PRODUCTION, PURIFICATION AND CHARACTERIZATION OF FUNGAL ALKALINE PROTEASE AND ITS APPLICATIONS

Thesis submitted to University of Pune

for the degree of

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

BY

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…… Dedicated To My Late Father

Dr. Bansilalji Khandelwal

TABLE OF CONTENTS

		Page No.
ACKNOWLEDGEMENTS		I
CERTIFICATE		III
DECLARATION BY THE RESEARCH SCHOLAR		IV
ABBREVIATIONS		V
ABSTRACT		VII
LIST OF PATENT FILED		XIV
LIST OF PUBLICATIONS		XIV
CONFERENCES /POSTERS/PRESENTATION		XV
CHAPTER 1:	**General introduction**	1-34
	Introduction	1
	References	29
CHAPTER 2:	**Isolation and identification of *Conidiobolus brefeldianus* and optimization and scale up of protease production**	35-160
Section 1:	**Isolation and identification of *Conidiobolus brefeldianus* strain**	35-49
	Abstract	35
	Introduction	36
	Materials and Methods	40
	Results and Discussion	43
	Conclusion	48
	References	49
Section 2:	**Optimization of media for protease production in shake flasks**	50-91
	Abstract	50
	Introduction	51
	Materials and Methods	60

	Results and Discussion	62
	Conclusion	87
	References	88
Section 3:	**Scale up of alkaline protease production in instrumented fermentors**	92-122
	Abstract	92
	Introduction	93
	Materials and Methods	98
	Results and Discussion	102
	Conclusion	120
	References	121
Section 4:	**Studies on crude alkaline protease and other associated enzymes**	123-160
	Abstract	123
	Introduction	124
	Materials and Methods	128
	Results and Discussion	135
	Conclusion	155
	References	156
CHAPTER 3:	**Purification and biochemical characterization of protease**	161-203
	Abstract	161
	Introduction	162
	Materials and Methods	166
	Results and Discussion	174
	Conclusion	199
	References	200

CHAPTER 4: Applications of crude protease from *C. brefeldianus* 204-273

Section 1: Application of protease in leather processing 204-238

Abstract 204

Introduction 205

Materials and Methods 218

Results and Discussion 220

Conclusion 235

References 236

Section 2: Application of protease in degumming of silk 239-273

Abstract 239

Introduction 240

Materials and Methods 247

Results and Discussion 251

Conclusion 270

References 271

ACKNOWLEDGEMENTS

The work presented in this thesis would not have been possible without my close association with many people. I take this opportunity to extend my sincere gratitude and appreciation to all those who made this Ph. D. thesis possible.

First, I would like to extend my sincere gratitude to my research guide Dr. (Mrs.) R. Seeta Laxman for introducing me to this exciting field of science and for her dedicated help, advice, inspiration, encouragement and continuous support, throughout my Ph.D. During our course of interaction, I have learnt extensively from her, including how to raise new possibilities, how to regard an old question from a new perspective, how to approach a problem by systematic thinking, data-driven decision making and exploiting serendipity. I owe her lots of gratitude for having me shown this way of research.

I am also grateful to Dr. Mala Rao, Dr. Yogesh Shouche, Dr. Sushma Gaikwad, Dr. Mahesh Kulkarni, and all the scientists of Biochemical Sciences Division for their invaluable help and critical suggestions.

I am grateful to Dr. Chandrababu, Mr. Sarvanan and Mr. Ramesh from CLRI, Chenaai for their help in dehairing experiments. Dr. M.K. Gouthaman from CLRI, Chennai is being thanked for his help in scale-up studies.

I express my heart-felt gratitude to Mrs. Snehal More and her family for being with me as an elderly figure through out. Her scientific inputs, personal helps and friendly nature has always made me feel at ease with her and I could always look back on her for any support during my course of Ph.D.

My heartfelt thanks to my seniors Dr. Shivshankar, Dr. Anis and Dr. Bhushan for their guidance and moral support. They always helped me out when I got any difficulties or queries regarding experiments. My special thanks to fellow lab mates, Atul, Shalaka, Ashwini, Priya, Dheeraj, Sudarshan, Sarvanan, Dhananjay, Rajkumar, Padmavati, Tanajee, Ishwar, Gyan, Vishnu, Deepali, Rupesh, Arvind, and Nilesh for

sharing a great relationship as compassionate friends. I will always cherish the warmth shown by them.

A special mention of thanks to my friends in NCL, Santosh, Atul, Arun, Shashidhar, Anil, Ejaj,, Yashwant and many more for their constant support and cooperation during my stay.

I express my special gratitude to my friends Trupti and Kamendra, I will never forget the moments, which we shared together during my stay at NCL.

I must thank the office staff, Indira Mohandasan for her kind support throughout my tenure at NCL. I acknowledge Giriji, Jagtapji, Ramakant and Trehanji who were always ready to give their timely help whenever required.

I owe my deepest gratitude towards my wife ***Gayatri*** *for her eternal support and understanding of my goals and aspirations. Without her help, I would not have been able to complete much of the work. I am thankful to my son* ***Aditya*** *for adding happiness and joy to my life.*

A special thanks to brothers Kishorbhau, Maheshbau and my sister Mangaljiji and all my family members for their constant support and love.

Words fall short if I try to thank my mother to whom I woe my deepest gratitude for her infallible love and support.

I gratefully acknowledge ***Council of Scientific and Industrial Research*** *for providing me financial support and* ***New millennium Indian Technology Leadership Initiative (NMITLI) program*** *of CSIR, under which the work was initiated and* ***Director, NCL*** *to allow me to carryout my research in this esteemed laboratory.*

HARISH KHANDELWL

CERTIFICATE

Certified that the work incorporated in the thesis entitled: "Production, purification and characterization of fungal alkaline protease and its applications", submitted by Mr. Harish Khandelwal, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411008, Maharashtra, India. Material obtained from other sources is duly acknowledged in the thesis.

Date: **Dr. (Mrs.) R. Seeta Laxman**

Place: Pune (Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Production, purification and characterization of fungal alkaline protease and its applications**", submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of Dr. R. Seeta Laxman. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Date: **Harish B. Khandelwal**

Place: Pune (Research Scholar)

ABBREVIATIONS

Abs	Absorbance
APS	Ammonium persulphate
ATFB	Alkali treated fungal biomass
BAPNA	N-α-benzoyl-DL-arginine-p-nitroanilide
BLAST	Basic Local Alignment Search Tool
BME	β- mercaptoethanol
BSA	Bovine serum albumin
°C	Degree Celsius
CF	Culture filtrate
Cm	Centimetre
CTAB	Cetyl trimethyl ammonium bromide
Da	Dalton
ddNTP	Dideoxy nucleotide triphosphate
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DFP	Diisopropylfluorophosphate
DMSO	Dimethyl sulphonic oxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DTNB	5, 5'-dithiobis 2-nitrobenzoic acid
EDTA	Ethylene diamine tetra acetic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
h	hour
kDa	Kilo Dalton
Km	Michaelis Menton Constant
L	liter
M	Molar
min	Minute
ml	Milliliter
mM	Milli molar
MTCC	Microbial Type Culture Collection

N-source	Nitrogen source
NBS	*N*-Bromosuccinimide
NCBI	National Center for Biotechnology Information
NCIM	National Collection of Industrial Microorganisms
nm	Nanometers
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride
RNAase	Ribonuclease
rpm	Revolutions per minute
SBTI	Soyabean trypsin inhibitor
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBE	Tris Borate EDTA
TEMED	N,N,N,N'-Tetramethylethylenediamine
TNBS	2,4,6-trinitrobenzenesulphonic acid
UV/Vis	Ultraviolet/visible spectroscopy
Vmax	Maximum velocity
WRK	Woodward's reagent
µl	Micro liter
ME	Malt extract
YE	Yeast extract
SBM	Soya bean meal

ABSTRACT

Introduction

Proteases belong to the class of hydrolases, which degrade proteins into small peptides and amino acids by catalyzing the reaction involved addition of water to cleave the peptide bond. They are metabolically important and are believed to be the most primitive enzymes in the biological evolution. Their physiological functions are versatile and varied, both at cellular and organelle level. They show wide diversity with respect to their properties like substrate specificity, mechanism of action, involvement of amino acids in the active site, pH and temperature dependency for activity and stability. Proteases can broadly divided into two major groups i.e. exopeptidases and endopeptidases depending on their site of action. Further, they are classified on the basis of the reaction type they catalyzed, chemical nature of the active site and the structural evolutionary relationship they share. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes. Proteases from plant, animal and microbial sources account for about 60% of total enzyme sales. Current world demand for proteases has led to an interest in microbial proteases because of their rapid growth, cost effectiveness, and the ease with which they can be genetically modified to generate high yielding and desirable protease producing strains. Most of the commercial proteases, mainly neutral and alkaline are of bacterial origin and mostly obtained from *Bacillus* species. Fungi are known to secrete variety of hydrolytic enzymes, among them proteases is one of the important groups. Enzymes of fungal origin are advantageous due to the ease of biomass removal during downstream processing. With emphasis on environmental related issues, proteases are expected to replace chemicals used in the conventional methods in various industrial processes. Despite the systematic application of recombinant DNA technology and protein engineering to alter the properties of enzymes, search for proteases with novel properties from microbial biodiversity and their exploitation in newer fields still continues.

