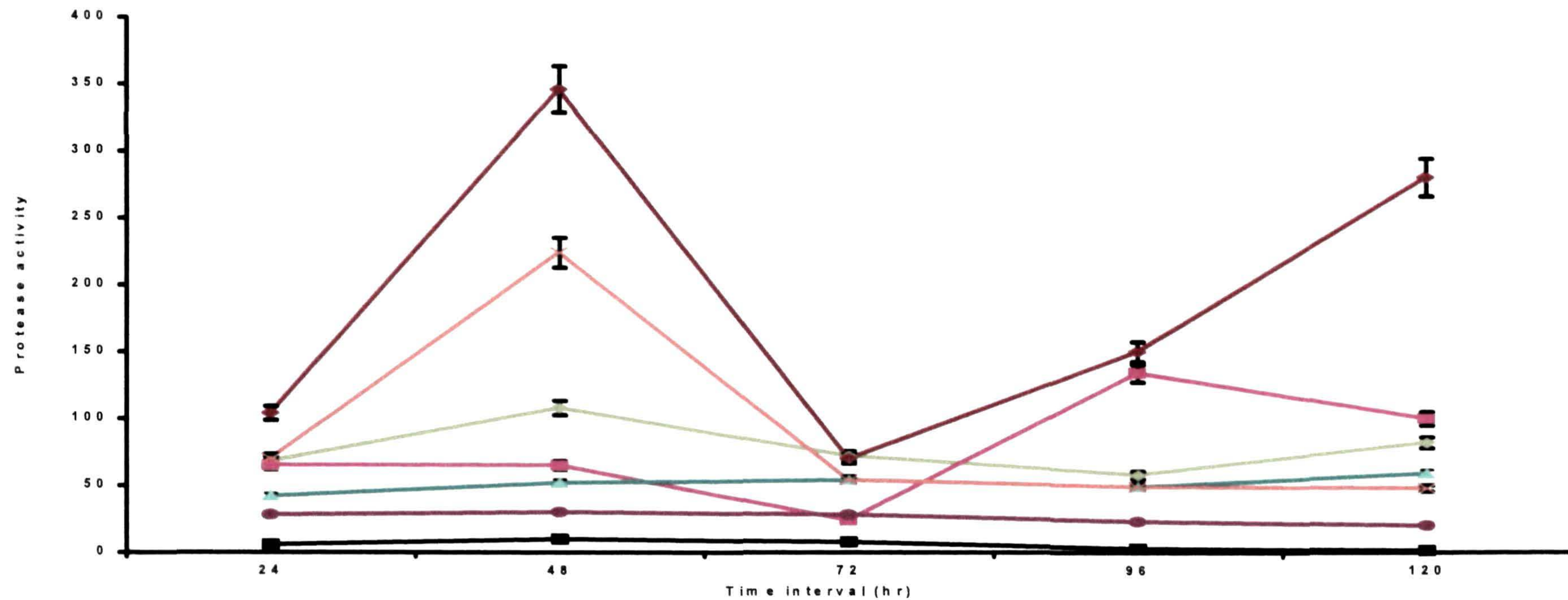


Fig.10.1 Screening of different agro-industrial waste residues such as MOC (◆), WB (■), RB (▲), IC(X), PP (◆), BL (●), and TL (■) for the production of alkaline protease by *Brevibacillus* sp.strain AS-S10-II at different time intervals. Values are mean ± S.D. of three experiments.

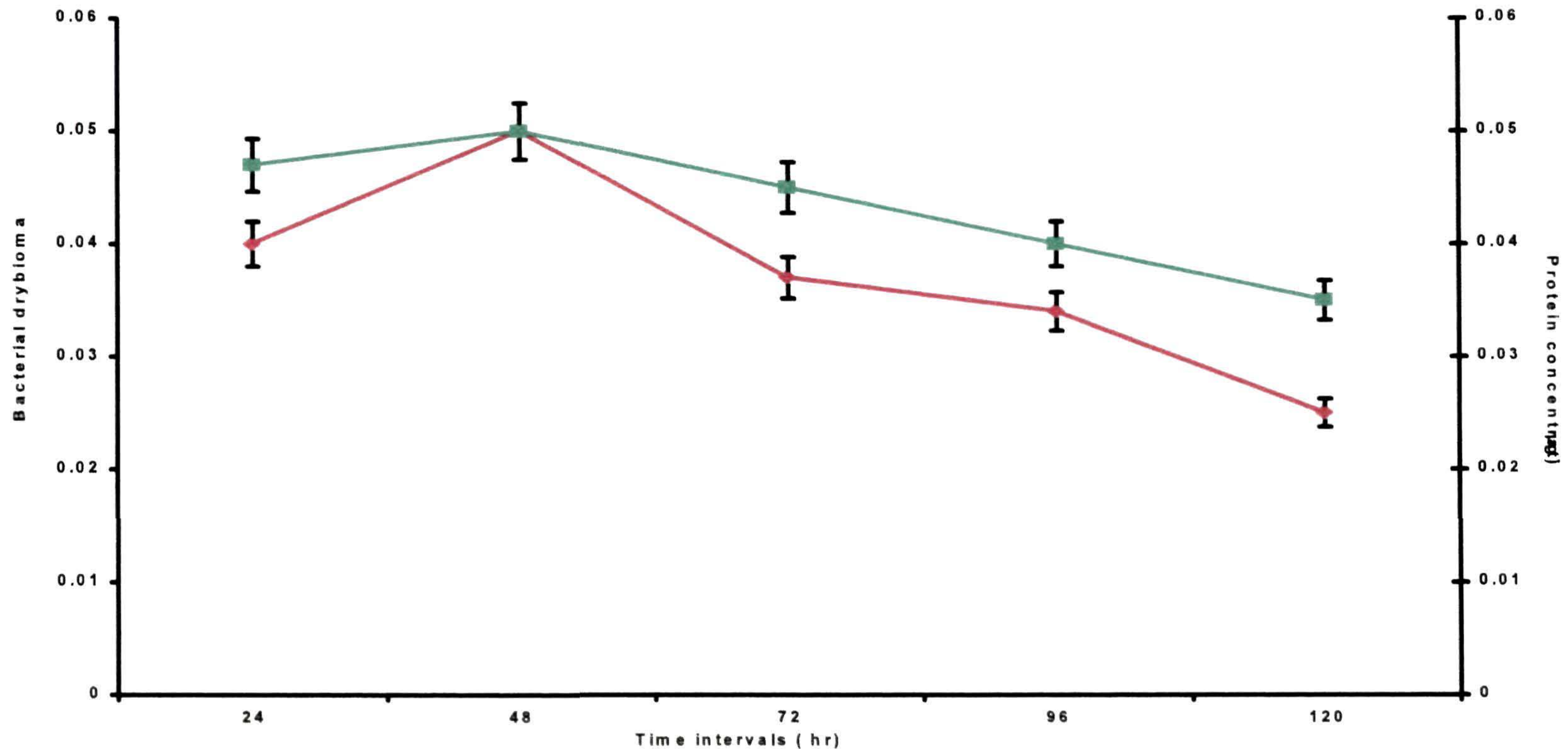


Fig.10.2 Kinetics of alkaline protease production by *Brevibacillus* sp. strain AS-S10-II in SSF using IC and PP substrates. Legends show bacterial dry biomass (■) and protein concentration (♦).Values are mean ± S.D. of three experiments.

10.1.2 Effect of initial moisture content of the substrate and moistening agent on protease production

The effect of initial moisture content on protease production is presented in Fig. 10.3. The highest enzyme production (300.0 U/ gds) was obtained at 100% initial moisture content. Further, increase in moisture content displayed a sharp decrease in protease production post 48h of incubation at 50°C. As compared to other moistening agents, distilled H_2O adjusted to pH 12.5 was found as most efficient moistening agent for protease production (Fig. 10.4).

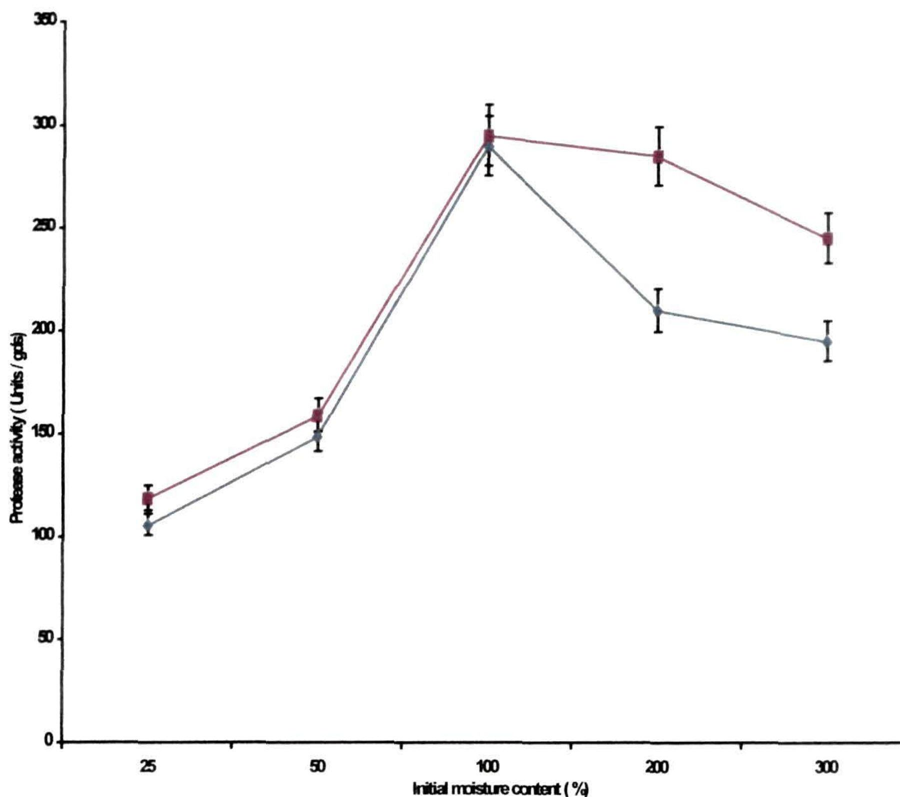


Fig.10.3 Influence of initial moisture content of the substrates [PP (■) or IC (♦)] on alkaline protease production. Values are mean ± S.D. of triplicate determinations post 48h incubation at 50°C. Moistening agent was distilled water, adjusted to pH 12.5 with 0.1N NaOH.

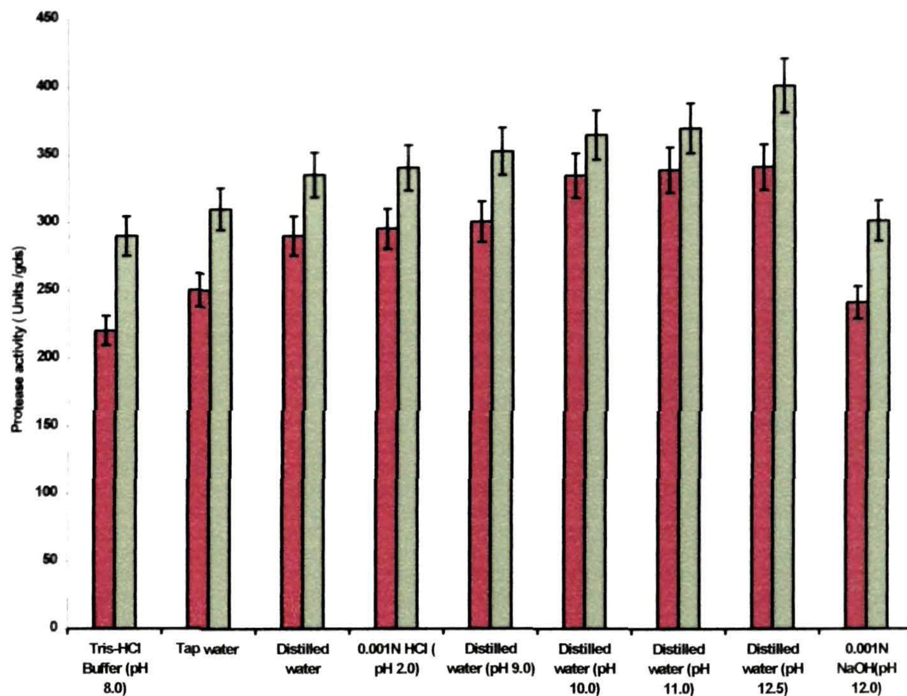


Fig.10.4 Influence of moistening agents on protease production using either IC (■) or PP (■) as a substrate. Values represent mean ± S.D of three experiments post 48h of incubation of *Brevibacillus* sp. strain AS-S10-II at 50°C.

10.1.3 Effect of inoculum's size on protease production

The inoculum size directly effects bacterial growth and protease production. An inoculum size of 0.5- 3.0 ml significantly accelerated the protease production with IC and PP substrates (Fig.10.5). Further increase in inoculum size displayed a sharp decrease in protease production due to competition among bacterial populations for nutrients.

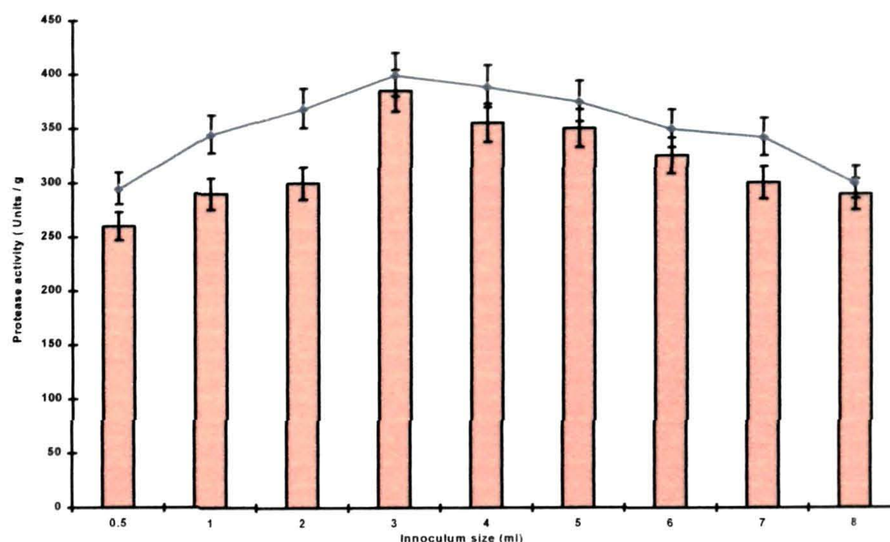


Fig.10.5 Influence of inoculum size on protease production by *Brevibacillus* sp. strain AS-S10-II using PP (♦) and IC (■) under SSF. Values are mean ± S.D. of triplicate determinations.

10.1.4. Effect of supplementation of co-carbon and co-nitrogen sources on protease production

It has been envisaged that co-carbon sources have tremendous potential to support bacterial growth and protease production. The influence of different co-carbon sources on protease production by *Brevibacillus* sp. strain AS-S10-II was tested under SSF systems. About 3.0 fold increase in protease production as well as bacterial growth was induced by 1.0 % w/v casein (900.0 U / gds) along with IC post 48h of incubation at 50°C by *Brevibacillus* sp. strain AS-S10-II (Fig. 10.6).

Effect of different nitrogen sources on bacterial growth and protease production by *Brevibacillus* sp. strain AS-S10-II was investigated. Maximum bacterial growth and protease production was observed in presence of 0.1 % (w/v) potassium nitrate along with IC substrate post 48h of incubation (Fig. 10.7). No significant protease production was observed

in presence of organic nitrogen sources as compared with inorganic nitrogen sources (Fig. 10.7).

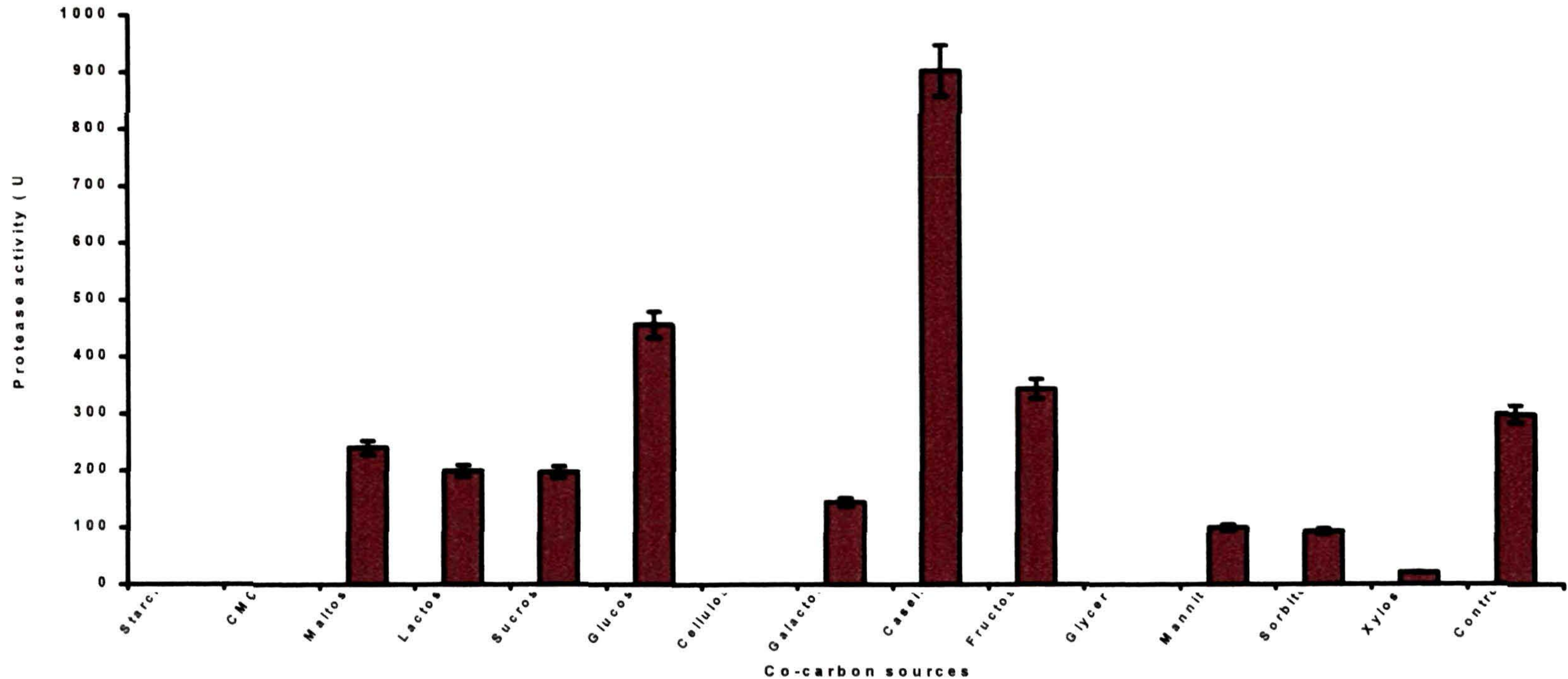


Fig.10.6 Effect of supplementation of co-carbon sources (10 % w/w) to IC (90.0 % w/w) (substrate) on protease production by *Brevibacillus* sp.strain AS-S10-II.Values are means ± S.D. of three determinations.

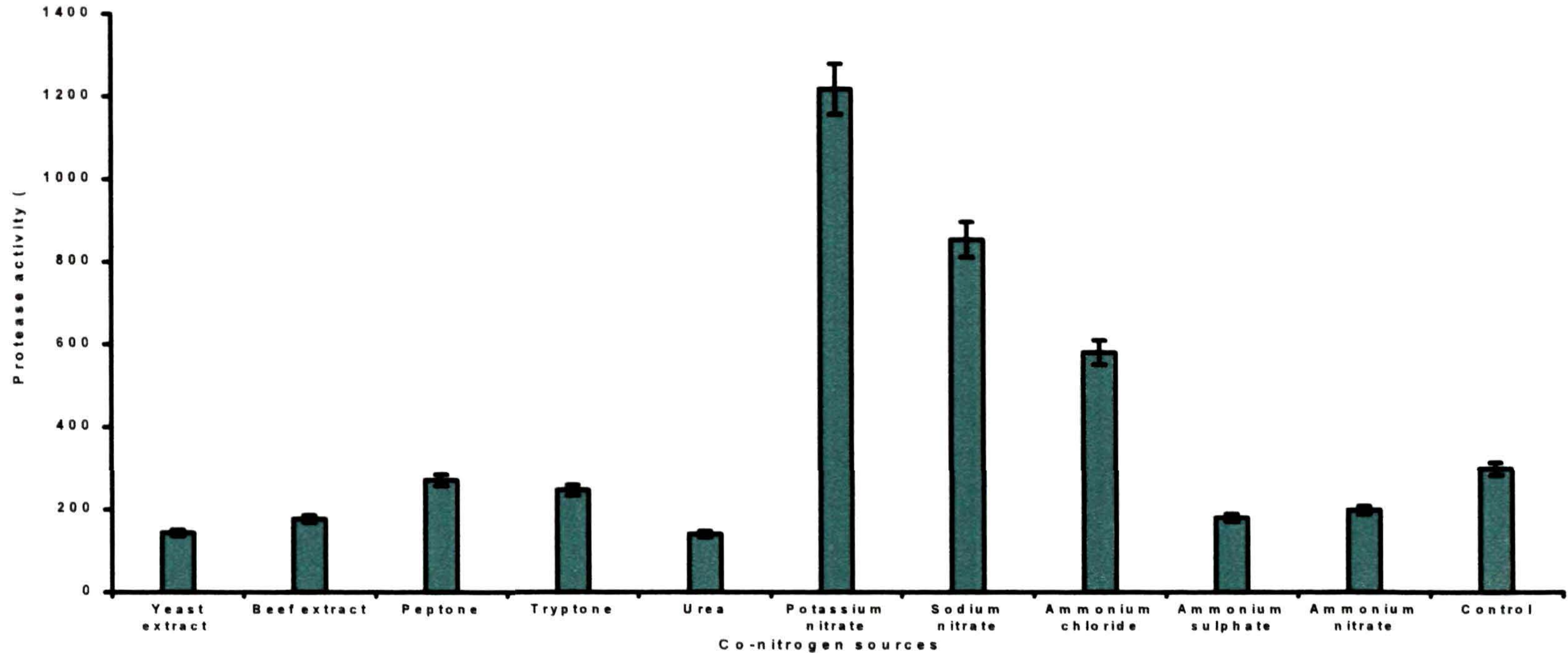


Fig.10.7 Effect of supplementation of co-nitrogen sources (1.0% w/w) to IC (99.0 % w/w) (substrate) on protease production by *Brevibacillus* sp. strain AS-S10-II.Values are means ± S.D. of three determinations.

10.1.5 Protease extraction from fermented matter

Ideal extraction medium was screened for recovery of protease from fermented matter. Maximum protease extraction was achieved in presence of distilled water containing 0.1 % v/v Triton-X-100 at pH 12.5 (Fig.10.8).

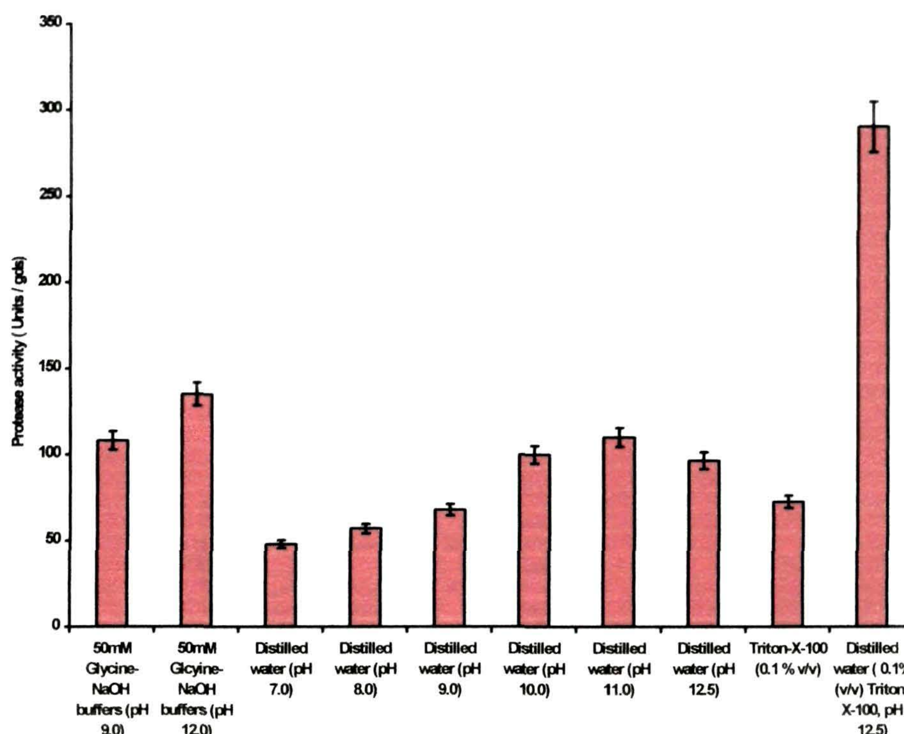


Fig. 10.8 Screening of best extraction medium for extraction of alkaline protease from fermented matter. Values are means ± S.D. of three determinations.

10.1.6 Batch fermentation for alkaline protease production

The influence of various combinations of IC and PP along with supplementation of co-carbon and co-nitrogen sources on protease production showed that when IC and PP powders were mixed in a ratio of 1:1 without any additional co- carbon and nitrogen sources, about 1.32 fold increase in protease yield ($p < 0.05$) was observed (Table10.1). Therefore, it could be inferred that the *Brevibacillus* sp. strain AS-S10-II can be used for protease production in industrial scale processes using IC and PP,

since it does not require any additional co-carbon and co-nitrogen sources. which may interfere with downstream processing.

Table 10.1 Influence of different combinations of co-substrates on alkaline protease production by *Brevibacillus* sp. strain AS-S10-II under solid-state fermentation. Values are mean ± S.D of triplicate determinations.

Substrate(s)	Protease activity (U/gds)
l.cylindrica (100%)	224.0 ± 11.2
Potato peel (100%)	346.0 ± 17.3
l.cylindrica (89%w/w)+ casein (10 % w/w) + potassium nitrate (1% w/w)	1500.00 ± 75.0
l.cylindrica (90%w/w) + Potato peel (10%)	1509.0 ± 75.5
l.cylindrica (80%w/w) + Potato peel (20 %w/w)	1636.0 ± 81.8
l.cylindrica (50%w/w) + Potato peel (50%w/w)	1982.0 ± 99.1
Potato peel (89% w/w) + casein (10 % w/w) + potassium nitrate (1%)	746.0 ± 37.3

10.2 Alkaline protease production under submerged fermentation

10.2.1 Effect of carbon source on protease production

Various carbon sources were used in the culture media (M9 minimal medium containing 1.0 %, w/v carbon sources) to observe their influence in protease production. Maximum alkaline protease production was observed in presence casein followed by glucose and than sucrose post 48h of

incubation at 50°C. Interestingly, this strain could not utilize maltose for growth and protease production (Fig.10.9).

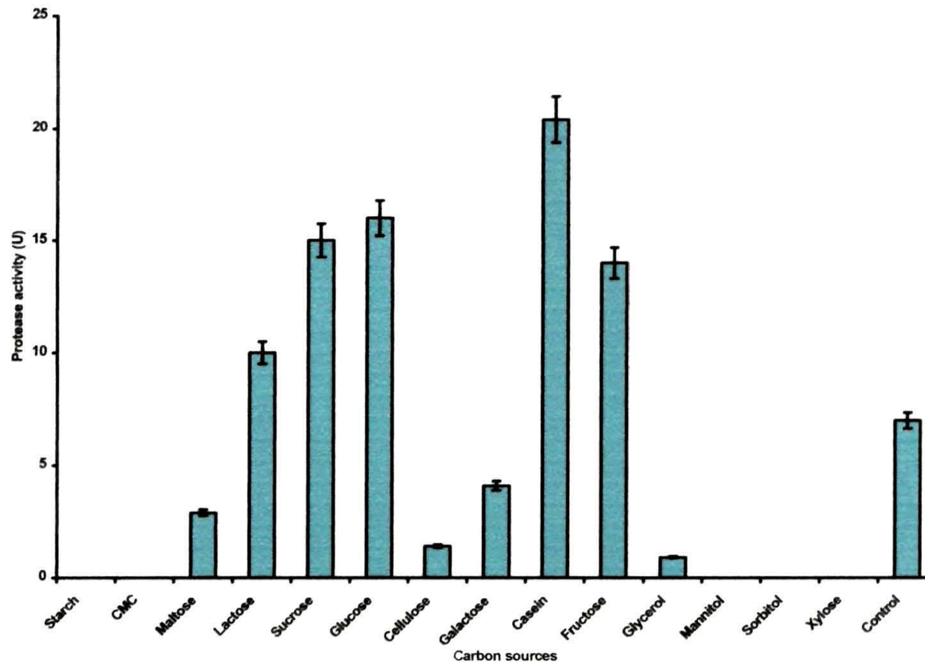


Fig.10.9 Effect of different carbon sources on alkaline protease production by *Brevibacillus* sp. strain AS-S10-II under SmF. Values are mean ± S.D. of three experiments.

10.2.2 Effect of nitrogen source on protease production

The effect of different nitrogen sources (0.1 % (w/w)) on protease production by *Brevibacillus* sp. strain AS-S10-II is illustrated in Fig. 10.10. The highest cell growth and protease production was achieved with 0.1 % (w/v) potassium nitrate (Fig. 10.10) and in it presence, 6.9 fold increase in protease production was observed compared with control (without nitrogen sources).

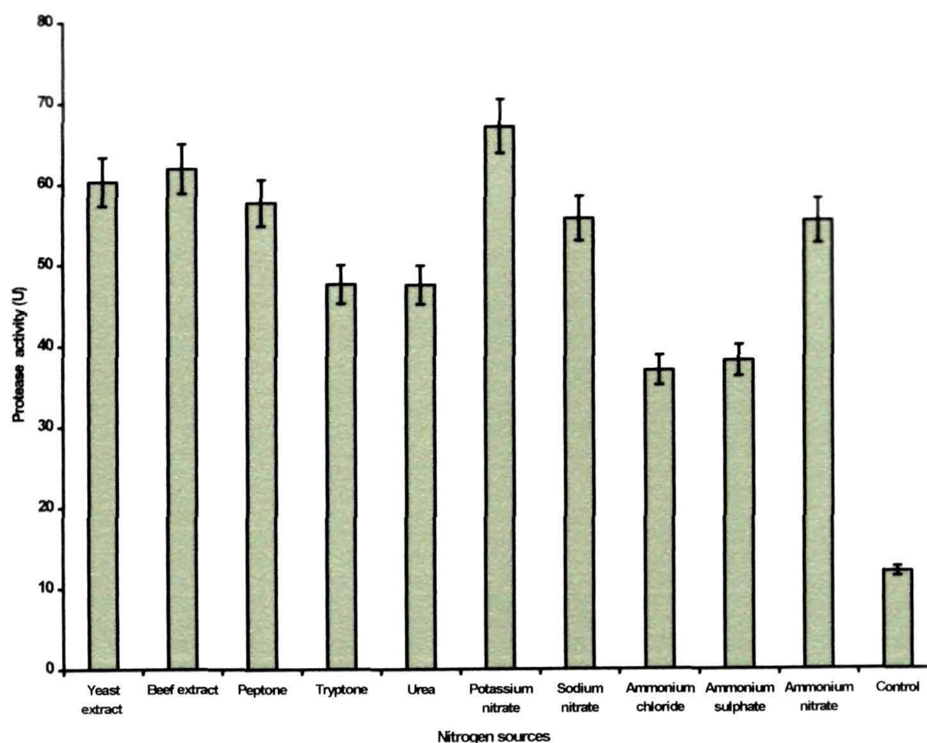


Fig.10.10 Effect of various nitrogen sources on alkaline protease production from *Brevibacillus* sp. strain AS-S10-II under SmF. Values are mean ± S.D. of three experiments.

10.2.3 Effect of pH on protease production

The pH of the medium strongly affects many enzyme processes and transport of various components across the cell membrane. Effect of initial pH of the medium has been illustrated in Fig. 10.11. Enzyme production gradually increased with increase in the pH of the medium from 5.0-12.0 and the maximum production (70.0 U) was recorded at pH 12.0, further increase in medium pH resulted decline in protease production.

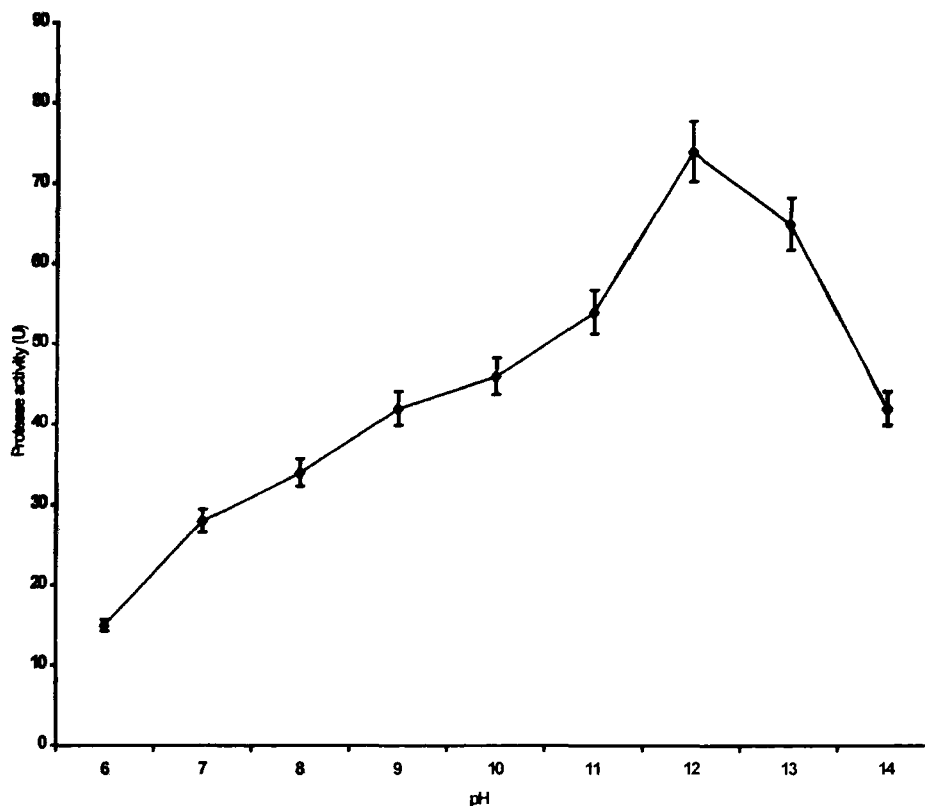


Fig.10.11 Effect of medium pH on alkaline protease production by *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.

10.2.4 Effect of temperature on protease production

The *Brevibacillus* sp. strain AS-S10-II displayed a linear increase in protease production upto 50°C and further increase in incubation temperature leading to decrease in protease secretion. Therefore, 50°C was found to be the optimum temperature for protease production by *Brevibacillus* sp. strain AS-S10-II.

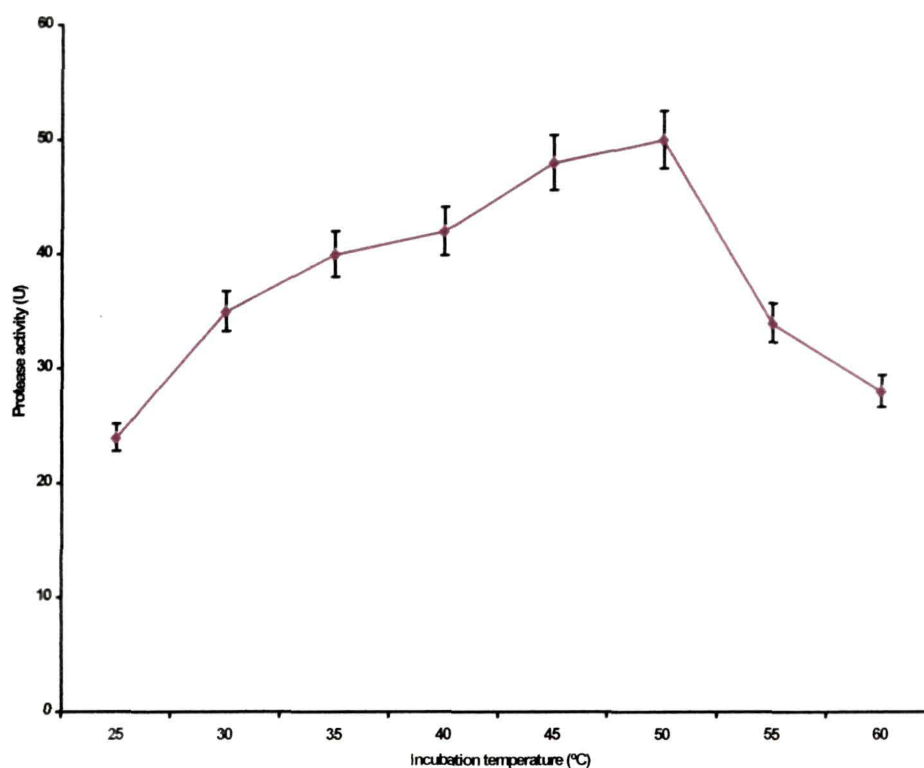


Fig.10.12 Effect of incubation temperature on alkaline protease production from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.

10.2.5 Kinetics of protease production

Protease production by *Brevibacillus* sp. strain AS-S10-II was optimized under different environmental and nutritional factors including temperature, pH, carbon sources, and nitrogen sources. Maximum protease production, increase in protein content and biomass formation was achieved in 48h of post incubation at 50°C growth temperature (Fig.10.13). Further, increase of incubation time lead a decline in protease production (Fig.10.13).

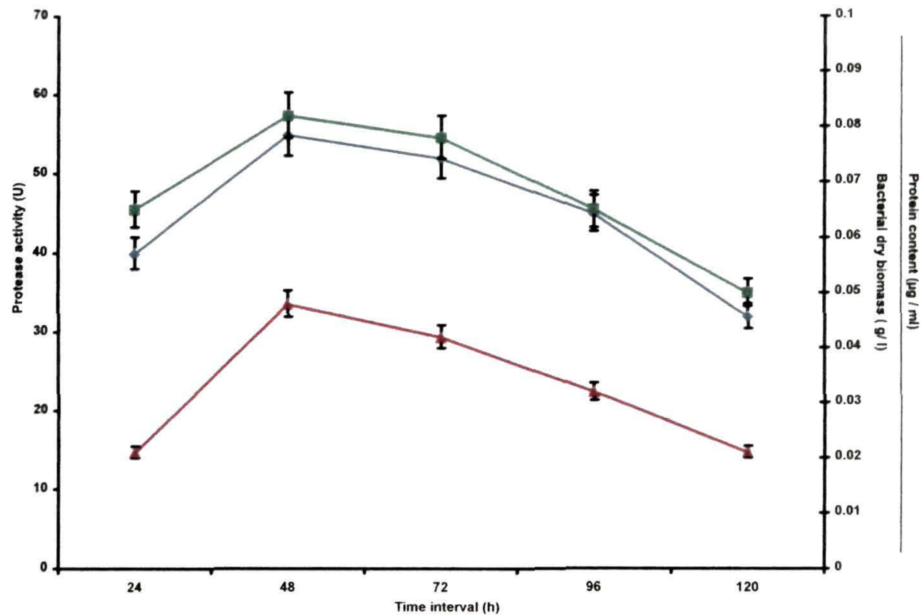


Fig. 10.13 Effect of incubation time on alkaline protease production (◆), bacterial dry biomass (■), and protein content (▲) from *Brevibacillus* sp. strain AS-S10-II under SmF condition. Values are mean ± S.D. of three experiments.

10.2.6 Effect of agitation rate on protease production

The effect of the agitation rate on the protease synthesis showed that maximum protease production was attained at 200 rpm (Fig.10.14) and further increase in agitation rate resulted in decline in protease production from *Brevibacillus* sp. strain AS-S10-II.

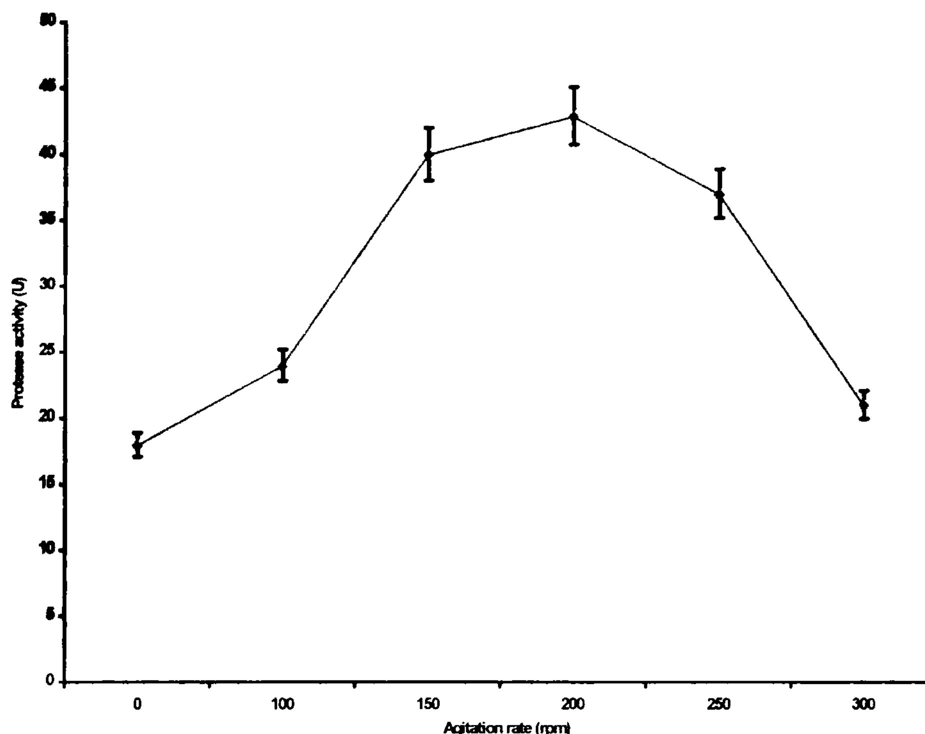


Fig.10.14 Effect of agitation rate on alkaline protease production from *Brevibacillus* sp. strain AS-S10-II under SmF. Values are mean ± S.D. of three experiments.

10.3 Statistical optimization of alkaline protease under submerged fermentation

10.3.1 Evaluation of factors effecting protease production using Plackett-Burman design

In the present study, maximum protease activity was estimated in the 48 h post-inoculated cell-free extract of *Brevibacillus* sp. strain AS-S10-II. Screening result reflected the significance of various factors on the β-keratinase activity (Table 10.2). The analysis of regression coefficients and t-value of six parameters showed that casein, potassium nitrate, and pH of the medium displayed a positive effect for protease production where as,

innoculum level, agitation rate and incubation time had a negative effect on enzyme production (Table10.2). Neglecting the variables which were insignificant, the model equation for keratinase production can be written as

$$Y = 25.31 + 16.04 X_1 + 11.06 X_2 + 25.97 X_3 \text{------}(10.1)$$

Where X_1 = pH of the medium, X_2 = casein level, and X_3 = potassium nitrate level.

For further optimization, casein KNO_3, and pH of the medium were chosen based in the calculated t and p values (Table 10.3). The innoculum level (% v/v) and incubation time (h) were kept at middle level.

Table 10.2 Plackett–Burman store design showing six variables with coded values along with the observed results for alkaline protease (β-keratinase) production by *Brevibacillus* sp. strain AS-S10-II.

pH	Casein (% w/v)	Incubation time (h)	Potassium nitrate (% w/v)	Innoculum size (% v/v)	Agitation rate (rpm)	Protease(β-keratinase) activity (Units)
1	-1	1	-1	-1	-1	23.56
1	1	-1	1	-1	-1	29.56
-1	1	1	-1	1	-1	11.12
1	-1	1	1	-1	1	25.68
1	1	-1	1	1	-1	27.56
1	1	1	-1	1	1	37.92
-1	1	1	1	-1	1	8.0
-1	-1	1	1	1	-1	11.44
-1	-1	-1	1	1	1	12.5
1	-1	-1	-1	1	1	22.32

pH	Casein (% w/v)	Incubation time (h)	Potassium nitrate (% w/v)	Innoculum size (% v/v)	Agitation rate (rpm)	Protease(β-keratinase) activity (Units)
-1	1	-1	-1	-1	1	15.0
-1	-1	-1	-1	-1	-1	12.0
0	0	0	0	0	0	25.3
0	0	0	0	0	0	25.3
0	0	0	0	0	0	25.3

Table 10.3 Statistical analysis of Plackett–Burman design showing coefficient values, t- and P-value for each variable for protease (β-keratinase) activity (p-value <0.05).

Terms	Co-efficient	SE-Coef	t-stat	p-value
Constant	25.31	0.7534	33.59	0.001
pH	16.04	0.9418	17.03	0.003
Casein (% w/v)	11.06	0.9967	11.09	0.008
Incubation time (h)	-6.89	0.9967	-6.91	0.020
Potassium nitrate (% w/v)	25.97	3.4577	7.51	0.017
Innoculum size (% v/v)	0.76	0.5651	1.35	0.308
Agitation rate (rpm)	-24.96	3.4731	-7.19	0.019

10. 3. 2 Statistical optimization of β-keratinase production in SmF

The results illustrated that maximum alkaline protease (β-keratinase) production of 910 U was achieved by employing RSM (Table 10.4). The overall second-order polynomial regression equation showing the empirical relationship between protease activity (Y) and three test variables in coded units can be represented by equation 10.2.

$$Y = 608.9 - 98.2C_1 + 356.8C_2 + 4528.0C_3 + 5.9C_1^2 - 178.3C_2^2 - 13208.6C_3^2 + 11.95C_1C_2 - 74.3C_1C_3 - 176.3C_2C_3 \quad \text{....} \quad (10.2)$$

The coefficients of the model including the significance of each coefficient as determined by t test and p-values showed that all the effects of C_1, C_2 and C_3, were significant ($p < 0.01$) and have a linear effect on β-keratinase production. The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the computed F value for linear regression was much greater than the tabulated (P) > F value (Table 10.5). The goodness-of-fit of the model was checked by determining the coefficient of determination (R^2) and adjusted R^2 (Table 10.6). The results showed that C_1 (pH of the medium) followed by C_3 (potassium nitrate level) had significant effect ($p<0.01$) on alkaline protease (β-keratinase) production by the bacterium under study (Table10.6). The validation of the statistically optimized condition for the production of alkaline protease (β-keratinase) by *Brevibacillus* sp. strain AS-S10-II was further verified by carrying out batch fermentation in a 5-l fermenter.