The work described in the thesis lays emphasis on optimization and scale up of protease production from a fungal strain isolated in our laboratory and identified as *Conidiobolus brefeldianus* on the basis of 18s rDNA sequence homology. The work was further extended to purify and characterize the enzyme. Finally, its application in leather and silk processing as an eco-friendly alternative is also explored.

Chapter 1: General Introduction

This chapter deals with general information of proteases and it provides concise review of literature on proteases and includes their occurrences, classification, catalytic behavior and mechanism of action. The chapter also focuses on production of proteases and their industrial applications.

Chapter 2: Isolation and identification of *Conidiobolus brefeldianus* and optimization and scale up of protease production

The ***second chapter*** deals with isolation, identification of the fungal isolate and optimization and scale up of protease production. This chapter is divided into four sections.

Section 1: **Isolation and identification of new strain of *Conidiobolus brefeldianus***

The newly isolated fungal strain was identified as a new strain of *Conidiobolus* sp. on the basis of morphological features. The organism grows rapidly and forcibly discharged large whitish conidia with basal papillae. Mycelium is coenocytic which becomes septate in later stages. Conidiophores are indistinguishable from mycelium. The organism is zygosporic and forms round, smooth, thick walled zygospores with two distinct wall layers and granular contents inside. The 18S rDNA sequence homology studies revealed that the new strain showed 99% homology with *Conidiobolus brefeldianus* AF 368506.1 and 98% homology with various strains of *Conidiobolus coronatus.* The new strain of *Conidiobolus brefeldianus* was deposited in Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India with accession number MTCC 5185. The nucleotide sequences of 18S rDNA have been deposited in the NCBI GenBank database and assigned accession numbers: FJ895304.

Section 2: **Optimization of media for protease production in shake flasks**

Optimization of protease production was initially studied in shake flasks, where effects of various physicochemical and nutritional factors such as pH, temperature, carbon & nitrogen sources, inducers, additives, their concentrations, age of stock etc. were investigated. Protease production was highest in GYE medium with soyabean meal (SBM) or skim milk. Among various agricultural residues, SBM was

found to be the best inducer. Additional nitrogen source did not show beneficial effect on production. Protease production was highest in the medium containing 2% glucose, 0.3% yeast extract and 3% SBM. Yeast extract in the medium was replaced by di-ammonium hydrogen phosphate (DAP) with 20% enhancement in activities. Similar activities were also obtained with commercial grade glucose and manure grade DAP. Protease production was observed in the pH range 5 to 10 and temperature range of 20 to 37°C, with optimal activities at pH 5.5-6 and 28°C. Optimum stock and inoculum age were found to be 2-3 days and 18-24 h respectively. Inoculum size of 10-15% gave maximum activities. Optimization resulted in more than four-fold increase in protease activities

Section 3: Scale up of protease production in instrumented fermentors

Optimization of process parameters for protease production by *C. brefeldianus* was initially studied in 7.5 L bioreactor with 5 L working volume. Agitation greatly influenced protease production both in terms of activity as well as fermentation time. Activities were low when the agitation was kept at fixed rate of 200 and 300 rpm irrespective of aeration rates. Further increase in agitation (400 and 500 rpm) resulted in increased protease activities. Glucose consumption was directly proportional to the rate of agitation and increased during the progress of fermentation. Similarly, biomass produced was more with higher agitation rates of 400 and 500 rpm compared to 200 and 300 rpm. Highest activities were obtained when the agitation rate was slowly increased from 250 to 450 rpm during the course of fermentation at both the aeration rates. Protease yields of 30 and 32 IU/ml were obtained at 0.5 and 1.0 vvm aeration respectively under these conditions. Production of protease was successfully scaled up in 75 and 700 L fermentor using commercial grade glucose and DAP. Maximum protease yield (40 IU/ml) was obtained within a period of 45 h in 700 L fermentor. Productivities increased from 700 IU/L/h for 7.5 L fermentor to 900 IU/L/h for 700 L fermentor. Crude enzyme was concentrated by various methods viz. ammonium sulphate precipitation, membrane concentration, lyophilization and spray drying with 70-90% recovery depending on the method applied. Ammonium sulphate precipitated and spray dried proteases were stable at ambient temperature for more than one and half years. The studies demonstrated that the alkaline protease production could be scaled up successfully in cost effective medium with yields similar to shake flasks and

the crude protease has very good shelf life indicating the potential of the enzyme for its commercialization.

Section 4: Studies on crude alkaline protease and other associated enzymes

The crude protease preparation of *C. brefeldianus* is active in the temperature range 30 to 60°C and pH range of 6-11 with optimum at 50ºC and pH 9. the enzyme was active in presence of Ca^{+2}, Cd^{+2}, Co^{+2}, K^{+}, Mg^{+2} and Mn^{+2} while Ni^{+2} and Zn^{+2} resulted in 35-40% inhibition. Cu^{+2} and Hg^{+2} totally inhibited the activity. The crude protease was active towards keratin azure, azocall, elastin-orcin, azocasein but did hydrolyze synthetic chromogenic collagen substrate. In addition, the crude enzyme exhibited various enzyme activities such as chondroitinase, laminarase and chitinase. It was fairly stable up to 12 h in presence of most of the organic solvents tested at 28°C, while at 37°C, it retained about 80, 70 and 60% of activity in presence of acetone, DMSO and methanol respectively. Protease is stable in presence of 1% non-ionic detergents, β-mercaptoethanol (BME), EDTA, 4 M urea and retained more than 30-40% activity in presence of 1% SDS. The protease retained 50-70% activity at 40ºC in presence of commercial detergents up to 1 h and effectively removed blood stains from cloth when applied in combination with commercial detergent. The culture was able to produce extra cellular lipase using various vegetable oils as inducers. The lipase was optimally active at pH 7 and 50°C.

Chapter 3: Purification and biochemical characterization of protease

C. brefeldianus secretes two proteases out of which the major protease was purified to homogeneity by salt precipitation and concentration, ion exchange and gel filtration chromatographic methods. There was 3.37 folds purification with a specific activity of 156 IU/mg and 54% yield. The molecular weight of the purified protease was found to be 30.19 kDa, 28.1 kDa and 27.8 kDa by SDS PAGE, gel filtration chromatography and MALDI-TOF respectively. Protease had an isoelectric point of 9.74. Protease was optimally active pH 9.0 and 50°C. The K_m and V_{max} of the protease were 2.5 mg/ml and 15.38 IU/ml respectively. Total inhibition of the protease was observed in presence of phenylmethylsulphonyl fluoride (PMSF), while L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) and *N*-tosyl-L-lysine chloromethyl ketone (TLCK) partially inhibited the enzyme. Protease was active in

presence of ethylene diamine tetra acetic acid (EDTA), iodoacetate, benzamidine and soyabean trypsin inhibitor (SBTI). The protease was stable in most of the metal ions tested except Hg^{+2} and Cu^{2} which inhibited completely and by 31.8% respectively**.** The protease showed highest activity with casein followed by hemoglobin and BSA. The protease was highly active towards Succ-Ala-Ala-Pro-Leu-pNA, followed by Succ-Ala-Ala-Pro-Phe-pNA, N-Bez-Arg-pNA and Succ-Ala-Ala-Ala-pNA in the order of catalytic efficiencies while it was inactive against Gly-Phe-pNA. Protease also showed esterase activity and hydrolyzed BAEE and BTEE. MALDI-TOF analysis of peptides obtained after hydrolysis of oxidized insulin B chain by the protease showed five major and three minor sites of cleavage. The amino acid modifier, phenylmethylsulphonyl fluoride (PMSF), diethyl pyrocarbonate (DEPC), Woodward's reagent (WRK) and N-bromosuccinimide (NBS) inhibited the protease, indicating the involvement of serine, histidine and aspartic acids for the activity of protease. Incubation of protease with casein prior to addition of PMSF offered protection against inhibition indicating the presence serine residue in the active site. Successive addition of NBS to the protease brought about progressive decrease in absorbance at 280 nm and simultaneous loss in activity and the number of tryptophan residues oxidized per mole of the enzyme was found to be 2.7. The sites of attack were identical to those of subtilisins but differed from site of attack for trypsin indicating it to be a subtilisin like serine protease.

Chapter 4: Applications of crude alkaline protease from *C. brefeldianus*

The ***fourth chapter*** deals with the application of crude *C. brefeldianus* protease in leather and silk industries. This chapter divided into two sections.

Section 1: Application of crude alkaline protease in leather processing

Application of alkaline protease from *C. brefeldianus* was tested as a substitute for conventional lime and sulfide dehairing in leather processing. Alkaline protease from *C. brefeldianus* was efficient in unhairing various types of skins and hides. The crude protease preparation was active towards keratin azure, elastin-orcin, azocasein and azocoll but did not show true collagenase activity. In addition, the crude enzyme exhibited other enzyme activities such as chondroitinase, laminarinase and chitinase. Complete hair removal of skin/hide by the protease was achieved in 16-

18 h. The dehaired pelt showed smooth and white appearance due to hair removal along with epidermal layer. In addition, the grain was clean and without damage in enzymatically dehaired pelts. The microscopic observation of the cross section of dehaired goat skin and cow hide showed absence of epidermis and hair shaft with empty follicles. Enzymatic dehairing resulted in complete and uniform fiber opening in the dermis and corium region. SEM pictures of the dehaired pelt and dyed crust revealed grain structure to be clean with more visible fiber separation compared to those obtained by conventional method. Physical properties viz. tensile strength, elongation and tear strength of dyed crust of enzymatically and conventionally dehaired pelts were comparable. Results were also validated on large scale with goat skins and cow hides.

Section 2: Application of protease in degumming of silk

Traditionally, degumming of silk is carried out with soap and alkali at higher temperature or by boiling at elevated temperature and/ or pressure for 1-2 h. It is a high resource consuming process and generates effluents with high impact on environment. In contrast, enzymatic degumming process is advantageous as it would save energy, water, chemicals and effluent treatment costs. Degumming of Chinese bivoltine silk with six indigenous microbial alkaline proteases was investigated and compared with commercial enzymes as well as with conventional degumming process. The enzymatic degumming was carried out between of 40 to 65°C while conventional alkali soap degumming was performed at 95°C. Among the six proteases tested, two fungal and two actinomycete proteases were promising which showed weight loss similar to conventional method (19.58 to 21.78%). *C. brefeldianus* and BOA-2 proteases were found to be best which showed complete degumming within 15-30 min and at low enzyme concentrations. Degummed fibers showed no significant difference in tensile strength or elongation at break indicating no damage to the fiber which was confirmed by scanning electron microscopic studies. Recovery of sericin from degumming liquid after enzymatic degumming and analysis by SDS–PAGE revealed the presence of three distinct proteins with molecular weights ranging from 19.6 kDa to 26.6 kDa or slightly higher. Enzymatic hydrolysis of pure sericin obtained from silk cocoons with *C. brefeldianus* protease was investigated. Analysis of peptides obtained after hydrolysis for 30 min with low protease concentrations showed a major peak corresponding to 1092 Da while with higher concentration,

major peptide formed was of lower molecular weight (516 Da). Effect of time on hydrolysis showed that 15 to 30 min was sufficient to hydrolyse the sericin to peptides with molecular weights in the range of 516-1092 Da.

List of patents

1. Laxman, R. S., **Khandelwal, H. B**., More, S. V., Kalal, K. M., Narasimhan, C. B. K., Palanivel, S., Balaram, P. **"**Enzymes from *Conidiobolus brefeldianus* and process for preparation thereof". 2011. PCT No. IB/2011/000516.

2. Laxman, R. S., Shankar, S., **Khandelwal, H. B**., More, S. V., Narasimhan, C. B. K., Palanivel, S., Balaram, P. **"**A novel fungal strain of *Beauveria* sp. and a process for preparation of enzymes thereof". 2011. PCT No. IB/2011/000178.

List of Publications

1. Enzymatic degumming of silk with microbial proteases.
   More, S.V., **Khandelwal H.B.,** Joseph, M.A. and Laxman, R.S. (Manuscript **accepted** in the *Journal of Natural Fibers*).

2. Eco-friendly enzymatic dehairing of skins and hides by *C. brefeldianus* protease.
   H. B. Khandelwal., S.V. More, K.M. Kalal and R. Seeta Laxman. (Shortly communicating to *New Biotechnology*)

3. Identification of alkaline protease producing fungus and properties of the protease. S. V. More**, H. B. Khandelwal,** S. Shankar and R. Seeta Laxman. (Shortly communicating to *The International Journal of Pharma and Biosciences*)

4. Optimization and scale up of alkaline protease production by *Conidiobolus brefeldianus* MTCC 5185.
   H. B. Khandelwal, S. V. More and R. Seeta Laxman. (Manuscript under preparation).

5. Purification and characterization of alkaline protease from newly isolated strain of *C. brefeldianus* MTCC-5185.
   H.B. Khandelwal and R. Seeta Laxman (Manuscript under preparation)

Posters

- Enzymatic degumming of silk with microbial proteases

  H. B. Khandelwal, S. V. More and R. Seeta Laxman

  National Seminar on Recent Advances in Molecular Microbiology & Microbial Technology, Warangal (INDIA). January 22-24, 2009.

- Preparation of alkaline protease from *Conidiobolus coronatus* and its properties

  S.V. More**,** S. Shankar**, H. B. Khandelwal** and R. Seeta Laxman

  International conference organized by Biotech Research Society in Emerging trends in Biotechnology at Banaras Hindu University, Varanasi (U.P.) India. December 5 - 8, 2010.

Presentation

- Degumming of silk with fungal protease and recovery of sericin from degumming waste.

  Golden Jubilee National Conference on 'Sericulture Innovations: Before And Beyond' organized by Central Sericulture Research & Training Institute. Mysore-570 008, Karnataka, India. January 28-29, 2011.

CHAPTER 1

GENARAL INTRODUCTION

Introduction

Enzymes have played an important role in many aspects of life since the dawn of time. In fact, they are vitally important to the existence of life itself. Civilizations have used enzymes for thousands of years without understanding what they were or how they work. Over the past several generations, science has unlocked the mystery of enzymes and has applied this knowledge to make better use of these amazing substances in an ever-growing number of applications. Enzymes play crucial roles in producing the food we eat, the clothes we wear, even in producing fuel for our automobiles. Enzymes are also important in reducing both energy consumption and environmental pollution and now modern biotechnology is opening doors that will further expand the use of enzymes into exciting new areas. The past few decades of the twentieth century have witnessed spectacular advances and betterment of living standards due to the beneficial integration of enzyme technology with scientific progress and rapid translation of laboratory findings into practical technologies and commercial scale manufacturing processes.

Importance of enzymes for life

Enzymes are proteins with highly specialized catalytic functions produced by all living organisms. Enzymes are essential for all metabolic processes and are responsible for many essential biochemical reactions in all living organisms viz. microorganisms, plants, animals and human beings but are not alive. Although like all other proteins, enzymes are composed of amino acids, they differ in function in that they have the unique ability to facilitate biochemical reactions without undergoing change themselves. This catalytic capability is what makes enzymes unique. Enzymes are natural protein molecules that act as highly efficient catalysts in biochemical reactions, that is, they help a chemical reaction take place quickly and efficiently. Enzymes not only work efficiently and rapidly but are also are biodegradable.

Nomenclature and classification of enzymes

Enzymes are identified by a common nomenclature system based on the description of what function it performs in the cell and ends with a common phrase- ase. The International Union of Biochemistry and Molecular Biology and the International Union of Pure and Applied Chemistry developed a nomenclature system wherein each enzyme is given an Enzyme Commission Number called as EC number. Accordingly,

classifications of enzymes based on the reaction they catalyze along with examples are listed in Table 1.1.