Table 10.4 Observed responses and predicted values of alkaline β-keratinase production by *Brevibacillus sp.* strain AS-S10-II. The observed values are average of triplicate determinations.

Run no.	Independent Variables			Y Response (Protease yield in Units)		Residual value
	C_1	C_2	C_3	Observed	Predicted value	
1	-1(9.0)	-1(0.5)	-1(0.05)	390.0	390.1	-0.135
2	1(11.0)	-1(0.5)	-1(0.05)	470.0	471.8	-1.802
3	-1(9.0)	1(1.5)	-1(0.05)	470.0	470.9	-0.985
4	1(11.0)	1(1.5)	-1(0.05)	635.0	635.7	-0.652

Run no.	Independent Variables			Y Response (Protease yield in Units)		Residual value
	C_1	C_2	C_3	Observed Value	Predicted value	
5				550.0	550.7	-0.733
6	1(11.0)	-1(0.5)	1(0.15)	580.0	580.4	-0.400
7	-1(9.0)	1(1.5)	1(0.15)	578.0	577.6	0.418
8	1(11.0)	1(1.5)	1(0.15)	689.0	690.3	-1.249
9	-2(8.0)	0(1.0)	0(0.10)	745.0	744.8	0.186
10	2(12.0)	0(1.0)	0(0.10)	910.0	908.2	1.772
11	0(10.0)	-2(0.25)	0(0.10)	295.0	293.8	1.158
12	0(10.0)	2(2.0)	0(0.10)	455.0	454.2	0.800
13	0(10.0)	0(1.0)	-2(0.025)	385.0	383.5	1.458
14	0(10.0)	0(1.0)	2(0.20)	565.0	564.5	0.501
15	0(10.0)	0(1.0)	0(0.10)	760.0	760.1	-0.056
16	0(10.0)	0(1.0)	0(0.10)	760.0	760.1	-0.056
17	0(10.0)	0(1.0)	0(0.10)	760.0	760.1	-0.056
18	0(10.0)	0(1.0)	0(0.10)	760.0	760.1	-0.056
19	0(10.0)	0(1.0)	0(0.10)	760.0	760.1	-0.056
20	0(10.0)	0(1.0)	0(0.10)	760.0	760.1	-0.056

R^2 = 100.0% R^2(pred) = 99.98% R^2(adj) = 99.9%

Table 10.5 Analysis of Variance (ANOVA) of alkaline protease produced by *Brevibacillus* sp. strain AS-S10-II.

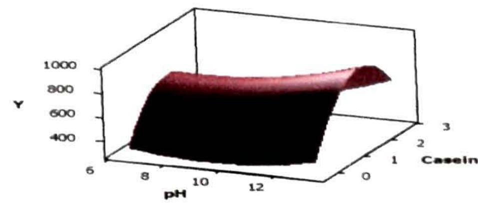
Source	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
Regression	9	518804	518804	57645	39372.17	0.000
Linear	3	102802	81112	27037	18466.75	0.000
pH	1	32235	5193	5193	3546.94	0.000
Casein	1	31040	25234	25234	17234.81	0.000

Source	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
Poatssium nitrate	1	39527	40628	40628	27749.73	0.000
Square	3	409747	409747	136582	93287.42	0.000
pH*pH	1	29665	7958	7958	5435.35	0.000
Casein * Casein	1	232698	268452	268452	183355.90	0.000
Potassium nitrate * Potassium nitrate	1	147384	147384	147384	100665.32	0.000
Interaction	3	6255	6255	2085	1423.97	0.000
pH*Casein	1	3444	3445	3445	2352.64	0.000
pH*Potassium nitrate	1	1352	1352	1352	923.43	0.000
Casein * Potassium nitrate	1	1458	1458	1458	995.83	0.000
Residual error	10	15	15	1		
Lack-of-Fit	5	15	15	3		
Pure error	5	0	0	0		
Total	19	518819				

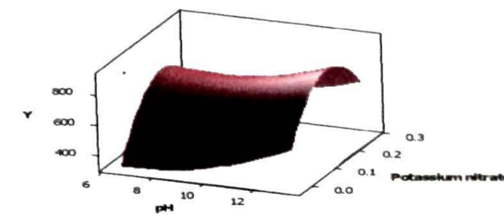
Table 10.6 Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for alkaline protease (β-keratinase) production by *Brevibacillus* sp. strain AS-S10-II in SmF under shake-flask study.($p<0.05$)

Terms	Co-ef	SE-Coef	t-Stat	p-value
Constant	608.9	8.9427	68.085	0.000
pH	-98.2	1.6486	-59.556	0.000
Casein	356.8	2.7182	131.281	0.000
Potassium nitrate	4528.0	27.1818	166.582	0.000
pH * pH	5.9	0.0797	73.725	0.000
Casein * Casein	-178.3	0.4163	-428.201	0.000

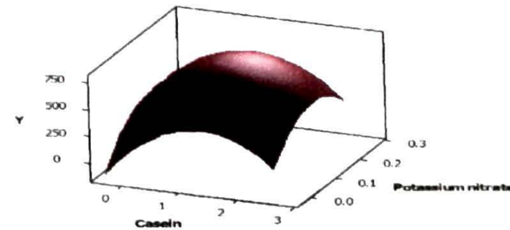
Terms	Co-ef	SE-Coef	t-Stat	p-value
Potassium nitrate * Potassium nitrate	-13208.6	41.6311	-317.278	0.000
pH * Casein	11.9	0.2445	48.504	0.000
pH * Potassium nitrate	-74.3	2.4446	-30.388	0.000
Casein * Potassium nitrate	-176.3	5.5876	-31.557	0.000



(a)

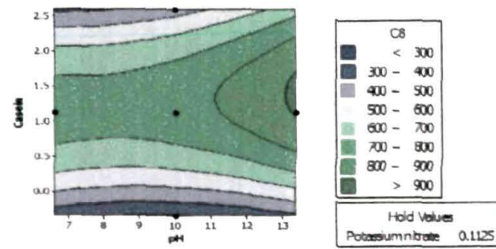


(b)

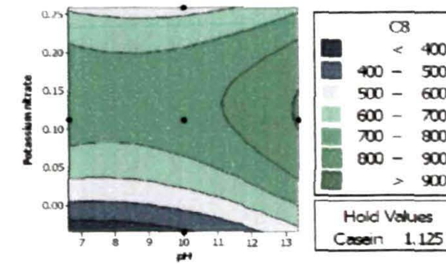


(c)

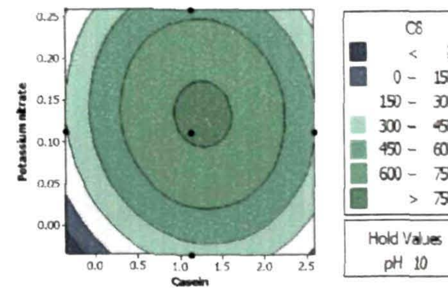
Fig.10.15 Response surface plots for alkaline protease production by *Brevibacillus* sp. strain AS-S10-II in SmF. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0 (b) pH of the medium vs concentration (%, w/v) of potassium nitrate, hold value C_2 = 0 and (c) concentration (%, w/v) of casein vs concentration of potassium nitrate (% w/v) hold value C_1 = 0. Values are mean ± S.D. of three experiments



(a)



(b)



(c)

Fig.10.16 Countor plots for alkaline protease production by *Brevibacillus* sp. strain AS-S10-II. The interaction between (a) pH of the medium and concentration (%, w/v) of casein, hold value C_3 = 0 (b) pH of the medium vs concentration (%, w/v) of potassium nitrate, hold value C_2 = 0 and (c) concentration (%, w/v) of casein vs concentration of potassium nitrate (% w/v) hold value C_1 = 0. Values are mean ± S.D. of three experiments.

10.3.3 Three dimensional response surface plots

Using three-dimensional (3-D) response surfaces which were plotted on the basis of the model equation, the interaction among variables and the optimum concentration of each factor for maximum protease (β-keratinase) production by *Brevibacillus* sp. strain AS-S10-II (Figs.10.15a-c) were investigated and determined. The β-keratinase production varied significantly upon changing the initial concentrations of casein and potassium nitrate. The three-dimensional plots showed that either with an increase in pH of the medium, or concentration of potassium nitrate β-keratinase production reached to a optimum value, whereas, further increase in their concentrations beyond the optimum value decreased the enzyme yield.

10.3.4 Countor plots

The 2D contour plots are the graphical representation of the regression equation generally used to visualize the relationship between the response and experimental levels of each variable and the type of interaction between the variables to deduce the optimum conditions. Fig. 10.16a clearly demonstrated the interaction of medium pH and casein level; a linear increase in pH of the medium and casein level displayed a significant increase in protease yield with an optimum pH 12.5 with 2.4 % (w/v) casein concentration. Interaction effects of potassium nitrate and medium pH was also studied using 2D countor plots, resulting a hyperparabolic pattern formation due to significant interaction, & yielding maximum protease production at pH 12.5 with 0.22 % (w/v) potassium nitrate level. (Fig.10.16b). Casein and potassium nitrate level demonstrated significant interaction due to circle formations in the centre of the system refering to the the point at which maximum protease production can be achieved (Fig.10.16c).

10.3.5 Validation of response surface model

The optimum levels of test variables for maximum β-keratinase yield found to be 1.4 % (w/v) of casein, 0.13% (w/w) and pH 12.5 for the production medium were as predicted by using the Minitab 16 Statistical Software® program. A significant higher optimal β-keratinase production (923.0 x 10^3 U/ l) was found using process controlled fermenter than that observed under the shake-flask study (910.0 x 10^3 U/ l) post 48 h of incubation.

10.4 Isolation and purification of an alkaline β-keratinase

The 80% acetone-precipitated fraction demonstrated maximum protease (β-keratinase activity). Subjecting this fraction to a gel filtration column three peaks exhibiting β-keratinase activity (Fig.10.17) were obtained. Maximum β-keratinase activity was observed for GF-I fraction (containing proteins in the molecular weight range of 80-85 kDa) which was found to be homogenous by SDS-PAGE. The protein displayed a single band of 83.2 kDa under both reduced and non-reduced conditions (Fig. 10.18) illustrating its monomeric nature. The protease activity of the 83.2 kDa purified protein was further confirmed by keratin zymography (Fig. 10.18). By RP-HPLC, the protein eluted as a single, sharp peak with retention time of 11.20 min showing the purity of preparation (Fig.10.19).This β-keratinase was named as Brevicarnase and a summary of purification of this enzyme from *Brevibacillus* sp.strain AS-S10-II is shown in Table 10.7.

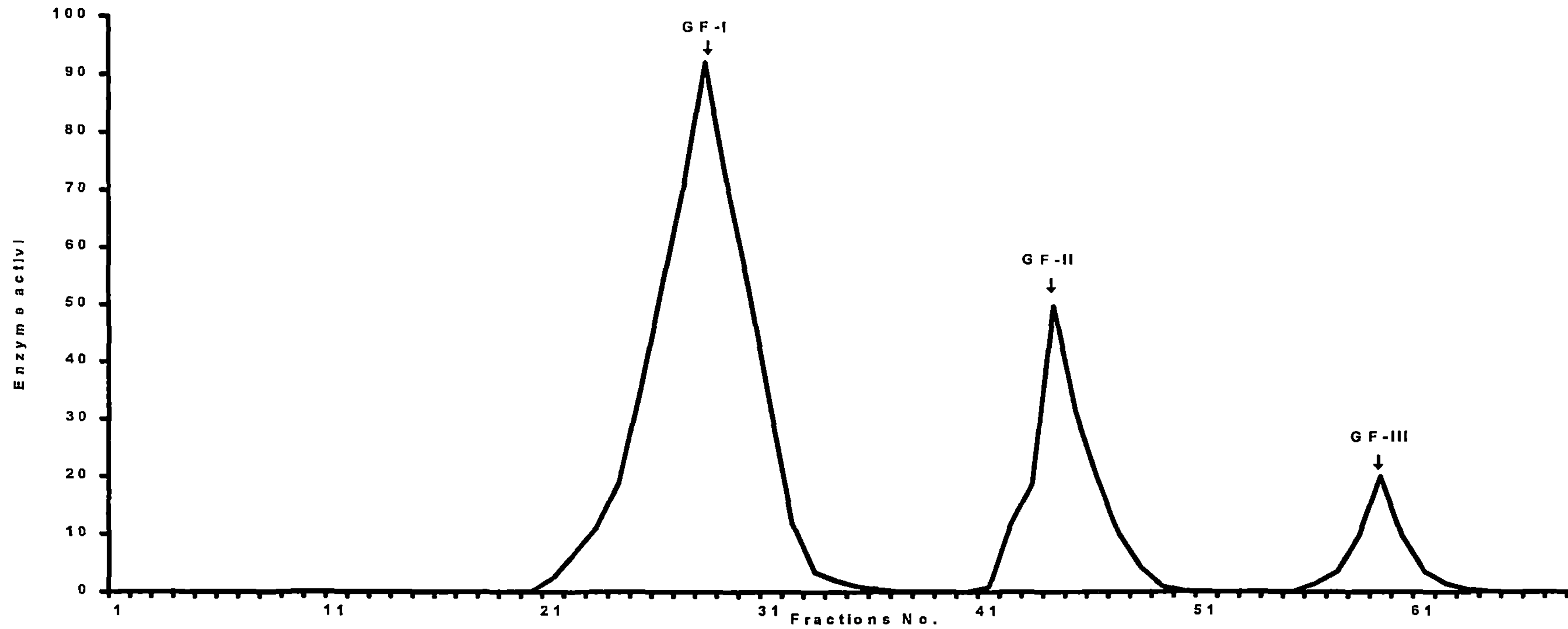


Fig.10.17 Gel–filtration pattern of 80% acetone precipitated protease (β-keratinase) fraction from *Brevibacillus* sp. strain AS-S10-II on Sephacryl S-200 column.

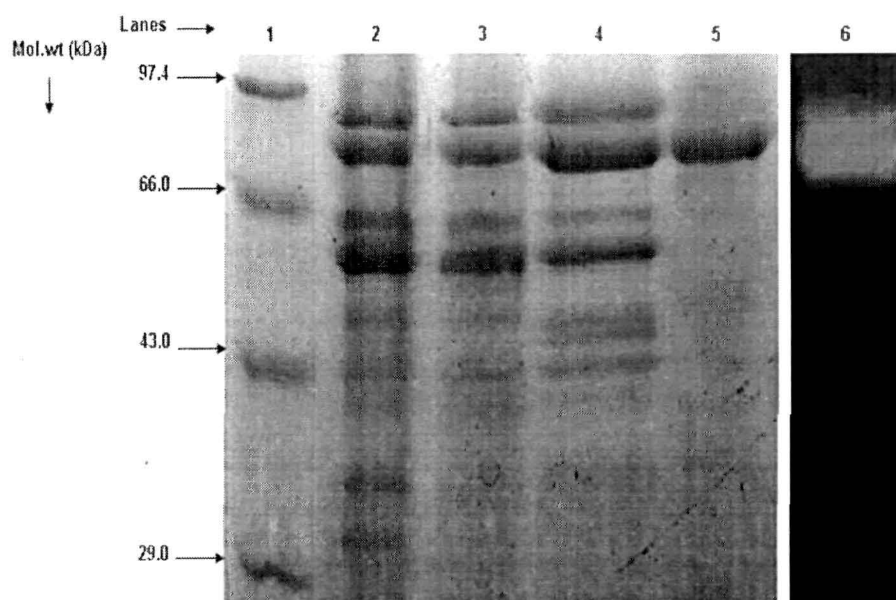


Fig. 10.18 SDS-PAGE (12.5%) of GF-I fraction from *Brevibacillus* sp. strain AS-S10-II. Lane 1 - protein molecular weight markers (kDa) :phosphorylase b (97.4), BSA (66.0), ovalbumin (43.0), carbonic anhydrase (29.0); Lane 2- crude protease under reduced condition (45.0µg); Lane3- crude protease non-reduced condition (45.0µg); Lane 4 – 80% acetone precipitate fraction (10.0µg); Lane 5- GF-I fraction under reduced condition (15.0 µg); Lane 6 – keratin zymographic study of GF-I fraction (15.0 µg).

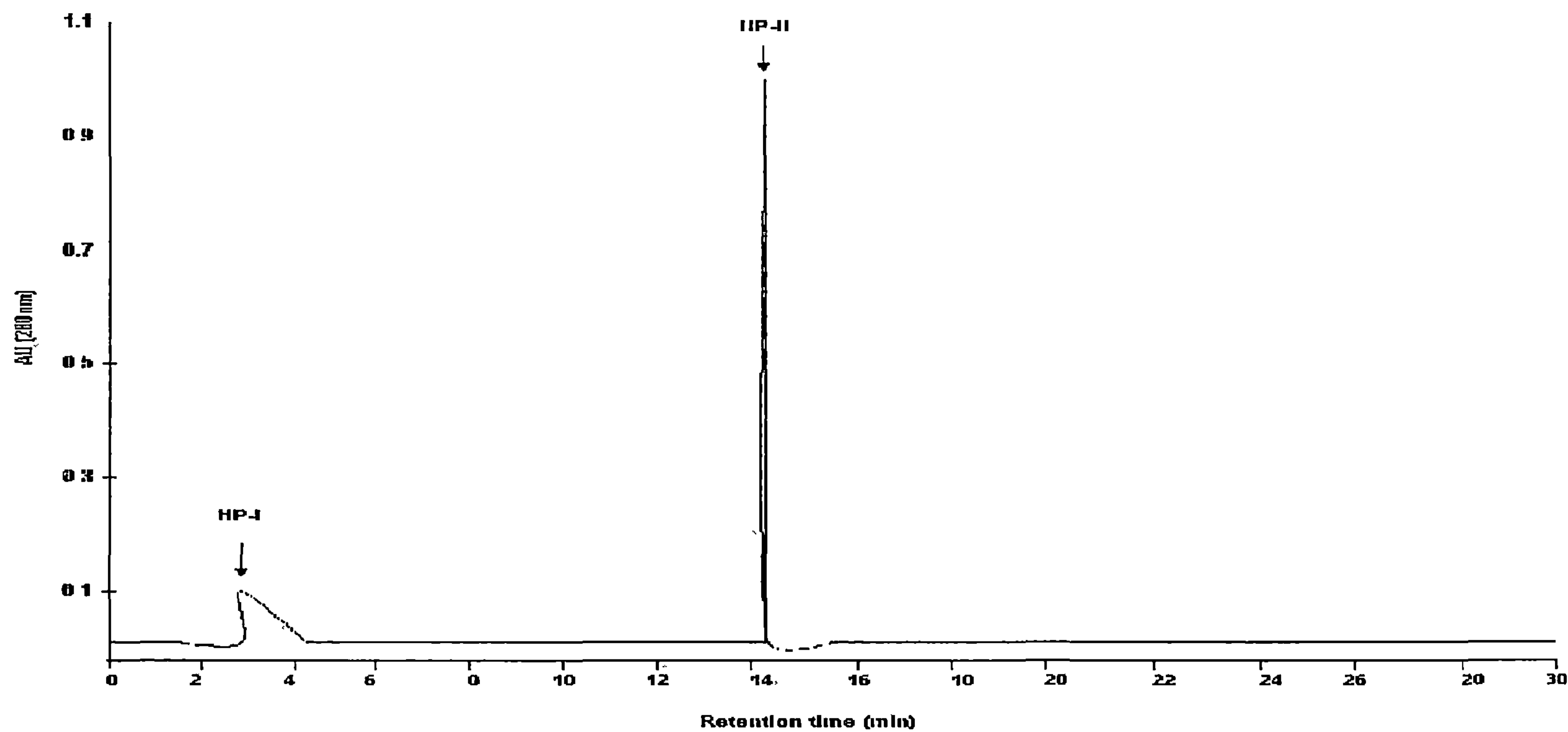


Fig.10.19 RP-HPLC of GF-I fraction, HP-I represents solvent peak.

Table 10.7 A summary of purification of Brevicarnase (an alkaline β-keratinase) from *Brevibacillus sp.* strain AS-S10-II. Data represent a typical experiment.

Step	Total protein (mg)	Total activity (Units)	Enzyme yield (%)	Specific activity (units/mg)	Purification (fold)
Cell-free supernatant	80.0	923.0	100	12.0×10^3	1
80% acetone precipitate fraction	8.0	840.0	91.0	105.0×10^3	9.0
GF-I (Brevicarnase)	2.5	540.0	58.5	216.0×10^3	18.0

10.5 Biochemical characterization

Before assessing the biotechnological potential of any enzyme, characterization of its biochemical properties pertinent to industrial application is utmost important and advantageous. Some of the biochemical properties of the enzyme are shown below.

10.5.1 Effect of pH and temperature on Brevicarnase

The Brevicarnase displayed optimum activity at 50°C (Fig.10.20) and remained active over a broad range of pH (5.0–14.0). Nevertheless, the protease showed a characteristic pH optima at 12.5 ± 0.5 (Fig.10.21).

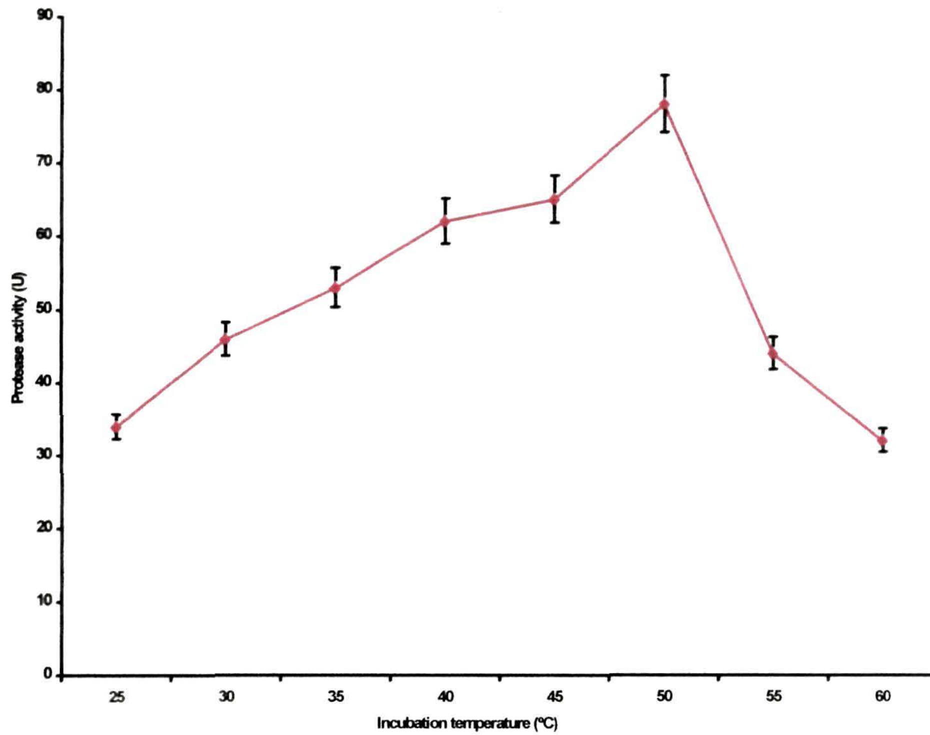


Fig. 10.20 Effect of incubation temperature on catalytic activity of Brevicarnase from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.

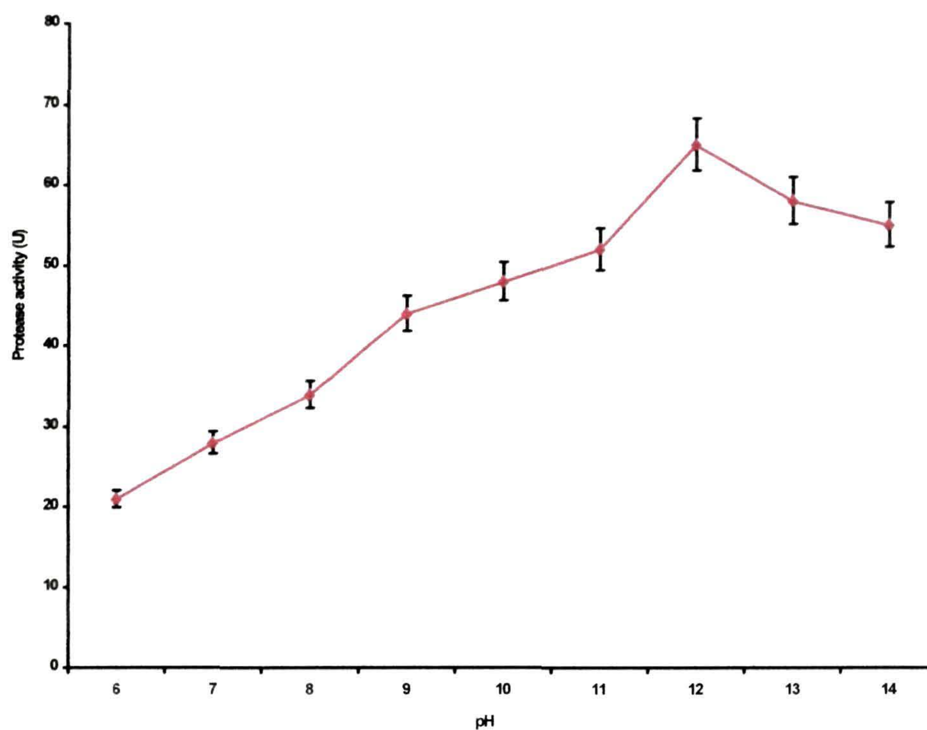


Fig.10.21 Influence of pH on Brevicarnase activity from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.

10.5.2 Heat-inactivation study of Brevicarnase

The Brevicarnase retained 95% of its original activity post-heating at 60°C for 30 min. However, further heating displayed a decrease in protease activity upto 80.0 % as shown in Fig.10.22.

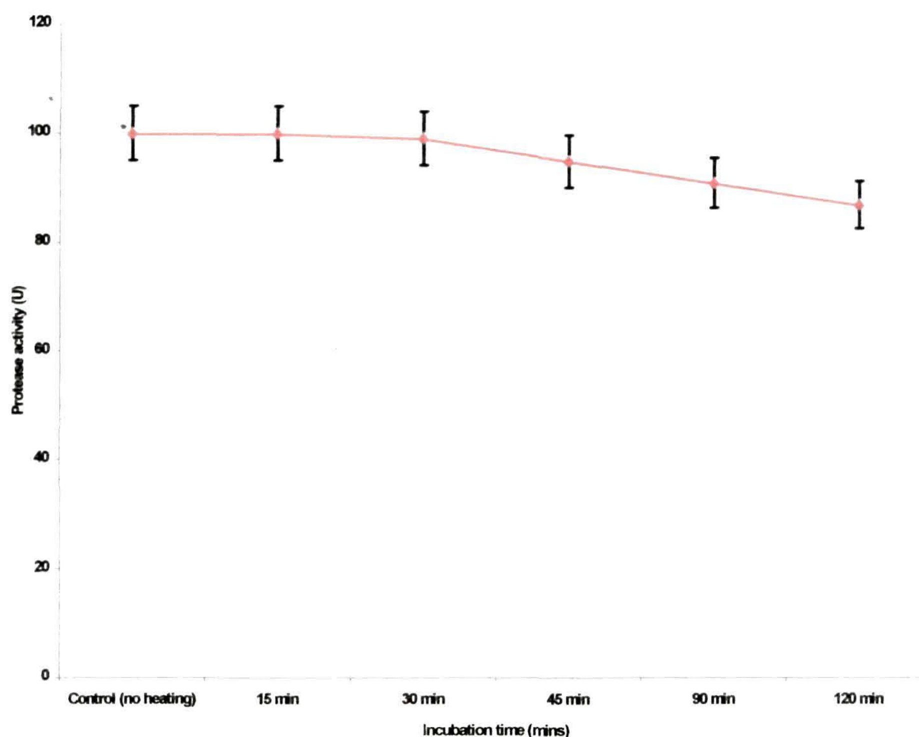


Fig. 10.22 Heat-inactivation study of Brevicarnase from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.

10.5.3 Effect of organic stabilizers on thermostability property of Brevicarnase

Different polyols were incubated with Brevicarnase at 60°C for 120 min. Results demonstrated that maximum stability of Brevicarnase was observed in presence of 5.0 % (v/v) of glycerol followed by mannitol (Fig.10.23). Among the tested stabilizers, glyercol (Fig.10.23) considerably increased the protease activity to about 1.6 fold higher as compared with the control (Fig.10.23).

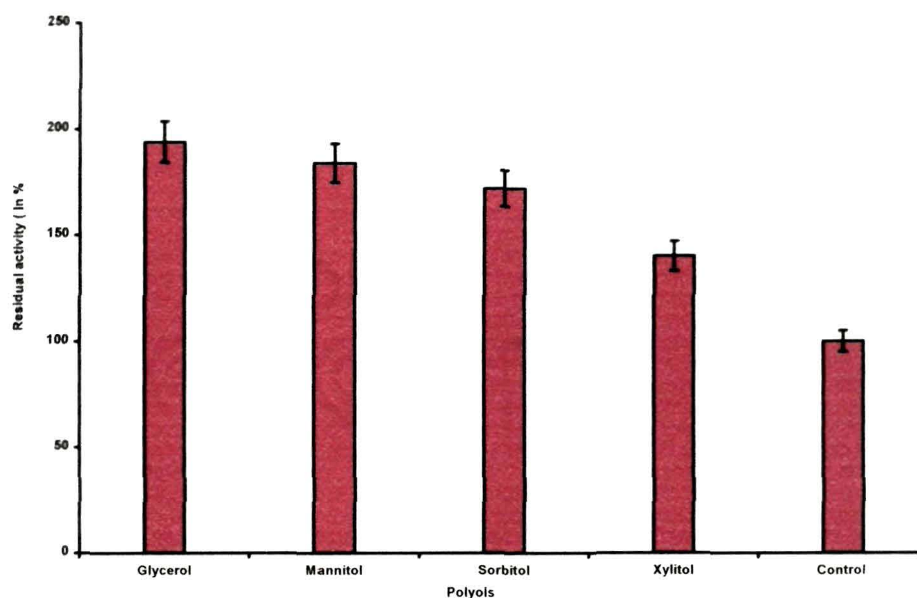


Fig.10.23 Influence of stabilizers on thermostability of Brevicarnase (post heating) at 60°C for 120 min. The enzyme without stabilizers (polyols) served as control (100% activity) and other values were compared with that. Values are mean ± S.D. of three experiments.

10.5.4 Substrate specificity study

The Brevicarnase hydrolyzed the a number of protein substrates in the following order of preference: chicken-feather keratin > casein > bovine serum albumin > bovine serum fibrinogen > Bovine serum globulin > hemoglobin. Gelatin and matrix protein collagen could not be hydrolyzed by Brevicarnase (Table 10.8).

Table 10.8 Biochemical characterization of Brevicarnase from *Brevibacillus* sp. strain AS-S10-II. Values are mean ± S.D. of three experiments.

Substrate specificity	**Specific activity (U / mg of protein)**
Raw chicken feather	216000.0 ± 899.0
Casein	7931.0 ± 397.0

Substrate specificity	Specific activity (U / mg of protein)
bovine serum albumin	7176.0 ± 359.0
bovine serum fibrinogen	6185.0 ± 434.2
Bovine serum globulin	1822.0 ± 91.0
Fibrin	1234.0 ± 24.68
Hemoglobin	602.0 ± 12.04
Gelatin	0
Human hair	0
Collagen	0

10.5.5 K_m and V_{max} values

The apparent Michaelis constant (K_m) and maximum velocity of enzyme catalyzed reaction (V_{max}) values for Brevicarnase towards keratin were determined as 0.16 mg / ml and 33.3 µmol/ min/ mg, respectively (Fig.10.24).

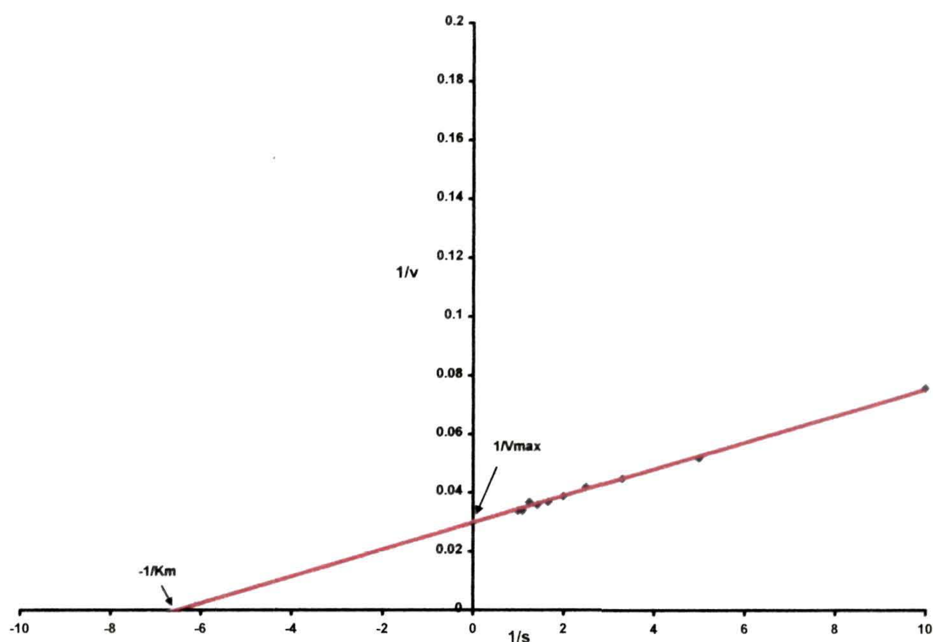


Fig. 10.24 Lineweaver-Burk plot to determine the K_m and V_{max} values of Brevicarnase . Values are mean ± S.D. of three experiments.

10.5.6 Effect of metal ions on Brevicarnase

In the present study, Fig.10.25 demonstrated the tested metal ions could not influence the keratinolytic activity of Brevicarnase, suggesting its *suitable application in laundry detergent formulation.*

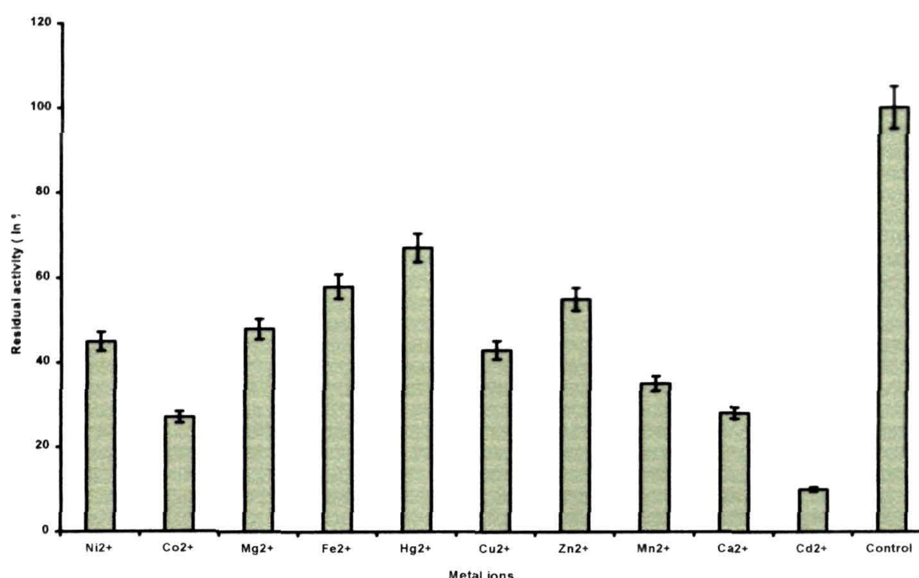


Fig. 10.25 Effect of metal ions on Brevicarnase activity . Values are mean ± S.D. of three experiments.

10.5.7 Effect of chemicals, inhibitors, chelators, surfactants, denaturing, oxidizing and bleaching agents on Brevicarnase

In order to evaluate the stability and the class / type of the isolated protease, Brevicarnase was incubated with various chemicals, inhibitors, chelators, surfactants, denaturing, oxidizing and bleaching agents at room temperature for 30 min followed by assay of protease activity. The Brevicarnase retained 97.0 ± 1.0% of their original activity in the presence of 2 mM EDTA, indicating non-metalloprotease in nature (Table 10.9). The *protease exhibited stability in presence of SDS (40mM), urea (8M), Tween* -20% (1% v/v) and Triton –X-100 (1%v/v). The tested metal ions could not

influence the protease activity (Fig.10.25). Brevicarnase was significantly affected by PMSF, IAA, 4-BPB inhibitors. This suggested predominant secretion of serine and cysteine like keratinase by *Brevibacillus* sp. strain AS-S10-II (Table 10.9). Further, a significant inhibition of protease activity in presence of DTT was also observed suggesting the presence of intra-molecular disulphide bonds in enzyme molecule.

Table 10.9 Effect of chemical inhibitors, chelator, metal ions, surfactants and urea on Brevicarnase activity. Values represent mean ± S.D of three determinations.

Metal ions/ reagents	Relative activity (%)
Control	100
Inhibitors	
PMSF (2mM)	9.6 ± 0.4
(4mM)	2.2 ± 0.2
4-pBPB (2mM)	11.3 ± 0.8
(4mM)	3.2 ± 0.5
IAA (2mM)	2.4 ± 0.1
(4mM)	0.0
Metal-ion chelator	
EDTA (2mM)	97.0 ± 1.0
(4mM)	91.0 ± 2.2
Anionic surfactant (SDS)	
20 mM	113.0 ± 5.5
40 mM	101.0 ± 2.1
60 mM	96.0 ± 1.1
Non-ionic surfactants (1% v/v)	
Trition-X-100	100.0 ± 3.0
Tween 20	125.0 ± 4.3
Tween 40	99 ± 4.2
Tween 60	95 ± 5.0

Metal ions/ reagents	Relative activity (%)
Urea (8 M)	81.0 ± 3.4
Oxidizing agents	
H_2O_2 (% v/v)	
5.0	109.0± 4.1
10.0	97.0 ± 2.3
Sodium perborate (% w/v)	
0.5	102.0± 6.6
1.0	96.0 ± 3.4
DTT	
4mM	11.0 ± 1.0

10.5.8 Effect of organic –solvents on Brevicarnase

The organic-solvent (at a final concentration of 20% v/v) stability of purified alkaline protease Brevicarnase is shown in Fig. 10.26. n-Hexane and xylene enhanced the protease activity whereas 2-propanol, methanol, ethanol, acetonitrile and benzene any affected the effect on enzyme stability. For example, in presence of benzene, the enzyme was destabilized and lost about 25.0 % of its original activity (Fig.10.26).

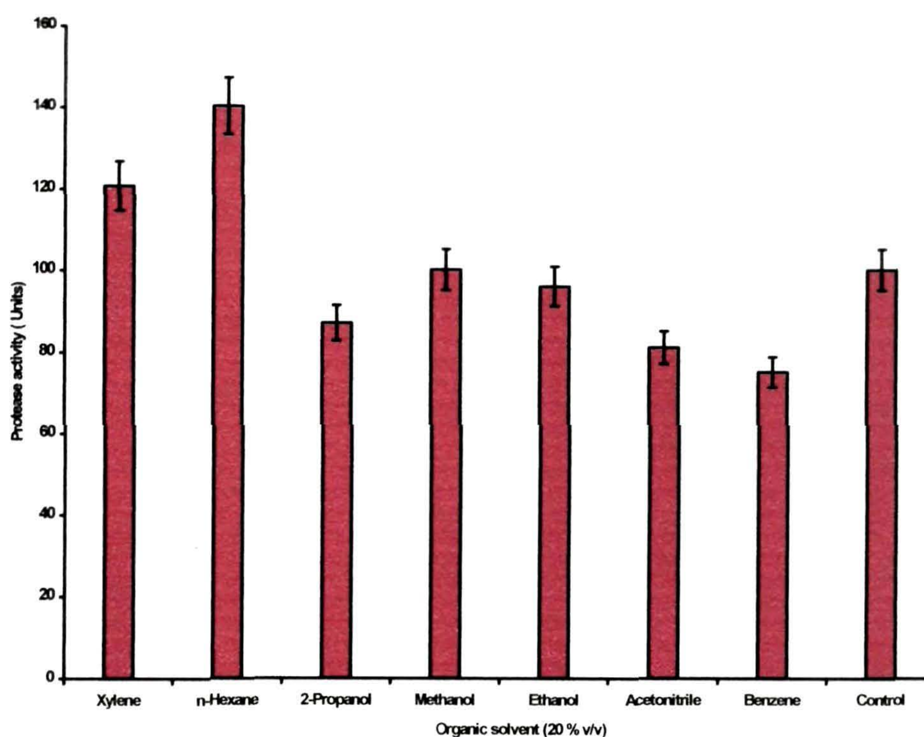


Fig.10.26 Organic - solvent stability of Brevicarnase. Enzyme activity in the absence of solvents was considered as 100% activity and other values were compared with that. Values are mean ± S.D. of three experiments.

10.5.9 Pharmacological properties of Brevicarnase from *Brevibacillus* sp. strain AS-S10-II

The pharmacological property of Brevicarnase is summarized in Table 10.10. Brevicarnase from *Brevibacillus* sp. strain AS-S10-II at a dose of 15 µg/ml induced 2.8 % hemolysis of the washed human erythrocytes and did not display any detrimental effect on goat liver, heart, lungs and kidney tissues (Table 10.10). Further, as shown in Table 10.10, Brevicarnase did not interfere with the normal clotting time of goat platelet- poor plasma (PPP).

Table 10.10 Pharmacological properties of Brevicarnase. Values are mean± S.D. of three experiments.

Pharmacological properties	Values
Hemolysis (% Hb released / 15.0 µg of Brevicarnase 90 min post incubation at 37 °C)	
Control (without Brevicarnase)	1.4 ± 0.1
Treatment	3.0 ± 0.5
Ca-Clotting time (s)	
Control (without Brevicarnase)	129.0 ± 6.5
Treated (with 15.0 µg / ml of Brevicarnase)	132.0 ± 7.0
In vitro tissue damaging activity (% hemoglobin release by 15.0 µg / ml of Brevicarnase 5 h post incubation at 37 °C)	
a) ***Heart***	
Control (without Brevicarnase)	0.09 ± 0.01
Treatment	0.27 ± 0.01
b) ***Lung***	
Control (without Brevicarnase)	0.13 ± 0.01
Treatment	0.36 ± 0.02
c) ***Liver***	
Control (without Brevicarnase)	0.16 ± 0.08
Treatment	0.28 ± 0.01
d) ***Kidney***	
Control (without Brevicarnase)	0.12 ± 0.05
Treatment	0.27 ± 0.01

10.6 Industrial applications of Brevicarnase

10.6.1. Detergent compatibility and stain removal potency of Brevicarnase

The Brevicarnase at a dose of 6.0 µg / ml retained 80- 160% of its original activity at 25, 37 and 45°C in the presence of laundry detergents in the following order: Henko® > Rin advanced® = Safed® > Fena Ultra® > Surf excel® =Ghari® >Ariel® >Sunlight® >Tide® >Wheel® (Fig.10.27).

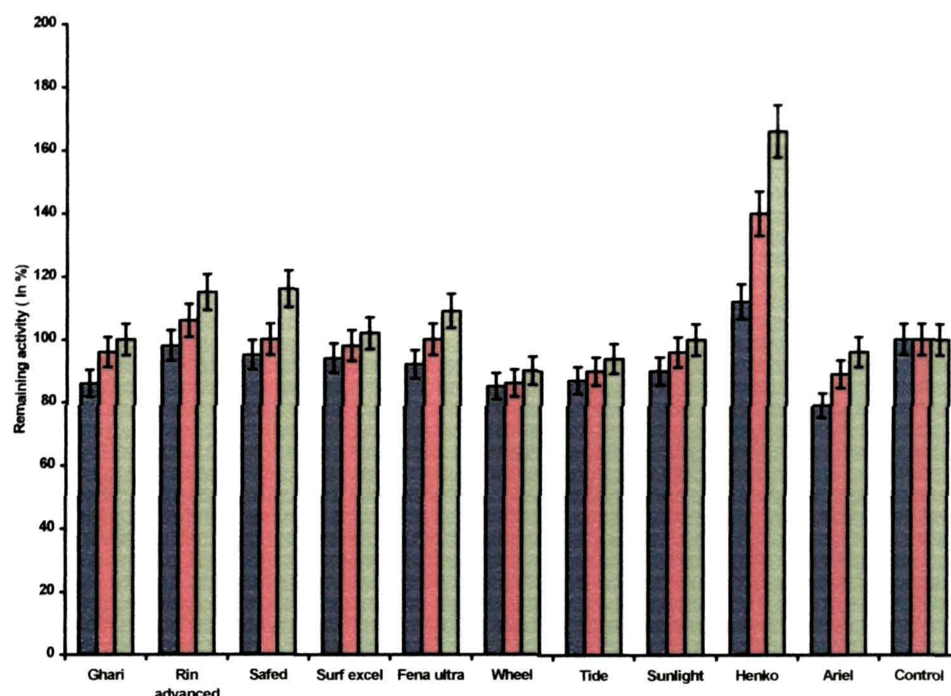


Fig.10.27 Detergent stability and compatibility of Brevicarnase (6.0 µg / ml) from Brevibacillus sp. strain AS-S10-II at the tested temperature ranges 25 (■), 37 (■) and 45 °C (■). Values are mean ± SD of triplicate determinations.

In order to study the wash performance property of Brevicarnase removal of blood stains from cotton fabrics were performed. Result demonstrated that a 1:1 (v/v) mixture of detergent: Brevicarnase solution exhibited significantly higher ($p < 0.05$) activity as compared to the detergents

solutions or Brevicarnase solution alone in the tested temperature shown in Fig. 10.28.