Table 1.1: Enzyme classless

Class	Chemical Reaction Catalyzed	Example
Oxidoreductase	Oxidation-reduction in which oxygen and hydrogen are gained or lost	Cytochrome oxidase, lactate dehydrogenase
Transferase	Transfer of functional groups, such as an amino group, acetyl group, or phosphate group	Acetate kinase, alanine deaminase
Hydrolase	Hydrolysis of bonds with addition of water	Amylase, Lipase, protease
Lyase	Removal of groups of atoms without hydrolysis	Oxalate decarboxylase, isocitrate lyase
Isomerase	Rearrangement of atoms within a molecule	Glucose-phosphate isomerase, alanine racemase
Ligase	Joining of two molecules (using energy usually derived from the breakdown of ATP)	Acetyl-CoA synthetase, DNA ligase

Proteases

Proteases are metabolically important and inseparable part of entire living beings. They are believed to be the most primitive enzymes in the biological evolution, as most of the digestive proteases of higher organisms have a common lineage with microbial origin. Their physiological functions are versatile and varied, both at cellular and organal level. The diversity of proteases is key factor in the maintenance of homeostasis of organism at one end and equally responsible to cause abnormal physiological conditions during many pathological states at the other. Proteases belong to the class of hydrolase, which degrade proteins into small peptides and amino acids by catalyzing the reaction involved addition of water to cleave the peptide bond. They show wide diversity with respect to their properties like substrate specificity, mechanism of action, involvement of amino acids in the active site, pH and temperature for activity and stability. The catalytic property of protease tends to make it attractive for wide range of applications in various processes of milk, meat, baking, brewing, detergent, textile and leather industry (Gupta *et al.,* 2002a; Bhaskar *et al.,* 2007; Sareen and Mishra, 2008; Jellouli *et al.,* 2009). They are widely exploited in pharmaceutical industry and in basic research as analytical tools (Rao *et al.,* 1998). Among them alkaline proteases, dominate the worldwide industrial applications.

Physiological significance of proteases

Proteases involved in numerous complex physiological functions. They are extremely important for conducting the essential metabolic and regulatory functions in all living forms. The modern methodologies and technologies of molecular biology and protein chemistry that developed during the period of past few decades have revealed new insights into the protein and gene structure of several protein precursors and their processing by regulating proteolysis. Proteases play vital role in many physiological processes such as maintenance of protein turn over, cell proliferation and migration, fertilization of egg by sperm, morphogenesis in development, release of hormones and peptides from precursor proteins, activation of zymogens, coagulation of blood, transport of proteins across cellular membranes, germination of seed, sporulation and discharge of conidia in fungi **(Rao *et al.,* 1998**). Dysfunction and dysregulation of physiologically important proteases can result in the onset of several pathological conditions such as neurodegenerative and cardiovascular diseases, arthritis and cancer. Consequently, there has been increasing interest in the identification and functional characterization of proteases. Many of these proteases and their substrates are considered as potential therapeutic targets and gaining attraction by pharmaceutical industry. For instance, the matrix metalloproteases (MMPs) have been identified as important targets for the treatment of cancer, arthritis and cardiovascular diseases and several MMPs inhibitors have been tested in clinical trials (Yan and Blomme, 2003).

Sources of protease

- ***Plant and animal***

  Being physiologically important, proteases are wide spread in all living organisms. They are ubiquitous and widely distributed in plants, animals and microbes. Plant proteases are involved in various aspects of plant physiology and development like seed germination, defense mechanism, programmed cell death, protein turnover and post translation modification (Rabade *et al.,* 2011). However, the number of industrially important proteases from plant origin is limited. The major plant proteases studied and currently in use, with an important role in food and pharmaceutical industries are papain, bromelain and ficin, extracted from papaya (*carica papaya),* pineapple *(Ananus comosus)* and fig (*Ficus carica*)

respectively. They are highly consumed in industry as a proofing agent during beer finishing operations in the brewing process, facilitates meat tenderization and used as biocatalyst for amino acid and peptide synthesis (Rowan *et al.,* 1990; Rabade *et al.,* 2011). Apart from applications in food processing and brewing, bromelin has several medicinally important qualities, which include anti-inflammatory, ani-thrombotic, fibrinoytic and anticancer functions (Chobotova *et al.,* 2010). Pancreatic trypsin, chymotrypsin, pepsin, and rennins are the well-known examples of animal proteases and usually released from their zymogens by autolysis or due to the proteolytic action of other enzyme (Rao *et al.,* 1998). Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins in all invertebrates and vertebrates. Because of its well defined specificity, trypsin is widely used in proteomics. It is also used in the production of microbial media ingredients, animal cell culture. Trypsin has also found applications to cure inflammation and to dissolve blood clots. In recent years, trypsin is used as target for controlling the insect pests by using trypsin inhibitors. Chymotrypsin is found in animal pancreatic extract and functionally activated by the action of trypsin from its zymogenic form. It is extensively used for diagnostic and analytical purposes. Rennin is a pepsin-like protease produced from the precursor 'prorennin' present in the stomachs of all nursing mammals. It cleaves a single peptide bond in *k*-casein to generate insoluble para-*k*-casein and C-terminal glycopeptides. It cleaves the peptide bond between phenyalanine and methionine, the specific linkage between the hydrophobic (para-casien) and hydrophilic (acidic glycopeptide) group of casein in milk, since they are joined by phenylalanine and methionine. The hydrophobic group would unite and would form a three dimensional network to trap the aqueous phase of the milk resulting in the formation of calcium phosphocaseinate. This brings about the extensive precipitation to produce a stable curd and cheese with good flavor (Nirmal *et al*., 2011).

- ***Microbial proteases***

Despite the importance of plant and animal proteases from industrial point of view, microorganisms represent an excellent source of proteases. They are easy to culture at large scale in relatively short time span by using established fermentation methods for abundant and regular supply. Being extra-cellular in

nature, they are directly secreted into the fermentation broth, thus simplify the downstream processing of enzyme extraction as compared to proteases obtained from plants and animals. In addition, microbial proteases have longer shelf life and storage stability (Gupta *et al.,* 2002a). Further, microorganisms offer an advantage of strain improvement by mutagenesis and genetic manipulation (Kumar and Takagi 1999). A major portion of worldwide sale of proteases is occupied by microbial origin and alkaline serine proteases are the most important group of commercial enzymes. Despite extensive research carried out on proteases, there is still need for proteases with novel properties that may have new biotechnological applications. With emphasis on environmental friendly technologies, enzymes especially proteases are expected to replace chemicals used in the conventional methods thus increasing their demand. Hence, in recent years, search is directed for proteases from microbial cultures isolated from diverse habitats or newer proteases with novel and unusual properties.

- ***Bacteria***

Although, proteases are produced by variety of bacteria such as *Pseudomonas aeruginosa* (Oh et al., 2000), *Flavobacterium, Staphylococcus aureus*, , *Achromobacter* and species belonging to *Streptomyces* (Lazim *et al.,* 2009; Azeredo *et al.,* 2004), majority of the commercial alkaline proteases are produced by strains belonging to *Bacillus* sp (Deng *et al.,* 2010). Alkaline proteases from *Bacillus* strains are mainly exploited as detergent additives because of their high pH and temperature stability and broad substrate specificity (Gupta *et al.,* 2002b). In addition, they are also useful in dehairing operations in leather industry (Madhavi *et al.,* 2011). On the other hand, neutral proteases of bacterial origin are useful in food industry for preparation of protein hydrolysates and in brewing industry.

- ***Fungi***

Fungi are known to secrete variety of hydrolytic enzymes, among them protease is important group of enzymes. One of the first known representatives of fungal proteases was proteinase K, an alkaline enzyme from *Engyodontium album* also known as *Tritirachium album* (Kotlova *et al.,* 2007). *Aspergillus* is the most

studied fungal strain for protease production (Malthi and Chakraborti 1991; Chakrabarti *et al.,* 2000; Anadan *et al.,* 2007; Hajji *et al.,* 2007; Chellapandi 2010; Sharma and De 2011; Kranthi *et al.,* 2012) and exploited for commercial production (www.amfep.org/ Amfep/09/68 OCT2009). However there are number of other genera apart from *Aspergillus* secreting proteases which have appeared in literature such as *Rhizopus* (Devi *et al.,*2011), *Penicillium* (Dahot 1993; Benito *et al.,* 2006; Patidar *et al., 2*005), *Fusarium* (Ueda *et al.,* 2007), *Trichoderma* (Kredics *et al.,* 2005) and *Beauveria* (Bidochka and Khachatourians, 1987; Shankar *et al.,* 2011; Rao *et al.,* 2006). Few genera such as *Conidiobolus* are less investigated as far as enzyme production is concerned and reports are meager (Phadatare *et al.,* 1993; Bhosale *et al.,* 1995; Laxman *et al.,* 2005; Bania *et al.,* 2006). Due to their hyphal nature which penetrates the substrate, fungi are most suited for production by solid state fermentation (SSF) and reports on protease production by submerged as well SSF are plenty (Tunga *et al.,* 2003; Germano *et al.,* 2003; Laxman *et al.,* 2005; Chellappan *et al.,* 2006; Rao *et al.,* 2006). Another advantage is that the downstream processing is easy for fungal enzymes because cell free enzyme can be obtained by simple filtration. Fungal proteases are active over broad pH range (4-11) and exhibit broad substrate specificity, consequently may find applications in diverse industrial processes.

Classification of proteases

Microbial proteases are classified into various groups, dependent on whether they are active under acidic, neutral, or alkaline conditions. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (IUBC 1992). Still they do not obey the common classification rules to get fit in the general system of enzyme nomenclature because of their vast structural and functional diversity. 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 respect to structure. Proteases are broadly subdivided into two major groups i.e. exopeptidases and endopeptidases depending on their site of action.

Exopeptidases act only near the ends of polypeptide chains at the N or C terminus (Rao *et al.,* 1998). Those acting at a free N terminus liberate a single amino acid

residue (aminopeptidases), a dipeptide (dipeptidyl- peptidases) or a tripeptide (tripeptidylpeptidases). The exopeptidases acting at a free C terminus liberate a single amino acid.

Aminopeptidase

Aminopeptidases are intracellular enzymes in general and classified as aminopeptidase N or aminopeptidase A, depending on their preference for neutral (uncharged) or acidic side chains respectively. Most of the aminopeptidases are metalloenzymes. Aminopepidase N is membrane bound mealloprotease functioning in endocytosis and as a signaling molecule. It has also been identified as cell surface marker for malignant cell and its level increases with progression of tumors in breast, ovarian and prostate cancer (Rojas *et al*., 2011).

Carboxypeptidase

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. 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.

Endopeptidases

Endopeptidases act preferentially in the inner regions of peptide chains away from the N and C termini. The presence of free α-amino or α-carboxyl groups has a negative effect on the activity of the enzyme. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, metalloproteases, and ATP-dependent proteases which require ATP for their activity (Menon and Goldberg 1987). The threonine and glutamic-acid proteases were described in 1995 and 2004, respectively. Depending on their amino acid sequences, proteases are grouped into different families and further classified into clans to accommodate sets of peptidases that have diverged from common ancestor. Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine,

cysteine, aspartic, metallo-, or unknown type, respectively (Rawlings and Barrett 1993; Rao *et al.,* 1998).

Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are widely distributed in nature and found in all life forms and several viruses, suggesting that they are of utmost important for physiological functions. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further, subdivided into about six clans with common ancestors. The primary structures of the members of four clans, chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia* D-Ala-D-Ala peptidase A (SE) are totally unrelated, suggesting that there are at least four separate evolutionary origins for serine proteases (Rawlings and Barrett 1994). Serine proteases show irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DIC), diisopropylfluorophosphate (DFP) , phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as *p*-chloromercuribenzoate (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. Serine proteases active in alkaline range are further grouped in serine alkaline proteases and they represent the largest subgroup of serine proteases. In commercial point of view, this group of protease is extremely important.

Types of serine proteases

Depending on structural and functional properties, a comprehensive account of subclasses of serine proteases is presented below.

- ***Chymotrypsin-like proteases***

  Chymotrypsin, a mammalian digestive protease is structurally similar to that of trypsin, elastase and thrombin. It specifically cleaves peptides at the carboxyl side of hydrophobic amino acids side chain of tyrosine, tryptophan and phenylalanine and most active at pH 8. The most important inhibitors of this class are *N*-tosyl-L-

lysine chloromethyl ketone (TLCK), L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), di-isopropylfluorophosphate (DFP) and soyabean trypsin inhibitor (SBTI). The molecular weight of these proteases is generally around 20 kDa (Gupta *et al.,* 2002a).

- ***Subtilisins and subtilisin like proteases***

Subtilisins are the second largest family of serine proteases. They are generally produced by B*acillus* sp., however production of subtilisin like proteases by fungi (Phadatare *et al*, 1997; Zou *et al.,* 2010) and *Streptomyces* (Suzuki *et al.,* 1997) are reported. Subtilisns are characterized by catalytic triad of the three amino acids namely aspartate, histidine, and serine. Subtilisins are specific for hydrolyzing aromatic or hydrophobic residues, such as tyrosine, phenylalanine and leucine. They are further divided in two types namely subtilisin Carlsberg and subtilisin Novo. The former is produced by *Bacillus licheniformis* and discovered in 1947 by Liderstrom, Lang and Ottesen at the Carlsberg laboratory. Subtilisin Novo, also known as BPN' or nagase is produced by *Bacillus amyloliquefaciens*. Subtilisin Carlsberg is widely used in detergents (Rao *et al.,* 1998). They are extremely susceptible to phenyl methyl sulphonyl fluoride, diisopropyl-fluorophosphate and potato inhibitor. They are most active around pH 10, with a molecular weight range of 15–30 kDa and an isoelectric point near 9 (Gupta *et al.* 2002a).

- ***Serine alkaline proteases***

As the name suggests, serine alkaline proteases are active in alkaline pH range and produced by several bacteria, molds, yeasts, and fungi. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or *N*-tosyl-L-lysine chloromethyl ketone (TLCK). Their substrate specificity is similar to but not rigid to that of chymotrypsin. Serine alkaline proteases hydrolyze a peptide bond which has the aromatic amino acid (tyrosine or phenylalanine) or leucine at the carboxyl side of the splitting bond. Their optimum pH is around 10 and isoelectric point is around 9. Their molecular mass falls in the range of 18-35 kDa (Gupta *et al,* 2002a). However some reports show exceptional molecular masses of serine alkaline protease as low as 6.8 kDa (Sutar *et al.,* 1991) and as high as 90 kDa (Kato *et al.,* 1992). Serine alkaline proteases

are produced by several species of bacteria, however *Bacillus* is predominant among them. Alkaline serine proteases are also produced by variety of fungal species (Nirmal *et al.,* 2010).

Aspartic proteases

Aspartyl proteases, also known as acid proteases or aspartyl proteinases are a widely distributed subfamily of proteolytic enzymes belonging to the enzyme family of endonucleases. Aspartyl proteases are known to exist in animals, plants, microbes and viruses (Canduri *et al.,* 1998). The subfamily of aspartyl proteases is characterized by having the highly conserved sequence of Asp-Thr-Gly (Pearl 1987). Aspartic proteases have been grouped into three families, namely pepsin, retropepsin and enzymes from para-retroviruses. Most of the aspartic proteases are optimally active at low pH (pH 3 to 4) and have their isoelectric points in the range of 3 to 4.5. Aspartyl proteases play an important role in many aspects of health and physiology, including blood pressure (renin), digestion (pepsin and chymosin), and in the maturation of the Human Immunodeficiency Virus (HIV I protease). Microbial aspartic proteases are broadly divided in to groups: (i) pepsin –like enzymes produced by *Aspergillus, Penicillum, Rhizopus* and *Neurospora* and (ii) rennin-like enzyme produced by *Mucor* sp. They are inhibited by diazoketone compounds like diazoacetyl-DL- norleucine methyl ester (DAN) and 1, 2–epoxy-3-(p-nitrophenoxy) propane (EPNP) in the presence of copper ions but insensitive to inhibitors such as diflurophosphate (DFP), p-chloromercuri benzoate e (pCMB) and metal ion chelator like ethylene diamine tetraacetate (EDTA) (Rao *et al*., 1998).