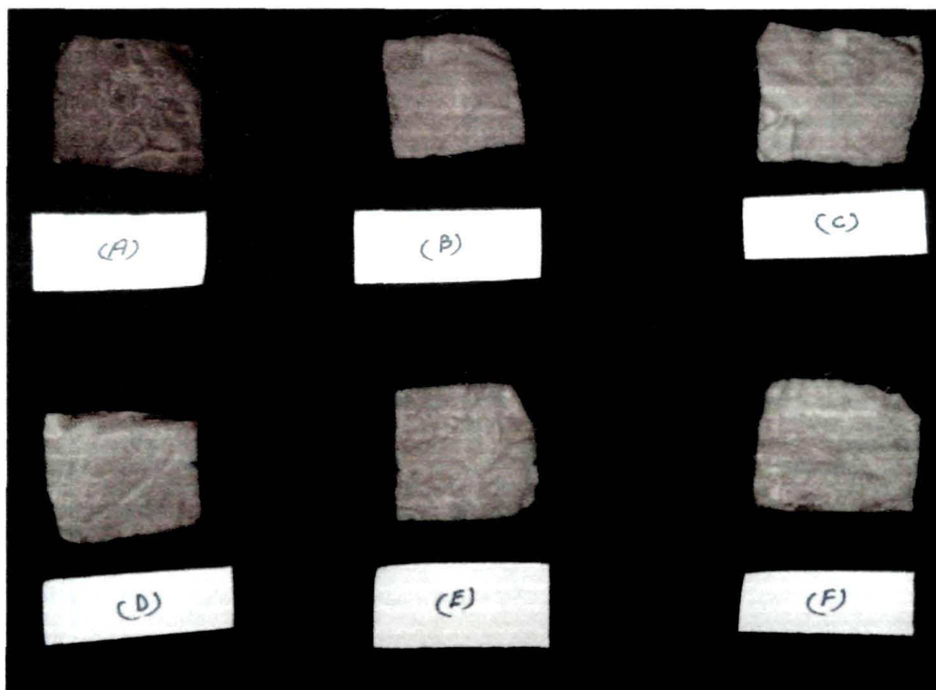
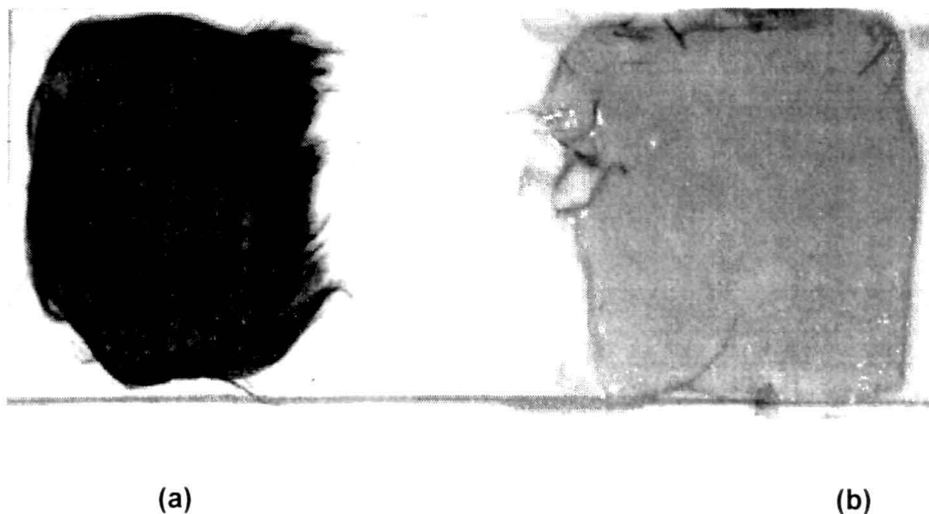


Fig.10.28 Wash performance study using blood stain containing cotton fabrics with Brevicarnase (6.0 µg / ml) at 37°C. From left (a) control (tap water with blood stain cloth), (b) Brevicarnase (6.0 µg / ml) with blood stain cloth, (c) unheated detergent with blood stain cloth, (d) heated detergent with blood stain cloth, (e) unheated detergent with Brevicarnase (6.0 µg / ml) with blood stain cloth, (f) heated detergent with Brevicarnase (6.0 µg / ml) with blood stain cloth. Values represent mean ± S.D of three experiments.

10.6.2 Dehairing activity

The dehairing effect of Brevicarnase from *Brevibacillus* sp.strain AS-S10-II showed a promising result as shown in Fig. 10.29. When the raw skin was incubated with the Brevicarnase at 37°C for 6h, the hairs could be pulled out easily. This effect was in correspondence with their protease activity.



(a) (b)

Fig. 10.29 Dehairing activity of Brevicarnase (a) goat skin incubated in 100 mM Glycine–NaOH buffer, pH 12.5 for 12 h at 37°C (control) and (b) enzymatically dehaired goat skin incubated with Brevicarnase (50 U / ml) for 6 h at 37°C.

CHAPTER XI

DISCUSSION

Chapter 11

Discussion

Micro organisms represent an attractive source of protease enzyme as they can be cultured in large quantities in a relatively short duration[7]. Micro-organisms secrete arrays of protease isoenzymes and each isoenzyme plays on important role in normal physiological processes of bacteria such as cell cycle regulation, cell growth and differentiation and sporulation[401,402]. The proteases could be intracellular and / extracellular. Intracellular proteases are vital to sustain various cellular and metabolic processes, such as sporulation and cell differentiation, protein turnover, and enzyme maturation. Extraceullar proteases carry out protein hydrolysis in fermented media and enable the cell to absorb and / or adsorb and utilize hydrolytic products[402]. Apart from their importance in maintaining the physiology of a living system, proteases are also found to be highly relevant in technical enzyme applications. Of these, their use in detergents is the most prominent with respect to market volume and tonnage[11].

During the last decade, numerous reports have been published on microbial alkaline proteases showing their major role in detergent industries[6,7]. Phylogenetic analysis has revealed that a great diversity exists among protease producing micro organisms. However, a majority of the microbial proteases of industrial significance are isolated from *Bacillus* species[6,7,23]. Undoubtedly, there is a dearth of reports on industrially important protease production from *Peanibacillus* species, *Brevibacillus* species and gram negative *Bacillus* species.

A close examination of literature evidences that many protease have been purified from diverse groups of micro organisms and a few of them are biotechnologically and industrially important. Nevertheless,

these are not sufficient to meet most of the industrial demands[201]. For example, the industrial applications of proteases in detergent or leather processing industries requires that the enzyme be stable at higher temperatures, ability to function at low washing temperature (for example at 23°C), detergent, surfactant and bleach-stable, and lack autoproteolyitc digestion tendency, besides being cost-effective. Considering the requirement of industry this strongly is undertaken, with an aim to isolate alkaline protease producing promising bacteria from the environmental samples of NE India, one of the biodiversity hot-spot regions of the world, and to explore the biotechnological potential of the isolated protease as a low-cost green-chemical. It is worthy to mention that limited reports are available on the production of alkaline protease from bacteria isolated form environmental samples of NE India.

11.1 Screening for alkaline protease producing bacterial isolates

In the present study, enrichment culture technique was used for the isolation of alkaline protease producing potential bacterial isolates from soil/ water samples of North-East India because of the efficacy of this technique for the isolation of a desired microorganisms (alkaline protease producing bacteria in the present study) from different environmental samples (even if that desired microbes are less abundant in that particular sample). Considering the industrial importance and hence the market demand of detergent-stable robust proteases, the preliminary screening was directed to search for detergent stable alkaline protease producing bacteria. In addition, some other important properties such as thermo-stability and storage stability of protease enzymes were also given a due consideration during the initial screening process. Since high level of enzyme yield (titer) is another crucial criteria for industrial application of that enzyme; therefore, the detergent-stable alkaline protease producing bacteria were further screened for their ability to produce high amount of alkaline-protease in a culture medium. Finally, five bacterial strains

namely *Brevibacillus* sp. AS-S10-II; *Bacillus subtilis* RM-01; *Bacillus* sp. AS-S20-I; *Bacillus licheniformis* AS-S24-I; *Peanibacillus tezpurensis* sp.nov. AS-S24-II and *Bacillus subtilis* DM-04 (previously isolated in our laboratory from petroleum contaminated soil samples) meeting the above criteria were selected for the production, isolation, purification, biochemical characterization and industrial applications of the purified protease enzymes.

11.2 Polyphasic approach: a unique system for taxanomic characterization of microorganisms

Microorganisms are tremendously diverse, both genetically and phenotypically. In the present study, polyphasic approach was applied for taxonomic identification of protease producing bacterial strains which was based on studying their morphological and biochemical properties supplemented with information obtained from molecular techniques. Advances in polyphasic approach for bacterial classification such as 16S rRNA gene sequencing and molecular fingerprinting techniques integrated with other molecular markers have become important tools in microbial systematic[403].

A number of criteria that include genotypic, chemotypic and phenotypic features used for polyphasic characterization of bacteria (Fig. 1) are discussed below[403,404].

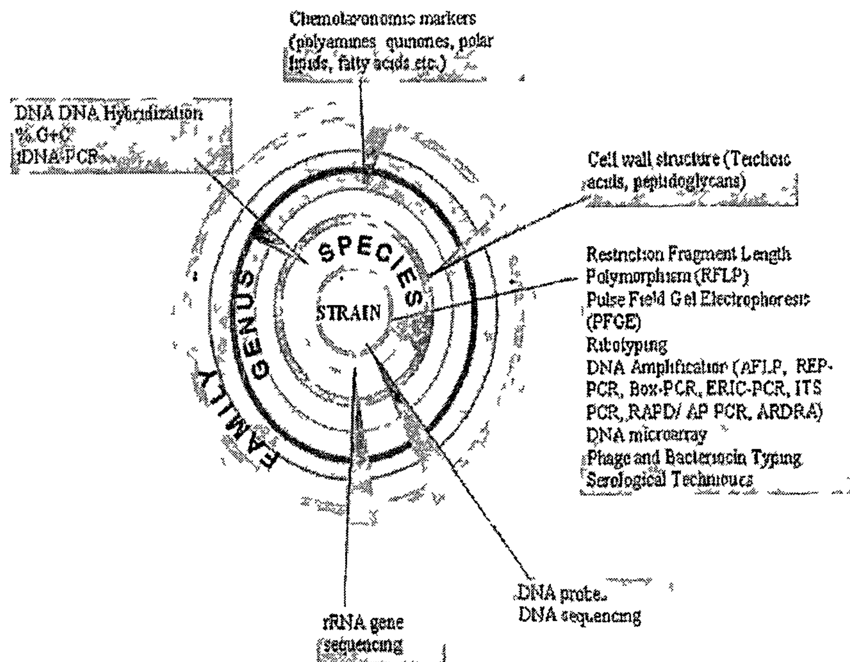


Fig. 11.1 Diagrammatic representation of techniques and markers used in modern polyphasic approach for resolving the bacterial hierarchy.

11.2.1 Phenotypic characterization of alkaline protease producing AS-S10-II isolate

Phenotypic data deserve special attention because of their impact on species delineation and also because it provides a description of new species on the basis of minimum number of phenotypic characteristics[405].

Phenotypic features of AS-S10-II isolates summarized in Table 11.1. AS-S10-II isolates share similar features like catalase, VP test, casein and starch hydrolysis, nitrate reductions tests with *Bacillus* KD-1014 strain[406] and *Bacillus* sp.[407] suggests that this isolate belongs to genus *Bacillus*. Considering the major disadvantages of phenotypic methods which are due to the conditional nature of gene expression wherein the same organism might show different phenotypic characters in different

environmental conditions[408,409], chemotaxonomic property of the isolate AS-S10-II was characterized.

Table 11.1 Distinctive phenotypic properties of isolate AS-S10-II and related species of *Bacillus* sp. ND: Not determined.

Character	AS-S10-II	*Bacillus* sp. KD 1014	*Bacillus* sp.KMM1918	*Bacillus* sp. 1044	*Bacillus* sp. 1124
Cell shape	Rods	Rods	Rods	Rods	Rods
Motility	+	+	Not determined	Not determined	Not determined
Spore shape	Spore forming	+	-do-	ND	Not determined
Gram stain	Positive	Positive	-do-	Positive	Positive
Growth temperature	30-60 °C range , optimum 45 °C	40-65 °C optimum temperature 45 °C	temperature 15-45 °C	Not determined	Not determined
Growth pH range	6.0-11.0, optimum 8.0	pH range 5.0-9.0, optimum pH 5.0	Not determined	Not determined	-do-
Growth in 5% (w/v) NaCl	-	Not determined	+	+	+
Growth in 7% (w/v) NaCl	-	-do-	+	-	+

Character	AS-S10-II	*Bacillus* sp. KD 1014	*Bacillus* sp.KMM1918	*Bacillus* sp. 1044	*Bacillus* sp. 1124
Growth at					
pH 5.5	-	+	+	+	+
50 °C	+	+	-	+	-
Catalase	+	+	Not determined	+	+
Voges-Proskauer Test	+	Not determined	-do-	+	+
Methyl red test	+	-do-	-do-	Not determined	Not determined
Acid from					
D-Glucose	+	-	+	+	Weakly positive
Mannitol	-	+	+	-	-
Lactose	-	-	-	-	-
Sucrose	-	+/-	Not determined	-	+
Gas from glucose	-	Not determined	-do-	-	-
Hydrolysis of					

Character	AS-S10-II	*Bacillus* sp. KD 1014	*Bacillus* sp.KMM1918	*Bacillus* sp. 1044	*Bacillus* sp. 1124
Casein	+	+	-	+	+
Gelatin	-	+	Not determined	+	+
Starch	+	+	-do-	+	+
Lipid	+	Not specified	-do-	-	Not determined
Urea	-	-	-do-	Not determined	-do-
Citrate Utilization	-	-	+	-	+
Indole formation	+	-	Not determined	Not determined	Not determined
Nitrate reduction	+	-	Not determined	+	+
H_2S production	+	Not specified	Not determined	Not determined	Not determined
References	Present study	Lee *et al*[406]	Ivanova *et al*[410]	Guerra-Cantera and Raymundo[407]	

11.2.2 Chemotaxonomic properties of isolate AS-S10-II

The term chemotaxonomy refers to the application of analytical methods for collecting information on different chemical constituents or chemotaxonomic markers of bacterial cells in order to group or organize them into different taxonomic ranks[403]. Identification of microorganisms by analyzing their cellular fatty acid methyl ester (FAME) is based on Similarity Index (SI) which is a numerical value expressing how closely the cellular fatty acid composition of an unknown bacterial sample compares with the mean fatty acid composition of the strains used to create the library entry listed as its match[411,412]. The database search presents the best matches and associated similarity indices. An exact match of the fatty acid make-up of the unknown sample to the mean of a library entry results in a similarity index of 1.000. The similarity index will decrease as each fatty acid varies from the mean percentage. Strains with a similarity of 0.700 or higher and with a separation of 0.200 or more between first and second choices are considered an excellent match[411,412]. If the similarity index is between 0.500 and 0.700 with a separation of 0.100 between the first and second choices, then the match is considered good. A Similarity Index between 0.300 and 0.500 could be an acceptable match, but would indicate an atypical strain. Values lower than 0.300 suggest that the species is not in the database, but those listed provide the most closely related species[411].

The analysis of fatty acid methyl ester (FAME) profiles by gas chromatography (GC) is a rapid and inexpensive technique that holds great promise in microbial identification. The iso and anteiso are major fatty acids components (60 to 98%) found within certain genera of micro-organisms and analysis of these classes of fatty acids has certain important implications for taxonomy and an evolutionary scheme for micro-organisms[413]. Based on the Sim Index (SI) of 0.689, and due to predominance of anteiso-branched $C_{15:0}$ and iso-$C_{15:0}$ fatty acids, the strain AS-S10-II was taxonomically identified as *Bacillus*

subtilis (MTCC 8960) by the Institute of Microbial Technology, Chandigarh (Table 11.2). However, the major qualitative and quantitative differences in the cellular fatty acid composition of AS-S10-II compared to other *Bacillus subtilis* is in absence of $C_{14:0}$, $C_{15:0}$,$C_{16:0}$, $C_{16:0}$ ω 7 cis alcohol, and $C_{16:1}$ ω11cis straight chain cellular fatty acid, suggesting that *Bacillus subtilis* AS-S10-II requires molecular level characterization for further conformation.

Table 11. 2 Percentage of cellular fatty acid composition of isolate AS-S10-II and related species of *Bacillus subtilis.*

Strains	Straight chain					Iso-branched							Anteiso-branched			References
	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$ ω 7 cis	$C_{16:1}$ ω11cis	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{17:1}$ w10c	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	
AS-S10-II	-	-	-	-	-	-	25.78	-	-	12.70	-	-	44.77	-	16.76	Present study
B. subtilis B-770	-	5.0	-	-	-	2.0	13.0	9.0	13.0	-	-	-	40.0	-	-	Kaneda,[413]
B. subtilis B-4	-	6.0	-	-	-	4.0	14.0	11.0	15.0	-	-	-	33.0	-	10.0	Kaneda,[413]
Bacillus subtilis	-	-	3.14	0.23	-	1.13	29.27	2.36	1.52	9.59	-	1.72	40.19	-	9.38	Roberts *et al*[414]
Bacillus subtilis JCM 1465	0.3	2.3	1.3	-	-	0.4	28.4	2.0	-	-	6.1	-	44.5	-	6.8	Shida *et al*[371]

11.2.3 Genotypic properties of strain AS-S10-II

Although FAME analysis is one of the methods to study bacterial diversity however, bacterial cellular fatty acid composition can be influenced by various factors such as temperature and nutrition[415]. In addition, individual fatty acids cannot be used to represent specific species because individuals can have numerous fatty acids and the same fatty acids can occur in more than one species[416]. Considering the above limitations, a number of approaches have been developed to study molecular microbial diversity. Among them, PCR targeted 16S rDNA gene and PCR-RFLP of 16S-23S ISR region have been extensively used to study prokaryote diversity as it allows identification of prokaryotes as well as the prediction of phylogenetic relationships[417].

Over the years, determination of the phylogenetic relationship or identification of bacteria based on 16S rDNA sequencing (ribotyping) has been widely accepted.. On the basis of the phylogenetic analysis, the strain AS-S10-II has been classified as a species of the genus *Brevibacillus* (Fig 4.1). The 16S-rDNA gene sequence was deposited in GenBank NCBI with accession No GU332637. The sequencing of 16S rRNA gene alone cannot discriminate among closely related species because of the highly conserved nature of this region[418]. However, the ISR of 16S-23S was reported to possess much greater variability in the DNA sequence and proved to be much more useful for the differentiation of closely related bacterial strains[22,361]. Due to dearth of reports on phenotypic and chemotypic properties of phylogenetically closed species for AS-S10-II strain were not compared.

On the basis of the data obtained from phenotypic, chemotaxonomic and phylogenetic analyses, the strain AS-S10-II could not be classified under a known species of the genus *Brevibacillus*, and the name of this new strain was proposed as *Brevibacillus* sp. strain AS- S10-II.

11.3 Phenotypic properties of isolate AS-S20-I

Study on phenotypic properties such as Gram staining of any microorganisms is the first step towards its identification and taxonomic classification[419]. Based on phenotypic properties of AS-S20-I, and according to Bergey's manual classification AS-S20-I belongs to genus *Kocuria*. A comparative analysis of phenotypic properties of AS-S20-I is displayed in Table 11.3.

Table 11.3 Some phenotypic properties of isolate AS-S20-I and related species of *Kocuria* sp. ND: Not determined.

Character	AS-S20-I	*K. varians*	*K.palustris*	*K.rhizophila*	*K.polaris*	*K.aegyptia*
Cell shape	Rods	Spherical arrange in tertrads	Spherical , tetrads and packets	Spherical , tetrads and packets	Coccoid, occurring in pairs	Coccoid, occur in pair, tetrads or clusters
Motility	+	ND	-	-	-	-
Spore shape	_	_	-	-	-	-
Gram stain	_	+	+	+	+	+
Optimum growth temperature	25- 55°C, optimum 45 -50 °C	22-37 °C	Not specified	Not specified	Optimum temperature 20.0°C	20-40°C , optimum 28.0°C
Growth pH	6.5 -11.0, optimum pH 9.0	4.3-5.9	-do-	-do-	Growing pH 7.0-12.0	5.0-12.0, optimum pH10.0-10.5
Growth in 7% (w/v) NaCl	_	+	+	+	-	-

Character	AS-S20-I	*K. varians*	*K.palustris*	*K.rhizophila*	*K.polaris*	*K.aegyptia*
Growth at		+				
pH 5.5	-	-	-	-	-	-
50 °C	+	+	-	-	-	-
Catalase	+	ND	+	+	+	+
Voges-Proskauer Test	+	ND	Not specified	Not specified	-	-
Methyl red test	+		Not specified	-do-	-	Not specified
		+				
Acid from						
D-Glucose	+	_	+	+	+	+
Mannitol	+	_	-	-	-	Not specified
Lactose	+	ND	+	-	-	-do-
Gas from glucose	-	-	-	-	-	Not specified

Character	AS-S20-I	*K. varians*	*K.palustris*	*K.rhizophila*	*K.polaris*	*K.aegyptia*
Hydrolysis of						
Casein	+	ND	-	-	-	Not specified
Gelatin	-	_	-	+	-	-do-
Starch	+	_	-	-	-	-do-
Lipid	+	ND		-	-	-do-
Urea	-	+	+	-		-
Citrate Utilization	+	ND	-	+	+	Not specifed
Nitrate reduction	+	_	+	-	Not specified	-

Character	AS-S20-I	*K. varians*	*K.palustris*	*K.rhizophila*	*K.polaris*	*K.aegyptia*
H_2S production	+	-	w+	Not specified	-do-	-
References	Present study	Stackebrandt *et al*[420]	Kovacs *et al*[421]		Reddy *et al*[422]	Li *et al*[423]

Results of the phenotypic properties prompted us to study on the chemotaxonomic properties of a bacterium is considered next to phenotypic characterization for the purpose taxonomic identification.

11.3.1 Chemotaxonomic characterization of isolate AS-S20-I

A comparison of cellular fatty acid composition of strain AS-S20-I with cellular fatty acid profile of other *kocuria* species show that fatty acid composition of AS-S20-I was more not similar to that of bacterium such as *Kocuria varians, Kocuria kristinae, Kocuria rosea, Kocuria erythromyxa, and Kocuria rosea* as shown in Table 11.6. Sim Index (SI) of 0.689 and because of the predominance of anteiso-branched $C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$ fatty acids, the strain AS-S20-I was taxonomically identified as *Kocuria varians* (MTCC 8961) by the Institute of Microbial Technology, Chandigarh.

A comparison of cellular fatty acid methyl esters (FAMEs) of strain AS-S20-I (Table 11.4) with the reported cellular fatty acid profiles for *Kocuria* strains demonstrated that majority of fatty acid are of anteiso-branched $C_{15:0}$ and $C_{17:0}$ class and minor fatty acids are $C_{17:0}$ of iso-branched fatty acid and $C_{18:0}$ of straight chain fatty acid.

Table 11.4 A comparison of percentage cellular fatty acid composition of isolate AS-S20-I with related species of *Kocuria*

Organism/ strain	Straight chain					Iso-branched							Anteiso-branched		References
	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$ w7c	$C_{16:1}$ w11c	$C_{18:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{17:1}$	$C_{17:1}$ w10c	$C_{18:1}$ H	$C_{15:0}$	$C_{17:0}$	
AS-S20-I	2.06	14.3	-	-	1.04	0.87	6.89	8.47	4.52	-	-	-	45.20	16.7	Present study
Kocuria varians	2.9	9.6	-	-	-	1.4	1.3	11.1	-	-	-	-	60 3	12.1	Kovacs *et al*[421]
Kocuria kristinae	1.0	0	-	-	-	2.6	1.6	1.7	9.3	-	-	-	70.1	-	
Kocuria rosea	1.5	1.6	5.9	-	-	1.8	7.6	1.6	-	-	-	-	70.9	4.3	
Kocuria erythromyxa	1.8	1.2	6.7	-	1.4	1.2	14.1	1.4	-	-	-	-	63.9	2.4	
Kocuria rosea	0.5	3.2	3.5	-	2.9	1.3	5.1	3 0	1.5	-	-	-	65.0	7.1	Reddy *et al*[422]

11.3.2 Genotypic characterization of strain AS-S20-I

The 16S rDNA-based phylogenetic analysis demonstrated 99.0% sequence similarity of strain AS-S20-I with the other species of the genus *Bacillus*, which suggested that the bacterium under study belongs to the genus *Bacillus*. The phylogenetic tree constructed from the sequence data by the neighbour-joining method (Fig.4.2) showed the detailed evolutionary relationship between the strain AS-S20-I and other closely related *Bacillus* sp. and it also demonstrated a distinct phylogenetic position of this strain within this genus. The *Bacillus* sp. strain BCL 23-1(EF026994) showing 99% 16S rDNA sequence identity represented the closest phylogenetic neighbours of the strain AS-S20-I (Fig. 4.2). It is worth mentioning that topologies near AS-S20-I estimated from the distance based methods (neighbour-joining and UPGMA), and the maximum-likelihood and parsimony analyses were essentially consistent. On the basis of the result obtained with phylogenetic analysis, the strain AS-S20-I may thus be classified as an unknown species of the genus *Bacillus*. Therefore, the selected bacterium taxonomically was identified as *B.* sp. strain AS-S20-I.The partial 16S-rDNA gene sequence of this strain was deposited in GenBank NCBI with accession No GU001817.

However, because of the highly conserved nature of the 16S-rRNA gene, its sequencing alone cannot discriminate between closely related species[360]. Sequencing of the 16S-23S gene is found to be more reliable as it is reported to possess greater variability in the DNA sequence, thus being more useful for the differentiation of closely related bacterial species. Based on phenotypic properties, AS-S20-I was taxonomically identified as a species of the genus *Kocuria*, but according to genotype properties it was identified as *Bacillus* species. The RFLP pattern of strain AS-S20-I was clearly distinct from the RFLP pattern of *B. subtilis* DM-03 and DM-04 strains[361], which reinforces that it would not be appropriate to classify the strain under study as *B.*

subtilis and therefore, it is proposed that the bacterium under study be named as *Bacillus* sp. AS- S20-I.

In the present study, results obtained by genotypic study for AS-S20-I strain couldn't compared with phylogenetically closest species *Bacillus* sp. strain BCL 23-1(EF026994), due to lack of phenotypic and chemotypic properties. Hence, AS-S20-I isolate was proposed as *Bacillus* sp. strain AS-S20-I.

11.4 Phenotypic properties of isolate AS-S24-I

In the present study, AS-S24-I isolates displayed rod shaped cells, gram variable, class of bacterial isolate. Based on phenotypic properties and according Bergey's standard classification system, AS-S24-I isolate were classified in genus *Paenibacillus* as shown in Table 11.5. A comparison of the phenotypic properties of AS-S24-I with other strains such as *Paenibacillus* species suggests AS-S24-I require further study at cellular level.

Table 11.5 Distinctive phenotypic properties of isolate AS-S24-I and related species of *Peanibacillus.*

Character	AS-S24-I	*Paenibacillus azotofixans*	*Paenibacillus lautus*	*Peanibacillus polymyxa*
Cell shape	Rods	Rods	Rods	Rods
Motility	+	Not specified	Not specified	Not specified
Spore shape	ND	Oval	Oval	Not specified
Gram stain	- /+	+	+	+
Growth temperature (ºC)	30-60 range , optimum 45	30-37	28-30	42.0
Growth pH range	6.0-11.0, optimum 7.0	Not specified	Not specified	6.8
Growth in 5% (w/v) NaCl	+	-	+	+
Growth in 7% (w/v) NaCl	+	-	+	+
Growth at				
pH 5.5	_	+	-	+
50 °C	+	-	-	-

Character	AS-S24-I	*Paenibacillus azotofixans*	*Paenibacillus lautus*	*Peanibacillus polymyxa*
Catalase	–	+	+	+
Voges-Proskauer Test	+	Not specified	Not specified	+
Methyl red test	+	-do-	-do-	-
Acid from				
D-Glucose	+	+	+	+
Mannitol	-	+	+	W+
Lactose	-		-	-
Gas from glucose	-	-	-	-
Hydrolysis of				

Character	AS-S24-I	*Paenibacillus azotofixans*	*Paenibacillus lautus*	*Peanibacillus polymyxa*
Casein	+	-	-	+
Gelatin	-	-	-	+
Starch	+	-	+	+
Lipid	-	-	-	-
Urea	-	-	+	-
Citrate Utilization	-	-	-	+
Indole formation	+	-	-	Not specified
Nitrate reduction	+	-	+	+
H_2S production	+	Not tested	Not tested	Not specified
References	Present study	Shida *et al*[371]		Guerra-Cantera and Raymundo[407]

To avoid difficulties in taxonomic characterization further examination was carried out based on variation in cellular fatty acids composition of isolate AS-S24-I.

11.4.1 Chemotaxonomic properties of isolate AS-S24-I

The cellular fatty acids composition of strain AS-S24-I suggested that it was likely to be a member of the genus *Paenibacillus* because of the predominance of anteiso-branched $C_{15:0}$ fatty acids amongst the member of this genus[413,414,424]. However, some major differences were observed in cellular fatty acids contents of AS-S24-I with respect to other strains of *Paenibacillus species* (Table 11.6). The percent distribution of iso and anteiso-branched $C_{15:0}$ in *Paenibacillus* strain was observed. In a sharp contrast, $C_{15:0}$ iso and ante-iso branched fatty acids represented 15 % and 68 % , respectively of the total cellular fatty acids pool in strain AS-S24-I. Moreover, strain AS-S-24-I did not possess $C_{16:0}$ straight chain fatty acid (Table 11.6). Based on Sim Index (SI) of 0.263 and because of the predominance of anteiso-branched $C_{15\,0,}$ iso-$C_{15:0}$ fatty acids, the strain AS-S24-I was taxonomically identified as *Paenibacillus polymyxa* (MTCC 9129) by the Institute of Microbial Technology, Chandigarh.

Table 11.6 Percentage cellular fatty acid composition of isolate AS-S24-I and related species of *Paenibacillus species.*

Organism/ strain	Straight chain		Iso-branched						Anteiso-branched			References
	$C_{14:0}$	$C_{16:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{17:1}$	$C_{17:1}$ w10c	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	
AS-S24-I	-	-	-	15.4	8.8	-	-	-	67.9	-	7.9	Present study
P.macerans ATCC 8244T	3.7	17.9	7.9	2.6	16.4	-	-	-	36.1	-	12.2	
P.sanguinis 2301083	-	10.1	1.7	6.8	13.4	5.8	-	-	44.2	-	6.8	Chou *et al*[425]
P.timonensis 2301032	1.3	10.6	8.2	10.1	15.5	1.9	-	-	43.4	-	4.0	
P.assamensis GPTSA 11	1.8	3.5	4.7	6.0	11.6	1.6	-	-	48.4	-	5.7	
P.chitinolyticus NBRC 15660	0.5	7.2	-	7.8	7.2	3.9	-	-	59.0	-	9.1	Chou *et al*[426]

11.4.2 Genotypic characterization of strain AS-S24-I

The 16S rDNA-based phylogenetic analysis demonstrated 100 % sequence similarity of AS-S24-I with the other *B. licheniformis* strains. The phylogenetic tree constructed from the sequence data by neighbour-joining method (Fig.4.3) showed that strain AS-S24-I share 100% 16S rDNA sequence identity with *B. licheniformis* (Accession no DQ171720). The topologies near strain AS-S24-I estimated from the distance (neighbour-joining and UPGMA) analyses were essentially consistent. Phylogenetically identified strain AS-S24-I could not compared with any species due to unavailability of phenotypic and chemotypic data , hence AS-S24-I strain proposed as *Bacillus licheniformis* strain AS-S24-I.

11.5 Phenotypic properties of isolate AS-S24-II

Present study, displayed rods shaped, endospore forming , gram positive bacterial isolate AS-S24-II. A comparative analysis of AS-S24-II isolate was summarized in Table 11.7. Based on phenotypic properties, AS-S24-II isolate belongs to genus *Bacillus licheniformis.*

Characters	AS-S24-II	*B.licheniformis NRRL NRS-1264*[T]	*B.licheniformis* JCM 2505	*B. licheniformis*
7% (w/v) NaCl	+	+	+	_
0.001% (w/v) Lysozyme	+	NT	NT	NT
Growth at				
pH 5.6	_	+	+	_
50 °C	+	+	+	+
Catalase	+	+	+	+
Voges-Proskauer Test	+	NT	+	+
pH in VP broth	< 6.0	-do-	NT	NT
Acid formation				
D-Glucose	+	+	+	+
Mannitol	_	+	+	+

Characters	AS-S24-II	*B.licheniformis NRRL NRS-1264*[T]	*B.licheniformis* JCM 2505	*B. licheniformis*
Lactose	+	–	-	NT
Sucrose	+	NT	NT	-do-
Glycerol	+	NT	NT	-do-
Gas from Glucose	_	-	-	NT
Hydrolysis				
Casein	+	+	+	NT
Gelatin	-	NT	+	NT
Starch	-	+	+	+
Lipid	-	NT	NT	NT
Urea	-	_	NT	NT

Characters	AS-S24-II	*B.licheniformis NRRL NRS-1264*[T]	*B.licheniformis* JCM 2505	*B. licheniformis*
Utilization of citrate	-	+	+	NT
Formation of indole	+	–	NT	–
Nitrate reduction	+	+	+	NT
Litmus milk reaction	+	NT	NT	NT
H2S production	+	NT	NT	NT
References	Present study	Roberts *et al*[414]	Guerra-Cantera and Raymundo[407]	Miranda *et al*[427]

11.5.1 Chemotaxonomic properties of isolate AS-S24-II

Based on the content of iso and anteiso fatty acids as major components of the fatty acid pool, the isolate AS-S24-II was identified as *Bacillus licheniformis* (MTCC). A comparison of cellular fatty acid distribution of *B.licheniformis* strain AS-S24-II with the reported *B.licheniformis* is presented in Table 11.8. Further , we also compared the cellular fatty acid composition of isolate AS-S24-II with reported fatty acid composition of *Bacillus licheniformis* (Table 11.8) shows a *qualitative as well as quantitative differences in composition of the* cellular fatty acid composition of AS-S24-II with either *B.licheniformis* .For example, straight chain C16:0 fatty acid content of AS-S24-II was found to be higher compared to *B.licheniformis*, similarly, anteiso-branched C17:0 fatty acid content also demonstrated a difference amongst all the strains. Therefore, based on chemotaxonomic properties, it was difficult to place the strain AS-S24-II is any recognized species of the genus *Bacillus* for example *B.licheniformis*.

Table 11.8 Percent cellular fatty acid composition of isolate AS-S24-II-and related species of *Paenibacillus* as determined by GC analysis.

Organism	**Straight-chain**				**Iso-branched**				**Anteiso-branched**			**References**
	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{18:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	
AS-S24-II	2.06	0.0	14.29	1.04	0.87	6.89	8.47	4.52	45.2	0.0	16.66	Present study
Bacillus licheniformis	-	-	-	-	-	29.5	3.9	3.0	36.0	4.0	1.0	Horani and Priest[428]
B.licheniformis NRRL NRS-1264	-	-	3.43	-	1.09	32.18	3.34	6.26	39.89	-	9.89	Palmisano *et al*[424]
B.licheniformis ATCC 12759	-	-	3.57	-	1.05	34.6	-	5.51	40.5	-	8.94	Gatson *et al*[429]
B. licheniformis MTCC 429	-	-	-	-	0.4	34.8	2.6	15.6	15.6	-	9.2	Shivaji *et al*[430]

11.5.2 Genotypic properties of strain AS-S24-II

The preceding morphological observations, biochemical tests and chemotaxonomic analysis could not establish a definitive taxonomic position for strain AS-S24-II; therefore, in order to establish the correct taxonomic position, molecular characterization of the strain was followed. Phylogenetic analysis reveals that great diversity exists amongst protease producing microorganisms, suggesting that protease production is an important survival tool for the producing microbes.

Using neighbor–joining and UPGMA methods the isolate AS-S24-II displayed closest species of genus *Peanibacillus* as *Paenibacillus lentimorbus* with 99 % homologous similarity. In contrast, no comparative anaylsis for AS-S24-II strain performed due to dearth of reports on closest species of AS-S24-II strain. Therefore, we proposed it as *Paenibacillus tezpurensis* sp.nov. strain AS-S24-II.

11.6 Phenotypic characterization of isolate AS-S18

The isolate AS-S24-II subjected to 31 phenotypic tests to provide more descriptive information that would help in recognizing their genus (Table 11.9). AS-S24-II were gram –positive , rod-shaped, catalase-positive, spore-formers, acid forming bacteria data suggests that it belongs to genus *Bacillus* (Table 11.9). A comparative analysis displayed by similar group of bacteria such as *Bacillus* sp. 1044 and *Bacillus* sp. 1124[407], *Bacillus* sp. KD 1014[406] and *Bacillus* sp.KMM1918[431], demonstrating that it belongs to *Bacillus* species.

Table 11.9 Phenotypic properties of AS-S18 with related genus of *Bacillus*.

Characters	AS-S10-II	*Bacillus* sp. KD 1014	*Bacillus* sp.KMM1918	*Bacillus* sp. 1044	*Bacillus* sp. 1124
Cell shape	Rods	Rods	Rods	Rods	Rods
Motility	+	+	Not specified	Not specified	Not specified
Spore shape	Spore forming	+	-do-	ND	Not specified
Gram stain	Positive	Positive	-do-	Positive	Positive
Growth temperature	30-60 °C range , optimum 45 °C	40-65 °C optimum temperature 45 °C	temperature 15-45 °C	Not specified	Not specified
Growth pH range	6.0-11.0, optimum 8.0	pH range 5.0-9.0, optimum pH 5.0	Not specified	Not specified	-do-
Growth in 5% (w/v) NaCl	-	Not determined	+	+	+
Growth in 7% (w/v) NaCl	-	-do-	+	-	+
Growth at					

Characters	AS-S10-II	*Bacillus* sp. KD 1014	*Bacillus* sp.KMM1918	*Bacillus* sp. 1044	*Bacillus* sp. 1124
pH 5.5	-	+	+	+	+
50 °C	+	+	-	+	-
Catalase	+	+	Not determined	+	+
Voges-Proskauer Test	+	Not determined	-do-	+	+
Methyl red test	+	-do-	-do-	Not specified	Not specified
Acid from					
D-Glucose	+	-	+	+	Weakly positive
Mannitol	-	+	+	-	-
Lactose	-	-	-	-	-
Sucrose	-	+/-	Not determined	-	+
Gas from glucose	-	Not determined	-do-	-	-
Hydrolysis of					

Characters	AS-S10-II	*Bacillus* sp. KD 1014	*Bacillus* sp.KMM1918	*Bacillus* sp. 1044	*Bacillus* sp. 1124
Casein	+	+	-	+	+
Gelatin	-	+	Not specified	+	+
Starch	+	+	-do-	+	+
Lipid	+	Not specified	-do-	-	Not specified
Urea	-	-	-do-	Not specified	-do-
Citrate Utilization	-	-	+	-	+
Indole formation	+	-	Not specified	Not specified	Not specified
Nitrate reduction	+	-	Not specified	+	+
H_2S production	+	Not specified	Not specified	Not specified	Not specified
References	Present study	Lee *et al*[406]	Ivanova *et al*[431]	Guerra-Cantera and Raymundo[407]	

11.6.1 Chemotaxonomic properties of AS-S18

Cellular fatty acid composition of AS-S18 isolates display iso and anteiso fatty acids are the major components of AS-S18 fatty acid pool, the isolate AS-S18 was identified as *Bacillus subtilis* (MTCC). A comparison of cellular fatty acid distribution of *Bacillus subtilis* (MTCC) with the reported *Bacillus subtilis* is presented in Table 11.10. AS-S18 shows a qualitative as well as quantitative differences in composition of the cellular fatty acid composition with *Bacillus subtilis* species For example, straight chain $C_{16:0}$ fatty acid content of AS-S18 was found to be higher than *Bacillus subtilis*[414] *and Bacillus subtilis* JCM 1465[371]. Therefore, based on chemotaxonomic properties, it was placed in genus *Bacillus* and was recognized as *Bacillus subtilis* strain RM-01.

Table 11.10 Percentage of cellular fatty acid composition of AS-S18 isolate with other related genus of *Bacillus*.

	Straight chain						Iso-branched								Anteiso-branched			References
	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$ ω 7 cis alcohol	$C_{17:0}$	$C_{18:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{17:1}$ w10c	C_{18} 1	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	
AS-S18	6.49	-	32.98	-	15.17	0.90	-	-	-	-	-	-	-	1.5	-	-	-	Present study
B. subtilis B-770	-	5.0	-	-	-		2.0	13.0	9.0	13.0	-	-	-	-	40.0	-	-	Kaneda[413]
B. subtilis B-4	-	6.0	-	-	-		4.0	14.0	11.0	15.0	-	-	-	-	33.0	-	10.0	
Bacillus subtilis	-	-	3.14	0.23	-		1.13	29.27	2.36	1.52	9.59	-	1.72	-	40.19	-	9.38	Roberts *et al*[414]
Bacillus subtilis JCM 1465	0.3	2.3	1.3	-	-		0.4	28.4	2.0	-	-	6.1	-	-	44.5	-	6.8	Shida *et al*[371]

11.6 Protease production under solid-state and submerged fermentation

On a large industrial scale, productions of secondary metabolites are mostly carried out by using SmF conditions, whereas the use of SSF for the production of commercially valuable metabolites is restricted mainly within Asian countries[434]. One of the probable reasons might be due to less acquaintance of SSF process by the scientists of the West compared to those in the East. In SSF the determination of parameters such as humidity, pH, free oxygen and dioxide of carbon, constitute a problem due to the lack of monitoring devices. Scaling up of SSF processes has been little studied and it presents several problems. Also the microorganisms studied are limited to those that grow in reduced levels of humidity[252, 433, 434]. In third world nations, the use of SSF is more dominant than SmF because of low production expense, less consumption of water and energy, less effluent problem and stability of the products due to less dilution in the media[432,435]. Besides, SSF technology holds a tremendous promise for employment generation particularly in developing countries. Furthermore, it has been reported that wild-type strains of microorganisms (fungi or bacteria) have a tendency for better performance in SSF system compared to genetically altered microorganisms, and thus further reducing the energy and cost requirement[72,254].

11.6.1 Protease production under solid-state fermentation using non-conventional and cheap sources of carbon and nitrogen: a cost-effective technology

The selection of an ideal agro-biotech waste for enzyme production in a solid-state fermentation process depends upon several factors, mainly related with cost and availability of the substrate material, and thus may involve screening of several agro-industrial residues[239]. In the present study, various agro-industrial residues viz. mustard oil cake (MOC), wheat bran (WB), rice bran (RB), *I. cylindrica* (IC) grass and banana leaves (BL) were used for alkaline protease production under SSF at

45°C with an aim to reduce the protease production cost. The present investigation relates to a simple, novel, low-cost process for the high-level production of alkaline protease from all the bacterial strains by using a weed *I. cylindrica* (commonly known as Ulukher in Assam) and potato peel (PP) as cheap sources of carbon and nitrogen under SSF. Although a number of solid substrates have been used for the production of bacterial proteases, neither IC grass nor PP has used for protease production in SSF or SmF systems. The major advantage of the present method of protease production is the commercial utilization of *I. cylindrical* grass, which otherwise remains unutilized and causes a severe problem in many areas of the world. This grass is considered as the top most of 10 notorious weeds in the world, as reported by 73 countries[436]. This grass is a nuisance wherever it is found, costing hundreds of million of dollars in control, with its only documented limited use in a thatch for building huts and roofs. Control of this grass by application of herbicides is an extremely complicated process and a costly measure[436]. On the other hand, large-scale cultivation of IC for commercial exploitation (for example protease production under SSF system) will be inexpensive, because it can grow in a wide range soil types with little to no special nutrient requirements. Similarly, the PP is also produced in huge amounts as a kitchen waste byproduct and generate effluent problem. Therefore, the utilization of PP and IC as fermentation substrates by microorganisms offers a low-cost microbial technology for obtaining proteolytic enzymes coupled with environment protection. Furthermore, the proteases produced by *B. subtilis* DM-04, *Brevibacillus* sp. AS-S10-II, *Bacillus* sp. AS-S20-I, *Bacillus licheniformis* AS-S24-I and *Paenibacillus tezpuresnsis* sp.nov. AS-S24-II in accordance with the present method is free of undesirable flavor that is advantageous for its use in food and pharmaceutical industries. The nature of the solid substrate in SSF system greatly influences the production of microbial enzymes. Although a number of substrates have been employed for cultivating different microorganisms, wheat bran has

been the preferred choice for microbial protease production, because it is proved to be superior as compared to many other substrates[257,437,438].

The present study documents that protease production by all the bacteria on a mixture of IC grass and PP (1:1 ratio) is much better than protease production on wheat bran or on other tested substrates, indicating that PP and IC in a proper combination influence the protease synthesizing mechanism in bacteria. In many cases, the time required for the optimum protease production by bacteria or fungus may be as long as 48 h to 9 days[75,439]. Kinetics study of the bacterial growth and protease production by all strains on IC and PP demonstrated that optimum protease production occurred within a short duration for *Bacillus subtilis* DM-04 (post 24 h of incubation whereas optimum growth was achieved post 48 h of incubation); *Brevibacillus* sp. AS-S10-II (post 24 h of incubation whereas optimum growth was achieved post 72 h of incubation), *Bacillus* sp. AS-S20-I (post 24 h of incubation whereas optimum growth was achieved post 60 h of incubation), *Bacillus licheniformis* AS-S24-I (post 24 h of incubation whereas optimum growth was achieved post 72 h of incubation) and *P.tezpurensis* sp.nov. AS-S24-II (post 24 h of incubation whereas optimum growth was achieved post 60 h of incubation). The results are similar to the kinetics of alkaline protease production by a *Bacillus* sp., under SSF condition by using lentil husk and wheat bran as substrates[257] Therefore, it may be assumed that like *B. sphaericus*[440], protease production by all the bacterial strains under study is both growth and non-growth associated. This observation is in contradiction to the earlier report demonstrating that alkaline protease production by *Bacillus* sp. is growth-associated[262]. The decline in enzyme activity of *B. subtilis* DM-04 post 24 h incubation might be due to slow denaturation and/or decomposition of protease[257].

A crucial factor in the SSF system that influences the microbial growth as well as product yield is the initial moisture content of the

substrate[262,441]. Increase in moisture level beyond optimum level is believed to reduce the porosity of solid-substrates, thus limiting oxygen transfer, while decrease in the moisture content from optimum moisture content of substrate causes reduction in the solubility of nutrients of the substrates resulting in a lower degree of swelling[275]. Since growth of microbes and product formation takes place at or near the surface of moist solid substrate[442]; therefore, for achieving maximum yield of the desirable product, it is crucial to optimize the moisture content that controls the water absorption (Wa) of the fermenting substrate[262]. For example, in the present study, 100% initial moisture contents of both the substrates (IC and PP) were found to be optimum for protease production by all the strains, whereas 30%, 40% and 140% initial moisture contents of wheat bran, lentil husk and green gram husk, respectively were found to be optimum concentrations for maximum protease production by *Bacillus* sp. in SSF[257,262]. However, depending upon the type of microorganism, the initial moisture content of the same substrate for optimum protease yield may vary. For example, Uyar and Baysal[257] reported 30% moisture level of wheat bran is optimum for alkaline protease production by *Bacillus* sp., whereas optimal moisture level was reported to be 74% with wheat bran for protease production by *Pseudomonas* sp.[443]. Similarily, alkaline protease production by *Mucor circinelloides* demonstrated about 20% moisture level requirement for optimum protease production using dhal husk[264]. In the present study, a decrease in protease production with a further increase in moisture content beyond 100% may be related to decrease porosity and/or air content of the substrate[444], which in turn interfered with the microbial activity.