Cysteine or thiol proteases

Cysteine proteases, which are mostly referred as thiol proteases in older literature, have been found in viruses, bacteria, protozoa, plants and mammals (Barrett *et al.,* 1986) and also reported in fungi **(**Liu *et al.,* 2010). About 20 families of cysteine proteases have been recognized. They have also been grouped into four broad classes based on their side chain specificity (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of cysteine and histidine (Cys-His or His-Cys) residues differ among the families **(**Barrett, 1994). For the catalytic activity of cysteine proteases,

presence of reducing agents such as HCN or cysteine is necessary. Cysteine proteases have neutral pH optima, however a few of them, e.g., lysosome proteases, are maximally active at acidic pH. Among all the lysosomal cysteine proteases, most extensively studied enzyme is cathepsin B. This enzyme has been isolated from various tissues. Cathepsin B shows two enzymatic activities, Cathepsin B1 is endopeptidase and now known as Cathepsin B (EC 3.4.22.1) and Cathepsin B2 (3.4.18.1) shows carboxypeptidase activity. Today the term Cathepsin is used for intracellular proteases, mostly localized in lysosomes, which are active at acidic pH values (Otta and Schrimeister, 1997) Cathepsin B can degrade β-amyloid precursor protein, accumulation of which may result in Alzheimer's disease, thus, Cathepsin B may play a pivotal role in the natural defense against Alzheimer's disease (Muller *et al.,* 2006). Papain is the best-known example of cysteine protease of plant origin having enormous commercial importance in food industry. It is monomeric polypeptide consisting of 212 amino acid residues with a molecular weight of 23406 and consists of three disulfide bridges (Mitchel *et al.,* 1970). Clostripain is a cysteine-activated protease found in culture filtrates of anaerobic bacterium *Clostridium histolyticum* responsible for gangrene. It is unique in its specificity for the carboxyl peptide bond of arginine and its dependence on thiol and calcium ions. Streptopain, the cysteine protease produced by human pathogen *Streptococcus sp.*, shows a broad specificity, including the cleavage of immunoglobulin G (IgG) in a manner similar to that of papain (Eriksson and Norgren, 2003). Cysteine proteases are susceptible to sulphahydryl compounds such as PCMB but remain unaffected by DFP and metal-chelating agents.

Metalloproteases

Metallproteases are characterized by the requirement of divalent metal ions to cleave peptide bond. This group includes enzymes from variety of origins such as collagenases from higher organisms, heamorrhagic toxin from snake venoms and thermolysisn from bacteria (Rao *et al.,* 1998). Based on their specificity metalloproteases can be divided into four groups (i) neutral (ii) alkaline (iii) *Myxobacter* I, and (iv) *Myxobacter* II. The neutral proteases show specificity for hydrophobic amino acids in the polypeptide chain while the alkaline proteases possess a very broad specificity. *Myxobacter* protease 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 (Rao *et al.*, 1998). Metalloproteases are important in many aspects of biology to maintain homeostatic control, ranging from cell proliferation, differentiation and remodeling of the extracellular matrix (ECM) to vascularization and cell migration (Chang and Verb, 2001), besides, they are also responsible for several pathopysiological states like rheumatoid arthritis and osteoarthritis (Murphy and Lee, 2005).

Threonine* and *glutamic acid proteases

The threonine and glutamic-acid proteases were not described until 1995 and 2004, respectively. Threnonine proteases are a family of proteolytic enzymes harbouring a threonine (Thr) residue within the active site. Recently a new protease family of glutamic acid proteases, termed eqolisins has been identified and it includes two fungal proteases, bringing the total number of recognized protease classes to five. However, glutamic acid proteases have not been reported in mammals (Lopez-Otin and Bond, 2008).

The MEROPS classification system

A classification scheme based on statistically significant similarities in sequence and structure of all known proteolytic enzymes was devised by Rawlings *et al.* (2007), and the database is termed as MEROPS. The classification system divides peptidases into clans based on catalytic mechanism and families on the basis of common ancestry. The MEROPS database is a manually curated information resource for proteases, their inhibitors and substrates. The database has been in existence since 1996 and can be found at http://merops.sanger.ac.uk. The MEROPS database provides a classification and nomenclature of proteolytic enzymes and their inhibitors that is widely used throughout the academic community. The classification system is based on hierarchical system where each hierarchy is the peptides or inhibitor unit at the bottom. The protein to which it belongs that has been most fully characterized biochemically is chosen as a representative called a 'holotype'. Sequences considered to represent the same protein but from different organisms (i.e. orthologues) are grouped as a single protein species according to the criteria set out by Barrett and Rawlings (2007). Over 66000 peptidase protein sequences have been classified into 50 clans and 184 families Over 26 000 serine peptidases are grouped into 13 clans and

40 families (Page and Cera, 2008). The database also includes proteolytic enzymes other than peptidases. Families of self cleaving proteins that utilize the peculiar chemistry of asparagines to break peptide bonds without hydrolysis are known as asparagine peptide lyases (Rawlings *et al.,* 2012).

Mechanism of action of proteases

Proteases catalyze amide (peptide) bond hydrolysis in protein or peptide substrates (Figure 1.1). The mechanism by which proteases bring about breakdown of peptide bond has been a subject of great interest to researchers as it forms a basis for modifying its activity to make it suitable for its biotechnological application. The catalytic site of proteases is flanked on one or both sides by specificity subsites, each able to accommodate the side chain of a single amino acid residue from the substrate. The general nomenclature of positions of cleavage sites on the substrate were formulated by Schechter and Berger (1967). The cleavage site is designated between P1-P1', incriminating the numbering in the N terminal direction of the cleaved peptide bond (P1, P2, P3, P4 ----etc.). In the same way, numbering is incremented from the cleavage site towards C terminus as P1', P2', P3', P4' and so on. In similar way, the corresponding binding sites on the enzyme are denoted with letter S. (Figure 1.2).

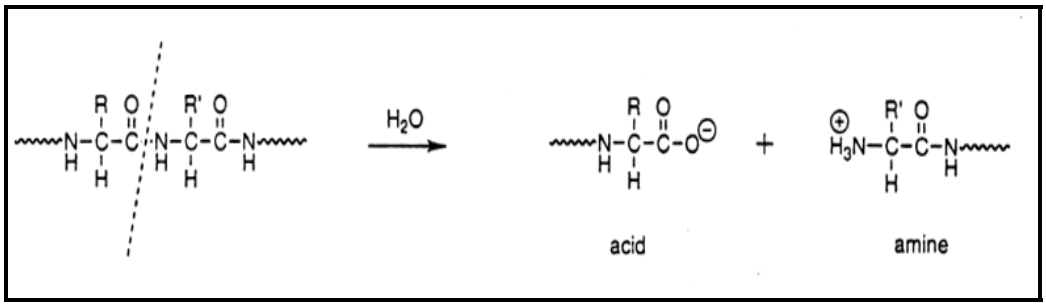

Figure 1.1: Hydrolysis of peptide bond

Protease: N Sn – S4 – S3 – S2 – S1--- K--- S1'- S2' – S3'- S4' – Sn' C

Substrate: N Pn - P4 – P3 – P2 – P1-- -¦- -- P1' – P2'- P3'- P4'- Pn' C

Figure 1.2: Nomenclature of the peptide and peptide substrate. K indicates catalytic site of peptide, ---¦--- indicates cleavage site on substrate. N and C indicates N and C terminus.

Mechanism of action of serine proteases

As the name in indicates, serine proteases are a class of proteolytic enzymes characterized by the presence of uniquely reactive serine side chain in the active site. They are extremely widespread in nature and have diverse functions. Kinetic studies performed with bovine chymotrypsin by various researchers has established the sequence of reactions that occurs when serine proteases catalyze the hydrolysis of peptide and ester bonds **(**Kraut, 1977). The catalytic action of serine protease depends on the interaction of nucleophile. In the classic trypsin and subtilisin families the catalytic triad consist of serine, histidine and aspartic acid residues and exhibits similar spatial arrangements, but the order of residues in the amino acid sequence is different (Polgar, 2005). The catalytic mechanism of serine proteases involve the nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the substrate, which is catalyzed by a histidine imidazole group as a general base. This leads to formation of tetrahedral intermediate, which breaks down to an acyl-enzyme, an imidazole base and amine product (elimination reaction) and then the peptide bond is cleaved (Figure 1.3). The acylation step is followed by deacylation through reverse reaction pathway in which water molecule acts as nucleophile instead of serine residue to release peptide and (Polgar, 2005; Rao *et al.,* 1998). Few other serine proteases such as peptidase A from *E. coli* and the repressor Lex A show distinctly different mechanism of action without involvement of this classic triad (Tanksale, 2001).