The inoculum concentration also plays a crucial role in enzyme production, and therefore, this parameter should also be given a due consideration. In the present study, 40% (v/w) of inoculum concentration (optical density at 600 nm 0.5±0.05) was found to be suitable for optimum protease production by *B. subtilis* DM-04 strain on

IC or PP, whereas Uyar and Baysal[257] reported a 20 and 25% (v/w) inoculum level requirement for the production of alkaline protease by a *Bacillus* sp. when grown on wheat bran and lentil husk, respectively. It was assumed that with the increase in inoculum level beyond 2.0ml the production of protease by *B.subtilis* DM-04 declines. Similarly, increase in inoculum level 3.0 ml *Brevibacillus* sp. AS-S10-II, inoculum level 1.5ml from *Bacillus* sp. AS-S20-I, inoculum level beyond 4.0ml from *Bacillus licheniformis* AS-S24-I and inoculum level beyond 2.0ml from *P.tezpurensis* sp. nov. AS-S24-II results in the decline of protease production which might be due to exhaustion of nutrients in the fermentation mash.

The choice of the nitrogen and carbon sources has a major influence on the yield of protease. Present observation as well as findings from other laboratories suggested that different bacteria have different preferences for either organic or inorganic nitrogen sources for growth and protease production, although complex nitrogen sources are usually used for alkaline protease production[262,442]. All strains used in the present study have shown a preference for both organic as well as inorganic nitrogen sources for protease production. In a sharp contrast to these observations, organic nitrogen sources like peptone and yeast extract were found to suppress the protease production of an alkaliphilic strain of *Arthrobacter ramosus* MCM B351[237]. In 2010, Murty and Naidu[445] described a partial inhibition of extracellular protease production from *Aspergillus oryzae* strain CFR05 in presence of glucose, a versatile source of carbon; however, present study shows that protease synthesis is enhanced ($P < 0.05$) by *B.lichenformis* strain AS-S24-I when glucose and some other carbohydrates are supplied as co-carbon source to the fermentation medium containing IC. This observation is in accordance with the report of Prakasham *et al*[262] describing no repressive effect of glucose on enzyme production by *Bacillus* sp. The data presented in the present study show that optimum protease production by *B.subtilis* DM-04, *B.subtilis* RM-01(Alkaline keratinase),

Brevibacillus sp. strain AS-S10-II, *Bacillus* sp. strain AS-S20-I, *Bacillus licheniformis* AS-S24-I and *Paenibacillus tezpurensis* sp. nov. strain AS-S24-II was enhanced significantly ($p<0.05$) when two best substrates (IC and PP) were mixed in a ratio of 1:1 and not by individual substrate. This observation may be well explained by considering the C: N ratio of individual substrate as well as their different combinations. It is well established that carbon-to-nitrogen ratio, carbon concentration as well as the amino acid composition of growth media/fermentation substrate influence the growth of microbe as well as enzyme production[446,447]. Since the carbon content of the individual substrate (IC or PP) or their different combination was approximately same; therefore, it may be concluded that an optimum C: N ratio of fermenting material (1:1 mixture of IC and PP in the present study) greatly influences the protease secretion by all the strains. Furthermore, from our results it appear that carbon and nitrogen sources present in IC and PP can serve as complete basal and standardized medium for protease production by bacteria. The current method does not require additional supplementation of co-carbon or co-nitrogen sources to IC and PP substrates and therefore, it can be considered as a cost effective process for protease production[72].

11.6.2 Chicken-feather degradation and keratinase production under solid-state fermentation

Microbial biodegradation of insoluble macromolecule like keratin, cellulose, collagen and lignin depends on the secretion of extracellular enzymes with the ablility to act on compact substrates. Keratin, a structural protein found in feathers, wool, hooves, and hair, is resistant to degradation by common proteolytic enzymes such as trypsin, pepsin, and papain because of its high degree of cross –linking[448,449]. Nevertheless, feathers do not accumulate in nature, which confirms the existence of feather degrading micro-organisms[182]. Studies on feather-degrading micro-organisms have focused either on animal diseases or on biotechnology for processing large amounts of waste by –products in

poultry-processing plants[296]. As keratinases from *Bacillus subtilis* have greater potential for applications in commercial processes, biotechnological studies have been focused on these strains[450,451].

Due to dearth of published reports on keratinase production in SSF, the optimum time taken by *B. subtilis* RM-01 for β-keratinase production in SSF could not be compared with the time course of β-keratinase production by other microorganisms under identical conditions. The fact *that the bacteria can grow and produce maximum β-keratinase at* lower moisture content of fermentable substrate offers significant advantages in reducing risk of contamination, since most bacterial species are unable to grow at reduced moisture level[452]. Increased moisture level is believed to reduce the porosity of substrate, thus limiting the oxygen transfer into the substrate[453]. Likewise, a lower moisture ratio leads to reduced availability of nutrients of the solid substrate, lower degree of swelling and a higher water tension[454]. The highest β-keratinase yield in SSF by using water instead of buffers is advantageous from economic point of view which advocates the cost-effectiveness of the present method. Similar reports are available on the use of distilled water or tap water as moistening liquid in SSF processes[452] and it might be reasonable to assume that high salt concentration of the buffer may have a negative impact on β-keratinase production by *B. subtilis* RM-01. The cross-linking of protein chains by cysteine bridges confers high mechanical stability and resistance to proteolytic degradation of keratins[31]. Since the alkaline pH possibly favors keratin degradation as higher pH modifies cystine residues to lathionine making it accessible for keratinase action[455]; therefore, it may be concluded that the alkaline pH of the moistening liquid supports maximum keratinase production and higher feather degradation by most microorganisms[450, 456].

The production of primary metabolites by microorganisms is determined by the availability of nutrients in the substrates. In the present study, β-keratinase production by *B.subtilis* RM-01 was inhibited by

monosaccharides which may be attributed to catabolite repression[7]. However, the choice of carbon source for growth and enzyme production has been shown to vary depending upon bacterial species. For example, glucose enhanced the protease production by *B. pseudofirmus* AL-89[457] and *B. sp*[262], whereas for *B. subtilis*[458] and *B. licheniformis*[459] protease production was suppressed by the addition of glucose. Present observation as well as findings from other laboratories suggested that different bacteria have different preferences for either organic or inorganic nitrogen for growth and keratinase production such as *Bacillus subtilis* KD-N2[328], *Bacillus subtilis* KD-N2[290], and *Bacillus halodurans* Strain PPKS-2[460]. *B. subtilis* RM-01 has shown a preference for inorganic nitrogen source compared to organic nitrogen for β-keratinase production.

11.6.3 Screening of process parameters influencing protease yield in submerged fermentation

At the industrial level, enzyme production is a fastest growing field of modern biotechnology. Annual world sales figures are close to 10^9 dollars with ever increasing number of patents and research articles related to this field[160,461]. About 90 % of industrial enzyme manufacturers produce enzymes using submerged fermentation (SmF) or liquid surface (SLF) fermentation techniques with enzyme titers in the range of grams per liter[462]. From technical aspects, enzyme production under submerged fermentation carry several advantages over other well know fermentation systems because of proper agitation and mixing of substrate, easy to control moisture level, dissolved oxygen content, pH level, aeration level, and proper monitoring of microbial growth etc in SmF[251,463,464]. Such levels are a prerequisite if specific compounds are to be considered as commodities because product recovery costs are inversely proportional to concentration in a fermentation broth.

Protease production in sufficient quantity is one of the important parameters which an enzyme should posses for industrial purposes. This is because around 30–40% of the production cost of the industrial enzymes was estimated to be accounted for the cost of the growth medium. Therefore, in the present study the low-cost growth substrates such as rice bran, banana leaf, and wheat bran and kitchen waste product are used to produce the bacterial proteases to greatly reduce the production costs. In addition, development of the economical fermentation method is also necessary to reduce the overall cost of the enzyme production[7,465,466].

It has been well established that medium components play an important role in protease production by bacteria[467,468]. In the present study, optimization of medium composition was done to balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly towards evaluating the effect of various carbon and nitrogen nutrient cost-effective substrates on the yield of enzymes, optimization of environmental parameters such as pH, temperature, aeration and agitation[7,466]. In addition, no defined medium has been established for the production of proteases from different microbial sources suggesting medium composition need to be optimized depending upon the types of protease producing microbes.

Amongst the tested carbon sources, casein was found to be the best carbon source for optimum growth as well as protease production by *Bacillus* sp. AS-S20-I, *Brevibacillus* sp. AS-S10-II and *Paenibacillus tezpurensis* sp. nov. AS-S24-II, whereas *B.subtilis* DM-04 demonstrated preferences of carbon sources IC: PP, *B.subtilis* RM-01 preferred maltose and *B.licheniformis* AS-S24-I preferred D-Glucose, as the best carbon source as described for a number of protease secreting *Bacillus* strains[262]. However, this result differed from previous observations

showing preference of maltose for optimum protease production by *Bacillus circulans* and *Bacillus pseudofirmus* SVB1 strains[92,239]. The difference in uptaking of carbon sources to enhance protease production is due to regulatory mechanism involved during selection of bacteria for growth and enzyme production[469].

Micro-organisms prefer both inorganic or organic forms of nitrogen for growth and other metabolic processes. Organic nitrogen is a complex nitrogen source composed of a spectrum of peptides and free amino acids. During fermentation, these are taken up from the medium by the cell and directly incorporated into proteins or transformed into other cellular nitrogenous constituents[470]. By contrast, the cell spends more energy and time in synthesizing amino acids for protein synthesis from inorganic nitrogen sources[470]. Among organic nitrogen sources, differences in protein and amino acid composition could have accounted for the differences in the production rates and yields observed. The choice of nitrogen source for growth and protease production by bacteria in the present study is in accordance with previous reports suggesting that each bacterium has its own choice for carbon and either inorganic or organic nitrogen sources for optimum growth and protease production[92,239]. For example, an increase in protease production by *Bacillus licheniformis* was observed in presence of ammonium sulphate and potassium nitrate[473]; whereas sodium nitrate was found to be stimulatory for alkaline protease production by *Rhizopus oryzae*[472].Prakasham *et al*[262] reported utilization of yeast extract for alkaline protease production by *Bacillus* sp. A significant increase in alkaline protease production by *Bacillus cereus* SV1 was observed in presence of ammonium chloride[473].

Variation in selection of nitrogen sources in *Bacillus* species is mainly because of the involvement of different regulatory precursors which are responsible for the uptake of low molecular weight source of nitrogen

present in growing media and activating precursors responsible for synthesis of extra-cellular proteases[469]. This phenomenon was explained in *Aspergillus nidulans* producing extra-cellular protease. According to them, when the *Aspergillus* strain was grown on media containing protein as sole nitrogen source, four extra-cellular proteases (*α, γ, ξ and ε*) are released into the medium and occur in the mycelium together with protease β in its active form. This regulation of proteases by nitrogen metabolites has been called 'ammonium repression'[474-476].

11.6.4 Optimization of process parameters for enhancing the protease yield: a statistical approach

11.6.4.1 Plackett-Burman design for preliminary screening

The biotechnological application of proteases / keratinases demands the production of these enzymes in sufficient amounts for commercial purposes and require optimized medium for enhanced enzyme yield. Classical approach of medium optimization is a time-consuming and labor-intensive process. The Plackett-Burman experimental design has proved to be a valuable tool for the rapid evaluation of the effects of the various medium components[382]. Since this design is a preliminary optimization technique, which tests only two levels of each factor, it can not provide the optimal quantity of each factor required for the optimum enzyme production. This technique, however, provides indications of how each factor tends to effect bacterial growth and enzyme production[382]. On the basis of the result obtained from Plackett-Burman analysis, and t-and p-value ($p< 0.05$) following factors significantly influences the protease / keratinase production under solid-state and submerged fermentation systems. Among the tested parameters, IC:PP level, beef extract level, and incubation time (h) were found to be significant factors for protease production by *B.subtilis* strain DM-04; casein level, ammonium sulphate and pH of the medium were found to be positively influencing factors for alkaline protease production by *P.tezpurensis* sp.nov. AS-S24-II; whereas maltose level, sodium nitrate

level and pH of the medium significantly influenced the keratinase production by *B.subtilis* strain RM-01, level of casein and KNO_3 as well as pH of the medium were found to significantly accelerate the bacterial growth as well as protease production by *Brevibacillus* sp. strain AS-S10-II. Increased protease production by *B.licheniformis* strain AS-S24-I post 48h of incubation was found to be influenced by glucose level, yeast extract level and pH of the medium casein level, whereas ammonium sulphate level and pH of the medium were found to be like positively influencing parameters for increased protease yield from *Bacillus* sp. strain AS-S20-I.

11.6.4.2 Statistical optimization using central composite design for protease production

Designing an appropriate fermentation medium is of critical importance in optimizing the product yield. However, the conventional experimental approaches for the optimization of media composition require a large number of experiments to study the influence of each factor of the media on enzyme production. On the contrary, statistical optimization of media components takes into account the interactions of variables in generating process responses[392]. The sample coefficient of determination (R^2) measures the closeness of fit of the sample regression equation to the observed value of Y[477]. Incase of a higher value of R^2 the regression accounts for a large proportion of the total variability in the observed value of *Y* which favors the regression equation model[392,477]. The high value of R^2 indicates that the statistical model presented in this study could explain the variability in the process responses of the alkaline protease /keratinase production from *B.subtilis* strain DM-04 (R^2 = 98.49%); *Brevibacillus* sp. strain AS-S10-II (R^2 = 100.00 %), *Bacillus* sp. strain AS-S20-I (R^2 = 100.00%), *Bacillus licheniformis* strain AS-S24-I (R^2 = 99.87%), *P.tezpurensis* sp.nov. strain AS-S24-II (R^2 = 99.40%) and *B.subtilis* strain RM-01 (R^2= 100.00%). The high value of R^2 advocates in favor of high significance of all the models. The significance of the predicted model (Eq. (3)) was

further evaluated by Student's *t* test and *p* values, and the larger magnitude of the *t* value and smaller *p* value vouches for the significance of the corresponding coefficient of all the regression models obtained in the present study for protease production.

The 3D response surface and the 2D contour plots were generally the graphical representations of the regression equation. The response surface plot is the theoretical three-dimensional plot showing the relationship between the response and the independent variables[478]. The two-dimensional display of the surface plot is called contour plot and in the contour plot, lines of constant response are drawn in the plane of the independent variables[478].

In all the experiments, the value of protease yield obtained by batch culture was slightly higher than the observed highest experimental value in shake-flask study as well as the predicted value of the protease yield by response surface method. This discrepancy might be due to slight variation in experimental conditions in a fermentor and EM flasks because the concentration of dissolved oxygen as well as the pH of the medium could be maintained at the desired level in a bioreactor but not in a flask. This may favor the higher protease production in less time in a bioreactor[88]. Although the protease yield by *B. subtilis* strain DM-04 in SmF was found to be lower than the reported yield of alkaline protease from *Bacillus* sp.[75] and *Bacillus* sp. RKY3[90]; however, use of cheaper substrates (IC and PP) for protease production could lead to a considerable reduction in enzyme production cost which may favor the commercialization of alkaline protease production by *B. subtilis* strain DM-04.

Alkaline protease yield by *Brevibacillus* sp. strain AS-S10-II and *Paenibacillus tezpurensis* sp. nov. strain AS-S24-II could not be compared with other *Brevibacillus sp or Paenibacillus sp.* because hardly any report is available on protease production by these strains. Nevertheless, a comparison showed that the yield of alkaline protease

by these strains (*Brevibacillus* sp. strain AS-S10-II and *P.tezpurensis* sp. nov. strain AS-S24-II) was significantly higher than the alkaline protease production by many other species of *Bacillus* such as *Bacillus* sp. RKY3[90] and *Bacillus licheniformis* BA17[227].

The optimum protease yield of 699 U from *Bacillus* sp. strain AS-S20-I was achieved by employing RSM and was higher than reported alkaline protease production by *B. subtilis* IH-72[480] and *Bacillus licheniformis* BA17[227] even under optimized conditions.The optimized protease yield by *Bacillus licheniformis* strain AS-S24-I reported here was significantly higher compared to the reported value of alkaline protease production by other strains of *B. licheniformis*[480-483]. This reinforces the industrial exploitation of the selected bacterium as a microbial cell factory for high-titre alkaline protease production. A comparison of RSM optimized protease yield by bacteria under study with the reported yield of protease from similar types of bacteria is presented in Table 11.11

Table 11.11 A comparison of statistically optimized alkaline protease yield by isolated strains and others bacteria.

Strains	Protease yield (Units)	References
Brevibacillus sp. AS-S10-II	942.0 ± 47.1	Present study
Bacillus sp. AS-S20-I	699.0 ± 35.0	-do-
P.tezpurensis sp.nov. strain AS-S24-II	583.0 ± 29.2	-do-
B. subtilis strain DM-04	508.0 ± 25.4	-do-
B.licheniformis strain AS-S24-I	779.0 ± 39.0	-do-
B. lichenformis	196.00	Nehete *et al*[480]
B. licheniformis	34.70	Lee and Chang[481]

Strains	Protease yield (Units)	References
B. subtilis	9.60	Haq *et al*[479]
Bacillus sp. RKY3	939.0	Reddy *et al*[90]
Colwellia sp. NJ341	183.21	Wang *et al*[225]
B. licheniformis BA17	11.99	Nikerel *et al*[227]

18.7 Isolation and purification of protease enzymes

Although most of the commercial applications of enzymes do not always need homogeneous preparation of the enzyme, however, a certain degree of purity is required, depending upon the final application, in industries such as fine chemicals, pharmaceuticals and cosmetics[484-488]. Besides, purification of the enzyme is a must for understanding the 3-D structure and the structure–function relationships of proteins[6,7]. For industrial purposes, the purification strategies employed should be inexpensive, rapid, high-yielding and amenable to large-scale operations. They should have the potential for continuous product recovery, with a relatively high capacity and selectivity for the desired product. Various strategies used for purification of microbial proteases have been reviewed by Gupta *et al*[7].

11.7.1 Isolation and purification of alkaline proteases from isolated strains

11.7.1.1 *B. subtilis* strain DM-04

In the present study, we report the purification of an alkaline protease (Bsubap-I) from *B.subtilis* DM-04 with molecular mass of 33.1 kDa. The molecular mass of Alzwiprase differed significantly from the molecular masses of the alkaline proteases isolated from other species of *Bacillus* (Table 11.12). Furthermore, based on its anionic nature at pH 7.0 and optimum activity in alkaline pH range, this purified protease was named as Bsubap-I (*Bacillus subtilis* alkaline protease-I).

Another neutral / zwitterionic protease was purified from *B.subtilis* strain DM-04 exhibiting maximum activity at pH 10.0 with a specific activity of 397.0U /mg of protein. It represented 57.0% of total extracellular proteases of *B. subtilis* strain DM-04. It is noteworthy to mention that when the extracellular proteases of *B. subtilis* strain DM-04 were separated on the basis of their overall net charge at pH 7.0, the zwitterionic proteases (which did not bind to cation and anion exchangers) were found to constitute a major proportion of extracellular protease secreted by *B.subtilis* strain DM-04, as compared to anionic and cationic proteases. Peak HP-I (Fig.5.31) demonstrated maximum protease specific activity (5 x 10^3 U/mg) and was found to be homogenous by 12.5% SDS-PAGE. SDS-PAGE of about 15 µg protein under both reduced and non-reduced conditions displayed a single band of 16.9 kDa suggesting its monomeric nature. This protein was subsequently named as Alzwiprase (Alkaline zwiterionic protease).

A comparison of molecular weight of proteases isolated in the present study with the other species of *Bacillus* and *Brevibacillus* is shown in Table 11.12.

Table 11.12 A comparison of molecular weight of isolated proteases (Bsubap-I and Alzwiprase (*B.subtilis* DM-04), Brevicarnase (*Brevibacillus* sp. AS-S10-II), Alkarnase (*B.lichenformis* AS-S24-I) and detergent stable alkaline protease (*P.tezpuresnsis* sp.nov. strain AS-S24-II)) with molecular weight of reported protease purified from *Bacillus* species.

Strain name	Molecular weight (kDa)'	References
Bacillus sp.	27.0	Margesin *et al*[489]
Bacillus subtilis NCIM No. 64	28.0	Kembhavi *et al*[490]
B.subtilis RM615	28.0	Moon *et al*[491]

Strain name	Molecular weight (kDa)	References
Bacillus sp. PS719	42.0	Hutadilok-Towatana *et al*[492]
Bacillus sp. SCB-3	134.0	Lee *et al*[315]
Bacillus subtilis PE-11	15.0	Adinarayana *et al*[134]
B.subtilis	37.0	Orhan *et al*[217]
Bacillus subtilis megatherium	45.0	Gerze *et al*[493]
Bacillus sp. TKU004	27.0 and 57.0	Wang *et al*[220]
Bacillus sp. PN-13	30.0	Ogino *et al*[181]
Bacillus licheniformis RSP-09-37	55.0	Sareen and Mishra[306]
Bacillus mojavensis A21	20.0	Haddar *et al*[96]
Bacillus pseudofirmus	30–32	Patel *et al*[209]
Bacillus licheniformis BA17	19.7	Ozturk *et al*[494]
Bacillus cereus	28.0	Doddapaneni *et al*[131]
B.subtilis AG-1	24.9 and 18.0	Ghafoor and Hasnain[495]
Bacillus sp. B001	28.0	Deng *et al*[310]
Bacillus licheniformis ER-15	58.0	Tiwary and Gupta[496]
Brevibacillus sp. AS-S10-II (Brevicarnase)	83.2	Present study
B.subtilis DM-04 (Bsubap-I)	33.1	Present study
B.subtilis DM-04 (Alzwiprase)	16.9	Present study

Strain name	Molecular weight (kDa)	References
Paenibacillus tezpurensis sp.nov.strain AS-S24-II	43.0	Present study

11.7.1.2 *P.tezpurensis* sp.nov. strain AS-S24-II

In the present study, we also report the purification of a detergent-stable alkaline protease from *P. tezpurensis* strain AS-S24-II, which represents less than 1% of total extra cellular protease activity. It is quite obvious that all the proteases secreted by a particular bacterium may not exhibit the identical property, for example high protease activity, thermo stability as well as detergent compatibility and therefore, all of them may not be equally important from an industrial perspective. Since our goal was to isolate and purify a detergent-stable alkaline protease for application in commercial laundry detergent formulations; therefore, in each purification step, we searched for the protease(s) showing best detergent stability rather than looking for protease demonstrating highest enzyme activity at alkaline pH. It is worthy to mention that, although the purified protease in the present study contributes a meager amount of overall extra cellular protease protein or activity, there is a dearth of report on alkaline proteases exhibiting comparable detergent stability as well as stain removal property as displayed by the purified protease from *P. tezpurensis* sp. nov. strain AS-S24-II. Since this is the first report on purification of a protease from *Peanibacillus* species, therefore we could not compare the molecular weight of this protease with other protease from *Peanibacillus* sp. Further, cloning of this protease gene and its high level expression in a suitable host may significantly improve the yield and therefore, holds a tremendous future prospect.

11.7.1.3 *Brevibacillus* sp.strain AS-S10-II

An alkaline protease from the strain *Brevibacillus* sp. AS-S10-II was purified from the cell-free culture supernatant to homogeneity using acetone precipitation, followed by gel filtration on Sephacryl S-200, which resulted in a 18.9 -fold increase in specific activity and 58.5% of protease recovery. The isolated protease demonstrated a high molecular mass of 83.2 kDa and SDS-PAGE analysis suggested monomeric nature of protease. This protease was named as Brevicarnase and molecular weight of Brevicarnase was found to be higher as compared to other isolated protease from *Bacillus* species (Table 11.12).

11.7.1.4 *Bacillus* sp.strain AS-S20-I

Partially purified protease from *Bacillus* sp. AS-S20-I demonstrated a molecular mass of 60.0 kDa. It was observed that the molecular weight of this protein was higher when compared to other proteases isolated from *Bacillus* sp.(Table 11.12).

11.7.1.6 *B.subtilis* strain RM-01

Isolation and down stream processes of extracellular β-keratinase were determined and the data presented here show, that a pronounced increase in specific proteolytic activity occurred after separation through CM-Cellulose column followed by sephacryl S-200 column and then reverse phase HPLC C_{18} nova pak column. The reverse phase HPLC C_{18} nova pak column separation resulted in elution of pure protease with 9.0 fold increase in specific activity, compared to crude enzyme. Moreover, HP-IV fraction was demonstrated to be the most detergent stable alkaline protease fraction. The SDS–PAGE analysis revealed that β-keratinase isolated from *B.subtilis* strain RM-01 had a mol ecular weight of 20.1 kDa and it was monomeric in nature. The molecular weight of the isolated β-keratinase differs from the reported molecular weight of alkaline keratinases isolated from other *Bacillus* species (Table 11.13).

Table 11.13 A comparison of molecular weight of alkaline β-keratinase (in the present study) with the reported molecular weight of the same enzyme purified from *B. subtilis* and other species of *Bacillus*.

Strain name	Molecular mass (kDa)	Reference
Bacillus licheniformis PWD-1	33.0	Lin *et al*[497]
Bacillus subtilis KS-1	25.4	Suh and Lee[498]
Bacillus licheniformis K-508	42.0	Rozs *et al*[499]
Bacillus sp. SCB-3	134.0	Lee *et al*[316]
B.lciheniformis strain HK-1	46.0	Korkmaz and Dincer[500]
Bacillus licheniformis FK14	35.0	Suntornsuk *et al*[501]
Bacillus licheniformis MSK103	26.0	Yoshioka *et al*[325]
Bacillus subtilis MTCC (9102)	64-69.0	Balaji *et al*[326]
Bacillus cereus DCUW	80.0	Ghosh *et al*[314]
Bacillus subtilis KD-N2	30.5	Cai *et al*[328]
Bacillus pumilis	65.0	Kumar *et al*[246]
Bacillus licheniformis RPk	32.0	Fakhfakh *et al*[329]
Bacillus subtilis RM-01 (alkaline β-keratinase)	20.1	Present study
B.licheniformis strain AS-S24-I (Alkarnase)	43.0	Present study

11.7.1.5 Alkarnase from *Bacillus licheniformis* strain AS-S24-I

The keratinolytic protease was purified from a culture supernatant (3 l) of *B.licheniformis* AS-S24-I. By SDS-PAGE, the apparent molecular weight of Alkarnase was estimated as 43.0 kDa. Further characterization demonstrated that Alkarnase exhibited a high level of

specificity for β-keratin substrates. A comparison of molecular mass of Alkarnase with other keratinase purified from other strains of *B.licheniformis* and other species of *Bacillus* is shown in Table 11.13.

11.8 Biochemical characterization of purified / partially purified proteases/ keratinase

Before assessing the biotechnological potential of any enzyme, characterization of biochemical properties pertinent to industrial application is of utmost importance and advantageous.

11.8.1 Effect of pH and incubation temperature on protease / keratinase activity

A survey of literature demonstrated that proteases with alkaline nature have novel stability[502]. Bacteria, actinomycetes and fungi have been extensively screened for industrial enzyme production and numerous processes and enzyme products have been developed over the years. The enzymes vary widely in their properties particularly with regard to temperature and pH optima for activity and stability. It is interesting to observe that even mesophilic strains secrete enzymes which are found to be stable at a temperature and / or pH values far above those required for optimal growth of the producing culture[503]. In our studies, Bsubap-I and Alzwiprase from *B.subtilis* DM-04 have demonstrated similar working pH properties. All proteases purified from *B.subtilis* RM-01 (alkaline β-keratinase), *P.tezpuresnsis* sp.nov. AS-S24-II, *Brevibacillus* sp. AS-S10-II, *B.licheniformis* AS-S24-I, and *Bacillus* sp. AS-S20-I (partially purified) exhibited proteolytic activity at an alkaline range of pH and a broad range of incubation temperature. This demonstrated their suitability for incorporation in commercial laundry detergent formulations which require the enzyme to be stable as well as active at higher alkaline pH and temperature regimes. A comparison of optimum pH for all purified proteases/ keratinase with previously reported proteases studied is shown in Table 11.14 and Table 11.15.

Table 11.14 A comparison of requirement of optimum temperature and pH for purified / partially purified proteases in the present study with reported alkaline proteases isolated from *Bacillus* species.

Strains	Optimum		References
	pH	Temperature (In°C)	
Bacillus sp.	11.0	60.0	Margesin et al., 1992[489]
Bacillus sp. B18	12.0-13.0	85.0	Fujiwara et al., 1993[504]
Bacillus licheniformis MIR 29	9.0	60.0	Ferrero *et al*[505]
B. subtilis Y-108	8.0	50.0	Yang *et al*[506]
Bacillus mojavensis	10.5	60.0	Beg *et al*[507]
Bacillus pseudofirmus	10.0 -11.0	45.0	Gupta et al[130]
Bacillus pseudofirmus	10.0-11.0	37.0	Patel *et al*[209]
Bacillus licheniformis RP1	10.0-11.0	65.0-70.0	Sellami-Kamoun *et al*[340]
Bacillus mojavensis A21	8.5	60.0	Haddar *et al*[96]
Bacillus mojavensis A21	8.0–10.0 and 10.0	60.0	
Bacillus sp. B001	10.0	60.0	Deng *et al*[310]
Bacillus licheniformis ER-15	11.0	70.0	Tiwary and gupta[496]
B.subtilis DM-04 (Bsubap-I)	10.10.5	45.0	Present study

Strains	Optimum		References
	pH	Temperature (In°C)	
B.subtilis DM-04 (Alzwiprase)	10.0	45.0	-do-
P.tezpurensis sp.nov. AS-S24-II	9.5	45.0 -50.0	-do-
Bacillus sp. AS-S20-I	9.0	45.0	-do-

Table 11.15 A comparison of requirement of optimum temperature and pH of purified keratinase in the present study with reported alkaline keratinase purified from *Bacillus* species.

Strains	Optimum		References
	pH	Temperature (in°C)	
Bacillus licheniformis PWD-1	7.5	50	Lin *et al*[497]
Bacillus subtilis KS-1	7.5	-	Suh and Lee[498]
Bacillus licheniformis K-508	8.5	52	Rozs *et al*[499]
Bacillus sp. SCB-3	7	40	Lee *et al*[316]
Bacillus licheniformis FK14	8.5	60	Suntornsuk *et al*[501]
Bacillus licheniformis MSK103	9–10	60–70	Yoshioka *et al*[325]
Bacillus cereus DCUW	8.5	50	Ghosh *et al*[314]
Bacillus pumilis	8.0	65	Kumar *et al*[246]

Strains	Optimum		References
	pH	Temperature (in°C)	
Bacillus subtilis KD-N2	8.5	55	Cai *et al*[328]
Bacillus subtilis MTCC (9102)	6	40	Balaji *et al*[326]
Bacillus licheniformis RPk	9.0	60	Fakhfakh *et al*[329]
B.licheniformis strain AS-S24-I (Alkarnase)	9.0	45.0	Present study
B.subtilis RM-01 (alkaline β-keratinase)	9.0	45.0	Present study
Brevibacillus sp. AS-S10-II (Brevicarnase)	12.5-13.0	45.0	Present study

11.8.2 Thermo stability study of purified / partially purified protease / keratinase

Microorganisms, like all living things, adapt to the conditions in which they have to live and survive. Thermophiles are reported to contain proteins which are thermostable and resist denaturation and proteolysis at a higher temperature[508]. Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms[509]. Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes[510-514]. Furthermore, thermostable enzymes are usually more resistant to inactivation by other denaturants such as detergents, organic solvents, chaotropic agents and oxidizing agents. In some cases thermostable enzymes also have proven extremely resistant to

proteolysis[46]. From an industrial point of view, thermostable enzymes can take advantages of the benefits of high temperatures systems. These are benefits such as increased substrate solubility, decreased viscosity of the medium and lower risk of microbial contamination or higher rates of concurrent non-enzymatic reactions[515-517].

Considering the above important aspects, we have examined the thermo-stability properties of proteases purified/ partially purified from all the strains under study. All these proteases demonstrated excellent thermo-stability properties as compared with the same property reported for alkaline proteases isolated from different bacterial sources, suggesting potential applications in heavy –duty laundry detergent formulation purpose.

11.8.3 Effect of polyols on stability of protease against heat-denaturation

The stability of enzymes and proteins *in vitro* condition remains a critical issue in their biotechnological application. Understanding the intricate balance of various factors responsible for the stability of proteins and enzymes in solution is not only an academic challenge but also has enormous implications for the pharmaceutical and biotechnology industries. Operational stability is of paramount importance for any bioprocess[519]. The effects of co-solvents on the stability of proteins are under investigation.

Several methods are employed to increase the stability of proteins in operational conditions, including chemical modification, use of stabilizing additives, derivatization, and modification with carbohydrates, amino acid substitution, mutagenesis, and genetic engineering of enzymes[519]. We investigated stabilization of purified / partially purified protease with co-solvent additives such as xylitol, sorbitol, glycerols and mannitol. All purified proteases demonstrated maximum stability in presence of glycerol except keratinase from *B.subtilis* RM-01 and

alkaline protease from *Bacillus* sp.AS-S20-I. Later proteases exhibited maximum stability in presence of sorbitol. The probable explanation for protease stability in presence of different polyols is because glycerol has lower dielectric constant and stronger electrostatic interaction properties as compared with other polyols[520,521].

The present study demonstrates, an increase in protease activity in the presence of polyols, which might be due to a change in the thermo-stabilization properties of the enzymes as a result of modification of the environment by the additives (polyols, PEG)[522,523]. These protective effects are due to the strengthening of the hydrophobic interactions inside protein molecules and by indirect action of polyols on water structure[524]. Hydrophobic interaction are generally considered to be the single major factor in stabilizing the three dimensional structure of proteins[525]. The effects of sugars and polyols on hydrophobic interaction and consequently on the thermal stability of proteins should also depend upon how they affect the structure of water. Hydrophobic interactions between pairs of hydrophobic groups are stronger in glycerol solution than pure water. It seems likely therefore that this mechanism by which sugars and polyols in general may stabilize proteins to heat denaturation[524,525].

11.8.4 Effect of inhibitors, surfactants and chelators on protease activity

Inhibition studies give insight into the nature of the enzyme, its cofactor requirements, and the nature of the active site i.c. presence of amino acid in the catalytic site of an enzyme[526]. Most of the alkaline proteases are found to be completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP) which was due to binding and subsequent inhibition of active site and it results in the complete loss of catalytic activity of proteases[527]. Further, 4-pBpB and IAA are responsible for binding to the histidine and cysteine residues respectively in the active site and that leads to inhibition of protease

activity[30]. Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) and Nα-Tosyl-L-Phenylalanine Chloromethyl Ketone (TPCK) are mainly used to characterize trypsin and chymotrypsin nature of alkaline protease and alkaline serine protease. Three dimensional structures of proteases mainly depend on the presence of sulphur containing amino acids and disulphide bonds. To classify the proteases belonging to cysteine protease family, iodoacetic acid (IAA) and dithiothreitol (DTT) are mainly used for categorization of protease family. Based on the inhibitor study, it appears that all purified/ partially purified proteases have similar catalytic domain, formed by the catalytic triad of serine, histidine and cysteine residues for enzyme-catalyzed reactions.

Due to the presence of different types of surfactants in any heavy duty laundry detergent, incorporating an enzyme in the detergent becomes a difficult task for the manufacturing industries[528]. To overcome this problem, it is important to investigate the surfactant stability of any protease in order to include it in commercial laundry detergent formulation.

Considering above criteria, we have investigated the effect of surfactants on all purified/partially purified alkaline protease. Inhibitors study illustrated that all the purified / partially purified proteases were unaffected by chelating agents. Moreover, the purified enzymes were found to be stable in both anionic and non-ionic surfactants, oxidizing and bleaching agents. These findings in this study were similar with previously reported surfactant stable proteases isolated from different species of *Bacillus* as shown in Table 11.16.

Table 11.16 A comparative study of effect of different surfactants, oxidizing and bleaching agen t's on purified alkaline proteases.

Strains	Surfactants, oxidizing and bleaching agent (% residual activity)								References
	SDS	Triton-X-100	Tween				H_2O_2	sodium perborate	
			20	40	60	80			
B. mojavensis	100	100	131	100	141	150	154.0	100.0	Beg and Gupta,[126]
Bacillus pseudofirmus	110.0	11.0	-	-	-	99.0	-	-	Patel *et al*[209]
B.cereus MCM B-326	0.72	-	-	-	-	79.64	121.91	62.30	Nilegaonkar *et al*[237]
B. licheniformis RSP-09-37	91.0	108	-	-	-	-	-	-	Sareen and Mishra[306]
Bacillus cereus TKU006	100	71.0	61.0	60	-	-	-	-	Wang *et al*[234]

Strains	Surfactants, oxidizing and bleaching agent (% residual activity)								References
	SDS	Triton-X-100	Tween				H_2O_2	sodium perborate	
			20	40	60	80			
Bacillus sp. HR-08	54.0	91.0	-	-	-	-	109.0	-	Moradian *et al*[236]
B.mojavensis A21	91.0	85.0	-	-	-	100.0	79.4	70.0	Haddar *et al*[96]
B.circulans	75.0	115.0	120.0	-	-	- -	105.0	-	Rao *et al*[308]
Bacillus mojavensis A21 (BM-01)	93.2	80.0	-	-	-	92.5	76.2	77.0	-do-
Bacillus sp. B001	101.0	110.0	112.0	-	-	111.0	99.0	N.T	Deng et al[310]

Strains	Surfactants, oxidizing and bleaching agent (% residual activity)								References
	SDS	Triton-X-100	Tween				H_2O_2	sodium perborate	
			20	40	60	80			
Bsubap-I (*B.subtilis* DM-04)	97.0	100.0	95.1	-	-	-	100.0	110.0	Present study
Alzwiprase (*B.subtilis* DM-04)	97.6	100.0	95.1	-	-	95.1	130.0	122.0	-do-
P.tezpurensis sp.nov. strain AS-S24-II (alkaline protease)	102.0	72.3	80.2	-	-	-	130.0	119.0	-do-
B.subtilis RM-01 (Alkaline β-keratinase)	100.0	87.4	82.9	-	-	100.0	110.0	108.0	-do-
Bacillus sp. AS-S20-I	98.4	100.0	99.3	-	-	95.2	140.0	129.0	-do-

Strains	Surfactants, oxidizing and bleaching agent (% residual activity)								References
	SDS	Triton-X-100	Tween				H_2O_2	sodium perborate	
			20	40	60	80			
Brevicarnase (*Brevibacillus* sp. AS-S10-II)	110.0	100.0	125.0	99.0	95.0	-	112.0	112.0	-do-
Alkarnase (*B.lichenformis* AS-S24-I)	113.0	100.0	125.0	99.0	95.0	-	109.0	102.0	-do-

11.8.5 Effect of metal ions on protease activity

Many enzymes incorporate divalent cations and transition metal ions within their structures to stabilize the folded conformation of the protein[529-531]. The commercial laundry detergent contains metal chelators as important ingredient to remove metal ions and decreases water hardness. The chelating agents also assist in stain removing property from fabrics[195]. Therefore, alkaline proteases with alkaline pH and non-metallic nature (metal-ion independent alkaline protease) are best for commercial laundry detergent formulations.

Considering the importance of the present study, we have examined the effect of metal ions on the proteolytic activity of the purified protease. Results demonstrated that Fe^{2+} ion stimulated the protease activity of Busbap-I and Alzwiprase, whereas all other purified / partially purified proteases displayed no specificity for any tested metal ions suggesting their non-metalloprotease nature. But in all purified/ partially purified proteases there was no significant increase in the protease activity in presence of Ca^{2+} and Mg^{2+}. Since water hardness depends on the presence of Ca^{2+} and Mg^{2+}, these enzymes were found to be applicable in laundry detergent formulations. Assessment of present data with the reported alkaline proteases demonstrated that most of the detergent stable alkaline proteases reported tilldate are metal dependent in nature especially for Ca^{2+} and Mg^{2+} as shown in Table 11.17.

Table 11.17 A comparison of influence of metal ions on purified / partially purified proteases in the present study with protease purified by other workers.

Strains	Metal ions dependency	References
Bacillus sp. PS719	Ca^{2+}	Towatana *et al*[532]
Bacillus brevis	Ca^{2+} and Na^{+}	Banerjee *et al*[533]
Bacillus sp. RGR-14	Mn^{2+}	Oberoi *et al*[195]
B.subtilis PE-11	Ca^{2+}, Mg^{2+}, and Mn^{2+}	Adinarayana *et al*[134]
Bacillus sp. HR-08 and KR-8102	Fe^{2+}	Moradian *et al*[236]
B. mojavensis A21	Ca^{2+}	Haddar *et al*[96]
Bacillus cereus	Cu^{2+}	Doddapaneni *et al*[131]
B. cereus SV1	Ca^{2+} and Mg^{2+}	Manni *et al*[473]
B.subtilis DM-04 (Bsubap-I)	Fe^{2+}	Present study
B.subtilis DM-04 (Alzwiprase)	Fe^{2+}	-do-

Strains	Metal ions dependency	References
P.tezpurensis sp.nov. strain AS-S24-II	No significant effect was observed	-do-
B.subtilis RM-01 (Alkaline β-keratinase)	Fe^{2+}	-do-
Bacillus sp. AS-S20-I	Non of the metal ions stimulated the protease activity	-do-
(Brevicarnase) Brevibacillus sp. AS-S10-II	-do-	-do-
(Alkarnase) B.licheniformis AS-S24-I	-do-	-do-

11.8.6 Kinetic study of purified / partially purified proteases

Enzymes display enormous catalytic power, accelerating reaction rates as much as 10^{16} over uncatalyzed levels, which is far greater than any synthetic catalysts can achieve, and enzymes accomplish these astounding feats in dilute aqueous solutions under mild conditions of temperature and pH[534].

Kinetics is the branch of science concerned with the rates of chemical reactions. The study of enzyme kinetics addresses the biological roles of enzymatic catalysts and how they accomplish their remarkable feats. In enzyme kinetics, we seek to determine the maximum reaction velocity that the enzyme can attain and its binding affinities for substrates and inhibitors[390,534]. Coupling with studies on the structure and chemistry of the enzyme, analysis of the enzymatic rate under different reaction conditions yields insights regarding the enzyme's mechanism of catalytic action [390,534]. Such information is essential to an overall understanding of metabolism.

The Michaelis-Menton equation, both in its original form and as modified by Briggs and Haldane, is derived with respect to a single-substrate enzyme-catalyzed reaction with one substrate-binding site per enzyme and involving the formation of a single intermediate complex[390]. The Michaelis-Menton equation has been found to be applicable to a great many enzyme-catalyzed reactions, and the constants V_{max} and K_m determined. V_{max} varies with the total concentration of enzyme present, but Km is independent of enzyme concentration and is characteristic of the system being investigated[390,534].

Understanding the significances of K_m and V_{max} values, we have characterized these kinetic properties for all the purified / partially purified protease with reference to best substrates for each protease; and all proteases in this study have demonstrated lower K_m values and

higher V_{max} values. A comparison of kinetic values of purified proteases with previously reported proteases from *Bacillus* species is shown in Table 11.18.

Table 11.18 A comparison of kinetic properties of purified/ partially purified protease in the present with the reported proteases purified from *Bacillus* species.

Strains	Kinetics		References
	K_m (mg /ml)	V_{max}	
Bacillus sp. SCB-3	0.17	1047.2 (U)	Lee *et al*[316]
Bacillus pseudofirmus	2.0	289.8 µg / min	Gupta *et al*[130]
Bacillus sp. TKU004	2.98	0.14 U/ml	Wang *et al*[220]
Vibrio fluvialis TKU005	2.21	0.26 U/ml	Wang *et al*[203]
Salinivibrio sp. strain AF-2004	1.4	264 U/mg	Heidari *et al*[201]
B.subtilis DM-04 (Bsubap-I)	6.5	320.0 ± 16.0 (µmol /min/mg)	Present study
B.subtilis DM-04 (Alzwiprase)	5.9	333.0 ± 16.7 (µmol /min/mg)	-do-
P.tezpurensis sp.nov. AS-S24-II	2.27	25.0 ±1.24 (µmol /min/mg)	-do-
B.subtilis RM-01 (Alkaline β-keratinase)	5.0	1.25 ± 0.063 (µmol /min/mg)	-do-
Bacillus sp. AS-S20-I	0.16	20.0 ± 0.4 (µmol /min/mg)	-do-
(Brevicarnase) Brevibacillus sp. AS-S10-II	0.16	33.3 ± 1.66 (µmol /min/mg)	-do-
(Alkarnase) B.licheniformis AS-S24-I	0.1	100 (µmol /min/mg)	-do-

11.8.7 Substrates specificity study of alkaline proteases

Alkaline proteases are ubiquitous in occurrence and constitute an important group of enzymes with diverse functions while sharing similar mechanism of catalysis. Their ability to hydrolyse proteins with varying specificities in a broad range of conditions renders them suitable for a number of industrial applications, such as detergent additives, leather tanning, production of protein hydrolysates, etc[6,7, 535]. Some of the microbial alkaline proteases are also able to hydrolyse insoluble proteins like keratin and elastin, which allow the production of value added products from renewable wastes like feathers[498,535]. Considering the important aspects of substrate specificity, the present study shows that the purified / partially purified alkaline proteases from bacterial strains isolated from soil samples of NE India exhibit board range specificity for insoluble and soluble proteinous substrates but with different rates of degradation which is advantageous for various industries. According to Beynon and Bond[30], specificity for different substrates by proteases is due to the limited proteolysis when a native protein is attacked by a protease under typical reaction conditions, not all the potential hydrolytic sites in a folded, native protein structure are cleaved and therefore, the proteolysis must be limited by higher-order structural phenomena. In many cases, only one or two bonds are cleaved over a few hours of incubation and sometimes no digestion can be measured over the limited time scale of the experiment. This defines the sequence-structure paradigm of limited proteolysis and several studies have been undertaken to discover those structural features that are responsible for limiting reaction.

Thermophilic bacteria are used in the decomposition of these hard-to-degrade animal proteins because in the elevated temperature range where thermophilic bacteria grow, such proteins tend to gain plasticity, resulting in more susceptibility to protease attack[537]. In the present study, alkaline β-keratinase producing thermophilic strain *B.subtilis* RM-01 demonstrated specificity for insoluble and hard-to-degrade animal proteins such as raw chicken feather keratin than human hair ,nail etc. This specificity may be due to the tight arrangement of protein chains in α-helices and β-sheets, respectively in human hairs than raw chicken feather keratin[31,537]. Furthermore, filament structures are

stabilized by their high degree of cross-linking of disulfide bonds, hydrophobic interactions, and hydrogen bonds [328,537].

A survey of literature shows that most of the proteases belong to the subtilisin family of serine proteases and cysteine protease, have higher activity on casein[538] and it is because of higher similar arrangement of catalytic triad of histidine, aspartate and serine residues in radically different α/β and β/β protein scaffolds[34].

11.8.8 Effect of organic solvents on protease activity

Proteases in organic synthesis have attracted a great deal of attention in recent years[539]. Under normal aqueous conditions, proteases catalyze the hydrolysis reaction, but the reverse actions of proteases have been found in water-restricted media such as synthesis of peptides and esters[540,541]. There are a great many advantages associated with the application of enzymes to organic synthesis including enantioselectivity, specificity, non-hazardous reaction conditions and so on[167,542]. These approaches require enzymes that are stable in the presence of organic solvents, but enzymes are generally very labile catalysts and easily lose their activities in non-aqueous media. Several current techniques such as medium engineering, substrate engineering and protein engineering have been attempted to improve enzymes for synthesis in organic solvent[167,539]. However, if enzymes were naturally stable and active in hostile environments, they would be very useful for organic synthesis.

Recently, solvent-tolerant bacteria as a relatively novel group of extremophilic microorganisms with unique ability to live in presence of organic solvents have attracted great attention of many researchers throughout the world. Some of these microbes have been found to be sources of solvent-stable enzymes, but only limited reports are available in the literature concerning the organic solvent-stable protease[170-173]. Considering the present requirement, we have explored the organic –solvent stability property of all isolated in the present study. Maximum stability was observed in presence of n-hexane and least stability was observed in presence benzene. Variation in organic

solvent stability may be assumed by the way in which solvents affect enzymatic activity i.e., interact directly with the essential water surrounding the enzyme molecule. Highly polar solvents are capable of rapidly absorbing the essential water molecule surrounding the enzyme, entailing the loss of its catalytic properties[543]. Generally, the enzymatic activity is low in polar solvents with log $P < 2$, is moderate in solvents with log $P = 2–4$, and is high in polar solvents with log $P>4$[173,543,544]. The organic solvent-stable PST-01 protease from *Pseudomonas aeruginosa* demonstrated presence of disulfide bonds in enzyme molecule which in turn is supposed to play an important role in the organic solvent stability of the protease[170,545] In the present study, proteases are found to contain disulfide bonds (because their activity was reduced in presence of DTT) and exerted organic solvent stability and therefore, reinforces the role of intramolecular disulfide bonds in maintaining the protease stability in presence of organic solvents.

11.8.9 Pharmacological properties of proteases

In their raw state, proteases from bacterial/fungal origin causes allergic reactions in the lung[546]. Proteolytic enzymes also cause irritation to skin, eyes and the respiratory tract. For 40 years, encapsulated enzymes have been used worldwide in detergent products, especially laundry formulations, and have increasing importance due to biodegradability and functionality at low temperatures, thus offering environmental benefits. However, many scientists have suggested that the inclusion of enzymes in such products leads to adverse skin reactions, including erythema, pruritus and exacerbation of eczema[546]. Therefore, in order to develop safer commercial laundry detergent formulation or for other biotechnological applications, it is extremely important to explore the pharmacological properties and toxicity of the isolated protease. It is worthy to mention that, all purified protease in the present study displayed no toxicity level at 2.5 fold higher concentration than the tested concentration for detergent compatibility and wash performance study, suggesting their suitability for incorporation in commercial laundry detergent formulations. Due to lack of reports on pharmacological properties of alkaline proteases, no comparison could be made with the present results.

11.9 Industrial applications of purified / partially purified proteases

Alkaline proteases account for a major share of the enzyme market all over the world[6,11,18,19]. On the basis of biochemical and pharmacological properties of purified / partially purified proteases, we have explored the possible industrial applications of all proteases, for example their application in laundry detergent, leather, and pharmaceutical industries. Present study demonstrated that all purified proteases lack milk -clotting activity. It might be due to alkaline nature of all purified proteases, because all reported milk-clotting proteases work in low pH range and they belongs to a class of aspartic protease[547].

11.9.1 *In vitro* blood clot lysis study

Since intravascular thrombosis due to fibrin aggregation in arteries is one of the main causes of cardiovascular diseases, a number of thrombolytic agents, such as streptokinase, urokinase, tissue-type plasminogen activator (t-PA), and single-chain urokinase-type plasminogen activator (scu-PA), have been developed for therapeutic use[335,548]. Of these, the first-generation fibrinolytic agents such as streptokinase and urokinase can mediate unwanted side reactions. For instance, both deplete plasminogen within the thrombus, converting circulating plasminogen to plasmin[550]. Streptokinase is also immunogenic as a result of which its use results in drug resistance, fever, and allergic reactions[335]. Second-generation agents such as t-PA and scu-PA are fibrin specific; however, they not only decrease the levels of circulating fibrinogen and plasminogen but also increase the risk of intracranial hemorrhage[550,551].These limitations have prompted the scientists to develop third-generation thrombolytic agents[552] that are either conjugates of t-PAs with monoclonal antibodies against fibrin, platelets, and thrombomodulin or chimeric plasminogen activators.

In addition, novel molecules from animal or bacterial origin possessing direct (plasmin like) fibrinolytic activity are screened for their thrombolytic activities[551,552].Several microbial serine proteases with direct fibrinolytic activity have been considered as therapeutic agents, and traditional fermented foods in Asia have been identified as effective sources of such thrombolytic agents. A strong direct-acting fibrinolytic enzyme (subtilisin NAT) originating from a *Bacillus subtilis* present in

Natto, a Japanese fermented soybean product, has been purified and studied extensively[548].

Considering the above drawbacks of presently available fibrinolytic and thrombolytic drugs, we examined the fibrin (ogen)lytic properties of partially purified alkaline protease from *Bacillus* sp. strain AS-S20-I isolated from soil samples of North east India. Based on biochemical and pharmacological properties of partially purified protease their mode of action on fibrin/fibrinogen was analyzed. According to their mechanism of action, direct fibrinolytic enzymes are usually classified into α-fibrinogenase, a based on specificity towards Aα-chain of fibrinogen and β-fibrinogenase, based on specificity towards Bβ- chains of fibrinogen. The fibrinolytic enzymes from *Bacillus* sp. strain AS-S20-I was identified to cleave the β-chain of fibrinogen and hence it may be called as β-fibrinogenase[553,554]. The fibrinolytic pattern of the isolated proteases differed from some other reported proteases in terms of their fibrinogenolytic pattern. FP84 is reported to degrade the Bβ-chains and slowly released γ-chain of fibrinogen[347], and fibrinolytic serine protease from *Bacillus subtilis* DC33 is found to preferentially degrades Aα-chains followed by Bβ- and γ-chains of fibrinogen[336]. A comparison of the fibrinolytic activities of the isolated protease showed that isolated proteases showed stronger fibrinolytic activity than earlier reported fibrinolytic proteases. These results indicated that proteases have strong proteolytic action on fibrin and thus suitable for thrombolytic therapy, and may also be used to prevent the formation of venous blood clots and treatment of cancer[548,553]. In support of above results, the fibrinolytic activity / caseinolytic activity (F/C) ratio and kinetic study for fibrin substrate (K_m and V_{max}) of partially purified protease from *Bacillus* sp. strain AS-S20-I displayed higher F/C ratio and K_m and V_{max} as compare with other reported fibrinolytic protease from *Bacillus* species as represented in Table 11.19 and Table 11.20. Further, direct-acting fibrinolytic proteases degrade fibrin clot similar to that of plasmin, and therefore,it is advantageous as compared to streptokinase & urokinase. Because of its direct fibrinolytic nature, these enzymes do not require the activation of plasminogen to active plasmin, thereby eliminating the severe concern such as associated with plasminogen

activation as platelet activation and depletion of several other clotting factors[548].

Table 11.19 Comparison of fibrinolytic / casinolytic (F/C) ratio of *Bacillus* sp strain AS-S20-I with other reported strains

Protease	Caseinolytic activity	Fibrinolytic activity	F/C %	References
Bacillus sp. strain CK 11-4	257	352	73.0 (40.0)	Kim *et al*[555]
B. licheniformis (type VIII)	423	41	9.7 (5.2)	
Subtilisin BPN' (type XXVII)	438	142	32.4 (17.5)	
B. amyloliquefaciens DC-4	72	138	1.92 (1.04)	Peng *et al*[556]
Bacillus sp. nov. SK006	59	110	1.86 (1.0)	Hua *et al*[557]
Staphylococcus strain AJ	57.70	59.92	1.03 (0.55)	Choi *et al*[558]
Subtilisin BPN' from *B. amyloliquefaciens*	102.62	53.36	0.52 (0.28)	-do-
Subtilisin Carlsberg from *B. licheniformis*	131.56	38.12	0.27 (0.15)	-do-
Bacillus sp. strain AS-S20-I	3830.0	7100.0	185.0 (100)	Present study

Table 11.20 Comparison of K_m and V_{max} of partially purified protease from *Bacillus* sp strain AS-S20-I with other reported strains

Protease	Km	Vmax	References
Rhizopus chinensis 12	0.226 mM	4.023×10^{-4} mM/s	Xiao-lan *et al*[559]
Bacillus subtilis DC33	0.21 mM	22.2 µmol/ min/ l	Wang *et al*[336]

Protease	Km	Vmax	References
Bacillus subtilis TP-6	259.0 µM	145.0 µmol mg^{-1} min^{-1}	Kim *et al*[335]
Bacillus subtilis LD-8547	0.521 mmol/L	0.049 mmol/min	Wang *et al*[341]
Bacillus sp. nov. SK006	0.45 µM	39.30 nmol/ min/ml	Hua *et al*[657]
Staphylococcus strain AJ	0.38 mM	19.73 s^{-1}	Choi *et al*[658]
Streptomyces sp. CS684	4.2 mg/ml	305.8 mg/min/mg	Simkhada *et al*[347]
Bacillus sp. strain AS-S20-I	0.2 mg / ml	25.0 µmol / min / mg	Present study

11.9.2 Alkaline proteases: a green chemical

Cleanliness has been an important consideration for human beings from time immemorial, but the relation between personal and environmental cleanliness is a less obvious one. Soap making dates back to about 1500 BC, the earliest records indicate that a combination of animal and vegetable oils with alkaline salts was used to form a soap-like material. In ancient India too, people used soap preparations made from plant or animal fats[560]. Modern technology has provided synthetic detergents that have slowly replaced soaps. The first detergents were used chiefly for hand dishwashing and fine fabric laundering. This was followed by the development of all-purpose laundry detergents introduced in the U.S. in 1946[560].

Detergents are household chemical cleaning compounds used for laundering and dishwashing. They contain wetting agents and emulsifiers, based on non-soap synthetic surfactants. Synthetic detergent powders consist of surface-active agents, builders and fillers[561]. In addition they have additives like anti re-deposition agents, optical fibre brighteners (whitening agents), bluing agents, bleaching agents, foam regulators, organic sequestering agents, enzymes, perfumers, and substances that regulate the density and assure crispness of the material they are used on (Table 11.21).

Table 11.21 General composition of detergents for laundry and dish-washing

Raw materials	Function in general	Types of compounds	Contents (w/w %)	
			Laundry	Dishwash
Builders	Washing alkalis Water softening Buffer action stabilizers Corrosion protection	Phosphates (sodium triphosphates Na-citrates Zeolites Soda Silicates Complex formers (NTA, etc.)	30-60	10-40
Surfactants	Emulsify particle interfacial activity reduce surface tension	Surfactants : Anionics Cationics Nonionic	10-30	1-4
Soap	Prevents overfoaming in the machine	Soap	1-5	0
Bleach –as peroxygen / activator	Oxidize polyphenolics form e.g., fruits, coffee,tea,wine and vegetables	Na-perborates Na-percarbonates Activators (TAED,etc.)	0.25	4.15

Raw materials	Function in general	Types of compounds	Contents (w/w %)	
			Laundry	Dishwash
Enzymes	Removal of starch –based stains	Amylases	0.4-1.0	1.0-3.0
	Removal of protein complexes	Proteases	0.4-2.0	0.5-2.0
	Removal of fat stains Textile color	Lipases	0.2-1.0	0.5
	brightening, softening, soil, removal , whiteness maintenance	Cellulases	1.0-3.0	0
Salt	Process aid to promote flow and loading properties (powders)	Na-sulfate	Balance to 100	Balance to 100
Perfume	To add a pleasant smell to the fabric	Various esters	0.1-0.5	0-0.1
Other organics	Graying inhibitors	Carboxyl methyl Cellulose	0.5-5.0	0-1.0
	Optical brighterners	Polycarboxylates		
	Solvents	Alcohols		

A study conducted to understand the Indian consumers knowledge of harmful effects of detergents on health and environment, showed that 77.6 percent of respondents had experienced some kinds of skin irritation due to detergents. Of these the majority comprised of dhobis (Washermen) and rural women. Conventional laundry detergents leave chemical residues on the clothes[560].These residues enter our bodies either through the skin or through the lungs. They cause many common health problems including allergies, skin infections and in rare cases, cancer. The fragrances used in laundry detergents can prove allergic and be highly irritating to lungs, causing serious health effects to people with asthma or chronic heart problems[560]. The usual result of a continuous and excessive exposure of the skin to detergents is drying, fissuring and dotting of the keratin layer leading to increased permeability that causes sensitization, which may develop into dermatitis. Elderly people are more susceptible to infections that may lead to developing eczema[560].

11.9.2.1 Laundry detergents consumption in India

Most laundry detergents in India are phosphate based. Phosphates are a major source of water pollution that has become the direct cause of 42 per cent of human and animal diseases. In India, per capita consumption of detergents in 1994 was 2.8 kg per annum. This is projected to rise to over 4 kg/capita by 2005. In rural areas the use of detergent bars is expected to grow 7-8 per cent annually. The figures are of concern because high quality detergents have as much as 35 per cent sewage treatment plant (STP) in them. The main problem is that of phosphate-based detergents promote eurtrophication of aquatic environments. Eutrophication or nutrient pollution is a process by which water bodies gradually age and become more productive. Any natural process like this might take thousands of years to progress but human activities accelerate this process tremendously. Sewage perhaps is a particular source of phosphorus when detergents containing large amounts of phosphates are drained during washing. The algal boom leads to consumption of the oxygen dissolved in water, creating hypoxic, and at times, near anoxic situation. This can lead to excessive eutrophication that kills the fish, cause odour and increase pathogenic animals.

11.9.2.2 Better options – Alkaline protease based cleaning powders

An environmentally superior detergent is the one that makes use of lesser chemical ingredients. The toxicity of detergents decreases by non-addition of additives like perfumes, colour and brightening agents. Minimal packaging can also reduce environmental harm substantially. Proteolytic enzymes function to degrade protein dirt such as egg, blood and gravy. Proteins are often denatured and aggregated by the washing process. The addition of proteolytic enzymes active under washing conditions greatly facilitates the degradation and subsequent removal of stubborn stains[11, 46,502].

Proteolytic enzymes incorporated into detergent formulations exhibit satisfactory catalytic activities in the presence of other detergents components and under standard washing conditions. Considering above mentioned drawbacks of available laundry detergents and present –day requirements, the present study offers highly stable purified / partially purified alkaline protease exhibiting stability against surfactant, oxidizing, bleaching and denaturing agents, working at a broad range of temperature and pH and ideal for incorporation in detergents (section 11.9.2, Table 11.21). The detergent compatibility properties of all purified / partially purified proteases demonstrated higher detergent compatibility than the reported alkaline proteases from other species of *Bacillus* as shown in Table 11.22.

Table 11.22 A comparison of detergent compatibility properties of purified / partially purified protease with previously reported proteases.

Micro-organisms	Detergent stability (In %)	References
Bacillus sp.	Rin supreme® (93.0) > Nirma ® (90.0) > Surf (ultra) ® (88.0) > Ariel® (87.0) > Surf (wash booster) ® (85.0) > Wheel® (68.0)	Oberoi *et al*[195]
B.clausii I-52	Hanspoon® (140.0) > Spark® (129.0) > Bright® (122.4) > Supertie® (118.9)	Joo and chang[187]
B.mojavensis A21	Axion®= Ariel®= Nadhif® (100.0)> New Det® (90.0)	Haddar *et al*[96]
B.mojavensis	Revel® (143.0) > Wheel® (130.0) > Nirma® (116.0) > Feha® (109.0) > Ariel® = Henko® = Rin® = Surf ®= Tide® (100.0)	Beg and Gupta[126]
B. laterosporus-AK1	Ariel® (75.0) > Henko® (63.0) > Surf excel ® (43.0) > Tide® (38.0)	Arulmani *et al*[312]
Bacillus subtilis PE-11	Wheel® (65.0) > Henko® = Nirma® (58.0) > Surf Excel® (56.0) > Rin® (53.0) > Ariel® (52.0) > Surf® (51.0)	Adinarayana *et al*[134]
B. circulans	Rin® (115.0) > Henko® (105.0) > Super wheel® (95.0) > Ariel® = Surf excel® (94.0) >Nirma® (93.0) > Surf® (85.0)	Rao *et al*[308]
Bacillus sp.	Tide® (80.0) > Cheer® (65.0) >Revel® (16.0) > Ariel® (11.4) > Wheel® (6.6)	Nascimento and Martins[562]
Bacillus licheniformis NH1	New Dex® (96.0) > Axion® (95.0) > Dixan® (93.0)> Schems® = Ariel® (80.0) > Nadhif® (64.0)	Hadj-Ali *et al*[563]
Haloalkaliphilic bacterium sp. AH-6	Wheel® (88.0) > Aerial® (86.0) > Nirma® (80.0) > Rin® (77.0) > Tide® (76.0) > Flash® (67.0) > Surf® (56.0)	Dodia *et al*[564]

Micro-organisms	Detergent stability (In %)	References
Bacillus pumilus KS12	Henko® (185.0) > Ariel® = Nirma® = Wheel® (183.0) >Fena® = Rin® (180)> Tide ® (179.0) > Surf® (160.0)	Rajput *et al*[565]
B.subtilis DM-04 (Bsubap-I)	Ghari® (120.0) > Rin advanced® = Safed® (99.0) > Surf excel® (79.0) > Fena Ultra® (67.0) > Wheel®= Tide® (65.0) > Sunlight® = Ariel® (58.0) > Henko® (55.0)	Present study
B.subtilis DM-04 (Alzwiprase)	Ghari® = Rin advanced® (118.0) > Safed® (115.0) > Surf excel® = Fena Ultra® = Wheel ® (100.0) > Tide® = Sunlight® (98.0) > Henko® (95.0) > Ariel® (92.0)	-do-
P.tezpurensis sp.nov. AS-S24-II	Sunlight® = Safed® (115.0) > Henko® = Ariel® (99.0) > Wheel ® = Tide®= Surf excel® (97.0) > Fena Ultra® (95.0)	-do-
B.subtilis RM-01 (Alkaline β-keratinase)	Rin advanced® (115.0) > Ghari® (105.0) > Henko® = Sunlight® (100.0) > Surf excel® = Fena Ultra®= Tide® (99.0) > Safed® (97.0) > Wheel ® (96.0) > Ariel® (94.0)	-do-
Bacillus sp. AS-S20-I	Safed® =Surf excel®=Sunlight® (100.0) > Ghari® (98.0) > Rin advanced® (95.0) > Wheel® (82.0) > Tide® (80.0) > Fena Ultra® (78.0) > Henko® = Ariel® (67.0)	-do-
(Brevicarnase) Brevibacillus sp. AS-S10-II	Henko® (159.0) > Rin advanced® = Safed® (118.0)> Fena Ultra® (115.0) > Surf excel® =Ghari® (100.0)>Ariel® (98.0) >Sunlight® (95.0) >Tide® (82.0) >Wheel® (80.0)	-do-

Micro-organisms	Detergent stability (In %)	References
(Alkarnase) *B.licheniformis* AS-S24-I	Henko (120.0)> Sunlight = Surf excel = Fena Ultra = Wheel = Rin advanced = Safed = Ariel (100.0) > Ghari (97.0)	-do-

Present study reinforces the use of alkaline protease based detergent as 'green detergents' that do not contain non-essential additives like *perfumes, colour and brightening agents in minimal packaging will* go a long way in ensuring a cleaner and healthier environment.

11.9.3 Antibacterial study

Antimicrobial peptides (AMPs) are usually synthesized by microorganisms as well as multicellular organisms, and they are part of the innate host defense mechanisms[566]. By their mode of production, AMPs fall into two categories; non-ribosomally synthesized peptides and ribosomally synthesized (natural) peptides. Whereas the first group is mostly produced by bacteria, the other is produced by all organisms including bacteria[567]. In another classification, electrostatic charge, the most important characteristic by which the action of AMPs is explained, is a trait that clusters AMPs into two main groups. The larger group consists of positively charged peptides, whereas the other group includes non-cationic peptides and it is divided into subgroups such as anionic peptides, aromatic peptides and peptides derived from oxygen-binding proteins[568]. Non-cationic peptides in comparison with the first group are scarce and very often in scientific texts, the term "antimicrobial peptide" only refers to cationic AMPs. The naturally derived AMPs are divided into peptides with β-sheet, α-helix, extended helix and loop structures; and among them the first two groups are more abundant in nature[569].

Mechanism of action for many of antimicrobial peptides (AMPs), including cecropin A, several defensins and magainins was investigated[570,571]. The killing mechanism found for most peptides investigated consists of attacks on the outer and inner membranes, ultimately resulting in lysis of the bacteria. Channel formation in artificial

membranes was demonstrated for cecropins, defensins and magainins[572]. Proline-arginine rich peptides, however, act differently, because the high concentration of proline is incompatible with amphipathic structure formation.

Extensive use of antibiotics in animal husbandry as therapeutics or growth promoters is widespread[573].Treatment of animals with antibiotics leads to antibiotic residue problems in veterinary products and possible transmission of antibiotic-resistant microbes to humans[574]. Due to the above-mentioned concerns and expectations, AMPs are suitable candidates applicable various fields. They are ubiquitous in nature, with high selectivity against target organisms and resistance against them is rarely observed[575]. Considerations such as increase in potency of anti-pathogen activity, reduction of their hemolytic effect or inhibition of their degradation by host protease, persuade scientists to use analog peptides or derivatives of the original AMPs that could be created by amino acid alterations[576].

On the basis of above limitation of commercially available antimicrobial agents used in food industries, aquaculture, animal husbandry, and agriculture sectors, we have investigated the antibacterial properties of purified proteases. Antibacterial strength of Bsubap-I and Alzwiprase displayed the hydrolysis of peptidoglycon layer present in cell wall of Gram positive and Gram –negative class of bacteria. Peptidoglycon is a polymer of β (1-4)-linked N-acetylglucosamine and N-acetylmuramic acid (MurNAc) cross-linked by short peptides containing alternating L- and D-amino acids[577], killing of Gram-negative bacteria by AMPs likely involves an additional step because, in Gram-negative bacteria, peptidoglycan is not exposed on the cell surface since it is located underneath the lipopolysaccharide (LPS) containing outer membrane. Killing of Gram-negative bacteria most likely involves initial binding of AMPs[578-580]. In Gram-positive bacteria, the initial binding of AMPs to bacteria may involve lipoteichoic acid (LTA), in addition to peptidoglycan, because LTA also inhibits killing of bacteria by AMPs[579,581]

Considering its antibacterial, biochemical and pharmacological properties its safe application as preservative for preventing or slowing microbial growth which is, the major reason of spoilage and poisoning of food products is warranted. As compounds of natural origin compounds, AMPs are advantageous options for use as new preservatives to prevent the problems that are encountered due to irregular use of conventional antibiotics, AMPs should therefore be studied properly and prudently for future applications as preservatives.

11.9.4 Alkaline protease as dehairing agent in leather industries

The story of leather is long and colourful. Many years before recorded history people wrapped themselves in dried animal pelts[582]. The fact that the skins turned stiff and rotted was a problem, but ways of softening and preserving the hides were discovered. This was the beginning of leather processing. At first the hides or skins were probably dried in air and sunlight. Later they may have been soaked in water and dried over a fire. Still later it was discovered that certain twigs, barks and leaves soaked with the hides in water helped to preserve them. Through archeologists findings, we know that primitive man used the skins of hunted animals for food as well as clothing. Nomadic tribes made shelters from the hides of larger animals, such as bison[582].

As civilization advanced, preserving hides and tanning them into leather became an important industry. In the 18th century tanning was an old and respectable trade and a tedious one. Nearly a year was spent manipulating a hide before it was delivered as leather to the saddle maker, harness maker or other craftsmen.

Present available conventional technology include several events during leather processing such as (a) pre-tanning or beam house operations, which clean the hides or skins; (b) tanning, which permanently stabilizes the skin or hide matrix; (c) post-tanning or finishing operations where aesthetic value is added[179]. At each stage, various chemicals are used and a variety of materials are expelled into the environment (Fig.11.2).

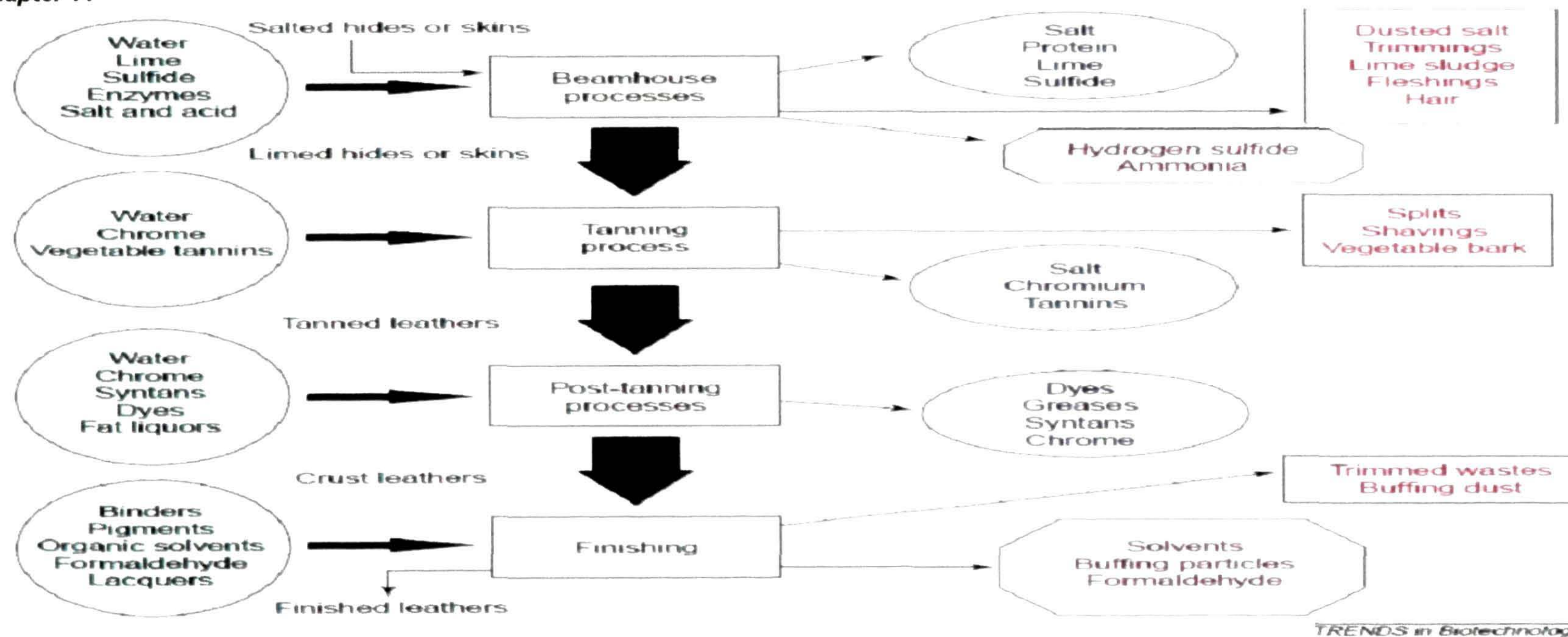


Fig.11.2 Inflow and outflow diagram for leather processing. Temporarily preserved (salted) hides and skins are treated with variety of chemicals in a water medium, through a series of unit processes and operations, to produce leathers. This leads to a variety of solid (red text) and air (purple text) pollutants at various stages of processing.

Current practices of pre-tanning and tanning processes discharge enormous amount of pollutants, which account for nearly 90% of the total pollution from a tannery wastewater. This comprises sulphides, sulphates, chlorides and other minerals. Further, toxic gases such as hydrogen sulphide and ammonia and solid wastes such as lime sludge are generated during the pre-tanning process. Dehairing (liming) and fibre opening (re-liming) alone contribute to 60–70% of the total pollution load in leather processing. The main objectives of this process are the removal of hair and flesh and splitting of fibre bundles by chemical and physical means. These processes employ lime and sodium sulphide, which generate severe pollution loads as well as possess unfavourable consequence to the environment.

Undertaking the consequences of conventional technology, many places in India tanneries industries closed such as 400 tannery industries closed in Tamil nadu ordered by Apex court during 1996[583]. About 418 in Uttar Pradesh got shutdown due to the heavy release of effluents on the river Ganga and the UP govt., decided not open the new leather tannery in Jajmau suburb[584].

The above mentioned drawbacks of conventional technology for leather processing and the restriction imposed by government on tannery industries in various states have promoted us to develop an eco-friendly, cheaper and cost-effective technology for sustainable development of society. The present study offers, matching standards for dehairing process using purified / partially purified proteases isolated from novel strains from North East India. In addition, no collagenolytic activity or minimum collagenase activity was observed in all purified proteases demonstrating the advantageous property for maintenance of skin texture during dehairing process.

All these properties supports the appropriate application of purified proteases in leather industry for dehairing process due to the following benefits : 1) significant reduction or complete elimination of the use of sodium sulfide, 2) total recovery of hair resulting good quality with good saleable value, and 3) creation of an ecologically conducive atmosphere for the workers. The chemical composition of fresh hides

and skins falls approximately within the following limits: water 60- 65%; protein 25-30; fats 5-10% and small amounts of minerals[179,585].

The potential use of protease enzymes in leather processing eliminates the use of chemicals such as sodium, lime and solvents which are responsible for pollution. Future generation might witness eco-labelled leather products emerging as niche products by the use of protease enzyme technology and the experience gained by the Indian leather industry in this area might greatly help us to emerge as a global leaders.

11.9.5 MNP-coupled alkaline β-keratinase mediated chicken feather hydrolysis (solid waste) for development of eco-friendly environment

Biological solid waste let out by the by-product industries is a matter of concern for all of us. Keratinous waste like horns, feather, nails, hoofs, scales, and wool are increasingly accumulating in the environment generated from poultry and meat processing plants, slaughter houses, tanneries, and other industries. Horn meal is available in large quantities. Keratins are proteins that form hard fibres and are components of epidermal and skeletal tissues. Due to the higher degree of cross-linking by disulfide bridges, hydrogen bonds and hydrophobic interactions, keratin is insoluble and poorly susceptible to digestion by enzymes such as trypsin, pepsin, and papain[586,587]. Keratin is the main component of feathers, representing nearly 90% of feather weight. Feather keratin shows an elevated content of the amino acids glycine, alanine, serine, cysteine and valine, but lower amounts of lysine, methionine and tryptophan[588,589]. The feathers constitute up to 10% of total chicken weight, reaching more than 7.7×108 kg/year as a by-product of the poultry industry. This excessive material is discarded in several cases, being a material of difficult degradation that may become an environmental problem. The feeding of biological waste to livestock is an accepted practice and has arisen because of the necessity to reduce costs both in terms of waste disposal and meat production from livestock[590].

Currently, some industries produce feather meal by steam pressure cooking, which require high-energy input. Feather meal has been used on a limited basis as an ingredient in animal feed, since it is deficient in methionine, histidine, and tryptophan[591,592]. Although feather meal has potential to be an important protein source for animal production, it presents low digestibility and variable protein quality and nutrient bioavailability[448]. Considering that animal feeds correspond to a significant part of the poultry production cost, it is relevant to investigate alternatives to the most expensive dietary ingredients. Since feathers have elevated keratin content, the use of this protein source should be considered.

Keratinases are a particular class of proteolytic enzymes that display the capability of degrading insoluble keratin substrates[593,594]. These enzymes are gaining importance in the last years, as several potential applications have been associated with the hydrolysis of keratinous substrates among other applications[296]. Unfortunately, its stability is poor and hence limits its practical applications[595].

Immobilization is one of the efficient methods to improve enzyme stability. Many organic and inorganic substances have been used as the support materials. Using magnetic nanoparticles as the support of immobilized enzymes has the following advantages: (1) higher specific surface area was obtained for the binding of a larger amount of enzymes, (2) lower mass transfer resistance and less fouling, and (3) the immobilized enzymes can be selectively separated from a reaction mixture by the application of a magnetic field[595,596].

Magnetite (Fe3O4) is one of the major magnetic materials now in common use. Due to its strong magnetism and low toxicity, its applications in biotechnology and medicine have gained significant attention[597,598]. Many bioactive substances, such as enzymes, proteins, antibodies, and anticancer agents, have been immobilized on it[597,599-601]. The immobilization is commonly accomplished through a surface coating with polymers, the use of coupling agents or crosslinking reagents, and encapsulation.

Recently, a new method for the direct binding of proteins such as bovine serum albumin via carbodiimide activation was reported[602]. This method is notable for its simplicity and high efficiency. The immobilization of alkaline keratinase on various supports has been investigated by several researchers[603-605].

A large number of nano-scaled carriers have been applied in the enzyme immobilization process[335]. However, from an industrial perspective there are several disadvantages of this process owing to the difficulty experienced in recovering and handling the nanoparticles at industrial level. Alternatively, the use of magnetic nanoparticles (MNPs) might overcome such problems because the use of a magnet allows a simple recovery of the catalyst post binding with MNP. In addition, the use of MNP as a support to immobilize the enzymes has several advantages such as higher specific surface areas resulting in binding of a large amount of enzyme, lower mass transfer resistance and less fouling [397]. It has been reported that amongst the MNPs, magnetite (Fe_3O_4) in particular has received a considerable attention particularly in the fields of biotechnology and medicine due to its strong magnetism and low toxicity[606].

An attempt has made in present study for improvement of nutritional quality of feather meal using MNP-coupled β-keratinase. Iron-oxide MNP-immobilized β-keratinase produce would find applications in nutritional improvement of livestock feed formulation from chicken-feather. The observed enhancement of β-keratinase activity post binding to Fe_3O_4 MNP was significantly higher than the reported increase in keratinase activity from *Aspergillus oryzae* after immobilization on various solid supports such as charcoal, silica gel, Dowex. DEAE-cellulose, Ca-alginate etc[322].

Morphological changes of the chicken feather keratin suggest that the maximum degradation was achieved by post treatment with Fe_3O_4 MNP immobilized β-keratinase than free β-keratinase. SEM observation showed that the large parts of the substrate had been completely digested. The complete degradation of hard keratin is possible only after denaturation by splitting the disulphide bonds. GC–MS confirm

that keratin degradation occurred as a result of action of two mechanisms, sulphitolysis and proteolysis[607]. Therefore, it might be reasonable to conclude that this type of treatment enhances release of nutritionally essential amino acids from chicken-feather keratin by iron-oxide MNP immobilized β-keratinase as compared to free β-keratinase, also the thermo-stability of Fe_3O_4 MNP immobilized β-keratinase offers a simple and inexpensive technology for bioconversion of poultry feather from a potent pollutant waste material to a nutritionally enriched feedstuff for livestock.

11.10 Conclusion

In the present study, process optimization, purification, biochemical and pharmacological characterization, and industrial applications of alkaline protease/ keratinase produced by *B.subtilis* DM-04, *B.subtilis* RM-01, *Brevibacillus* sp. strain AS-S10-II, *Bacillus* sp. strain AS-20-I, *Bacillus licheniformis* strain AS-S24-I, and *P.tezpurensis* sp.nov. AS-S24-II growing under thermophilic growth condition (45°C) were characterized. One out of six bacteria , viz. *B.subtilis* DM-04 was previously isolated in our laboratory from petroleum-oil contaminated soil samples, whereas *B.subtilis* RM-01 was isolated from poultry waste material. All other strains viz. *Brevibacillus* sp. strain AS-S10-II, *Bacillus* sp. strain AS-20-I, *Bacillus licheniformis* strain AS-S24-I, and *P.tezpurensis* sp.nov. AS-S24-II isolated from soil samples of Northeastern part of India.

Optimized alkaline protease production by all the strains were demonstrated in presence of *Imperata cylindrica* and potato peel under solid-state fermentation without any additional co-carbon and nitrogen sources. Raw chicken feather exhibited best carbon and nitrogen sources for alkaline keratinase production by *B.subtilis* strain RM-01. All strains exhibited distinct preferences for the carbon and nitrogen source, and pH for alkaline protease production under submerged fermentation. For example, D-glucose demonstrated the preferable carbon source for protease production from *Bacillus licheniformis* strain AS-S24-I; casein demonstrated best carbon source for protease production from *Brevibacillus* sp. strain AS-S10-II, *Bacillus* sp. strain AS-20-I, and *P.tezpurensis* sp.nov. strain AS-S24-II under submerged

fermentation; maltose served as the best carbon source for alkaline keratinase production from *Bacillus subtilis* strain RM-01 under solid-state fermentation system. Similarly, all strains demonstrated preference for either organic or inorganic nitrogen sources. For example, *B.subtilis* DM-04 had a preference for beef extract; *B. licheniformis* strain AS-S24-I preferred yeast extract as the best organic nitrogen sources, whereas inorganic nitrogen sources such as potassium nitrate was preferred by *Brevibacillus* sp. strain AS-S10-II; ammonium sulphate was choice for *P.tezpurensis* sp.nov. strain AS-S24-II, and *Bacillus* sp. strain AS-S20-I. Statistically optimized fermentation conditions leads to formulation of a cheaper, low-cost and effective fermentation medium for alkaline protease production at industrial scale using IC: PP as substrates.

Biochemical properties of all purified/ partially purified proteases/keratinase demonstrates that all protease / keratinase belongs to serine protease family and they showed activity at alkaline pH and thermo-stability property. All proteases demonstrated excellent stability in presence of surfactants, bleaching and oxidizing agents suggesting their suitable applications in laundry detergent formulation. Organic solvent stability of purified proteases suggested suitable applications in peptide synthesis.

In the present study, pharmacological and detergent stability properties suggest the suitable applications of purified proteases in commercial laundry detergent formulations. All the purified proteases were devoid of collagenase activity suggesting their application in leather industry as dehairing agents. Antibacterial activity of Bsubap-I and Alzwiprase suggested their futuristic applications in food industries as preserving agents. Thrombolytic activity of partially purified protease from *Bacillus* sp. AS-S20-I strain advocated its application as a safe thrombolytic agent for the treatment of thrombosis and may be cancer. Feather degradation properties of alkaline β-keratinase from *B.subtilis* RM-01 strains suggest low cost feed formulation with high content of essential amino acids. Present study offer cheaper, cost-effective, eco-friendly technology for the development of clean and green environment.

1. Eggleston, G. *Advances in the Industrial Application of Enzymes on Carbohydrate-Based Materials* (ACS Symposium Series; American Chemical Society: Washington, DC, 2007)

2. Mahmoud, D.A.R., Helmy, W.A. Potential Application of Immobilization Technology in Enzyme and Biomass Production (Review Article). *J. Appl. Sci. Research* **5(12),** 2466-2476 (2009)

3. Hasan, F. *et al.* Enzymes used in detergents: Lipases. African J Biotechnol. **9(31),** 4836-4844 (2010)

4. Soetaert, W., Vandamme, E.J. *Industrial Biotechnology: Sustainable growth and economic success* (Wiley-VCH, Weinheim, 1st edition, 2010)

5. http://www.biotechcorp.com.my/Documents/AboutBiotechCorp/industrial-cover-and-inside-final.pdf., The Malaysian Industrial Biotechnology Sector. Access on 19th August 2010

6. Kumar, C.G., Takagi, H. Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnol. Adv.* **17,** 561–594 (1999)

7. Gupta, R. *et al.* An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl. Microbiol. Biotechnol.* **60,** 381–395 (2002)

8. Beilen, V., J.B., Li, Z. Enzyme technology: an overview. *Curr. Opin. Biotechnol.* **13,** 338–344 (2002)

9. Krik, O. *et al.* Industrial enzyme applications. *Curr. Opin. Biotechnol.* **13,** 345–435 (2002)

10. Haki, G.D., Rakshit, S.K. Developments in industrially important thermostable enzymes: a review. *Bioresource Technol.* **89,** 17–34(2003)

11. Maurer, KH. Detergent proteases. *Curr. Opin. Biotechnol.* **15,** 330–334 (2004)

12. http://www.thefreelibrary.com/BCC+Research+Repor+Indicates+Global+Market+for+Industrial+Enzymes...-a0172107942, BCC Research Report Indicates Global Market for Industrial Enzymes Worth $2.7 Billion by 2012. Accessed on 10 July, 2010

13. Rajan M. Global market for industrial enzymes to reach $2.4 million by 2009 Business Communications Company, Inc. RC-147U Enzymes for Industrial Applications; 2004. http://www.bccresearch.com/editors/RC- 147U.html, Accessed on 10 July, 2010

14. Garrett, J.B. *et al.* Enhancing the thermal tolerance and gastric performance of a microbial phytase for use as a phosphate-mobilizing monogastric-feed supplement. *Appl. Environ. Microbiol.* **70,** 3041–3046 (2004)

15. Afinah, S. *et al.* Review Article Phytase: application in food industry. *Int.l Food Research J.* **17,** 13-21 (2010)

16. Chandel, A.K. *et al.* Industrial enzymes in bioindustrial sector development: An Indian perspective. *J Commer. Biotechnol.* **13(4),** 283–291(2007)

17. http://biospectrumindia.ciol.com/content/BSTOP20/110061423.asp Biospectrum, June,2010. Accessed on 12th July, 2010

18. Godfrey, T., West, S.I. *Introduction to industrial enzymology. In: Industrial enzymology* (2nd Edition, Stockton Press, New-York, États-Unis, 1-8, 1996)

19. Kalisz, H.M. Microbial proteinases. *Adv. Biochem. Eng. Biotechnol.* **36**, 1–65 (1988)

20. Wiseman, A. *Handbook of enzyme biotechnology*. (Ellis Horwood Ltd., New York, 274–379, 1985)

21. Hasan, F. *et al.*Industrial applications of microbial lipases. *Enzyme Microbial Technol.* **39**, 235-251(2006)

22. Rai, S.K. *et al.* Characterisation of a detergent-stable alkaline protease from a novel thermophilic strain *Paenibacillus tezpurensis* sp. nov. AS-S24-II. *Appl. Microbiol. Biotechnol.* **85**,1437–1450 (2010)

23. Rao, M.B. *et al.* Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiol. Mol. Biol. R.* **62(3)**, 597–635 (1998)

24. Bankus, J. M., and Bond, J. S. In *Proteolytic Enzymes: A Practical Approach* (Oxford University Press, Oxford, 312, 2001)

25. Poldermans, B. *Proteolytic enzymes in industry: production and applications* (VCH Publishers, Weinheim, Germany, p108-123, 1990)

26. Barett, A.J. Proteolytic enzymes: serine and cysteine peptidases. *Methods Enzymol.* **244**, 1–15 (1994)

27. Menon, A.S., Goldberg, A.L. Protein substrates activate the ATP-dependent protease La by promoting nucleotide binding and release of bound ADP. *J. Biol. Chem.* **262**, 14929–14934, (1987)

28. Argos, P. A sensitive procedure to compare amino acid sequences. *J. Mol. Biol.* **193**, 385–396 (1987)

29. Rawlings, N.D., Barrett, A.J. Evolutionary families of peptidases. *Biochem. J.* **290**, 205–218 (1993)

30. Beynon, R., Bond, J.S. *Proteolytic enzymes* 2nd edition practical approach (Oxford university press, 2001)

31. Voet, D., Voet, J.G. *Biochemistry*, 3rd Edition (John Wiley & Sons, 2005)

32. Cerny, G. Studies on the aminopeptidase test for the distinction of gram-negative from gram-positive bacteria. *Eur. J. Appl. Microbiol. Biotechnol.* **5,** 113–122 (1978)

33. Gupta, R., Beg, Q.K., Lorenz, P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.* **59,** 15–32 (2002)

34. Siezen, R.J., Leunissen, J.A.M. Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci.* **6,** 501–523 (1997)

35. Lehninger, A.L. Nelson, D.L. Cox, M.M. *Lehninger principles of Biochemistry*, 5th edition (W. H. Freeman 2008)

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

37. Bone, R., Shenvi, A.B., Kettner, C.A., Agard, D.A. Serine protease mechanism: structure of an inhibitory complex of .alpha-lytic protease and a tightly bound peptide boronic acid. *Biochem.* **26 (24),** 7609–761(1987)

38. Brenner, S. The molecular evolution of genes and proteins: a tale of two serines. *Nature* **334,** 528–530 (1988)

39. Boguslawski, G., Shultz, J.L., Yehle, C.O. Purification and characterization of an extracellular protease from *Flavobacterium arborescens*. *Anal. Biochem.* **132,** 41–49 (1983)

40. Mizuno, K., Matsuo, H. A novel protease from yeast with specificity towards paired basic residues. *Nature* **309,** 558–560 (1984)

41. Phadatare, S.U., Srinivasan, M.C., Deshpande, V.V. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): enzyme production and compatibility with commercial detergents: *Enzyme Microbial Technol.* **15,** 72–76 (1993)

42. Lindberg, R.A., Eirich, L.D., Price, J.S., Wolfinbarger, L.Jr., Drucker, H. Alkaline protease from *Neurospora crassa. J. Biol. Chem.* **256,** 811–814 (1981)

43. Wiseman, A. *Handbook of enzyme biotechnology 3rd edition* (Prentice hall, Ellis horwood, 1999)

44. Glazer, A.N., Nikaido, H. *Microbial enzymes. In: Glazer AN, Nikaido H, editors. Microbial Biotechnology* (W.H. Freeman and Co., New york, 24–263, 1995)

45. Tewari, J.P. *et al. Advances in microbial biotechnology* (A.P.H. publishing corporation, New delhi, 1999)

46. Walsh, G. *Proteins: Biotechnology and Biochemistry* (John Wiley & sons, 2001)

47. Phadatare, S., Rao, M., Deshpande, V. A serine alkaline protease from the fungus *Conidiobolus coronatus* with a distinctly different structure than the serine protease subtilisin Carlsberg. *Arch. Microbiol.* **166,** 414–417 (1997)

48. Sielecki, A.R., Fujinaga, M., Read, R.J., James, M.N.G. Refined structure of porcine pepsinogen at 1.8A resolution. *J.Mol.Biol.* **219,** 671-692 (1991)