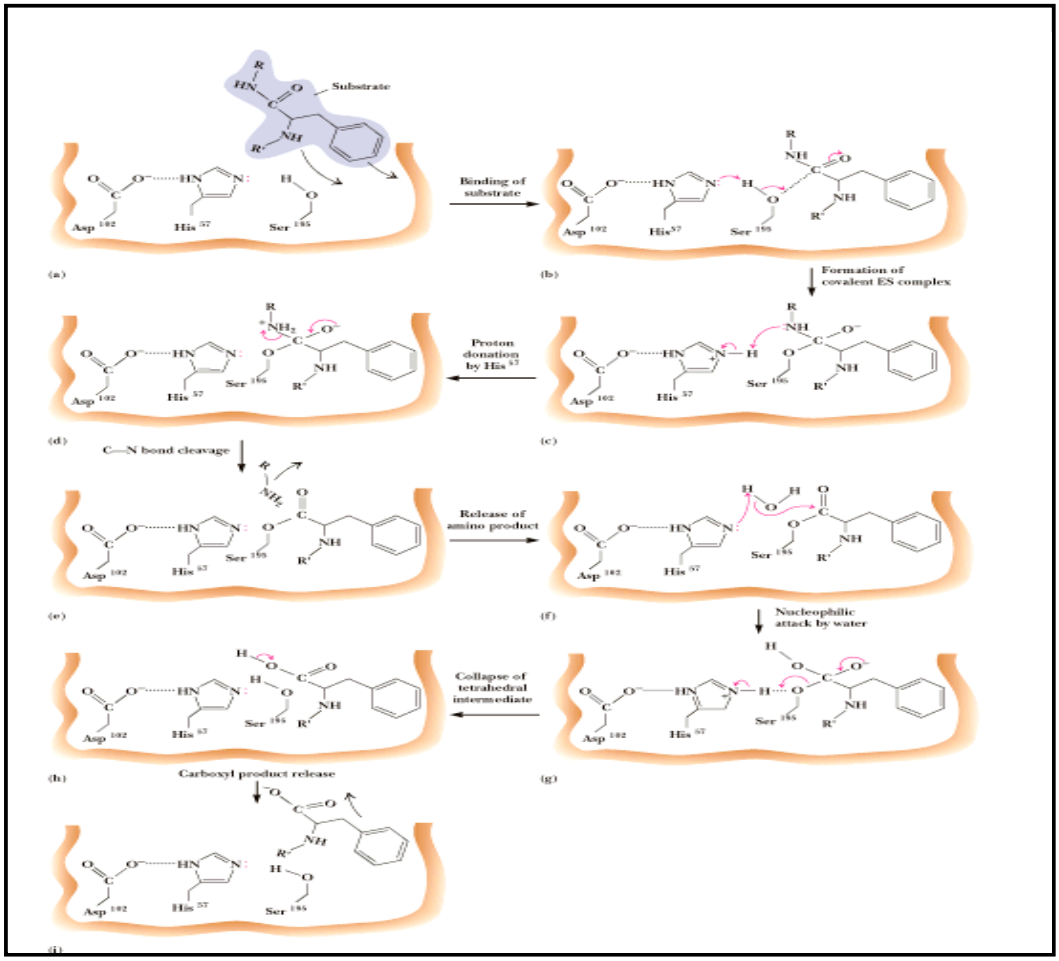

Figure 1.3: Mechanism of action of serine proteases
(http://info.bio.cmu.edu/courses/03231/Protease/SerPro.htm)

Mechanism of action of aspartic proteases

Aspartic proteases group include several important enzymes such as pepsin, rennin, chymosin, cathepsin D and proteases isolated from several fungi. The amino acid sequences of these enzyme show maximum homology around the active site residues. The tertiary structures of pepsin and microbial proteases show a remarkable resemblance. Aspartic proteases differ from serine and cysteine proteases with respect catalytic action. The catalysis of serine and cysteine proteases involve the covalent intermediate (ester and thioester respectively), whereas aspartic protease catalysis do not have intermediate. A general acid-base catalytic mechanism has been proposed for the hydrolysis of proteins by aspartic protease and the phenomenon is supported by crystallographic and kinetics studies. The pKa values of Asp residues are crucial for aspartic protease action. One Asp residue has relatively low pKa value than the other. Deprotonated Asp act as general base, accepting a proton from H_2O and forms OH^- in

the transition state, other Asp (general acid) donates proton and facilitated formation of tetrahedral intermediate (Figure 1.4). 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. Majority of aspartic proteases cleave the bond in peptides of at least six residues with hydrophobic amino acids at P1 and P1' positions. The substrate-binding cleft is large enough to accommodate polypeptide of about seven amino acids (Polgar, 1987; Rao *et al.,* 1998**)**.

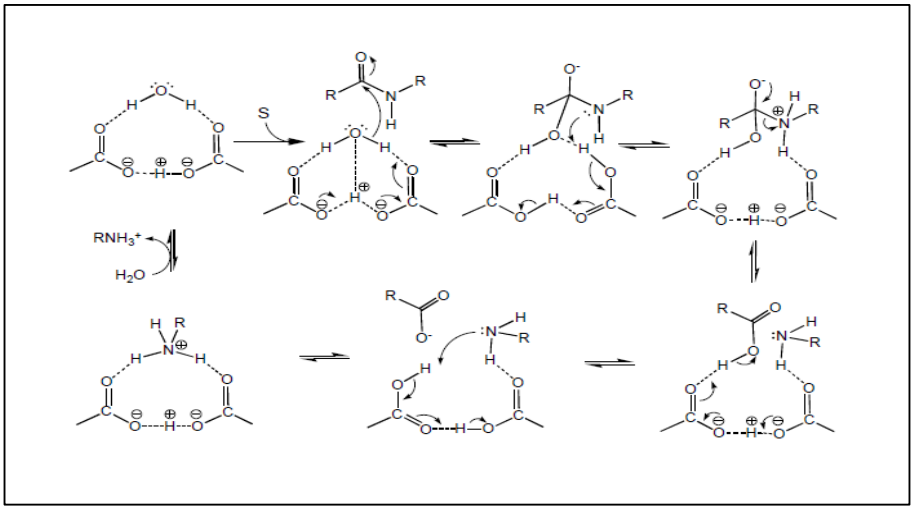

Figure 1.4: Aspartic protease mechanism

Mechanism of action of cysteine proteases

The mechanism of action of cysteine proteases is very similar to that of serine proteases. The essential cysteine and histidine play the same role as serine and histidine respectively as in serine proteases. The catalytic dyad formed by the cysteine and histidine residues exists as an ion pair (-S^-…H^+ Im-) in the pH interval 3.5 -8.0. Formation of an intermediate S-acyl-enzyme moiety is a fundamental step in hydrolysis. This intermediate is formed by nucleophilic attack of the thiolate group of the cysteine residue on the carbonyl group of the hydrolyzed peptide bond with a release of C-terminal fragment of the cleaved product. In the next step, a water molecule reacts with intermediate, the N-terminal fragment is released and the regenerated free enzyme molecule can begin a new catalytic cycle (Grzonka *et al.,* 2001**).** Bond cleavage specificity of some of the known protease is given in Table 1.2.

Table 1.2: Specificities of some of the proteases from diverse sources

Enzyme	E.C. No.	Peptide bond cleaved at
Trypsin	3.4.21.4	-Lys (or Arg) ↓-----
Chymotrypsin,	3.4.21.1	-Trp (or Tyr, Phe, Leu)↓---
Pepsin	3.4.23.1	- Phe (or Tyr, Leu) ↓---Trp (or Phe, Tyr)
Papain	3.3.22.2	- Phe (or Val, Leu)- Xaa↓----
Thermolysin	--	- Leu (or Phe) ↓----
Staphylococcus V8 protease	--	-Asp (or Glu) ↓----

The arrow indicates the site of action of the protease. Xaa- any amino acid residue.