49. Page, R.D.M. TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12,** 357–358 (1996)

50. Chakraborti, S. *et al.* Regulation of matrix metalloproteinases: An overview. *Mol. Cell. Biochem.* **253,** 269–285 (2003)

51. Hooper, N.M. Families of zinc metallo-proteases. *FEBS Lett.* 354, l-6 (1994)

52. Browner, M.F. *et al.* Crystal structures of matrilysin-inhibitor complexes. *Biochem.* **34 (20),** 6602–6610, (1995)

53. Bryan, P.N. *et al.* Proteases of enhanced stability: Characterization of a thermostable variant of subtilisin. *Proteins: Struct. Funct. Gen.* **1,** 326–34 (1986)

54. Windhorst, S. *et al.* The major extracellular protease of the nosocomial pathogen Stenotrophomonas maltophilia: characterization of the protein and molecular cloning of the gene. *J. Biol. Chem.* **277,** 11042–11049 (2002)

55. Nonaka, T. *et al.* The crystal structure of an oxidatively stable subtilisin-like alkaline serine protease, KP-43, with a C-terminal β-barrel domain. *J. Biol. Chem.* **279,** 47344–47351(2004)

56. Piao, S. *et al.* Crystal structure of the serine protease domain of prophenoloxidase activating factor-I. *J. Biol. Chem.* **282,** 10783–10791(2007)

57. Yamagata, Y. *et al.* Functional changes of dextran-modified alkaline proteinase from alkalophilic *Bacillus* sp. *Enzyme Microbial Technol.* **16,** 99–103 (1994)

58. http://www.protease.org/structure.php?start=1200 three dimensional structure of trypsin. Accessed on 12th April 2010

59. Fastrez, J., Fersht, A.R. Demonstration of the acyl-enzyme mechanism for the hydrolysis of peptides and anilides by chymotrypsin. *Biochem.* **12,** 2025–2034 (1973)

60. Jany, K.D., Mayer, B. Proteinase K from *Tritirachium album Limber I*. Molecular mass and sequence around the active site serine residue. *Biol. Chem. Hoppe-Seyler* **366,** 485–92 (1985)

61. Austew, B. M., Smith, E.L. Action of staphylococcal proteinase on peptides of varying chain length and composition. *Biochem. Biophys. Res. Commun.* **72,** 411–417 (1976)

62. Blundell, T. L., Cooper, J.B., Sali, A., Zhu, Z. Comparisons of the sequences, 3-D structures and mechanisms of pepsin-like and retroviral aspartic proteinases. *Adv. Exp. Med. Biol.* **306,** 443–453 (1991)

63. Keil, B. *Specificity of proteolysis.* (Springer-Verlag KG, Germany, 1992)

64. Holmes, M. A., Matthews, B.W. Binding of hydroxamic acid inhibitors to crystalline thermolysine suggests a pentacoordinate zinc intermediate in catalysis. *Biochem.* **20,** 6912–6920 (1981)

65. Polgar, L.Common feature of the four types of protease mechanisms. *Biol. Chem. Hoppe-Seyler* **371,** 327–331 (1990)

66. Moon, S.H., Parulekar, S.J. Some observations on protease production in continuous suspension cultures of *Bacillus firmus. Biotechnol. Bioeng.* **41,** 43–54 (1993)

67. Aunstrup, K. Proteinases. *In: Rose AH (ed) Microbial enzymes and bioconversions* (Academic Press, New York, pp 50–114, 1980)

68. Chakraborty, R., Srinivasan, M. Production of a thermostable alkaline protease by a new *Pseudomonas* sp. by solid substrate fermentation. *J. Microb. Biotechnol.* **8,** 7–16 (1993)

69. Malathi, S., Chakraborty, R. Production of alkaline protease by a new *Aspergillus flavus* isolate under solid-substrate fermentation

conditions for use as a depilation agent. *Appl. Environ. Microbiol.* **57,** 712–16 (1991)

70. George, S. *et al.* Production of protease by *Bacillus amyloliquefaciens* in solid-state fermentation and its application in the unhairing of hides and skins. *Process Biochem.* **30,** 457–62 (1995)

71. Beg, Q.K., Saxena, R.K., Gupta, R. De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operation. *Process Biochem.* **37,** 1103-1109 (2002)

72. Mukherjee, A.K., Adhikari, H., Rai, S.K. Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using Imperata cylindrica grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation. *Biochemical Eng. J.* **39,** 353–361(2008)

73. Varela, H. *et al.* Effect of medium composition on the production by a new *Bacillus subtilis* isolate of protease with promising unhairing activity. *World J Microbiol. Biotechnol.* **12,** 643– 645 (1996)

74. Hameed, A., Keshavarz, T., Evans, C.S. Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. *J. Chem. Technol. Biotechnol.* **74,** 5–8 (1999)

75. Puri, S., Beg, Q.K., Gupta, R. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. *Curr. Microbiol.* **44,** 286–290 (2002)

76. Rahmana, R.N.Z.R.Abd., Geoka, L.P., Basrib, M., Salleha, A.B. An organic solvent-tolerant protease from *Pseudomonas aeruginosa*

strain K Nutritional factors affecting protease production. *Enzyme Microbial Technol.* **36,** 749–757 (2005)

77. Potumarthi, R., Subhakar, Ch., Pavani, A., Jetty, A. Evaluation of various parameters of calcium-alginate immobilization method for enhanced alkaline protease production by *Bacillus licheniformis* NCIM-2042 using statistical methods. *Bioresource Technol.* **99,** 1776–1786 (2008)

78. Shivanand, P., Jayaraman, G. Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. *Process Biochem.* DOI:10.1016/j.procbio.2009.05.010

79. Hubner, U., Bock, U., Schugerl, K. Production of alkaline serine protease subtilisin Carlsberg by *Bacillus licheniformis* on complex medium in a stirred tank reactor. *Appl. Microbiol. Biotechnol.* **40,** 182–188 (1993)

80. Mao, W., Pan, R., Freedman, D. High production of alkaline protease by *Bacillus licheniformis* in fed-batch fermentation using a synthetic medium. *J. Ind. Microbiol.* **11,** 1–6 (1992)

81. Van Putten, A.B. *et al.* Improvement of the production of subtilisin Carlsberg alkaline protease by *Bacillus licheniformis* by on-line monitoring and control in a stirred tank reactor. *J Biotechnol* **49,** 83–93 (1996)

82. Frankena, J., Verseveld, H.W. van., Stouthamer, A.H. A continuous culture study of the bioenergetic aspects of growth and production of exocellular protease in *Bacillus licheniformis. Appl. Microbiol. Biotechnol.* **22,** 169–176 (1985)

83. Frankena, J., Koningstein, G.M., Verseveld, H.W. van., Stouthamer, A.H. Effect of different limitations in chemostat cultures on growth and production of exocellular protease by *Bacillus licheniformis*. *Appl. Microbiol. Biotechnol.* **24,** 106–112(1986)

84. De Coninck, J., Bouquelet, S., Dumortier, V., Duyme, F., Denantes, IV. Industrial media and fermentation processes for improved growth and protease production by *Tetrahymena thermophila*. *J. Ind. Microbiol. Biotechnol.* **24,** 285–290 (2000)

85. Adinarayana, K., Ellaiah, P. Response surface optimization of the critical medium components for this production of alkaline protease by a newly isolated *Bacillus* sp. *J. Pharmacy Pharmaceutical Sci.* **5,** 272–227 (2002)

86. Tari,C., Genckal, H., Tokatl, F. Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. *Process Biochem.* **41,** 659–665 (2006)

87. Oskouie, S.F.G.,Tabandeh, F., Yakhchali, B., Eftekhar, F. Enhancement of alkaline protease production by *Bacillus clausii* using Taguchi experimental design. *African J. Biotechnol.* **6(22),** 2559-2564, (2007)

88. Saran, S., Isar, J., Saxena, R.K. Statistical optimization of conditions for protease production from *Bacillus* sp. and its scale-up in a bioreactor. *Appl. Biochem. Biotechnol.* **141(2-3),** 229-239(2007)

89. Guangrong, H., Dehui, D., Weilian, Hu., Jiaxin, J. Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 with a statistical method. *African J. Biotechnol.* **7(8),** 1115-1122, (2008)

90. Reddy, L.V.A.,Wee, Y.J., Yun, J.S., Ryu, H.W. Optimization of alkaline protease production by batch culture of *Bacillus* sp. RKY 3 through Plackett-Burman and response surface methodological approaches. *Bioresource Technol.* **99,** 2242–2249 (2008)

91. Wang, Q. *et al.* Optimization of cold-active protease production by the psychrophilic bacterium *Colwellia* sp. NJ341 with response surface methodology. *Bioresource Technol.* **99,**1926–1931(2008)

92. Sen,S., Dasu, V.V., Mandal, B. Medium development for enhanced production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1. *Asia-Pacific J Chemical Eng.* DOI: 10.1002/apj.417 2009

93. Abdel-Fattah, Y.R., El-Enshasy, H.A., Soliman, N.A., El-Gendi, H. Bioprocess development for production of alkaline protease by *Bacillus pseudofirmus* Mn6 through statistical experimental designs. *J. Microbiol. Biotechnol.* **19(4),** 378–386 (2009)

94. Amid, M. *et al.* Optimization of serine protease extraction from mango peel (Mangifera Indica Cv. Chokanan). *Food Chem.* **124(2),** 666-671 (2011)

95. Esposito, T.S. *et al.* Fish processing waste as a source of alkaline proteases for laundry detergent., *Food chem.* **112(1),** 125-130 (2009)

96. Haddar, A. *et al.* Two detergent stable alkaline serine proteases from *Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent additive. *Bioresource Technol.* **100(13),** 3366-3373 (2009)

97. Takagi H. Protein engineering on subtilisin. *Int. J. Biochem.* **25,** 307–12 (1993)

98. Tsai, Y.C., Yamasaki, M., Yamamoto-Suzuki ,Y., Tamura, G. A new alkaline elastase of an alkalophilic *Bacillus. Biochem. Int.* **7,** 577–83 (1983)

99. Mukhopadhyay, A., Chakrabarti, S.K., Bajpai, P.K. Treatment and clarification of fermented broth in bacterial enzyme production. *Biotechnol. Techniques* **4,** 121–26 (1990)

100. Boyer, E.W., Byng, G.S. *Bacillus proteolyticus* species which produce an alkaline protease (US Patent No. 5518917, 1996)

101. Strathmann, H. The use of membranes in downstream processing. *Food Biotechnol.* **4,** 253–72 (1990)

102. Bohdziewicz J, Bodzek M. Ultrafiltration preparation of pectinolytic enzymes from citric acid fermentation. *Process Biochem.* **29,** 99–107(1994)

103. Bohdziewicz J. Ultrafiltration of technical proteolytic enzymes. *Process Biochem.* **29,** 109–18(1994)

104. Bohdziewicz J. Ultrafiltration of technical amylolytic enzymes. *Process Biochem.* **31,** 185–91 (1996)

105. Han, J., Park, C.H., Ruan, R. Concentrating alkaline protease, subtilisin, using a temperature-sensitive hydrogel. *Biotechnol. Lett.* **17,** 851–52 (1995)

106. Bell, D.J., Hoare, M., Dunnill, P. The formation of protein precipitates and their centrifugal recovery. *Adv. Biochem. Eng. Biotechnol.* **26,** 1–72 (1983)

107. Fujiwara, N., Masui, A., Imanaka, T. Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp. *J. Biotechnol.* **30,** 245–56 (1993)

108. Kumar, C.G., Tiwari, M.P., Jany, K.D. Purification and characterization of two alkaline proteases from an alkalophilic *Bacillus* sp. *Zeitschrift für Ernährungs-Wissenschaft.* **36,** 48 (1997)

109. Tsuchiya, K., Seki, K., Arai, T., Masui, T. Substrate specificity of alkaline proteases from *Cephalosporium* sp. KSM388. *Biosci. Biotechnol. Biochem.* **57,** 1803–4(1993)

110. Keay, L., Wildi, B.S. Proteases of the genus *Bacillus.* I. Neutral proteases. *Biotechnol. Bioeng.* **12,** 179–212 (1970)

111. Kobayashi, T., Hakamada, Y., Hitomi, J., Koike, K., Ito, S. Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. *Appl. Microbiol. Biotechnol.* **45,** 63–71(1996)

112. Ong, P.S., Gaucher, M. Production, purification and characterization of thermomycolase, the extracellular serine protease of the thermophilic fungus *Malbranchea pulchella* var. *sulfurea. Can. J. Microbiol.* **22,** 165–76 (1976)

113. Manachini, P.L., Fortina, M.G., Parini, C. Thermostable alkaline protease produced by *Bacillus thermoruber*—a new species of *Bacillus. Appl. Microbiol. Biotechnol.* **28,** 409–13 (1988)

114. Bockle, B., Muller, R. Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feather. *Appl. Environ. Microbiol.* **63,** 790–95 (1997)

115. Larcher, G. *et al.* Purification and characterization of a fibrinogenolytic serine proteinase from *Aspergillus fumigatus* culture filtrate. *FEBS Lett.* **308,** 65–69(1992)

116. Roe,S. *Protein purification applications a practical approach* (Oxford university press, 2001)

117. Simpson, R.J. *Purifying proteins for proteomics : A laboratory manual* (Cold spring harbor Laboratory Press, New York, 2003)

118. Guerrier, L., Righetti, P.G., Boschetti, E. Reduction of dynamic protein concentration range of biological extracts for the discovery of low-abuhdance proteins by means of hexapeptide ligand library. *Nat. Protoc.* **3(5),** 883-90(2008)

119. Mazzola, P.G.*et al.* Liquid–liquid extraction of biomolecules: an overview and update of the main techniques. *J. Chem. Technol. Biotechnol.* **83**, 143–157 (2008)

120. Burgess, R.R. Deutscher, M.P. *Methods in Enzymology, vol 463 : In, Guide to protein purification 2nd edition* (Academic press,2009)

121. Brennan, J.D., Benjamin, D., DiBattista, E., Gulcev, M.D. Using sugar and amino acid additives to stabilize enzymes within sol-gel derived silica. *Chem. Mater.* **15,** 737-745 (2003)

122. Iyer, P.V., 'Ananthanarayan, L. Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochem.* **43(10),** 1019-1032(2008)

123. Shetty, J.K., Patel, C.P., Nicholson, M.A. Purified alkaline protease concentrate and method of preparation. (European. Patent Appl. EP 0549048, 1993)

124. Stoner, M.R. *et al.* Protease autolysis in heavy- duty liquid detergent formulations: effects of thermodynamic stabilizers and protease inhibitors. *Enzyme Microbial Technol.* **34,** 114–125 (2004)

125. Alam, S.I. *et al.* Purification and characterisation of extracellular protease produced by *Clostridium* sp. from *Schirmacher oasis*, Antarctica. *Enzyme Microbial Technol.***36(5-6),**824-831(2005)

126. Beg, Q.K. Gupta, R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microbial Technol.* **32,** 294-304 (2003)

127. Chellappan, S. Production, purification and partial characterization of a novel protease from marine *Engyodontium album* BTMFS10 under solid state fermentation. *Process Biochem.***41(4),** 956-961(2006)

128. Hajji, M., Rebai, A., Néji Gharsallah, N., Nasri, M. Optimization of alkaline protease production by *Aspergillus clavatus* ES1 in *mirabilis jalapa* tuber powder using statistical experimental design. *Appl. Microbiol. Biotechnol.* **79,** 915–923 (2008)

129. Joo, H.S. *et al.* Simple methods for alkaline protease purification from the polychaeta, *Periserrula leucophryna*. *Process Biochem.* **37,** 299–303 (2001)

130. Gupta, A. *et al.* One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. *J. Chromatogr. A.* **1075,** 103–8(2005)

131. Doddapaneni, K.K. *et al.* Purification and characterization of a solvent and detergent-stable novel protease from *Bacillus cereus. Microbiol.Research* **164(4),** 383-390 (2009)

132. Dodia, M.S. *et al.* Purification and stability characteristics of an alkaline serine protease from a newly isolated haloalkaliphilic bacterium sp. AH-6. *J. Ind. Microbiol. Biotechnol.* **35,** 121–131 (2008)

133. Miyali, T., Otta, Y., Nakagawa, T., Watanabe, T., Niimura, Y., Tomizuka, N. Purification and molecular characterization of

subtilisin-like alkaline protease BPP-A from *Bacillus pumilus* strain MS-1. *Lett. Appl. Microbiol.* **42(3),** 242-7(2006)

134. Adinarayana, K., Ellaiah, P., Prasad, D.S. Purification and partial characterization of thermostable serine alkaline protease from a newly Isolated *Bacillus subtilis* PE-11. *AAPS PharmSciTech.* **4(4),**1-9(2003)

135. Abidi, F., Limam, F., Nejib, M.M. Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. *Process Biochem.* **43(11),** 1202-1208 (2008)

136. Tang, X.X. *et al.* Purification and characterization of an alkaline protease used in tannery industry from *Bacillus licheniformis. Biotechnol. Lett.* **26,** 1421–1424, (2004)

137. Chung, C. H., Goldberg, A.L. The product of the lon (capR) gene in Escherichia coli is the ATP dependent protease, protease La. *Proc. Natl. Acad. Sci.* **78,** 4931–4935 (1981)

138. Hoffman, T. Food related enzymes. *Adv. Chem. Ser.* **136,** 146–185 (1974)

139. Kornberg, A. *et al.* Origin of proteins in sporulation. *Annu. Rev. Biochem.* **37,** 51–78 (1968)

140. Esposito, R. E., Klapholz, S. *Meiosis and ascospore development, In J. N. Strathern, E. W. Jones, and J. R. Broach, (ed.), The molecular biology of the yeast Saccharomyces. Life cycle and inheritance* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 211-287, 1981)

141. North, M. J. Comparative biochemistry of the proteinases of eukaryotic microorganisms. *Microbiol. Rev.* **46,** 308–340 (1982)

142. Phadatare, S. U., Srinivasan, M.C., Deshpande, V.V. Evidence for controlled autoproteolysis of alkaline protease: a mechanism for physiological regulation of conidial discharge in *Conidiobolus coronatus*. *Eur. J. Biochem.* **205,** 679–686 (1992)

143. Phadatare, S., Rao, M., V. Deshpande, V. A serine alkaline protease from the fungus *Conidiobolus coronatus* with a distinctly different structure than the serine protease subtilisin Carlsberg. *Arch. Microbiol.* **166,** 414–417 (1997)

144. Postemsky, C. J., Dignam, S.S., Setlow, P. Isolation and characterization of *Bacillus megaterium* mutants containing decreased levels of spore protease. *J. Bacteriol.* **135,** 841–850 (1978)

145. Leighton, T. J.,Stock, J.J. Biochemical changes during fungal sporulation and spore germination. I. Phenyl methyl sulfonyl fluoride inhibition of macroconidial germination in *Microsporum gypseum*. *J. Bacteriol.* **101,** 931–940 (1970)

146. Jackson, D. P., Cotter, D.A. Expression of proteolytic enzymes during *Dictyostelium discoideum* spore germination. *Arch. Microbiol.* **137,** 205–208 (1984)

147. O'Day, D. H. Acid protease activity during germination of microcysts of the cellular slime mold *Polysphodylium pallidum*. *J. Bacteriol.* **125,** 8–13 (1976)

148. Roberts, J. W., Roberts, C.W., Mount, D.W. Inactivation and proteolytic cleavage of phage repressor in vitro in an ATP-dependent reaction. *Proc. Natl. Acad. Sci.* **74,** 2283–2287 (1977)

149. Lecadet, M. M., Lescourret, M., Klier, A. Characterization of an intracellular protease isolated from *Bacillus thuringiensis* sporulating

cells and able to modify homologous RNA polymerase. *Eur. J. Biochem.* **79,** 329–338 (1977)

150. Gottesman, S., Maurizi, M.R. Regulation by proteolysis: energy-dependent proteases and their targets. *Microbiol. Rev.* **56,** 592–621(1992)

151. Van Melderen, L. *et al.* ATP-dependent degradation of Ccd A by Lon protease. *J. Biol. Chem.* **271,** 27730–27738 (1996)

152. Suzuki, C. K. *et al.* ATP-dependent proteases that also chaperone protein biogenesis. *Trends Biochem. Sci.* **22,** 118–123 (1997)

153. Pearson, K.K., Karatan, E. Biofilm development in bacteria. *Adv.Appl.Microbiol.* **57,** 79-111 (2005)

154. Nelson, J.M., Griffin, E.G. Adsorption of invertase. *J. Am. Chem. Soc.* **38 (5),** 1109–1115 (1916)

155. Sumner, J.B. Denaturation of urease without inactivation. *Science* **108(2807),** 410 (1948)

156. Grubhofer, N., Schleith, L. Protein coupling with diazotized polyaminostyrene. *Hoppe Seylers Z Physiol. Chem.* **297,** 108–112 (1954)

157. Guisan, J.M. *Immobilization of Enzymes and Cells, Second Edition* (Humana Press Inc, 2006)

158. Sumantha, A. Rice bran as a substrate for proteolytic enzyme production. *Brazilian Arch. Biol. Technol.* **49(5),** 843-851(2006)

159. Podar, M., Reysenbach, A.L. New opportunities revealed by biotechnological explorations of extremophiles. *Curr. Opin. Biotechnol.* **17,** 250–255 (2006)

160. Kumar, D. *et al.* Microbial proteases and application as laundry detergent additive.*Research J. Microbiol.* **3(12),** 661-672 (2008)

161. Neklyudov, A.D., Ivankin, A.N., Berdutina, A.V. Production and Purification of Protein Hydrolysates (Review). *Appl. Biochem. Microbiol.* **36(4),**317-324(2000)

162. Ward, O. P. *Proteinases, In W. M. Fogarty (ed.), Microbial enzymes and*

biotechnology (Applied Science Publishers, London, England, p-251-305, 1983)

163. Dalev, P. An enzyme-alkaline hydrolysis of feather keratin for obtaining a protein concentrate for fodder. *Biotechnol. Lett.* **12,** 71–72 (1990)

164. Dalev, P.G. Utilisation of waste feathers from poultry slaughter for production of a protein concentrate. *Bioresource Technol.* **48,** 265–67(1994)

165. Cheng, S.W. Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis. Biosci. Biotechnol. Biochem.* **59,** 2239–43 (1995)

166. Bordusa, F.Proteases in organic synthesis. *Chem. Rev.* **102,** 4817–4867 (2002)

167. Kumar, D., Bhalla, T.C. Microbial protease in peptide synthesis: approaches and applications. *Appl. Microbiol. Biotechnol.* **68,** 726–736 (2005)

168. Gupta, M.N., Roy, I.Enzymes in organic media forms, functions and applications. *Eur. J. Biochem.* **271,** 2575–2583 (2004)

169. Klibanov, A.M.Improving enzymes by using them in organic solvents. *Nature* **409,** 241–246 (2001)

170. Ogino, H., Yasui, K., Shiotani, T., Ishihara, T., Ishikawa, H. Organic solvent-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme. *Appl. Environ. Microbiol.* **61,** 4258–4262 (1995)

171. Geok, L.P., Razak, C.N.A., Rahman, R.N.Z.A., Basri, M., Salleh, A.B. Isolation and screening of an extracellular organic solvent-tolerant protease producer. *Biochem. Eng. J.* **13,** 73–77 (2003)

172. Gupta, A., Khare, S.K. A protease stable in organic solvents from solvent tolerant strain of *Pseudomonas aeruginosa. Bioresource Technol.* **97,** 1788–1793 (2006)

173. Rahman, R.N.Z.A., Mahamad, S., Salleh, A.B., Basri, M. A new organic solvent tolerant protease from *Bacillus pumilus* 115b. *J. Ind. Microbiol. Biotechnol.* **34,** 509–517 (2007)

174. Bergmann, M., Frankel-Conrat, H. The role of specificity in the enzymic synthesis of proteins: synthesis with intracellular enzymes. *J. Biol. Chem.* **119,** 707–720, (1937)

175. Clapes, P., Pera, E., Torres, J.L. Peptide bond formation by the industrial protease, neutrase, in organic media. *Biotechnol. Lett.* **19,** 1023–1026 (1997)

176. Isono, Y., Nakajima, M. Enzymic peptide synthesis using a microaqueous highly concentrated amino acid mixture. *Process Biochem.* **36,** 275–278 (2000)

177. Kise, H., Hayakawa, A., Noritomi, H. Protease catalyzed synthetic reactions and immobilization–activation of the enzyme in hydrophilic organic solvents. *J. Bacteriol.* **14,** 239–254 (1990)

178. Morihara, K. Using protease in peptide synthesis. *Trends Biotechnol.* **5,** 164–170 (1987)

179. Thanikaivelan, P., Rao, R.J., Nair, U.B., Ramasami, T. Progress and recent trends in biotechnological methods for leather processing. *Trends in Biotechnol.* **22(4),** (2004)

180. Ozgunay, H., Colak, S., Mutlu, M.M., Akyuz, F. Characterization of leather industry wastes. *Polish J. Environ. Stud.* **16(6),** 867-873 (2007)

181. Ogino, H., Otsubo, T., Ishikawa, H. Screening, purification, and characterization of a leather-degrading protease. *Biochemical Eng. J.* **38,** 234–240 (2008)

182. Onifade, A.A., Al-Sane, N.A., Al-Musallam, A.A., Al-Zarban, S. Potentials for biotechnological applications of keratin- degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresource Technol.* **66,** 1–11 (1998)

183. Brandelli, A. Bacterial keratinases: useful enzymes for bio-processing agroindustrial wastes and beyond. *Food Bioprocess Technol.* **1,** 105–116 (2008)

184. Ichida, J.M. *et al.* Bacterial inoculum enhances keratin degradation and biofilm formation in poultry compost. *J. Microbiol. Methods*, **47,** 199–208 (2001)

185. Takami, H., Nakamura, F., Aono, R., Hirishiri, K. Degradation of human hair by a thermostable alkaline proteinase from alkalophilic *Bacillus* sp. no. AH-101. *Biosci. Biotechnol. Biochem.* **56,** 1667–1669 (1992)

186. Saeki, K., Ozaki, K., Kobayashi, T., Ito, S. Detergent Alkaline Proteases: Enzymatic Properties, Genes, and Crystal Structures. *J. Biosci. Bioeng.* **103(6),** 501–508 (2007)

187. Joo, H-S., Chang, C-S. Production of an oxidant and SDS-stable alkaline protease from an alkaophilic *Bacillus clausii* I-52 by submerged fermentation: Feasibility as a laundry detergent additive. *Enzyme Microbial Technol.* **38(1-2),** 176-183 (2006)

188. Basketter, D.A., English, J.S., Wakelin, S.H., White, I.R. Enzymes, detergents and skin: facts and fantasies. *Br. J. Dermatol.* **158,** 1177- 1181 (2008)

189. http://pubs.acs.org/cen/coverstory/87/8704cover.html. The greening game report. Accessed on 18th July 2010

190. Bech, L.M., Branner, S., Breddam, K., Groen, H. Oxidation stable detergent enzymes. US Patent 5,208, 158 (1993)

191. Gupta, R., Gupta, K., Saxena, R.K., Khan, S. Bleach-stable, alkaline protease from *Bacillus* sp. *Biotechnol. Lett.* **21,**135– 138 (1999)

192. Kumar, C.G., Malik, R.K., Tiwari, M.P. Novel enzyme-based detergents: an Indian perspective. *Curr. Sci.* **75,** 1312–1318 (1998)

193. Aehle, W., Sobek, H., Amory, A., Vetter, R., Wilke, D., Schomburg, D. Rational protein engineering and industrial application: structure prediction by homology and rational design of protein-variants with improved "washing performance" – the alkaline protease from *Bacillus alcalophilus. J. Biotechnol.* **28,** 31–40 (1993)

194. Beg, Q.K., Saxena, R.K., Gupta, R. Kinetic constants determination for an alkaline protease from *Bacillus mojavensis* using response surface methodology. *Biotechnol. Bioeng.* **78,** 289–295 (2002)

195. Oberoi, R., Beg, Q.K., Puri, S., Saxena, R.K., Gupta, R. Characterization and wash performance analysis of an SDS-resistant alkaline protease from a *Bacillus* sp. *World J. Microbiol. Biotechnol.* **17,** 493–497 (2001)

196. Showell, M.S. *Enzymes, detergent. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol 2.* (Wiley, New York, pp 958–971, 1999)

197. Hasan, A.K.M.Q., Tamiya, E. Cold-active protease CP70. Patent WO9727313 , (1997)

198. Kitayama, M. New low-temperature alkaline protease. Patent JP4271781, (1992)

199. Nielsen, M.H., Jepsen, S.J., Outtrup, H. Enzymes for low temperature washing. *J. Am. Oil. Chem. Soc.* **58,** 644–649 (1981)

200. Moreira, K.A., Porto, T.S., Teixeira, M.F.S., Porto, A.L.F., Filho, J.L.L. New alkaline protease from *Nocardiopsis* sp.: partial purification and characterization. *Process Biochem.* **39(1),** 67-72 (2003)

201. Karbalaei-Heidari, H.R., Ziaee, A.A., Amoozegar, M.A. Purification and biochemical characterization of a protease secreted by the *Salinivibrio* sp. strain AF-2004 and its behavior in organic solvents. *Extremophiles* **11,** 237–243 (2007)

202. Alam, S.I., Dixit, A., Reddy, G.S.N., Dube, S., Palit, M., Shivaji, S., Singh, L. C. *Clostridium schirmacherense* sp.nov. an obligately anaerobic, proteolytic, psychrophilic bacterium isolated from lake sediment of Schirmacher Oasis, Antarctica. *Int. J. Syst. Evol. Microbiol.* **56,** 715-720 (2006)

203. Wang, S.L., Chio, Y-H., Yen, Y-H., Chuan-Lu Wang, C.Lu. Two novel surfactant-stable alkaline proteases from *Vibrio fluvialis* TKU005 and their applications. *Enzyme Microbial Technol.* **40,** 1213–1220 (2007)

204. Chu, W-H. Optimization of extracellular alkaline protease production from species of *Bacillus. J. Ind. Microbiol. Biotechnol.* **34(3),** 241-245 (2007)

205. Lazim, H., Mankai, H., Slama, N., Barkallah, I., Limam, F. Production and optimization of thermophilic alkaline protease in solid-state fermentation by *Streptomyces* sp. CN902. *J. Ind. Microbiol. Biotechnol.* **36,** 531–537(2009)

206. Liang, T-W., Lin, J.J., Yen, Y-H., Wang, C-L., Wang, S-L. Purification and characterization of a protease extracellularly produced by *Monascus purpureus* CCRC31499 in a shrimp and crab shell powder medium. *Enzyme Microbial Technol.* **38(1-2),** 74-80 (2006)

207. Lee, S.H., Chung, C.W., Yu, Y.J., Rhee, Y.H. Effect of alkaline protease-producing *Exiguobacterium* sp. YS1 inoculation on the solubilization and bacterial community of waste activated sludge. *Bioresource Technol.* **100(20),** 4597-4603 (2009)

208. Jellouli, K. *et al.* Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio metschnikovii* J1. *J. Ind. Microbiol. Biotechnol.* **36,** 939–948 (2009)

209. Patel, R.K., Dodia, M.S., Joshi, R.H., Singh, S.P. Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. *Process Biochem.* **41,** 2002–2009 (2006)

210. Shanmughapriya, S. *et al.* Optimization of extracellular thermotolerant alkaline protease produced by marine Roseobacter sp. (MMD040). *Bioprocess Biosyst. Eng.* **31,** 427–433 (2008)

211. Vidyasagar, M., Praskash, S.B., Sreeramulu, K. Optimization of culture conditions for the production of haloalkaliphilic thermostable protease from an extremely halophilic archaeon *Halogeometricum* sp. TSS101. *Lett. Appl.Microbiol.* **43(4),** 385-91 (2006)

212. Shikha, Sharan, A., Darmwal, N.S. Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. *Bioresource Technol.* **98(4),** 881-885 (2007)

213. Kumar, C.G., Joo, H-S, Koo, Y-Mo, Paik, R.S., Chang, C-S. Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate. *World J.Microbiol. Biotechnol.* **20,** 351–357 (2004)

214. Emtiazi, G., Nahvi,I., Maal, B.K. Production and immobilization of alkaline protease by *Bacillus polymyxa* which degrades various proteins. *Int. J. Environ. Sci.* **62(1),** 101-107 (2005)

215. Demir, Y., Alayli, A., Yildirim, S., Demir, N. Identification of protease from *Euphorbia amygdaloides* latex and its use in cheese production. *Preparative Biochem. Biotechnol.* **35(4),** 291-299 (2005)

216. Rahman, RNZAbd, Geok, L.P., Basri, M., Salleh, A.B. Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresource Technol.* **96(4),** 429-436 (2005)

217. Orhan, E., Omay, D., Guvenilir, Y. Partial purification and characterization of protease enzyme from *Bacillus subtilis* and *Bacillus cereus*. *Appl. Biochem. Biotechnol.* **121–124,**183–94(2005)

218. Wang. S.L., Chen, Y.H., Wang, C.L., Yen, Y.H., Chern, M.K. Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. *Enzyme Microbial Technol.* **36,** 660– 665 (2005)

219. Wang, R.B., Yang, J.K., Lin, C., Zhang, K.Q. Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Dactylella shizishanna. Lett. Appl. Microbiol.* **42,** 589–594 (2006)

220. Wang, S.L., Kao, T.Y., Wang, C.L., Yen, Y.H., Chern, M.K., Chen, Y.H. A solvent stable metalloprotease produced by *Bacillus* sp.TKU004 and its application in the deproteinization of squid pen for beta-chitin preparation. *Enzyme Microbial Technol.* **39,** 724–31 (2006)

221. Genckal, H., Tari, C. Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats. *Enzyme Microbial Technol.* **39,** 703–710 (2006)

222. Moradian F., Khajeh, K., Naderi-Manesh, H., Ahmadvand, R., Sajedi, RH, Sadeghizadeh,M. Thiol-dependent serine alkaline proteases from *Bacillus* sp. HR-08 and KR-8102 :Isolation, production, and characterization. *Appl. Biochem. Biotechnol.* **134(1),** 77-87 (2006)

223. Chi, Z., Ma, C., Wang, P., Li, H.F. Optimization of medium and cultivation conditions for alkaline protease production by the marine

yeast *Aureobasidium pullulans*. *Bioresource Technol.* **98(6),** 1238-1245 (2006)

224. Zhu, W., Cha, D., Cheng, G., Peng, Q., Shen, P. Purification and characterization of a thermostable protease from a newly isolated *Geobacillus* sp. YMTC 1049. *J Enzyme Microbial Technol.* **40,** 1592–1597 (2007)

225. Wang, Q., Hou, Y., Xu,Z., Maio, J., G, Li. Optimization of cold-active protease production by the psychrophilic bacterium *Colwellia* sp. NJ341 with response surface methodology. *Bioresource Technol.* **99,** 1926-1931(2007)

226. Manni, L. *et al.* An oxidant- and solvent-stable protease produced by *Bacillus cereus* SV1: application in the deproteinization of shrimp wastes and as a laundry detergent additive. *Appl. Biochem. Biotechnol.* **160(8),** 2308-2321 (2008)

227. Nikerel, I.E., Ates, O., Oner, E.T. Effect of bioprocess conditions on growth and alkaline protease production by halotolerant *Bacillus licheniformis* BA17. *Appl. Biochem. Microbiol.* **44(5),** 487-492 (2008)

228. Jellouli, K., Bayoudh, A., Manni, L., Rym, A., Nasri, M.: Purification, biochemical and molecular characterization of a metalloprotease from *Pseudomonas aeruginosa* MN7 grown on shrimp wastes. *Appl. Microbiol. Biotechnol.* **79,** 989–999 (2008)

229. Tang, X.Y., Pan, Y., Li, S., He, B.F. Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. *Bioresource Technol.* **99,** 7388–7392(2008)

230. Valasaki, K. *et al.* Purification and kinetics of two novel thermophilic extracellular proteases from *Lactobacillus helveticus*, from kefir with possible biotechnological interest. *Bioresource Technol.* **99(13),** 5804-5813 (2008)

231. Wang, S.L. *et al.* Bioconversion of squid pen by *Lactobacillus paracasei* subsp *paracasei* TKU010 for the production of proteases and lettuce growth enhancing biofertilizers. *Bioresource Technol.* **99(13),** 5436-5443 (2008)

232. Wang, S.L., Hsu, W.T., Liang, T.W., Yen, Y.H., Wang, C.L. Purification and characterization of three novel keratinolytic metalloproteases produced by .*Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium. *Bioresource Technol.* **99,** 5679–5686 (2008)

233. Wang, S.L., Yang, C-H, Liang, T-W., Yen, Y-H. Optimization of conditions for protease production by *Chryseobacterium taeanense* TKU001. *Bioresource Technol.* **99(9),** 3700-3707 (2008)

234. Wang, S.L., Chao, C.H., Liang, T.W., Chen, C.C. Purification and characterization of protease and chitinase from *Bacillus cereus* TKU006 and conversion of marine wastes by these enzymes. *Marine Biotechnol.* **11(3),** 334-344 (2009)

235. Wan, M-Y., Wang, H-Y., Zhang, Y-Z., Feng, H. Substrate specificity and thermostability of the dehairing alkaline protease from *Bacillus pumilus Appl.Biochem.Biotechnol.* **159(2),** 394-403 (2009)

236. Moradian, F., Khajeh, K., Naderi-Manesh, H., Sadeghizadeh, M. Isolation, purification and characterization of a surfactants-, laundry detergents- and organic solvents-resistant alkaline protease from *Bacillus* sp. HR-08. *Appl. Biochem. Biotechnol.* **159,** 33–45 (2009)

237. Nilegaonkar, S.S., Kanekar, P.P., Sarnaik, S.S., Kelkar, A.S. Production, isolation and characterization of extracellular protease of an alkaliphilic protease of an alkaliphilic strain of *Arthrobacter ramosus*, MCM B-351 isolated from the alkaline lake of Lonar, India. *World J. Microbiol. Biotechnol.* **18,** 785–789 (2000)

238. Gupta, A. *et al.* One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. *J. Chromato. A.* **1075,** 103–108 (2005)

239. Prakasham, R.S., Subba, Rao Ch., Sreenivas, Rao R., Sarma, P.N. Alkaline protease production by an isolated *Bacillus circulans* under solid-state fermentation using agro-industrial waste: process parameters optimization. *Biotechnol. Prog.* **21,** 1380–8 (2005)

240. Laxman, R.S. *et al.* Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus*. *Process Biochem.* **40,** 3152–3158 (2005)

241. Naidu, K.S.B., Devi, K.L.Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. *J. Biotechnol.* **4,** 724-726 (2005)

242. Kasana, R.C., Yadav, S.K. Isolation of a Psychrotrophic *Exiguobacterium* sp. SKPB5 (MTCC 7803) and Characterization of Its Alkaline Protease. *Curr. Microbiol.* **54(3),** 224-229 (2007)

243. Bhaskar, N., Sudeepa, E.S., Rashmi, H.N., Selvi, A.T. Partial purification and characterization of protease of *Bacillus proteolyticus*-CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresource Technol.* **98,** 2758– 2764 (2007)

244. Zambare, V.P., Nilegaonkar, S.S., Kanekar, P.P. Production of an alkaline protease by *Bacillus cereus* MCM B-326 and its application

as a dehairing agent *World J. Microbiol. Biotechnol.* **23,** 1569–1574 (2007)

245. Jaswal, R.K., Kocherand, G.S., Virk, M.S. Production of alkaline protease by *Bacillus circulans* using agricultural residues: A statistical approach. *Indian J. Biotechnol.* **7,** 356-360 (2008)

246. Kumar, GA., Swarnalatha, S., Sairam, B., Sekaran, G. Production of alkaline protease by *Pseudomonas aeruginosa* using proteinaceous solid waste generated from leather manufacturing industries. *Bioresource Technol.* **99(6),** 1939-44 (2008)

247. Joshi, R.H., Dodia, M.S., Singh, S.P. Production and optimization of a commercially viable alkaline protease from a haloalkaliphilic bacterium *Biotechnol. Bioprocess Eng.* **13,** 552-559 (2008)

248. Mahanta, N., Gupta, A., Khare, S.K.Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation using Jatropha curcas seed cake as substrate. *Bioresource Technol.* **99,** 1729–1735 (2008)

249. Sindhu, R., Suprabha, G.N., Shashidhar, S. Optimization of process parameters for the production of alkaline protease from *Penicillium godlewskii* SBSS 25 and its application in detergent industry. *African J. Microbiol. Research*, **3(9),** 515-522 (2009)

250. Soccol, C.R., Vandenberghe, L.P.S. Overview of applied solid-state fermentation in Brazil. *Biochemical Eng. J.* **13(2-3),** (2003)

251. Couto, S. R., Sanroman, M. A. Application of solid-state fermentation to food industry - A review. *J. Food. Eng.* **76(3),** 291-302 (2006)

252. Aguilar, N.C. *et al.* Perspectives of Solid State Fermentation for Production of Food Enzymes. *American J. Biochem. Biotechnol.* **4 (4),** 354-366 (2008)

253. Singhania, R.R., Patel, A.K., Soccol, C.R. Pandey, A. Recent advances in solid-state fermentation. *Biochemical Eng. J.* **44,** 13–18 (2009)

254. Barrios-Gonzalez, J., Gonzalez, H., Mejia, A. Effect of particle size, packing density and agitation on penicillin production in solid state fermentation. *Biotechnol. Adv.* **11,** 539– 547 (1993)

255. Germano, S., Pandey, A., Osakud, C.A., Rochaa, S.N., Soccol, C.R. Characterization and stability of proteases from *Penicillium* sp. produced by solid-state fermentation. *Enzyme Microbial Technol.* **32,** 246–251(2003)

256. Agrawal, D., Patidar, P., Banerjee, T., Patil, S. Production of alkaline protease by *Penicillium* sp. under SSF condition and its application to soy protein hydrolysis. *Process Biochem.* **39,** 977–81(2004)

257. Uyar, F., Baysal, Z. Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid-state fermentation. *Process Biochem.* **39,** 1893–1898 (2004)

258. Sandhya, C., Sumantha, A., Szakacs, G., Pandey, A. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid state fermentation. *Process Biochem.* **40,** 2689–2694 (2005)

259. Elibola, M., Moreira, A.R. Optimizing some factors affecting alkaline protease production by a marine bacterium *Teredinobacter turnirae*

under solid substrate fermentation. *Process Biochem.* **40,** 1951–1956 (2005)

260. Wang, S., Chen, Y., Wang, C., Yen, Y., Chern, M. Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. *Enzyme Microbial Technol.* **36,** 660-665 (2005)

261. Valeria, F.S., Castilho, L.R., Bon, E.P.S., Freire, D.M.G. High-yield *Bacillus subtilis* protease production by solid-state fermentation. *Appl. Biochem. Biotechnol.* **311,** 121-124 (2005)

262. Prakasham, R.S., Rao, S.Ch., Sarma, P.N. Green gram husk—an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation. *Bioresource Technol.* **97,** 1449–1454 (2006)

263. Wu T.Y., Mohammad, A.W., Md. Jahim J., Anuar N. Investigations on protease production by a wild-type *Aspergillus terreus* strain using diluted retentate of pre-filtered palm oil mill effluent (POME) as substrate. *Enzyme Microbial Technol.* **39,** 1223-1229 (2006)

264. Sathya, R., Pradeep, B.V., Angayarkanni, J., Palaniswamy, M. Production of milk clotting protease by a local isolate of *Mucor circinelloides* under SSF using agro-industrial wastes. *Biotechnol. Bioprocess Eng.* **14,** 788-794 (2009)

265. Haddar, A. *et al.* Low-cost fermentation medium for alkaline protease production by *Bacillus mojavensis* A21 using hulled grain of wheat and sardinella peptone. *J. Biosci. Bioeng.* **110(3),** 288-94 (2010)

266. Gupta, R., Beg, Q.K., Lorenz, P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.* **59,** 15–32 (2002)

267. Lynd, L.R., Weimer, P.J., Zyl, W.H.V., Pretorius, I.S. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. R.* **66(3),** 506–577 (2002)

268. Hongzhang, C., Hui, W., Aijun, Z., L. Zuohu, L. Alkaline protease production by solid-state fermentation on polyurethane foam. *Chem. Biochem. Eng. Q.* **20 (1),** 93–97 (2006)

269. Prakasham, R.S., Sreenivas Rao, R., Hobbs, P.J. Current trends in biotechnological production of xylitol. *Curr. Trend. Biotechnol. Pharm.* **3,** 8–36 (2009)

270. Reddy, G.B. et al. Optimization of production of alkaline protease from Bacillus megaterium NCIM-2087 through solid-state fermentation. *The IUP Journal of Biotechnol.* **3(3),** 46-55 (2009)

271. Paranthaman, R., Alagusundaram, K., Indhumathi, J. Production of protease from rice mill wastes by *Aspergillus niger* in solid state fermentation. *World J.Agri. Sci.* **5 (3),** 308-312 (2009)

272. Agrawal, D., Patidar, P., Banerjee, T., Patil, S. Alkaline protease production by a soil isolate of *Beauveria felina* under SSF condition: parameter optimization and application to soy protein hydrolysis. *Process Biochem.* **40,** 1131–1136 (2005)

273. Aijun, Z., Hingzhang, C., Zuohu, L. Air pressure pulsation solid state production of alkaline protease by *Bacillus pumilus* 1.1625. *Process Biochem.* **40,** 1547–51 (2005)

274. Sumantha, A., Larroche, C., Pandey, A. Microbiology and industrial biotechnology of food-grade proteases. *Food Technol. Biotechnol.* **44(2),** 211-220 (2006)

275. Divakar, G., Sunitha, M., Vasu, P., Shanker, U.P., Ellaiah, P. Optimization of process parameters for alkaline protease production under solid-state fermentation by *Thermoactinomyces thalpophilus* PEE 14. *Indian J.Biotechnol.* **5,** 80-83 (2006)

276. Anandan, D., Marmer, W.N., Dudley, R.L. Isolation, characterization and optimization of culture parameters for production of an alkaline protease isolated from *Aspergillus tamari. J. Ind. Microbiol. Biotechnol.* **34,** 339–347 (2007)

277. Vishwanatha, K.S., Rao, A.G.A., Singh, S.A. Production and characterization of a milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341. *Appl. Microbiol. Biotechnol.* **85(6),** 1849-1859 (2010)

278. Merheb-Dini, C., Gomes, E., Boscolo, M., Silva, R.da. Production and characterization of a milk-clotting protease in the crude enzymatic extract from the newly isolated *Thermomucor indicae-seudaticae* N31 (Milk-clotting protease from the newly isolated *Thermomucor indicae-seudaticae* N31. *Food Chem.* **120,** 87–93 (2010)

279. Kumar, R., Balaji, S., Uma, T.S., Mandal, A.B., Sehgal, P.K. Optimization of influential parameters for extracellular keratinase production by *Bacillus subtilis* (MTCC9102) in solid state fermentation using horn meal—A biowaste management. *Appl. Biochem. Biotechnol.* **160(1),** 30-39 (2010)

280. Gioppo, N.M.R. *et al.* Influence of the carbon and nitrogen sources on keratinase production by *Myrothecium verrucaria* in submerged

and solid state cultures. *J. Ind. Microbiol. Biotech.* **36,** 705–711 (2009)

281. Morsy, M., Gendy, El, A. Keratinase production by endophytic *Penicillium* spp. under solid-state fermentation using rice straw. *Appl.Biochem.Biotechnol.* **162(3),** 780-794 (2010)

282. Ramesh, S., Rajesh, M., Mathivanan, N. Characterization of a thermostable alkaline protease produced by marine *Streptomyces fungicidicus* MML1614. *Bioprocess Biosyst. Eng.* **32,** 791–800 (2009)

283. Kalaiarasi, K., Sunitha, P.U. Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. *African J. Biotechnol.* **8 (24),** 7035-7041(2009)

284. Esakkirai, P., Immanuel, G., Sowmya, S.M., Iyapparaj, P., Palavesam, A. Evaluation of protease-producing ability of fish gut isolate *Bacillus cereus* for aqua Feed. *Food Bioprocess Technol.* **2(4),** 383-390 (2009)

285. Singh, S.K., Agarwal, U.S., Saxena, R. P. Optimization of process parameters for milling of green gram (Phaseolus aureus). *J. Food Sci.Technol.* **41(2),** 124- 30 (2004)

286. Zhou, Li-H. *et al.* Optimization of mycelial biomass and protease production by *Laccocephalum mylittae* in submerged fermentation. *African J. Biotechnol.* **8(8),** 1591-1601(2009)

287. Tari, C., Genckal, H., Tokatl, F. Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. *Process Biochem.* **41,** 659–665 (2006)

288. Amid, M. *et al.* Optimization of serine protease extraction from mango peel (Mangifera Indica Cv. Chokanan). *Food Chem.* 10.1016/j.foodchem.2010.06.027

289. Ramnani, R., Gupta, R. Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG1 using statistical methods involving response surface methodology. *Biotechnol. Appl. Biochem.* **40,** 191–196 (2004)

290. Cai, C., Zheng, X. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology *J. Ind. Microbiol. Biotechnol.* **36,** 875–883 (2009)

291. Thys, R.C.S., Guzzon, S.O., Olivera, F.C., Brandelli, A. Optimization of protease production by *Microbacterium* sp. in feather meal using response surface methodology. *Process Biochem.* **41,** 67–73 (2006)

292. Khan, S. *et al.* Response surface optimization of effective medium constituents for the production of alkaline protease from a newly isolated strain of *Pseudomonas aeruginosa. Indian J. Exp. Biol.* **44,** 151-156 (2006)

293. Singh, A.K., Chhatpar, H.S. Optimization of protease production by *Streptomyces* sp. A6 using statistical approach for reclamation of shellfish waste. *World J. Microbiol. Biotechnol.* **26(9),** 1631-1639 (2010)

294. Liu, S., Fang, Y., Lv, M., Wang, S., Chen, Li. Optimization of the production of organic solvent-stable protease by *Bacillus sphaericus* DS11 with response surface methodology. *Bioresource Technol.* **101(20),** 7924-7929 (2010)

295. Tatineni, R. *et al.* Purification and characterization of an alkaline keratinase from *Streptomyces* sp. *Bioresource Technol.* **99(6),** 1596-1602 (2008)

296. Gupta, R., Ramnani, P. Microbial keratinases and their prospective applications: An overview. *Appl. Microbiol. Biotechnol.* **70,** 21-33 (2006)

297. Brandelli, A., Daniel J. Daroit, D.J., Riffel, A. Biochemical features of microbial keratinases and their production and applications. *Appl. Microbiol. Biotechnol.* **85,** 1735–1750 (2010)

298. Marangoni, A.G. *Enzyme kinetics . A modern approach* (John wiley & sons, inc, 2003)

299. Bisswanger. *Enzyme kinetics- Principles and methods* (John wiley & sons, inc., 2008)

300. Zhou, L. Purification, Characterization and *in vitro* Anthelmintic Activity of a Neutral Metalloprotease from *Laccocephalum mylittae* . *Chinese J. Chemical Eng.* **18(1),** 122-128(2010)

301. Hayet, B.K., Rym, N., Ali, B., Sofiane, G., Moncef, N. Low molecular weight serine protease from the viscera of sardinelle (*Sardinella aurita*) with collagenolytic activity: purification and characterization. *Food Chem.* DOI: 10.1016/j.foodchem.2010.06.096

302. Xu, J., Jiang, M., Sun, H., He, B.F. An organic solvent-stable protease from organic solvent-tolerant *Bacillus cereus* WQ9-2: Purification, biochemical properties, and potential application in peptide synthesis. *Bioresource Technol.* **101,** 7991–7994 (2010)

303. Doddapaneni, K.K., Radhika, T.,.Ravichandra, P., Narasu, L.M. Optimization of media constituents through response surface methodology for improved production of alkaline proteases by

Serratia rubidaea. *J. Chemical Technol. Biotechnol.* **82(8),** 721-729 (2007)

304. Abidi, F., Limam, F., Marzouki, M.N.Purification and characterization of an alkaline protease prot 1 from *Botrytis cinerea* biodetergent catalyst assay. *Appl. Biochem. Biotechnol.* **141,** 361-376 (2007)

305. Pena-Montes, C., Gonzalez, A., Castro-Ochoa, D., Farres, A. Purification and biochemical characterization of a broad substrate specificity thermostable alkaline protease from *Aspergillus nidulans*. *Appl. Microbiol. Biotechnol.* **78,** 603–612 (2008)

306. Sareen, R., Mishra, P. Purification and characterization of organic solvent stable protease from *Bacillus licheniformis* RSP-09–37. *Appl. Microbiol. Biotechnol.* **79,** 399–405 (2008)

307. Zhu, H-Y, Tian, Y., Hou, Y-H., Wang, T-H. Purification and characterization of the cold-active alkaline protease from marine cold-adaptive *Penicillium chrysogenum* FS010. *Mol. Biol. Rep.* **36,** 2169–2174(2009)

308. Rao, S. Ch., Sathish, T., Ravichandra, P., Prakasham, R.S. Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. *Process Biochem.* **44,** 262–268(2009)

309. Moreira, F.G. *et al.* Production of hydrolytic enzymes by the plant pathogenic fungus *myrothecium verrucaria* in submerged cultures. *Brazilian J. Microbiol.* **36,** 7-11 (2005)

310. Deng, A., Wu, Jie., Zhang, Y., Zhang, G., Wen, T. Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. *Bioresource Technol.* **101,** 7100–7106 (2010)

311. Olivera, N., Sequeiros, C., Sineriz, F., Breccia J.D.Characterization of alkaline proteases from a novel alkali-tolerant bacterium *Bacillus patagoniensis* . *World J. Microbiol. Biotechnol.* **22(7),** 737-743 (2005)

312. Arulmani, M. *et al.* Purification and partial characterization of serine protease from thermostable alkalophilic *Bacillus laterosporus*-AK1. *World J Microbiol. Biotechnol.* **23,** 475–481(2006)

313. Kuddus, M., Ramteke, P.W. Purification and properties of cold-active metalloprotease from *Curtobacterium luteum* and effect of culture conditions on production. *Chinese J. Biotechnol.* **24(12),** 2074-2080(2008)

314. Ghosh, A., Chakrabarti, K., Chattopadhyay, D. Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. *J. Ind. Microbiol. Biotech.* **35,** 825–834 (2008)

315. Jaouadi, B. *et al.* Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp. strain AB1 with high stability in organic solvents. *Bioresource Technol.* **101,** 8361–8369 (2010)

316. Lee, H., Suh, D.B., Hwang, J.H., Suh, H.J. Characterization of a keratinolytic metalloprotease from *Bacillus* sp. SCB-3. *Appl. Biochem. Biotechnol.* **97,** 123–133 (2002)

317. Xie, F. *et al.* Purification and characterization of four keratinases produced by *Streptomyces* sp. strain 16 in native human foot skin medium. *Bioresource Technol.* **101,** 344–350 (2010)

318. Bressollier, P., Letourneau, F., Urdaci, M., Verneuil, B. Purification and characterization of a keratinolytic serine proteinase from

Streptomyces albidoflavus. *Appl. Environ. Microbiol.* **65,** 2570–2576 (1999)

319. Lin, X., Lee, C.G., Casale, E.S., Shih, J.C.H. Purification and characteriztion of a keratinase from a feather-degrading *Bacillus licheniformis* strain. *Appl. Environ. Microbiol.* **58,** 3271–3275 (1992)

320. Suh, H.J., Lee, H.K. Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-1. *J. Protein Chem.* **20,** 165–169 (2001)

321. Rozs, M., Manczinger, L., Vágvölgyi, C., Kevei, F. Secretion of a trypsin-like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*. *FEMS Microbiol. Lett.* **205,** 221–224 (2001)

322. Farag, A.M., Hassan, M.A. Purification, characterization and immobilization of a keratinase from *Aspergillus orizae*. *Enzyme Microbial Technol.* **34,** 85–93 (2004)

323. Anbu, P., Gopinath, S.C.B., Hilda, A., Lakshmipriya, T., Annadurai, G. Purification of keratinase from poultry farm isolate-*Scopulariopsis brevicaulis* and statistical optimization of enzyme activity. *Enzyme Microbial Technol.* **36,** 639–647 (2005)

324. Suntornsuk, W. *et al.* Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium. *World J. Microbiol. Biotechnol.* **21,** 1111–1117 (2005)

325. Yoshioka, M. *et al.* Characterization of a proteolytic enzyme derived from a *Bacillus* strain that effectively degrades prion protein. *J. Appl. Microbiol.* **102,** 509–515 (2007)

326. Balaji, S. *et al.* Purification and characterization of an extracellular keratinase froma hornmeal-degrading *Bacillus subtilis* MTCC (9102). *World J. Microbiol. Biotechnol.* **24,** 2741–2745 (2008)

327. Wang, S.L., Hsu, W.T., Liang, T.W., Yen, Y.H., Wang, C.L. Purification and characterization of three novel keratinolytic metalloproteases produced by *Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium. *Bioresource Technol.* **99,** 5679–5686 (2008)

328. Cai, C.G., Chen, J.S., Qi, J.J., Yin, Y., Zheng, X.D. Purification and characterization of keratinase from a new *Bacillus subtilis* strain. *J. Zhejiang Univ. Sci. B* **9,** 713–720 (2008)

329. Fakhfakh, N., Kanoun, S., Manni, L., Nasri, M. Production and biochemical and molecular characterization of a keratinolytic serine protease from chicken feather-degrading *Bacillus licheniformis* RPk. *Can. J. Microbiol.* **55,** 427–436 (2009)

330. Gasparin, M.G.F. *et al.*Purification and characterization of an efficient poultry feather degrading-protease from *Myrothecium verrucaria. Biodegradation,* 20(5), 727-736 (2009)

331. Halling, P.J., Dunnill, P. Magnetic supports for immobilized enzymes and bioaffinity adsorbents. *Enzyme Microbial Technol.* **2(1),** 2-10 (1980)

332. Wu, J., Wang, J-L., Li, M-H., Lin, J-P., Wei, D-Z.Optimization of immobilization for selective oxidation of benzyl alcohol by *Gluconobacter oxydans* using response surface methodology. *Bioresource Technol.* **101(23),** 8936-8941 (2010)

333. Sadjadi, M.S., Farhadyar, N., Zare, K. Synthesis of bi-metallic Au–Ag nanoparticles loaded on functionalized MCM-41 for immobilization of alkaline protease and study of its biocatalytic activity. *Supperlatt. Microstruct.* **46(4),** 563-571 (2009)

334. Bakhitar, S., Estiveira, RJ., Hatti-Kaul. Substrate specificity of alkaline protease from alkaliphilic feather-degrading *Nesterenkonia* sp. AL20. *Enzyme Microbial Technol.* **37,** 534-540 (2005)

335. Kim, S.B. *et al.* Purification and characterization of a fibrinolytic subtilisin-like protease of *Bacillus subtilis* TP-6 from an Indonesian fermented soybean, Tempeh. *J.Ind. Microbiol. Biotechnol.* **33(6),** 436-44 (2006)

336. Wang, C.T. *et al.* Purification and characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional Douchi. *J. Ind. Microbiol. Biotechnol.* **33,** 750–758 (2006)

337. Wang, H.Y. *et al.* Screening and mutagenesis of a novel *Bacillus pumilus* strain producing alkaline protease for dehairing. *Lett. Appl.Microbiol.* **44(1),** 1-6 (2007)

338. Kim, J-B. *et al.* Identification of a fibrinolytic enzyme by *Bacillus vallismortis* and its potential as a bacteriolytic enzyme against *Streptococcus* mutans. *Biotechnol. Lett.* **29,** 605–610 (2007)

339. Nilegaonkar, S.S., Zambare, V.P., Kanekar, P.P., Dhakephalkar, P.K., Sarnaik, S.S. Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326.*Bioresource Technol.* **98(6),** 1238-1245 (2007)

340. Sellami-Kamoun, A., Haddar, A., Ali, N., Ghorbel-Frikha, B., Nasri, M. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. *Microbiol.Research,* **163(3),** 299-306 (2008)

341. Wang, S.H., Zhang, C., Yang, Y.L., Diao, M., Bai, M.F. Screening of a high fibrinolytic enzyme producing strain and characterization of

the fibrinolytic enzyme produced from *Bacillus subtilis* LD-8547. *World J. Microbiol. Biotechnol.* **24,** 475–482 (2008)

342. Hua, Y., Jiang, B., Mine, Y., Mu, W. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste. *J.Agr.Food.Chem.* **56,** 1451-1457(2008)

343. Cui, L. *et al.* A novel fibrinolytic enzyme from *Cordyceps militaris*, a Chinese traditional medicinal mushroom. *World J.Microbiol. Biotechnol*, **24,** 483-489(2008)

344. Kumar, A.G., Nagesh, N., Prabhakar, T.G., Sekaran, G. Purification of extracellular acid protease and analysis of fermentation metabolites by synergists sp. utilizing proteinaceous solid waste from tanneries. *Bioresource Technol.* **99(7),** 2364-72(2008)

345. Rovati, J.I., Delgado, O.D., Lucıa I. C. Figueroa, L.I.C., Farin, J.I. A novel source of fibrinolytic activity: *Bionectria* sp., an unconventional enzyme-producing fungus isolated from Las Yungas rainforest (Tucuman, Argentina). *World J. Microbiol. Biotechnol.* DOI 10.1007/s11274-009-0142-z

346. Shieh, C.J., Phan Thi, L. A., Shih, I. L. Milk-clotting enzymes produced by culture of *Bacillus subtilis* natto. *Biochemical Eng. J.* **43,** 85–91(2009)

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

348. Deng, Z. *et al.* Purification and characterization of a novel fibrinolytic enzyme from the polychaete, *Neanthes japonica* (Iznka). *Bioresource Technol.* **101,** 1954–1960 (2010)

349. Choudhary, R.B., Jana, A.K., Jha, M.K. Enzyme technology applications in leather processing. *Indian J. Chemical Technol.* **11,** 659-671 (2004)

350. Lin, X., Shih, J.C.H., Swaisgood, H.E. Hydrolysis of feather keratin by immobilized keratinase. *Appl. Environ. Microbiol.* **62,** 4273–4275 (1996)

351. Riffel, A., Lucas, F., Heeb, P., Brandelli, A. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. *Arch. Microbiol.* **179,** 258–265 (2003)

352. Friedrich, J., Gradisar, H., Vrecl, M., Pogacnik, A. In vitro degradation of porcine skin epidermis by a fungal keratinase of *Doratomyces microsporus. Enzyme Microbial Technol.* **36,** 455–460 (2005)

353. Ulfig, K., Plaza, G., Terakowskip, M., Janda-Ulfig, K. Sewage sludge open-air drying effects on keratinolytic, keratinophilic and actidione- resistant fungi. *Rocz Panstw Zakl Hig* **57,** 371-379(2006)

354. Grazziotin, A., Pimentel, F.A., de Jong, E.V., Brandelli, A. Nutritional improvement of feather protein by treatment with microbial keratinase. *Anim. Feed Sci. Technol.* **126,** 135–144 (2006)

355. Tapia, D.M.T., Simoes, M.L.G. Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater. *African J. Biotechnol.* **7,** 296–300 (2008)

356. Tamilmani, P., Umamaheswari. A., Vinayagam, A., Prakash, B. Production of an extra-cellular feather-degrading enzyme by

Bacillus licheniformis isolated from poultry farm soil in Namakkal District (Tamilnadu). *Int. J. Poult. Sci.* **7(2),** 184–188 (2008)

357. Matsui, T., Yamada, Y., Mitsuya, H., Shigeri, Y., Yoshida, Y., Saito, Y., Matsui, H., Watanabe, K. Sustainable and practical degradation of intact chicken feathers by cultivating a newly isolated thermophilic *Meiothermus ruber* H328. *Appl. Microbiol. Biotechnol.* **82,** 941–950 (2009)

358. Khardenavis, A.A., Kapley, A., Purohit, H.J. Processing of poultry feathers by alkaline keratin hydrolyzing enzyme from *Serratia* sp. HPC 1383. *Waste Manage.* **29,** 1409–1415 (2009)

359. Vesela, M., Friedrich, J. Amino acid and soluble protein cocktail from waste keratin hydrolyzed by fungal keratinase of *Paecilomyces marquandii. Biotechnol. Bioprocess Eng.* **14(1),** 84-90 (2009)

360. Prakash, P., Jayalakshmi, S.K., Sreeramulu, K. Production of keratinase by free and immobilized cells of *Bacillus halodurans* strain PPKS-2: partial characterization and its application in feather degradation and dehairing of the goat skin. *Appl. Biochem. Biotechnol.* **160(7),** 1909-1920(2010)

361. Mukherjee, A.K., Das, K. Correlation between diverse cyclic lipopetides production and regulation of growth and substrate utilization by *Bacillus subtilis* strain in a particular habitat. *FEMS Microbiol. Ecol.* **54,** 479–489 (2005)

362. www.epa.gov/oust/cat/mason.pdf .Preparation of soil sampling protocols: sampling techniques and strategies. Accessed on 9th April, 2010

363. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193,** 276–275 (1951)

364. Das, K., Doley, R., Mukherjee, A.K. Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular α-amylase from *Bacillus subtilis* DM-03, isolated from the traditional fermented food of India. *J. Biotechnol. Appl. Biochem.* **40,** 291–298 (2004)

365. Mukherjee, A.K. Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis* strains in laundry detergent formulations. *Lett. Appl. Microbiol.* **45,** 330–335 (2007)

366. Aneja, K.R. *Experiments in microbiology, plant pathology, tissue culture and mushroom cultivation,. 2nd edition* (Wishwa prakashan, New age International (p) Limited, New delhi, 1996)

367. Cappuccino, J.G., Sherman, N. *Microbiology: A Laboratory Manual 4th edition* (Addison-Wesley Longman, Inc, 1999)

368. Castenholz, R.W., Garrity, G.M. *Bergey's Manual of Systematic Bacteriology Volume 1: The Archaea and the Deeply Branching and Phototrophic Bacteria*_(Springer 2nd ed., 2001)

369. Staley, J.T. *et al. Bergey's Manual® of Systematic Bacteriology: Volume Two: The Proteobacteria (Part C) (Bergey's Manual of Systematic Bacteriology*)(Springer-Verlag, 2nd edition, 2005)

370. Pandey, K.K., Mayilraj, S., Chakrabarti, T. *Pseudomonas indica* sp. nov., a novel butane-utilizing species. *Int. J. Syst. Evol. Microbiol.* **52,** 1559–1567 (2002)

371. Shida, O., Takagi, H., Kadowaki, K., Nakamura, L.K., Komagata, K. Transfer of *Bacillus alginolyticus, Bacillus chondroitinus, Bacillus*

curdlanolyticus, Bacillus glucanolyticus, Bacillus kobensis, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus. Int. J. Syst. Bacteriol.* **47,** 289– 298 (1997)

372. Mukherjee, A.K. Fatty acid composition of four potential aquatic weeds and their possible use as fish-feed neutraceuticals. *Food Chem.* **123,** 1252–1254 (2010)

373. Yuste, L. *et al.* Characterization of bacterial strains able to grow on high molecular mass residues from crude oil processing. *FEMS Microbiol. Ecol.* **32(1),** 69-75 (2000)

374. Ausubel, F.M., Brent, R., Kinston, R.E., Moorse, D.D., Seidman, J.D., Smith, J.A., Struhl, K. *Short protocols in molecular biology.* (Wiley, New York, pp 212–214, 1995)

375. Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173(2),** 697-703 (1991)

376. Tamura, K., Dudley, J., Nei, M., Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24,** 1596–1599 (2007)

377. Saitou, N., Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4,** 406–425 (1987)

378. Thompson, J.D., Higgins, D.G., Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22,** 4673–4680 (1994)

379. Winker, S., Woese, C. R. A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. *Syst. Appl. Microbiol.* **14,** 305-310 (1991)

380. Krishna, P., Arora, A., Reddy, M.S. An alkaliphilic and xylanolytic strain of actinomycetes Kocuria sp. RM1 isolated from extremely alkalin ebauxite residue sites. *World J.Microbiol. Biotechnol.* **24(12),** 3079-3085(2008)

381. Pettersson, B., Rippere, E.K., Yousten, A.A., Priest, F.G. Transfer of *Bacillus lentimorbus* and *Bacillus popilliae* to the genus *Paenibacillus* with emended descriptions of *Paenibacillus lentimorbus* comb. nov. and *Paenibacillus popilliae* comb.nov. *Int. J. Syst. Bacteriol.* **49,** 531–540 (1999)

382. Plackett, R.L., Burman, J.P. The design of optimum multifactorial experiments. *Biometrika* **33,** 305–325 (1946)

383. Laemmli, U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227,** 680–685 (1970)

384. Mazotto, A.M. *et al.* Keratinolytic activity of *Bacillus subtilis* AMR using humanhair *Lett. Appl. Microbiol.* **50,** 89–96 (2010)

385. Thangam, E.B., Rajkumar, S.G. Purification and characterization of alkaline protease from *Alkaligenes faecalis. Biotechnol. Appl. Biochem.* **35,** 149–154 (2002)

386. Kim, S.H., Choi, N.S., Lee, W.Y. Fibrin zymography: a direct analysis of fibrinolytic enzymes on gels. *Anal. Biochem.* **263,**115–6 (1998)

387. Kleiner, D.E., Stetler-Stevenson, W.G. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem.* **218,** 325–329 (1994)

388. Tatineni, R. *et al.* Purification and characterization of an alkaline keratinase from *Streptomyces* sp. *Bioresource Technol.* **99,** 1596-1602 (2008)

389. Mukherjee, A.K., Maity, C.R. The composition of *Naja naja* venom samples from three districts of West Bengal, eastern India. Comp. *Biochem. Physiol.* **119A,** 621–627 (1998)

390. Palmer, T. *Enzymes: biochemistry, biotechnology and clinical chemistry*. (Horwood, 2004)

391. Rossi, L.M., Quach, A.D., Rosenzweig, Z. Glucose oxidase–magnetite nano-particle bio-conjugate for glucose sensing. *Anal. Bioanal. Chem.* **380(4),** 606-613 (2004)

392. Haaland, P.D. *Experimental design in biotechnology* (statistics: A series of textbooks and monographs, CRC Press; 1 edition, 1999)

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

394. Doley, R., Mukherjee, A.K. Purification and characterization of an anticoagulant phospholipase A2 from Indian monocled cobra (*Naja kaouthia*) venom. *Toxicon* **41,** 81–91 (2003)

395. Huang, Q., Peng, Y., Li, X., Wang, H., Zhang, Y. Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus. Curr. Microbiol.* **46,** 169–173 (2003)

396. Bulet, P. *et al.* Insect immunity: Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. *J. Biol. Chem.* **266,** 24520-24525 (1991)

397. Ehret-Sabatier, L. Characterization of novel cysteine-rich antimicrobial peptides from scorpion blood. *J. Biol. Chem.* **271,** 29537-29544 (1996)

398. Haeberli,S., Kuhn-Nentwig, L., Johann Schaller, J., Nentwig, W. Characterization of antibacterial activity of peptides isolated from the venom of the spider *Cupiennius salei (Araneae: Ctenidae)*. *Toxicon* **38,** 373-380 (2000)

399. Ramnani, P., Gupta, R. Keratinases vis-à-vis conventional proteases and feather degradation. *World J. Microbiol. Biotechnol.* **23,** 1537–1540 (2007)

400. Williams, C.M., Richter, C.S., Mackenzie, J.M., Shih, J.C.H. Isolation, identification and characterization of a feather-degrading bacterium. *Appl. Environ. Microbiol.* **56,** 1509–1515 (1990)

401. Priest, F. G. Extracellular enzyme synthesis in the genus *Bacillus. Bacteriol. Rev.* **41,** 711-753 (1977)

402. Brar, S.K. *et al. Bacillus thuringiensis* proteases: production and role in growth sporulation and synergism. *Process Biochem.* **42,** 773–790 (2007)

403. Prakash, O. *et al.* Polyphasic approach of bacterial classification – An overview of recent advances. *Indian J. Microbiol.* **47,** 98–108 (2007)

404. Cohan, F.M., Perry, E.B. A systematics for discovering review the fundamental units of bacterial diversity. *Curr. Biol.* **17,** 373–386 (2007)

405. Wayne, L. G. *et al.* Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37,** 463- 464 (1987)

406. Lee, K.D., Kim, J.H., Kim, H. Isolation and characterization of *Bacillus* sp. KD1014 producing carboxymethyl-cellulase. *The J. Microbiol.* **34(4),** 305-310, (1996)

407. Guerra-cantera, M.A.R.V., Raymundo, A.K. Utilization of a polyphasic approach in the taxonomic reassessment of antibiotic- and enzyme-producing *Bacillus* sp. isolated from the Philippines. *World J. Microbiol. Biotechnol.* **21,** 635-644 (2005)

408. On, S.L.W., Holmes, B. Reproducibility of tolerance tests those are useful in the identification of *compylobacteria. J. Clin. Microbiol.* **29,**1785–1788 (1991)

409. On, S.L.W., Holmes, B. Assessment of enzyme detection tests useful in identification of *campylobacteria*. **30,** 746–749 (1992)

410. Ivanova, E.P. *et al.* Phenotypic diversity of *Pseudoalteromonas citrea* from different marine habitats and emendation of the description. *Int. J. Syst. Bacteriol.* **48,** 247–256 (1998)

411. Brown, B.J., Leff, L.G. Comparison of fatty acid methyl ester analysis with the use of API 20E and NFT strips for identification of aquatic bacteria. *Appl. Environ. Microbiol.* **62(6),** 2183–2185 (1996)

412. Quezada, M., Buitron, G., Moreno-Andrade, I., Moreno, G., pez-Marin, M.L.M.Lo. The use of fatty acid methyl esters as biomarkers to determine aerobic, facultatively aerobic and anaerobic communities in wastewater treatment systems. *FEMS Microbiol. Lett.* **266,** 75–82 (2007)

413. Kaneda, T. Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol. Rev.* **41,** 391–418 (1977)

414. Roberts, M.S., Nakamura, L.K., Cohan, F.M. *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. *Int. J. Syst. Bacteriol.* **44**, 256-264 (1994)

415. Graham, P. H. *et al.* Differences among strains of *Bradyrhizobium* in fatty acid-methyl ester analysis. *Can. J. Microbiol.* **41,** 1038–1042 (1995)

416. Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J. Determinants of soil microbial communities: effects of agricultural management management, season, and soil type on phospholipid fatty acid profiles. *Microb. Ecol.* **36,** 1–12 (1998)

417. Pace, NR. A Molecular View of Microbial Diversity and the Biosphere.
Science **276 (5313),** 734 – 740 (1997)

418. Stackebrandt, E., Goebel, B.M. Taxonomic note: a place for DNA–DNA reassociation and 16S-rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44,** 846–849 (1994)

419. Gram, H.C. U ber die isolierte Farbung der Schizomyceten in Schnittund Trockenpra paraten. *Fortschr. Med.* **2,** 185–189 (in German) (1884)

420. Stackebrandt, E., Koch, C., Gvozdiak, O., Schumann, P. Taxonomic dissection of the genus *Micrococcus: Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *Int. J. Syst. Bacteriol.* **45,** 682–692 (1995)

421. Kovacs, G. *et al. Kocuria palustris* sp. nov. and *Kocuria rhizophila* sp. nov., isolated from the rhizoplane of the narrow-leaved cattail (*Typha angustifolia*). *Int. J. Syst. Bacteriol.* **49,** 167–173 (1999)

422. Reddy, G.S.N. *et al. Kocuria polaris* sp. nov., an orange-pigmented psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. *Int. J. Syst. Evol. Microbiol.* **53,** 183–187 (2003)

423. Li, W-J. *et al. Kocuria aegyptia* sp. nov., a novel actinobacterium isolated from a saline, alkaline desert soil in Egypt. *Int. J. Syst. Evol. Microbiol.* **56,** 733–737 (2006)

424. Palmisano, M.M., Nakamura, L.K., Duncan, K.E., Istock, C.A., Cohan, F.M. *Bacillus sonorensis* sp.nov., a close relative of *Bacillus licheniformis*, isolated from soil in the sonoran desert, Arizona. *Int. J. Syst. Evol. Microbiol.* **51,** 1671-1679(2001)

425. Chou, J-H. *et al. Paenibacillus fonticola* sp.nov., isolated from a warm spring. *Int. J. Syst. Evol. Microbiol.* **57,** 1346-1350 (2007)

426. Chou, J-H. *et al. Paenibacillus contaminants* so.nov. isolated from a contaminated laboratory plates. *Int. J. Syst. Evol. Microbiol.* **59,** 125-129 (2009)

427. Miranda, C.A.C., Martins, O.B., Clementino, M.M. Species-level identification of Bacillus strains isolates from marine sediments by conventional biochemical, 16S-rRNA gene sequencing and inter-tRNA gene sequence lengths analysis. *Antonie van Leeuwenhoek* **93,** 297-304 (2008)

428. Horani, H.K., Priest, F.G. Thermotolerant varieties of *Bacillus licheniformis* isolated from desert environments. *J Appl. Bact.* **77,** 392-400(1994)

429. Gatson, J.W. *et al. Bacillus tequilensis* sp.nov., isolated from a 2000-year-old Mexican shaft-tomb is closely related to *Bacillus subtilis. Int. J. Syst. Evol. Microbiol.* **56,** 1475-1484 (2006)

430. Shivaji, S. *et al. Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. *Int. J. Syst. Evol. Microbiol.* **56,** 1465–1473 (2006)

431. Ivanova, E.P. *et al.* Characterization of *Bacillus* strains of marine origin. *Int.Microbiol.***2,** 267-271 (1999)

432. Holker, U., Lenz, J. Solid-state fermentation-are there any biotechnological advantages? *Curr. Opin. Microbiol.* **8,** 301–306 (2005)

433. Nigam, P., Singh, D. Solid-state (substrate) fermentation systems and their applications in biotechnology. *J. Basic Microbiol.* **34,** 405–423 (1994)

434. Robinson, T., Singh, D., Nigam, P. Solid-state fermentation: A promising microbial technology for secondary metabolite production. *Appl. Microbiol. Biotechnol.* **55,** 284-289 (2001)

435. Das, K., Mukherjee, A.K. Characterization of biochemical properties and biological activities of biosurfactants produced by *Pseudomonas aeruginosa* mucoid and non-mucoid strains isolated from hydrocarbon-contaminated soil samples. *Appl. Microbiol. Biotechnol.* **69,** 192–199 (2005)

436. Gaffney, J.F. Ecophysiological and technological factors influencing the management of cogon (*Imperata cylindrica*), Dissertation, Agronomy Department, University of Florida, Gainesville, FL, 1996

437. Babu, K.R., Satyanarayana, T. Production of bacterial enzymes by solid- state fermentation. *J. Ind. Sci. Res.* **55,** 464–467 (1996)

438. Kaur, S., Vohra, R.M., Kapoor, M., Beg, Q.K., Hoondal, G.S. Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus* sp. P-2. *World J. Microbiol. Biotechnol.* **17 (2),** 125–129 (2001)

439. Aikat, K., Bhattacharyya, B.C. Protease extraction in solid-state fermentation of wheat.bran by a loçal strain of *Rhizopus oryzae* and growth studies by the soft gel technique. *Process Biochem.* **35,** 907–914 (2000)

440. Singh, J., Vohra, R.M., Sahoo, D.K. Enhanced production of alkaline protease by *Bacillus sphaericus* using fed-batch culture. *Process Biochem.* **39,** 1093–1101(2004)

441. Ramachandran, S. *et al.* Coconut oil cake—a potential ray material for the production of α-amylase. *Bioresource Technol.* **93,** 169–174 (2004)

442. Pandey, A., Soccol, C.R., Mitchell, D. New developments in solid-state fer- mentation. I. Bioprocesses and applications. *Process Biochem.* **35,** 1153–1169 (2000)

443. Chakraborty, R., Srinivasan, M.J. Production of a thermo stable alkaline protease by a new *Pseudomonas* sp. by solid-substrate fermentation. *J. Microbiol. Technol.* **8,** 7–16 (1993)

444. Gautam, P., Sahu, A., Pandey, A., Szackaes, G., Soccol, C.R. Microbial production of extracellular phytase using polystyrene as inert support. *Bioresource Technol.* **83,** 229–233 (2002)

445. Murthy, P.P., Naidu, M.M. Protease production by *Aspergillus oryzae* in solid-state fermentation utilizing coffee by-products. *World Appl. Sci. J.* **8(2),** 199-205 (2010)

446. Marshall, E.K., David, A.S., Mark, J.A. Carbon-to-nitrogen ratio, carbon concentration, and amino acid composition of growth media influence conidiation of *Helminthosporium solani. Mycologia* **90,** 406–413 (1998)

447. Salwanis, Md.Z.W.W. *et al.* Production of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp.TS1-1: optimization of carbon and nitrogen con- centration in the feed medium using central composite design. *Biochemical Eng. J.* **33,** 26–33 (2007)

448. Papadopoulos, M.C., El Boushy, A.R., Roodbeen, A.E., Ketelaars, E.H. Effects of processing time and moisture content on amino acid composition and nitrogen characteristics of feather meat. *Anim. Feed Sci. Technol.* **14,** 279–290 (1986)

449. Shih, J.C.H. Recent development in poultry waste digestion and feather utilization-a review. *Poultry.Sci.* **72,** 1617-1620 (1993)

450. El-Refai, H.A., AbdelNaby, M.A., Gaballa, A., El-Araby, M.H., Abdel Fattah, A.F. Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. *Process Biochem.* **40,** 2325-2332 (2005)

451. Grazziotin, A., Pimentel, F. A., de Jong, E. V., Brandelli, A. Nutritional improvement of feather protein by treatment with microbial keratinase. *Animal Feed Sci.Technol.* **126,** 135–144 (2006)

452. Das, K., Mukherjee, A.K. Comparison of lipopeptide biosurfactants production by *Bacillus subtilis* strains in submerged and solid state fermentation systems using a cheap carbon source: some industrial

applications of biosurfactants. *Process Biochem.* **42,** 1191–1199 (2007)

453. Raimbault, M., Alazard, D. Culture method to study fungi growth in solid fer- mentation. *Appl. Microbiol. Biotechnol.* **9,**199–209 (1980)

454. Ikasari, L., Mitchelli, D.A. Leaching and characterization of Rhizopus oligosporous acid protease from solid-state fermentation. *Enzyme Microbial Technol.* **19,** 171–175 (1994)

455. Friedrich, A.B., Antranikian, G. Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilic anaerobic species of the order Thermatogales. *Appl. Environ. Microbiol.* **61,** 3705–3710 (1996)

456. Anbu, P., Gopinath, S.C.B., Hilda, A., Lakshmipriya, T., Annadurai, G. Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis. Bioresource Technol.* **98,** 1298–1303 (2007)

457. Gessesse, A., R, H-K., Gashe, B.A., Mattiasson, B. Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme Microbial Technol.* **32,** 519–524 (2003)

458. Kole, M.M., Draper, L., Gerson, D.F. Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentration. *J. Chem. Technol. Biotechnol.* **41,** 197–206 (1998)

459. Frankena, J., Koningstein, C.M., Van verseveld, H.W., Stouthamer, A.H. Effect of different limitations in chemostat cultures on growth and production of exo- cellular protease by *Bacillus licheniformis. Appl. Microbiol. Biotechnol.* **24,** 106–112 (1986)

460. Prakash, P., Jayalakshmi, S.K., Sreeramulu, K. Production of keratinase by free and immobilized cells of *Bacillus halodurans* strain PPKS-2: partial characterization and its application in feather degradation and dehairing of the goat skin. *Appl. Biochem. Biotechnol.* **160,** 1909–1920 (2010)

461. Viniegra-González, G. *et al.* Advantages of fungal enzyme production in solid state over liquid systems. *Biochemical Eng. J.* **13,** 157-167 (2003)

462. Aguilar, C.N., Favela-Torres, E., Viniegra- González, G., Augur, C. Culture conditions dictate protease and tannase production in submerged and solid-state cultures *Aspergillus niger* Aa-20. *Appl. Biochem. Biotechnol.* **102-103,** 407-414(2002)

463. Krishna, C. Solid-state fermentation systems-an overview. *Crit. Rev. Biotechnol.* **25(1/2),** 1-30 (2005)

464. Singhania, R.R., Patel, A.K., Soccolc, C.R., Pandey, A. Recent advances in solid-state fermentation. *Biochemical Eng. J.* **44,** 13–18 (2009)

465. González, G.V., Favela-Torres, E., Aguilar, C.N., Romero-Gomez, S., Díaz-Godinez, G., Augur, C. 2003. Advantages of fungal enzyme production in solid state over liquid systems. *Biochemical. Eng. J.* **13,** 157-167 (2003)

466. Holker, U.M., Hofer, M., Lenz, J. Biotechnological advantages of laboratory-scale solid state fermentation with fungi. *Appl. Microbiol. Biotechnol.* **64,** 175-186(2004)

467. Wang, Y., Lee, M. Influence of culture and nutritional condition on the production of protease from Thermophilic strain *Aspergillus*

species NTIJ-FC-671. *J.Chinese Agric.Chemical.Society.* **34,** 732-742 (1996)

468. Nehra, K.S., Dhillon, S., Chaudhary, K., Singh, R. Production of alkaline protease by *Aspergillus* species under submerged and solid state fermentation. *Ind.J. Microbiol.* **42,** 43-47 (2002)

469. Priest F.S. Extracellular enzyme synthesis in the genus *Bacillus. Bacteriol. R* **41(3),** 711-753 (1977)

470. Mongi, F., Edward, C., William, H., Gwang- Hoon, G., Almadidy, A. Influence of culture parameters on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021. *World J. Microbiol . Biotechnol.* **21,** 855-862 (2005)

471. Sinha, N, Satyanarayana, T. Alkaline protease production by thermophilic *Bacillus licheniformis. Indian J. Microbiol.* **31(4),** 425 – 430 (1991)

472. Banerjee, R., Bhattacharyya, B. Extracellular alkaline protease by newly isolated *Rhizopus oryzae. Biotechnol. Lett.* **14,** 301-304 (1992)

473. Manni, L. *et al.* An oxidant- and solvent-stable protease produced by Bacillus cereus SV1: Application in the deproteinization of shrimp wastes and as a laundry detergent additive. *Appl. Biochem. Biotechnol.* **160,** 2308–2321(2010)

474. Cohen, B.L. Ammonium repression of extracellular protease in *Aspergillus nidulans. J. General Microbiol.***71,** 293- 299 (1972)

475. Cohen B.L. The neutral and alkaline proteases of *Aspergillus nidulans. J. General Microbiol. **77,** 521-528 (1973)*

476. Pateman, J.A., Kinghorn, J.R., Etta Dunn, E., Forbes, E. Ammonium regulation in *Aspergillus nidulans.* J. Bacteriol. 114(3), 943–950 (1973)

477. Daniel, W.W. *A foundation for analysis in the health sciences. Hypothesis testing. In: Daniel WW (ed) Biostatistics, 7th edn.* (Wiley, New York, pp 166–167,2000)

478. Bas, D., Boyac, I.H. Modelling and optimization I: Usability of response surface methodology. *J. Food Eng.* **78,** 836-845 (2007)

479. Haq Iul, Mukhtar H. Fuzzy logic control of bioreactor for enhanced biosynthesis of alkaline protease by an alkalophilic strain of *Bacillus subtilis. Curr. Microbiol.* **52,** 149-152 (2006)

480. Nehete, P.N., Shah, V.D., Kothari, R.M. Profiles of alkaline protease production as a function of composition of the slant, age, transfer and isolate number and physiological state of culture. *Biotechnol. Lett.* **7,** 413–418 (1985)

481. Lee, Y.H., Chang, H.N. Production of alkaline protease by *Bacillus licheniformis* in an aqueous two-phase system. *J. Ferment. Bioengin.* **69,** 89-92 (1990)

482. Calik, P., Calik, G., Ozdamar, T.H. Oxygen transfer effects in serine alkaline protease fermentation by *Bacillus licheniformis:* Use of citric acid as the carbon source. *Enzyme Microbial Technol.* **23,** 451-461 (1998)

483. Potumarthi, R., Ch, S., Jetty, A. Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. *Biochemical Eng. J.* **34,** 185-192 (2007)

484. Antonian, E. Recent advances in the purification, characterization and structure determination of lipases. *Lipids* **23,** 1101-1 106 (1988)

485. Taipa, M.A., Moura-Pinto, P., Cabrat, J.M.S.Identification and characterization of *chromobacterium viscosum* lipase. *Biotechnol. Lett.* **8(1),** 27-32 (1992)\

486. Aires-Barros, M.R., Taipa, M.A., Cabral, J.M.S. *Isolation and purification of lipases. In: Wooley P, Petersen SB (eds) Lipases — their structure, biochemistry and application.* (Cambridge University Press, Cambridge, pp 243–270, 1994)

487. Saxena, R.K., Sheoran, A., Giri, B., Davidson, S. Purification strategies for microbial lipases. *J. Microbiol. Methods* **52,** 1–18 (2003)

488. Palekar, A.A., Vasudevan, P.T., Yan, S.Purification of lipase: a review. *Biocat. Biotransform.* **18,** 177–200(2000)

489. Margesin, R., Palma, N., Knauseder, F., Schinner, F. Purification and characterization of an alkaline protease produced by a psychrotropic *Bacillus* sp. *J. Biotechnol.* **24,** 203–6 (1992)

490. Kembhavi, A.A., Kulkarni, A., Pant, A. Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No-64. *Appl. Biochem. Biotechnol.* **38 (1-2),** 83-92 (1993)

491. Moon, S.Y., Oh, T.K., Rho, H.M. Purification and characterization of an extracellular alkaline protease from *Bacillus subtilis* RM615. *Korean Biochem. J.* **27(4),** 323-329 (1994)

492. Hutadilok-Towatana, N., Painupong, A., Suntinanalert, P. Purification and characterization of an extracellular protease from

alkaliphilic and thermophilic *Bacillus* sp. PS719. *J. Biosci. Bioeng.* **87(5),** 581-587 (1999)

493. Gerze, A., Omay, D., Guvenilir, Y. Partial purification and characterization of protease enzyme from *Bacillus subtilis* and *Bacillus megatherium. Appl. Biochem. Biotechnol.* **121-124,** 335-45 (2005)

494. Ozturk, S., Ozeren-Morgan, M., Dilgimen, A.S., Denizci, A.A., Arikan, B., Kazan, D. Alkaline serine protease from halotolerant *Bacillus licheniformis* BA17. *Ann. Microbiol.* **59(1),** 83-90 (2009)

495. Ghafoor, A., Hasnain, S. Production dynamics of *Bacillus subtilis* strain AG-1 and EAG-2, producing moderately alkaline proteases. *African J. Microbiol. Research,* **3(5),** 258-263 (2009)

496. Tiwary, E., Gupta, R. Medium optimization for a novel 58kDa dimeric keratinase from *Bacillus licheniformis* ER-15: biochemical characterization and application in feather degradation and dehairing of hides. *Bioresource Technol.* **101(15),** 6103-10 (2010)

497. Lin, X., Lee, C.G., Casale, E.S., Shih, J.C.H. Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain. *Appl. Environ. Microbiol.* **58,** 3271–3275 (1992)

498. Suh, H.J., Lee, H.K. Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-1. *J. Protein Chem.* **20,** 165–169 (2001)

499. Rozs, M., Manczinger, L., Vágvölgyi, C., Kevei, F. Secretion of a trypsin-like thiol protease by a new keratinolytic strain of *Bacillus licheniformis. FEMS Microbiol. Lett.* **205,** 221–224 (2001)

500. Korkmaz, H., Hür, H., Dinçer, S. Characterization of alkaline keratinase of *Bacillus licheniformis* strain HK-1 from poultry waste. *Ann. Microbiol.* **54,** 201–211(2004)

501. Suntornsuk, W. *et al.* Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium. *World J. Microbiol. Biotechnol.* **21,** 1111–1117 (2005)

502. Saeki, K. *et al.* A novel species of alkaliphilic *Bacillus* that produces an oxidatively stable alkaline serine protease. *Extremophiles* **6,** 65–72 (2002)

503. Srinivasan, P.T., Viraraghavan, T., Subramanian, K.S. Aluminium in drinking water: An overview. *Water SA* **25 (1),** 47-55 (1999)

504. Fujiwara, N., Masui, A., Imanaka, T. Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp. *J. Biotechnol.* **30,** 245–56 (1993)

505. Ferrero, M.A., Castro, G.R., Abate, C.M., Baigori, M.D., Sineriz, F. Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: isolation, production and characterization. *Appl. Microbiol. Biotechnol.* **45,** 327–32 (1996)

506. Yang, Y., Jiang, L., Zhu, L., Wu, Y., Yang, S. Thermal stable and oxidation resistant variant of subtilisin E. *J. Biotechnol.* **81,** 113–118 (2000)

507. Beg, Q. K., Saxena, R.K., Gupta, R. Derepression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. *Process Biochem.* **37,** 1103-1109 (2002)

508. Kumar, S., Nussinov, R. How do thermophiles deal with heat? *Cell. Mol. Life Sci.* **58,** 1216–1233 (2001)

509. Saboto, D. *et al.* The b-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: enzyme activity and conformational dynamics at temperatures above 100°C. *Biophys. Chem.* **81,** 23–31 (1999)

510. Leuschner, C., Antranikan, G. Heat stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. *World J. Microbiol. Biotechnol.* **11,** 95–114 (1995)

511. Fredrich, A., Antrakian, G. Keratin degradation by *Fervido-bacterium pennavorans*, a novel thermophilic anaerobic species of the order *Thermotogales. Appl. Environ. Microbiol.* **62,** 2875– 2882 (1996)

512. Diane, W., Stephan, R., Wolfgang, Z. Application of thermostable enzymes for carbohydrate modification. In: Contribution of the Fourth International Workshop on Carbohydrate as Organic Raw Materials, March 20–21, WUV-Universitatverlag, Vienna 1997

513. Zeikus, J., Lee, C., Lee, Y., Saha, B. Thermostable saccharides: new sources, uses and biodesigns. In: Kalegoris, E., Christakopoulos, D., Kekos, D., Macris, B. (Eds.), Studies on the solid-state production of thermostable endoxylanases from *Thermoascus aurantiacus*: Characterization of two isoenzymes. *J. Biotechnol.* **60,** 155–163, (1998)

514. Zeikus, J., Vielle, C., Savachenko, A.Thermozymes: biotechnology and structure-function relationships. *Extremophiles* **2,** 179– 183 (1998)

515. Mozhaev, V. Mechanism-based strategies for protein thermostabilization. *Trends Biotechnol.* **11,** 88–95 (1993)

516. Krahe, M., Antranikian, G., Markel, H. Fermentation of extremophilic microorganisms. *FEMS Microbiol. Rev.* **18,** 271– 285 (1996)

517. Kumar, H.D., Swati, S. *Modern Concepts of Microbiology, second revised ed.* (Vikas Publishing House Pvt. Ltd., New Delhi, 2001)

518. Gangadhara., P, RK., V. Prakash. Influence of polyols on the stability and kinetic parameters of invertase from *Candida utilis*: correlation with the conformational stability and activity. *Protein J.* **27(7-8),** 440-9 (2008)

519. Fagain O. Understanding and increasing protein stability. *Biochem. Biophys. Acta* **1252,** 1–14 (1995)

520. Street, T.O., Bolen, D.W., Rose, G.D. A molecular mechanism for osmolyte-induced protein stability. *Proc. Natl. Acad. Sci.* **103,** 13997– 14002 (2006)

521. Taravati, A. *et al.* Various effect of sugars & polyols on the protein structure and function: Role as osmolyte on protein stability. *World Appl. Sci. J.* **2(4),** 353-362 (2007)

522. Asther, H., Meunier, J.C. Increased thermal stability of *Bacillus licheniformis* α-amylase in the presence of various additives. *Enzyme Microbial Technol.* **12,** 902–5 (1990)

523. Gonzalez, G., Gonzalez, C., Merino, P. Thermostabilization of *Cucurbita ficifolia* protease in the presence of additives. *Biotechnol. Lett.* **14,** 919–24 (1992)

524. Ghorbel B. Sellami-Kamoun A. Nasri M.Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microbial Technol.* **32(5),** 513-518 (2003)

525. Fersht, A. R. *Enzyme Structure and Mechanism.* (Freeman: Reading, England, 1977)

526. Sigma, D.S., Moser, G. Chemical studies of enzyme active sites. *Ann. Rev. Biochem.* **44,** 889–931 (1975)

527. Gold, A.M., Fahrney, D. Sulfonyl fluorides as inhibitors of esterases. II. Formation and reactions of phenyl methane sulfonyl alpha-chymotrypsin. *Biochem.* **3,** 783–91(1964)

528. Jones, M.N., Manley, P. Interaction between lysozyme and n-alkyl sulphates in aqueous solution. *J. Chemical Soci. Faraday Transactions* **76(1),** 654-664 (1980)

529. Pan, T., Lin, S. Fermentative production of alkaline protease as detergent additive. *J. Chinese Biochem. Soc.* **20,** 49–60 (1991)

530. Steele, D.B., Fiske, M.J., Steele, B.P., Kelley, V.C. Production of a low molecular weight, alkaline active, thermostable protease by a novel spiral-shaped bacterium, *Kurthia spiroforme* sp. nov. *Enzyme Microbial Technol.***14,** 358–60 (1992)

531. Donaghy, J.A., McKay, A.M. Production and properties of an alkaline protease by *Aureobasidium pullulans. J. Appl. Bacteriol.* **74,** 662–66 (1993)

532. Towatana, N.H., Painupong, A., Suntinanalert, P. Purification and characterization of an extracellular protease from alkaliphilic and thermophilic *Bacillus* sp. PS719. *J. Biosci. Bioeng.* **87,** 581–587 (1999)

533. Banerjee, U.C., Sani, R.K., Azmi, W., Soni, R. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Proc. Biochem.* **35,** 213-219 (1999)

534. Garrett, R.H., Grisham, C.M. *Biochemistry* (Brooks Cole; 2 edition, 1999)

535. Anwar, A., Saleemuddin, M. Alkaline proteases: A review. *Bioresource Technol.* **64,** 175-183 (1998)

536. Bockle, B., Galunsky, B., Muller, R. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. *Appl. Environ. Microbiol.* **61,** 3705–3710 (1995)

537. Suzuki, Y., Tsujimoto, Y., Matsui, H., Watanabe, K. Decomposition of extremely hard-to-degrade animal proteins by thermophilic bacteria. *J. Biosci. Bioeng.* **102,** 73–81 (2006)

538. Sangali, S., Brandelli, A. Feather keratin hydrolysis by a *Vibrio* sp., strain kr2. *Appl. Environ. Microbiol.* **89,** 735– 743 (2000)

539. Bordusa, F. Proteases in organic synthesis. *Chem. Rev.* **102,** 4817– 4867 (2002)

540. Zaks, A., Klibanov, A.M. Enzymatic catalysis in organic media at 100°C. *Science* **224,** 1249–1251 (1984)

541. Gupta, M.N., Roy, I.Enzymes in organic media forms, functions and applications. *Eur. J. Biochem.* **271,** 2575–2583 (2004)

542. Klibanov, A.M. Improving enzymes by using them in organic solvents. *Nature* **409,** 241–246 (2001)

543. Shuang, Li., Bingfang, He., Zhongzhong, Bai., Pingkai,Ouyang. A novel organic solvent-stable alkaline protease from organic solvent–tolerant *Bacillus licheniformis* YP1A. *J. Mol.Cat. B : Enzymatic.* **56(2-3),** 85-88 (2009)

544. Laane, C., Tramper, J., Lilly, M. D., Eds. *Biocatalysis in Organic Media* (Elsevier: Amsterdam, 1987)

545. Ogino, H., Ishikawa, H. Enzymes which are stable in the presence of organic solvents. *J. Biosci. Bioeng.* **91,** 109–116 (2001)

546. Basketter, D.A., English, J.S., Wakelin, S.H., White, I.R. Enzymes, detergents and skin: facts and fantasies. *Br. J. Dermatol.* **158,** 1177- 1181 (2008)

547. Visser, S. Proteolytic Enzymes and Their Relation to Cheese Ripening and Flavor: An Overview. *J. Dairy Sci.* **76(1),** 329-350 (1993)

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

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

550. Torr, S.R., Nachowiak, D.A., Fujii, S., Sobel, B.E. Plasminogen steal and clot lysis. *J. Am. Coll. Cardiol.* **19,**1085–1090 (1992)

551. Lijnen, H.R., Van Hoef B, Collen,.D. Interaction of staphylokinase with different molecular forms of plasminogen. *Eur. J. Biochem.* **211,** 91–97 (1993)

552. Schleuning, W.D. Vampire bat plasminogen activator DSPAalpha-1 (desmoteplase): a thrombolytic drug optimized by natural selection. *Haemostasis* **31,** 118-122 (2001)

553. Lu Q, Clemetson JM, Clemetson KJ. Snake venoms and hemostasis. *J.Thromb. Haemost.* **3,** 1791-9 (2005)

554. Mukherjee. A.K. Correlation between the phospholipids domains of the target cell membrane and the extent of *Naja kaouthia* PLA2-induced membrane damage: Evidence of distinct catalytic and

cytotoxic sites in PLA_2 molecules. *Biochimica et Biophysica Acta* **1770,** 187–195(2007)

555. Kim, W. *et al.* Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. *Appl. Environ. Microbiol.* **62,** 2482–2488 (1996)

556. Peng, Y., Huang, Q., Zhang, R-H.,Zhang, Y-Z. Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi,a traditional Chinese soybean food. *Compar. Biochem. Physiol. Part B.* **134,** 45–52 (2003)

557. Hua, Y., Jiang, B., Mine, Y., Mu, W. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste. *J. Agr. Food Chem.* **56,** 1451–1457 (2008)

558. Choi, N.S. *et al.* Purification and characterization of a novel thermoacid-stable fibrinolytic enzyme from *Staphylococcus* sp. strain AJ isolated from Korean salt-fermented Anchovy-joet. *J. Ind. Microbiol. Biotechnol.* **36,** 417–426 (2008)

559. Xiao-lan, Liu., Lian-xiang, Du., Fu-ping, Lu., Xi-qun, Z., Jing, X. Purification and characterization of a novel fibrinolytic enzyme from *Rhizopuschinensis* 12. *Appl. Microbiol. Biotechnol.* **67,** 209–214 (2005)

560. http://www.indiatogether.org/environment/articles/tlink-1002.htm) Detergents under scrutiny. (Accessed on 20th July, 2010)

561. Olsen, H.S., Falholt, P. The role of enzymes in modern detergency. *J. Surf. Det.* **1(44),** 555-567 (1998).

562. Nascimento, W.C.A., Martins, M.L.L. Studies on the stability of protease from *Bacillus* sp. and its compatibility with commercial detergent. *Brazilian J. Microbiol.* **37,** 307-311 (2006)

563. Hadj-Ali, N.El *et al.* Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. *Enzyme Microbial Technol.* **40,** 515–523(2007)

564. Dodia, M.S., Bhimani, H.G., Rawal, C.M., Joshi, R.H., Singh, S.P. Salt dependent resistance against chemical denaturation of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. *Bioresource Technol.* **99,** 6223–6227 (2008)

565. Rajput, R., Sharma, R., Gupta, R. Biochemical characterization of a thiol-activated, oxidation stable keratinase from *Bacillus pumilus* KS12. *Enzyme Research,* 1-7 (2010)

566. Brown, K.L., Robert, E.W. Hancock. cationic host defense (antimicrobial) peptides. *Curr. Opin. Immun.* **18,** 24–30 (2006)

567. Hancock, R.E.W., Chapple, D.S. Peptide Antibiotics. *Antimicrobial Agents and Chemo.* **43(6),** 1317-1323 (1999)

568. Vizioli, J., Salzet, M. Antimicrobial peptides from animals: focus on invertebrates. *Trends Pharmacological Sci.* **23(11),** 494-496 (2002)

569. Powers, J.P.S., Hancock, R.E. The relations between peptide structure and antibacterial activity. *Peptides* **24,** 1681–1691(2003)

570. Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N., Miyajima, K. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. *Biochimica. Et. Biophysica. Acta* **1327,** 119-130 (1997)

571. Bechinger, B. Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. *J. Membr. Biol.* **156(3),** 197–211 (1997)

572. Park, C.B., Kim, H.S., Kim, S.C. Mechanism of action of the antimicrobial peptide buforin II: Buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Re.s Commun.* **244,** 253-257(1998)

573. Khachatourians, G.G. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *J.A.M.C.* **159 (9)** 1129-1136 (1998)

574. Molina, M.P., Althaus, R.L., Molina, A., Fernandez, N. Antimicrobial agent detection in ewe's milk by the microbial inhibitor test brilliant black reduction test-BRT AiMÂ®. *Int. Dairy J.* **13,** 821-826 (2003)

575. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature,* **415(6870),** 389-395 (2002)

576. Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. *Biochim. Biophys. Acta* **1696,** 1–14 (2004)

577. Dziarski, R., Gupta, D. The peptidoglycan recognition proteins (PGRPs). *Genome Biol.* **7(232),** 1-13 (2006)

578. Mahamoud, A., Chevalier, J., Alibert-Franco, S., Kern, W.V., Pages J-M. Antibiotic efflux pumps in Gram-negative bacteria : the inhibitor response strategy. *J. Antimicro. Chemother.* DOI: 10.1093/jac/dk1493

579. Wang, M. *et al.* Human peptidoglycan recognition proteins require zinc to kill both Gram-positive and gram-negative bacteria and are

synergistic with antibacterial peptides. *The J. Immunol.* **178,** (116-3125) 2007

580. Nasri, N.A.Md. *et al.* Anitbacterial properties of tualang honey and its effect in burn wound management: a comparative study. *BMC Complement. Alter. med.* **10(31),** (1-7) 2010

581. Umamaheswari, A., Nuni, A., Shreevidya, R. Evaluation of antibacterial activity of *Boerhaavia diffusa L.leaves. Int.J.Green Pharmacy.* DOI: 10.4103/0973-8258.63879

582. http://www.contractleathers.com/HistoryandProcess.pdf. History and Process of Leather. (Accessed on 24th July 2010)

583. http://biospectrumindia.ciol.com/content/bioFocus/104021201.asp. Superstore of leather tech.(Accessed on 24th July 2010)

584. http://www.financialexpress.com/news/No-new-tanneries-in-Kanpur-suburb-UP-govt/623048/).No new tanneries in Kanpur suburb: UP govt.(Accessed on 24th July 2010)

585. Bhavan, S., Rao, J.R., Nair, B.U. A potential new commercial method for processing leather to reduce environmental impact. *Environ. Sci. Pollut. Res.* **15,** 293–295 (2008)

586. Cohlberg, J.A.The structure of α-keratin. *Trends in Biochemical Sci.* **18,** 360–362 (1993)

587. Steinert, P. M. Structure, function, and dynamics of keratin intermediate filaments. *Journal of Investigative Dermat.* **100,** 729–734 (1993)

588. Harrap, B.S., Woods, E.F. Species differences in the proteins of feathers. *Comparative Biochem. Physiol.* **20(2),** 449-452(1967)

589. Cherry, J.P., Young, C.T., Shewfelt, A.L. Characterization of protein isolates from keratinous material of poultry feathers. *J. Food Sci.* **40,** 331–335 (1975)

590. El-Boushi, A.R.Y., van der Poel, F.B. *Handbook of Poultry Feed from Waste. Processing and Use.* (Kluwer Academic Publishers, Dordrecht, 2000)

591. Luong, V.B., Payne, C.G. Hydrolysed feather protein as a source of amino acids for laying hens. *Br. Poult. Sci.* **18,** 523–526 (1977)

592. Liu, J.K., Waibel, P.E., Noll, S.L.Nutritional evaluation of blood meal and feather meal for turkeys. *Poult.Sci.* **68,** 1513–1518 (1989)

593. Casarin, F., Cladera-Olivera, F., Brandelli, A. Use of poultry by product for production of keratinolytic enzymes. *Food Bioprocess Technol.* **1,** 301–305 (2008)

594. Brandelli, A. Bacterial keratinases: useful enzymes for bio-processing agroindustrial wastes and beyond. *Food Bioprocess Technol.* **1,** 105–116 (2008)

595. Wang, J. J., Swaisgood, H. E., Shih, J. C. H. Production and characterization of bio-immobilized keratinase in proteolysis and keratinolysis. *Enzyme Microbial Technol.* **32,** 812– 819 (2003)

596. Halling, P.J., Dunnill, P. Magnetic supports for immobilized enzymes and bioaffinity adsorbents. *Enzyme Microbial Technol.* **2,** 2-10 (1980)

597. Dresco, P.A., Zaitev, V.S., Gambino, R.J., Chu, B. Preparation and properties of magnetic and polymer magnetite nanoparticles. *Langmuir* **15,** 1945-1951 (1999)

598. Roath, S. Biological and biomedical aspects of magnetic fluid technology. *J. Magn.Mater.* **122,** 329-334(1993)

599. Löster, K., Seidel, S., Kirstein, D., Schneider, F., Noll, F. Novel antibody coating of a magnetizable solid phase for use in enzyme immunoassays. *J.Immunol. Meth.* **148,** 41-47(1992)

600. Schütt, W. *et al.* Applications of magnetic targetting in diagnosis and therapy-possibilities and limitations: a mini-review. *Hybridoma* **16,** 109-117(1997)

601. Shinkai, M., Honda, H., Kobayashi, T. Preparation of fine magnetic particles and application for enzyme immobilization. *Biocatalysis* **5,** 61-69 (1991)

602. Mehta, R.V., Upadhyay, R.V., Charles, S.W., Ramchand, C.N. Direct binding of protein to magnetic particles. *Biotechnol. Bioeng.* **11,** 493-496 (1997)

603. Cochrane, F.C., Petach, H.H., Henderson W. Application of tris(hydroxymethyl) phosphine as a coupling agent for alcohol dehydrogenase immobilization. *Enzyme Microbial Technol.* **18,** 373-378(1996)

604. Ooshima, H., Genko, Y., Harano,Y. Stability of immobilized yeast alcohol dehydrogenase. *Biotechnol. Bioeng.* **23,** 2851-2862 (1981)

605. Schöpp,W., Grunow, M. Immobilization of yeast ADH by adsorption onto polyaminomethylstyrene. *Appl. Microbiol. Biotechnol.* **24,** 271-276 (1986)

606. Liang, Y.Y., Zhang, L.M. Bioconjugation of papain on superparamagnetic nanoparticles decorated with carboxymethylated chitosan. *Biomacromolecules* **8,** 1480-1486 (2007)

607. Kunert, J. Biochemical mechanism of keratin degradation by the actinomycetes Streptomyces fradiae and the fungus *Microsporum gypseum*: a comparison. *J Basic Microbiol.* **29,** 597–604 (1989)

Appendix-I

(A)Screening media for protease producing bacteria

Composition	Concentration (gm/l)
Skim milk agar	4.0
Yeast extract	2.5
Glucose	1.0
Agar	20.0
pH	8.0/10.0
Distilled water	1000 ml

Note:Adjust the media pH separately followed by addition of skim milk , agar and yeast extract.

(B)Composition of M9 Media for protease production

(I) M9 Medium composition	Concentration (gm/l)
Na_2HPO_4	6.0
KH_2PO_4	3.0
NH_4Cl	1.0
NaCl	0.5
$MgSO_4.H_2O$	0.246
$CaCl_2.7H_2O$	0.014
Carbon source	1.0
(II) Macro-nutrient composition	
$FeSO_4.7H_2O$	1.0 mg l^{-1}
$CuSO_4.5H_2O$	50.0 μg l^{-1}
H_3BO_3	10.0 μg l^{-1}
$MgSO_4.5H_2O$	10.0 μg l^{-1}
$ZnSO_4.7H_2O$	70.0 μg l^{-1}
MoO_3	10.0 μg l^{-1}

Add 1ml of macro –nutrient in 1000 ml of M9 production medium

(C) Carbohydrate fermentation medium compositions

(i) Phenol red broth	Concentration (gm/l)
Protease peptone	10.0

Beef extract	1.0
Sodium chloride	5.0
Sucrose/Lactose/fructose/D-glucose/ Mannitol	5.0
Phenol red	0.018

(D) Nutrient Agar composition	Concentration (gm/l)
Peptic digest of animal tissue	10.0
Meat extract	10.0
NaCl	5.0
Agar	15.0

(E) Litmus milk composition	Concentration (gm/l)
Skim milk powder	100.0
Litmus	0.50
Sodium sulphite	0.50

(F) Urea broth	Concentration (gm/l)
Yeast extract	0.1
Monopotassium phosphate	9.1
Dipotassium phosphate	9.5
Urea	20.0
Phenol red	0.01

(G) Tryptone broth	Concentration (gm/l)
Casein enzymatic hydrolysate	10.0
Sodium chloride	5.0

(H) Simmon citrate agar (SIM agar)	Concentration (gm/l)
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
NaCl	5.0
Bromothymol blue	0.08

Agar	15.0

(I) Nitrate broth	**Concentration (gm/l)**
Peptic digest of animal tissue	5.0
Meat extract	3.0
Potassium nitrate	1.0
NaCl	30.0

(J) Indole nitrate broth	**Concentration (gm/l)**
Casein enzymic hydrolysate	20.0
Disodium phosphate	2.0
Dextrose	1.0
Potassium nitrate	1.0
Agar	1.0

(K) Methyl red –voges proskauer broth	**Concentration (gm/l)**
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0

(L) Triple sugar iron agar	**Concentration (gm/l)**
Peptic digest of animal tissue	10.0
Casein enzymic hydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferric ammonium citrate	0.30
NaCl	5.0
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.0

(M) Nutrient Broth

	Concentration (gm/l)
Peptic digest of animal tissue	10.0
Meat extract	10.0
NaCl	5.0

(N)SDS-PAGE gel electrophoresis composition

(i) Resolving buffer (8X)	Concentration (gm/l)
1.5 M Tris-Cl (pH 8.8)	18.17

(ii) Stacking buffer (4X)	Concentration (gm/l)
0.5M Tris-Cl (pH 6.8)	6.06

Note : Adjust the pH 8.8 with 6N HCl. Make up the final volume to 100 ml with distilled water

(iii) Acrylamide mixture	Concentration (gm/l)
30 % acrylamide	30.0
0.8% bis-acrylamide	0.8

Note : Dissolve in 100ml of warm deionized water to facilitate dissolution of bis-acrylamide. Store in amber colour bottle at 4°C

(iv) Reservoir buffer (pH 8.3, 1X)	Concentration (gm/l)
Tris-Cl	3.0
Glycine	14.4
10% SDS	10.0

Note : Dissolve in 1000ml of distilled water adjusted to pH to 8.3

(v) Sodium dodecyl sulfate (SDS)	Concentration (gm/l)
10 % SDS	10.0

(vi) Ammonium per sulphate (APS)	Concentration (gm/l)
10% APS	0.2

(vii) Staining solution **Volume (ml / concentration)**

Methanol	40.0
Glacial acetic acid	10.0
Distilled water	50.0
Commassie brilliant blue	0.4 (gm/ ml)

(viii) Destaining solution

Composition	Volume (ml)
Methanol	40
Glacial acetic acid	10
Distilled water	50

(ix) Loading buffer (50 ml , 3X)

Composition	Amount / volume
Tris-Cl (pH 6.8)	1.296
SDS	3.0
Glycerol	1.5 ml
Bromophenol blue	3.0

Note : 1ml of loading buffer mixed with 30µl of 2-mercaptoethanol (3%)

(x) Resolving gel composition

(12.5%)

Components	Volume (ml)
Distilled water	2.3
1.5 M Tris-Cl (pH 8.8)	5.0
Acrylamide mixture	8.3
10% SDS	0.2
1 % APS	1.5
Glycerol	0.8
TEMED	0.025

(xi) Stacking gel (4X)

Components	Volume (ml)
Distilled water	5.4
0.5M Tris-Cl (pH 6.8)	2.5

10% SDS	0.1
Acrylamide mixture	1.3
1 % APS	0.7
Glycerol	0.4
TEMED	0.015

(O) Chemicals composition for chromosomal DNA isolation

(i) Solution I	**Concentration (gm %)**
50mM glucose	0.9
25mM Tris-Cl	0.30
10mM EDTA	0.37

(ii) Solution II	**Concentration (gm %)**
0.2N NaOH	0.0079
SDS	1.0

(iii) Solution III	**Volume (ml)**
5M Potassium acetate	60.0
Glacial acetic acid	11.5
Distilled water	28.5

(iv) Loading dye	**Concentration (gm %)**
Bromophenol blue	0.25
Xylene cyanol	0.25
Sucrose	40.0

List of publications

LIST OF PUBLICATIONS FROM Ph.D THESIS

1.**Rai, S.K.,** Mukherjee, A.K. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. Biochemical Eng. J. 48 (2010) 173–180

2. **Rai, S.K.,** Roy, J.K., Mukherjee, A.K. Characterisation of a detergent-stable alkaline protease from a novel thermophilic strain *Paenibacillus tezpurensis* sp. nov. AS-S24-II. Appl. Microbiol. Biotechnol. 85(5), (2010) 1437-50.

3. **Rai, S.K.,** Mukherjee, A.K. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04.Bioresourc. Technol. 100 (2009) 2642–2645

4.**Rai, S.K.,** Konwarh, R., Mukherjee, A.K. Purification, characterization and biotechnological application of an alkaline β-keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate Biochemical Eng. J. 45 (2009) 218–225

5. Mukherjee, A.K., Adhikari, H., **Rai, S.K.** Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation. Biochemical Eng. J. 39 (2008) 353–361

PAPER PRESENTED IN SEMINAR / SYMPOSIUM / CONFERENCES

(A) International level

1. **Rai, S.K.,** Mukherjee, A.K. (2009) ***International Symposium on Envirnomental Pollution Ecology and Human Health (ISEPEHH-2009).*** 25-27 July 2009, organized by S.V.University, Tirupati, India

2.Rai, S.K., Mukherjee, A.K. (2009) ***INSA Platinum Jubilee International Symposium on Research in Molecular Medicine Based on Natural Resources and Traditional Knowledge*** 21-23 November 2009, organized by National chemical Laboratory (NCL), Pune, India

3. Mukherjee, A.K., **Rai, S.K.,** Bhuyan, P (2009) ***INSA Platinum Jubilee International Symposium on Research in Molecular Medicine Based on Natural Resources and Traditional Knowledge*** 21-23 November 2009, organized by National chemical Laboratory (NCL), Pune, India

4. Mukherjee, A.K., Rai, S.K. (2007) **In:** ***II International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2007),*** 28 November - 1 December 2007, University of Sevilla, Spain.

(B) National level

1. Rai, S.K., Mukherjee, A.K. (2009) ***New Biology section of 96th Indian Science Congress,*** 3-7 January, 2009, organized by North-Eastern Hill University, Shillong, India.

2. Rai, S.K., Mukherjee, A.K. (2008) ***National conference on Recent Trends in Bioscience and Industrial Application,*** December, 2008, organized by K. J. Somaiya College of Arts and science, Mumbai.

3. Rai, S.K., Mukherjee, A.K. (2008) ***New Biology section of 95th Indian Science Congress.*** 3-7 January, 2008, organized by Andhra University, Visakhapatnam, India.

LIST OF PUBLICATIONS APART FROM Ph.D WORK

1. Konwarh, R., Karak, N., **Rai, S.K.,** Mukherjee, A.K., Polymer-assisted iron oxide magnetic nanoparticle immobilized keratinase. Nanotechnology 20 (2009) 225107, doi:10.1088/0957-4484/20/22/225107

2. Mukherjee, A.K., Borah, M., **Rai, S.K.** To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations. Biochemical Eng. J. 43 (2009) 149–156

3. Mukherjee, A.K., Kumar, T.S., **Rai, S.K.,** Roy, J.K. Statistical optimization of *Bacillus alcalophilus* α-amylase immobilization onto iron-oxide (Fe_3O_4) magnetic nanoparticles. Biotechnology and Bioprocess Engineering Journal (2010) (In press).

PAPER PRESENTED IN SEMINAR / SYMPOSIUM / CONFERENCES

National level

1. Roy, J.K., **Rai, S.K.,** Mukherjee, A.K. (2009) ***New Biology section of 96th Indian Science Congress,*** 3-7 January, 2009, organized by North-Eastern Hill University, Shillong, India.

2. Bhuyan, P., Nag, A., **Rai, S.K.,** Mukherjee, A.K. (2009) ***New Biology section of 96th Indian Science Congress,*** 3-7 January, 2009, organized by North-Eastern Hill University, Shillong, India.

3. **Rai, S.K.,** Saikia, D., Mukherjee. A.K. (2007) ***In: Proceedings of Seminar on Bioelectronics, (Ed., Drs J.C. Dutta and R.Chutia),*** Bioelectronics Division, Dept. of Electronics and Communication Engineering, Tezpur University, Tezpur, pp: 55-56 (2007).

2. Bhuyan, P., Nag, A., **Rai, S.K.,** Mukherjee, A.K. (2009) ***New Biology section of 96th Indian Science Congress,*** 3-7 January, 2009, organized by North-Eastern Hill University, Shillong, India.

3. **Rai, S.K.,** Saikia, D., Mukherjee. A.K. (2007) ***In: Proceedings of Seminar on Bioelectronics, (Ed., Drs J.C. Dutta and R.Chutia),*** Bioelectronics Division, Dept. of Electronics and Communication Engineering, Tezpur University, Tezpur, pp: 55-56 (2007).

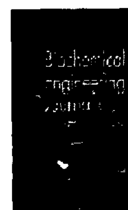
Biochemical Engineering Journal 48 (2010) 173–180

ELSEVIER

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej



Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04

Sudhir K. Rai, Ashis K. Mukherjee*

Department of Molecular Biology and Biotechnology, Tezpur University, Napaam, Tezpur 784028, Assam, India

ARTICLE INFO

Article history:
Received 1 July 2009
Received in revised form 3 September 2009
Accepted 9 September 2009

Keywords:
Enzyme
Submerged
Microbial
Bacillus subtilis
Chromatography
Protease
Kinetics
Solid-state fermentation
Optimization

ABSTRACT

Optimum protease production of 518 U by *Bacillus subtilis* DM-04 in submerged fermentation was attained by response surface method. An alkaline protease, exists as zwitterionic form at pH 7.0 was purified to 23.5-fold by a combination of cation and anion exchange chromatography, ethanol precipitation followed by reverse-phase HPLC. The purified protease (Alzwiprase) contributes 29.0% of overall extracellular proteases of *B. subtilis* DM-04, has a subunit molecular mass of 16.9 kDa and exists as a monomer. It shows optimum activity at 45 °C and pH 10.0, respectively. The K_m and V_{max} values of Alzwiprase towards casein were determined as 59 μM and 336 μg min^{-1}, respectively. Irreversible inhibition of enzyme activity of Alzwiprase with serine protease inhibitors demonstrates that it belongs to serine protease family, more particularly endopeptidase K and/or subtilisin-like protease. The significant stability and compatibility towards organic solvents, urea, surfactants, commercial laundry detergents as well as excellent stain removal and dehairing properties of Alzwiprase hold a tremendous promise for its industrial application.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The enzymes are considered as "green chemicals" owing to their eco-friendly in nature and possess wide range of applications from industrial sector to house-hold products. Microbial proteases represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sale in the world and they are the leaders of the industrial enzyme market worldwide [1]. Microbial proteases are classified as acidic, neutral and alkaline depending on the pH at which they show maximum activity. Amongst these, alkaline proteases find a wide range of applications in laundry detergent, textile, food processing, pharmaceuticals, and leather, paper and pulp industries [1–3].

Since the enzyme based detergents can function better at room temperature (~23 °C) and possess pollution-alleviating capacity over conventional synthetic detergents; therefore, alkaline proteases have made their way as key-ingredients in detergent formulations [2,3]. It is worthwhile to mention that there are many parameters involved in the selection of an ideal detergent protease, such as compatibility with the detergent components, good activity at relevant washing pH and temperature, compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and half-life. This has stimulated the scientists to search for newer microbial proteases with novel properties that can further improve the wash performance of enzyme based laundry detergents.

In our previous study, we report the purification, characterization, physiological significance and industrial application of a 33.1 kDa anionic, alkaline serine protease (Bsubap-I) from *Bacillus subtilis* DM-04 [3]. It is to be noted that a bacterium produces arrays of protease isoenzymes for its survival and growth and many of these proteases may be explored for the industrial purpose [3]. Our subsequent study has shown that apart from secreting the anionic and cationic proteases, the same bacterium produces another major group of proteases, which is zwitterionic in nature at pH 7.0 (protease does not bind to any cation or anion exchanger at pH 7.0) and holds a tremendous promise for industrial application. This has prompted us to purify, characterize and explore the possible biotechnological potential of an organic solvent-stable protease which exists as zwitterionic form at pH 7.0 (Alzwiprase) but shows optimum activity at pH 8.0 from *B. subtilis* DM-04. Further, we also improved the protease yield in SmF by statistical optimization of process parameters using response surface method. Characteriza-

* Corresponding author. Tel.: +91 3712 267007x5405; fax: +91 3712 267005/267006.
E-mail address: akm@tezu.ernet.in (A.K. Mukherjee).

1369-703X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2009.09.007

Appl Microbiol Biotechnol
DOI 10.1007/s00253-009-2145-y

Characterisation of a detergent-stable alkaline protease from a novel thermophilic strain *Paenibacillus tezpurensis* sp. nov. AS-S24-II

Sudhir K. Rai · Jetendra K. Roy · Ashis K. Mukherjee

Received: 25 June 2009 / Revised: 25 June 2009 / Accepted: 13 July 2009
© Springer-Verlag 2009

Abstract An alkaline-protease-producing bacterial strain (AS-S24-II) isolated from a soil sample in Assam is a Gram-stain-positive, catalase-positive, endospore-forming rod and grows at temperatures ranging from 30 °C to 60 °C and salinity ranging from 0% to 7% (*w/v*) NaCl. Phenotypic characterisation, chemotaxonomic properties, presence of *Paenibacillus*-specific signature sequences, and ribotyping data suggested that the strain AS-S24-II represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus tezpurensis* sp. nov. (MTCC 8959) is proposed. Phylogenetic analysis revealed that *P. lentimorbus* strain DNG-14 and *P. lentimorbus* strain DNG-16 represent the closest phylogenetic neighbour of this novel strain. Alkaline protease production (598×10^3 U l^{-1}) by *P. tezpurensis* sp. nov. in SmF was optimised by response surface method. A laundry-detergent-stable, Ca^{2+}-independent, 43-kDa molecular weight alkaline serine protease from this strain was purified with a 1.7-fold increase in specific activity. The purified protease displayed optimum activity at pH 9.5 and 45–50 °C temperature range and exhibited a significant stability and compatibility with surfactants and most of the tested commercial laundry detergents at room temperature. Further, the protease improved the wash performance of detergents, thus demonstrating its feasibility for inclusion in laundry detergent formulations.

The 16S rDNA sequence to GenBank and the accession number are bankit1191281 FJ804507, and the strain is deposited in Microbial Type Culture Collection (MTCC 8959).

S. K. Rai · J. K. Roy · A. K. Mukherjee (✉)
Department of Molecular Biology and Biotechnology,
School of Science and Technology, Tezpur University,
Tezpur 784 028 Assam, India
e-mail: akm@tezu.ernet.in

Keywords Alkaline protease · Detergent compatibility · *Paenibacillus tezpurensis* · Response surface · Serine protease · Submerged fermentation

Introduction

Microbial proteases, representing one of the three largest groups of industrial enzymes, account for approximately 60% of the total enzyme sale. Amongst the different types of proteases, alkaline proteases particularly those isolated from the genus *Bacillus* find a wide range of applications in laundry, dishwashing, textile, food processing, pharmaceuticals, leather, paper and pulp industries (Anwar and Saleemuddin 1998; Gupta et al. 2002). Owing to the better cleansing properties of enzyme-based detergents at lower washing temperature and pollution-alleviating capacity over conventional synthetic detergents (Krik et al. 2002; Mukherjee et al. 2008), alkaline proteases have made their way as key ingredients in detergent formulations (Maurer 2004).

It is well known that exploration of microbial diversity is the major driving force for the development of biotechnological products and processes. The northeastern part of India is considered as one of the mega biodiversity zones of the world and, by screening the soil samples of this region, we have isolated a potent bacterial strain capable of secreting significant amount of protease in the culture medium at 50 °C temperature. Taxonomic identification of the strain by polyphasic approach showed that this is a novel species belong to the genus *Paenibacillus*, and this strain was subsequently named as *Paenibacillus tezpurensis* sp. nov. There are hardly few reports available on protease production by *Paenibacillus* which are ubiquitous in nature, and the proteases from this genus may find novel industrial

Published online: 11 August 2009

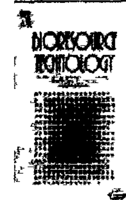
Bioresource Technology 100 (2009) 2642-2645

ELSEVIER

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech



Short Communication

Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04

Sudhir K. Rai, Ashis K. Mukherjee *

Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784 028, Assam, India

ARTICLE INFO

Article history:
Received 11 August 2008
Received in revised form 24 November 2008
Accepted 25 November 2008
Available online 10 January 2009

Keywords:
Bacillus subtilis
Serine alkaline protease
Dehairing activity
Antibacterial specificity
Organic solvent stable protease

ABSTRACT

An organic solvent stable, alkaline serine protease (Bsubap-I) with molecular mass of 33.1 kDa, purified from *Bacillus subtilis* DM-04 showed optimum activity at temperature and pH range of 37-45 °C and 10.0-10.5, respectively. The enzyme activity of Bsubap-I was significantly enhanced in presence of Fe^{2+}. The thermal resistance and stability and of Bsubap-I in presence of surfactants, detergents, and organic solvents, and its dehairing activity supported its candidature for application in laundry detergent formulations, ultrafiltration membrane cleaning, peptide synthesis and in leather industry. The broad substrate specificity and differential antibacterial property of Bsubap-I suggested the natural ecological role of this enzyme for the producing bacterium.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Proteases are important from an industrial perspective due to their wide scale applications in the detergents, food, pharmaceuticals, chemicals, leather, paper and pulp and silk industries (Krik et al., 2002; Mukherjee et al., 2008). Owing to the better cleansing properties of protease based detergents and pollution-alleviating capacity over conventional synthetic detergents (Krik et al., 2002; Mukherjee et al., 2008), alkaline proteases have made their way as key-ingredients in detergent formulations. A survey of literature showed that extracellular proteases were largely responsible providing nutrients to the bacteria (Brar et al., 2007) and therefore, an attempt was made to explore the possible ecological significance of a major extracellular protease from *Bacillus subtilis* DM-04, a strain isolated form a soil sample of Assam, North-east India (Mukherjee and Das, 2005). In this study, we report the purification, characterization and explore the biotechnological potential and ecological significance of a alkaline serine protease (Bsubap-I) from *B. subtilis* DM-04 (Mukherjee and Das, 2005).

2. Methods

2.1. Microorganisms

The microbes viz. *Escherichia coli*, *B. subtilis* DM-04, *Bacillus licheniformis*, *Kocuria varians*, and *Paenibacillus polymyxa* were isolated from the soil samples of Assam and taxonomically identified *Staphylococcus aureus* and *Klebesilla pneumoniae* were obtained from Microbial Type Culture Collection, IMTECH, Chandigarh

2.2. Isolation and purification of acidic protease

Batch fermentation with *B. subtilis* DM-04 was carried out by using potato peel (PP) and *Imperata cylindrica* (IC) grass as substrates (Mukherjee et al., 2008). Cell-free culture supernatant (48 h post inoculation) equivalent to 100 mg protein was applied to a CM-cellulose column (1 cm × 20 cm) and the unbound proteins (showing major protease activity) were re-fractionated on a DEAE-Sephadex A-50 column (1.5 cm × 9.0 cm) by eluting stepwise using phosphate buffers of increasing molarities and decreasing pH values at room temperature (~25 °C). The fractions showing high caseinolytic activity were pooled, desalted, concentrated and further fractionated on a Sephacryl S-200 column (1.5 cm × 60 cm) with 20 mM K-phosphate buffer (pH 7.0). Fractions showing the high protease activity were pooled and protein content was determined (Lowry et al., 1951). The homogeneity of preparation and molecular mass of native protein was determined by 12.5% SDS-PAGE, with or without reduction of protein(s) with β-mercaptoethanol (Lammeli, 1970). Purity of protein was also checked by reverse-phase high performance liquid chromatography (RP-HPLC) of gel-filtration fraction (GF-II) on Waters reverse-phase Nova-Pak C_{18} column (3.9 mm × 300 mm). Protein was eluted with a linear gradient from 5% to 70% acetonitrile containing 0.1% TFA. Casein (1% w/v) zymography was applied to determine the proteolytic activity of the purified protease as described by Thangam and Rajkumar (2002).

* Corresponding author. Mobile: +91 9957 184351; fax: +91 3712 267005/267006.
E-mail address: akm@tezu.ernet.in (A.K. Mukherjee).

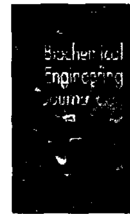
0960-8524/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2008.11.042

Biochemical Engineering Journal 45 (2009) 218–225

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej



Purification, characterization and biotechnological application of an alkaline β-keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate

Sudhir K. Rai, Rocktotpal Konwarh, Ashis K. Mukherjee*

Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784 028, Assam, India

ARTICLE INFO

Article history:
Received 3 February 2009
Received in revised form 30 March 2009
Accepted 1 April 2009

Keywords:
Bioreactor systems
Solid-state
Enzyme
Microbial
Keratinase
Waste treatment
Response surface method

ABSTRACT

We report the application of response surface methodology (RSM) for optimization of the media composition for β-keratinase production by a feather degrading *Bacillus subtilis* strain RM-01 in solid-state fermentation using chicken-feather as substrate. The optimized culture conditions for 5.0 g of chicken-feathers moistened with distilled water (1:1, w/v, adjusted to pH 8.0) supplemented with maltose (10%, w/w) and sodium nitrate (1.25%, w/w) as best co-carbon and co-nitrogen sources, respectively resulted in 5-fold increase of β-keratinase production post-72 h of incubation at 50 °C compared to β-keratinase production under un-optimized condition. The purified β-keratinase was a 20.1 kDa molecular weight serine protease requiring Fe^{2+} for enzyme activity, showed restricted substrate specificity for β-keratin, and exhibited a significant stability and compatibility with surfactants, and most of the tested commercial laundry detergents, thus demonstrating its feasibility for inclusion in laundry detergents formulations.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Amongst the industrially important enzymes, microbial keratinases are stimulating tremendous interests in the enzyme-market owing to the fact that there is a great demand for developing biotechnological alternatives for recycling of keratin-wastes, for example unused chicken-feather to useful value added products [1–3]. Keratinase-mediated degradation of feathers in the niche of environmental biotechnology provides a viable alternative to alkali hydrolysis and steam pressure-cooking [1]. New avenues for commercial exploitation of keratinase have yielded better animal feed additives from feather hydrolysates of keratin degrading bacteria [1]. Besides, keratinases are finding applications for the manufacturing of biodegradable plastics, nitrogenous fertilizers, glues, cosmetic, medicine, and foils [2,3].

Despite these, the industrial application and hence the market demand of keratinases as compared to other industrial enzymes is still in the stage of infancy. Therefore, effort should be directed to reduce the keratinase production cost by using the low cost waste material(s) as fermentable substrate(s). During the past couple of years, solid-state fermentation (SSF) has shown a tremendous potential in the industrial production of a myriad of value-added products with advantages over submerged fermentation in terms of low production expense, saving of water and energy, less effluent problem and stability of products due to less dilution in the media [4–6]. Besides, SSF technology holds a tremendous promise for employment generation particularly in developing countries. Furthermore, it has been reported that wild-type strains of microorganisms (fungi or bacteria) have a tendency to do better performance in SSF systems compared to genetically altered microorganisms, and thus reducing the energy and cost requirement even further [7]. However, unlike for production of other biotechnologically important microbial enzymes in SSF, a perusal of literature shows that till date, production of bacterial keratinase in SSF has never been attempted which prompted us to initiate the present study.

In this study, we report the β-keratinase production by a feather degrading *Bacillus subtilis* RM-01 in SSF using chicken-feather as substrate, biochemical characterization and some application of the purified β-keratinase. Response surface methodology (RSM) was used to optimize the media composition for maximal β-keratinase yield in SSF with in an effort to reduce the production cost. To the best of our knowledge, this is the first report on keratinase production as well as statistical optimization of fermentation condition to improve the keratinase yield in SSF.

* Corresponding author. Tel.: +91 9957184351 (M); fax: +91 3712 267005/267006 (O).
E-mail address: akm@tezu.ernet.in (A.K. Mukherjee).

1369-703X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2009.04.001

ELSEVIER

Biochemical Engineering Journal 39 (2008) 353–361

Biochemical Engineering Journal

www.elsevier.com/locate/bej

Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation

Ashis K. Mukherjee*, Hemanta Adhikari, Sudhir K. Rai

Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India

Received 14 May 2007; received in revised form 27 September 2007; accepted 29 September 2007

Abstract

Among the different agro-industrial waste products and kitchen waste materials, viz. mustered oil cake, wheat bran, rice bran, *Imperata cylindrica* grass, banana leaves, potato peels and used tea leaves screened as substrates/solid supports for the production of alkaline protease by a thermophilic strain of *Bacillus subtilis* DM-04 under solid-state fermentation, potato peel followed by *I. cylindrica* grass supported maximum protease production. Further, potato peel and *I. cylindrica* grass mixed in a ratio of 1:1 (w/w) significantly ($P < 0.05$) enhanced the protease production by *B. subtilis* DM-04 compared to individual substrate. Among the tested nitrogen compounds, 0.1% (w/w) beef extract followed by yeast extract served as the best co-nitrogen sources for protease production by *B. subtilis* DM-04 strain on *I. cyindrica* grass. Distilled water adjusted to pH 8.0 was more efficient moistening agent for protease production. The crude protease displayed optimum activity at 37–45 °C temperature range and showed characteristics pH optima at pH 8.0–9.0. The protease from *B. subtilis* DM-04 retained 67% of its original activity post-heating at 60 °C for 15 min. Among the tested protein substrates, casein served as the most preferred substrate for the enzyme. Crude protease exhibited a significant stability and compatibility with most of the tested commercial laundry detergents, demonstrating its feasibility for inclusion in laundry detergent formulation.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Alkaline protease; *Bacillus subtilis*; *Imperata cylindrica*; Laundry detergent formulation; Potato peel; Solid-state fermentation, Ulukher

1. Introduction

Proteases catalyze the cleavage of peptide bonds in other proteins, are the class of enzymes having tremendous applications in both physiological and commercial fields. Among all the different commercial enzymes, microbial protease in particular, represents about 60% of all the industrial enzyme's sales in the world due to their applications in several industrial sectors like in the detergent, food, pharmaceuticals, chemicals, leather, paper and pulp and silk industries [1]. Proteases are classified into acid, neutral and alkaline proteases on the basis of pH range in which their activities are optimum [2]. Among all proteases, alkaline proteases are primarily used as detergent additives [1,3] and they accounted for 40% of the total worldwide enzyme sales and 89% of the total protease sale. Nowadays, the use of enzyme (protease)-based detergents is preferred over the conventional synthetic ones in view of their cleaning properties, better performance at lower washing temperature and the alleviation of pollution [3]. The first detergent containing the bacterial enzyme was introduced in the market in 1956 under the trade name Bio-40 and today, a significant share of the enzyme market is captured by subtilisins and/or alkaline proteases from *Bacillus* species [1].

At present, the overall cost of enzyme production is very high (due to high cost of substrates and mediums used); therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view. Further, proteases produced by using commercial medium have undesirable flavor, which are unsuitable for application

* Corresponding author. Tel.: +91 3712 267172; fax: +91 3712 267005/267006.

E-mail address: akm@tezu.ernet.in (A.K. Mukherjee).

1369-703X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2007.09.017