Microbial protease production

Proteases emerged as an industrially important group of enzymes which accounts about 60% share of total enzyme market (Rao *et al.,* 1998). Protease market has experienced notable growth, owing to their characteristic degradative and synthetic functions and growing steadily due to strong demand from various industries. Bacteria are the most dominant group of alkaline protease producer and genus *Bacillus* being the most prominent source exploited for industrially important proteases (Gupta *et al.,* 2002a). However, fungi elaborate a wider variety of enzymes than bacteria. For example, *Aspergillus oryzae* produces acid, neutral and alkaline proteases. Enzymes of fungal origin are advantageous due to the ease of cell removal during downstream processing. In addition, fungi are suited better for solid-state fermentation. There are plenty of reports available on fungal protease production by solid-state fermentation (Kranthi *et al*., 2012; Chutmanop *et al.,* 2008; Haq and Mukhtar, 2004). Besides solid-state fermentation, fungal proteases are also produced by submerged fermentation in shake flasks (Devi *et al*., 2011; Ire *et al.*, 2011) as well as in fermentor (M'hir *et al*., 2012; Kamath *et al*., 2010; Rao *et al*., 2006; Laxman *et al*., 2005). Protease production is greatly influenced by nutritional factors (carbon and nitrogen source, inducers and metal ions) and physical factors (pH, temperature, aeration).

Literature survey on various aspects of microbial protease production is presented in Section 2 of Chapter 2 which deals with optimization of media in shake flasks.

Applications of proteases

Over the past several years, we have experienced the unique capabilities of enzymes and due to emerging scientific knowledge and environmental concern, applied these amazing substances in an ever-growing number of applications. Proteases constitute the largest product segment in the global industrial enzyme market. They find wide range of applications in food, detergent, leather, textile and pharmaceutical industries. Proteases are one of the three important commercial enzymes, amyloglucosidases and glucose isomerases being the other two. For the application in food, leather and detergents industry crude protease preparation is generally used while for pharmaceutical and medicinal applications proteases are needed in pure forms. Applications of protease in diverse fields are described below.

Detergent

Detergents constitute the major end use segment for industrial enzymes. Demand for detergent enzymes, however, is likely to be affected by the fluctuating prices of raw materials and continuous innovations by the manufactures to cut down on the costs (www.allaboutfeed.net/news/global-enzyme-market). One of the major applications of microbial proteases is in detergent industry, which accounts for around 25% of the total worldwide sale of enzymes. Though the first protease to be used in commercial detergents was pancreatic trypsin way back in 1913, the first detergent containing bacterial protease was introduced only in 1956 under the trade name Bio-40. Novo Industry introduced Alcalase, an alkaline protease produced by *Bacillus licheniformis* (BIOTEX). All the proteases presently used in the market are serine proteases produced by *Bacillus* strains. The other proteases for detergent applications are Maxatase (Gist Brocades), Esperase and Savinase T produced by alkalophilic *Bacillus sp* (Novo Industry). An ideal detergent should have broad substrate specificity and capable of removing variety of stains such as blood, sweat and food etc. In addition, the protease should be active at alkaline pH (pH of the detergent solution), wide temperature range, and stable at high pH, in presence of chelating and oxidizing agents, which are common components of detergents (Rao *et al.,* 1998; Kumar and Takagi, 1999). Protease having iso-electric pH (pI) that coincides with the pH of

detergent solution is considered to be most suitable for detergent application. A variety of bacterial proteases active and stable at alkaline pH also stable in oxidizing agents, bleach and SDS are found to be suitable for detergent applications (Kumar and Takagi, 1999; Gupta *et al.,* 2002a). The alkaline protease from *B. clausii* I-52 is significant for industrial perspective because of its ability to function in broad pH and temperature ranges in addition to its tolerance and stability in presence of an anionic surfactant like SDS and oxidants like peroxides and perborates. The enzymatic properties of this protease therefore suggest its suitable application as additive in detergent formulations (Joo *et al.,* 2003). Some fungal proteases are also reported to be suitable for detergent application (Phadatare *et al.,* 1993; Tanksale *et al.,* 2001; Hajji *et al.,* 2007). The global trend has been to reduce wash temperatures and ban phosphates. To compensate for the reduced cleaning ability at lower temperatures, detergent manufacturers have turned to enzymes for help and have introduced several classes of enzymes into their products. A lower wash temperature significantly reduces the energy needed to do a load of laundry. For example, in northern Europe wash temperatures have been reduced from about 90°C to 40 - 60°C. The energy input is dramatically reduced and thanks to enzymes, the same wash performance is maintained. In addition, the reduction in phosphate load to rivers and lakes is believed to lower the human-induced decline of these systems.

Food

The use of proteases in the food processing dates back to antiquity. They have been routinely used for various purposes such as cheese making, baking and meat tenderization (Rao *et al.,* 1998). Protein hydrolysates produced by hydrolyzing animal and plant proteins have well defined peptide profiles and extensively used in therapeutic dietary products and infant food formulations. These protein hydrolysates also play an important role in blood pressure regulation. In recent years, there has been substantial interest in developing enzymatic methods for the hydrolysis of soya protein, gelatin, casein, whey and other proteins in order to prepare protein hydrolysates of high nutritional value (Sumantha *et al.,* 2006). Carreno (1991) used 'Takabate' an alkaline protease produced by *Bacillus liqueniformis* for the hydrolysis of fish proteins. Alkaline protease from *B. amyloliquefaceins* is also used to produce methionine rich hydrolysate from chickpea protein (Kumar and Takagi, 1999). The

alkaline elastase and thermophilic alkaline protease are used as meat tendering enzymes. Proteases are added in dough during the preparation of bread, to modify wheat gluten and milk proteins. Endo and exo proteinases from *Aspergillus oryzae* are used to modify wheat gluten by limited proteolysis resulting in the improvement of dough elasticity permitting easier machining and consequent increase in loaf volume, better grain, symmetry and texture. Proteases are also used in biscuit, cracker and cookie manufacture. The protease from *Mucor miechi* and *M. pusillus* are used as substitutes of calf rennet in cheese manufacture. During ripening, cheese proteins are cleaved by the proteases at various sites and protein network, which forms the structural component of fresh cheese curd, looses parts of its original structure leading to alterations in rheological properties of cheese. The fungal proteases are used for centuries for preparation of soy sauce and other soy products. The alkaline and neutral proteases from *Aspergillus* play an important role in the processing of soy sauce. Proteolytic modification of soy proteins helps to improve their functional properties. The treatment of soy proteins with Alcalase at pH 8 results in soluble hydrolysates with high solubility, good protein yield, and low bitterness (Rao *et al.,* 1998). A protease from *Pseudomonas fluorescens* R098 was able to hydrolyze the peptides found in cheese, which are responsible for bitterness and thus can be used as debittering agent in cheese making (Koka and Weimer 2000). They can also be used for the fortification of fruit juices or soft drink and in manufacturing protein rich therapeutic diets (Kumar and Takagi, 1999). The cereal foods are also treated with proteolytic enzymes to modify their proteins resulting in better processing including improved product handling, increased drying capacity and lower power requirements. To prevent development of undesirable haze in beer and ale when these beverages are cooled, proteolytic enzymes are added during the finishing operation to "chillproof" these beverages. An immobilized preparation of thermolysin, a protease from *B. thermoproteolyticus* is used for enzymatic synthesis of non-caloric artificial sweetener Aspartame (Rao *et al.,* 1998).

Leather processing

In leather industry, raw skins or hides undergo series of operations before converting in finished leather. Dehairing of skins or hide is the major step of these operations, wherein the hair, epidermis, some portion of noncollagenous proteins and other

cementing materials is removed from the skin. Conventional method of dehairing involves the use of high proportions of lime and sulfide. Dehairing by this method is governed by the phenomenon of hair burning, which takes place due to the chemical reaction of lime and sulfide on hair root. This method leads to create environmental and safety hazards. The generation of pollution is significantly high in the pre-tanning operations compared to the post-tanning operations. The chemicals mainly responsible for pollution in pre-tanning processes are lime, sodium sulphide, and caustic soda apart from common salt and degreasing chemicals. In fact, one third of the pollution caused by the leather industries results from the wastes generated during dehairing operations (Kamini *et al.,* 1999). Considering theses issues, eco-friendly approach of leather processing using enzymes, predominantly alkaline proteases is preferred. Proteases find their use in the soaking, dehairing and bating stages of leather making process (Gupta *et al.,* 2002a). Bating is very important process in which enzymes have been successfully used for centuries. Bating process in leather manufacturing can not be replaced by chemical method. In the earlier method of bating infusion of animal dung was used for softening dehaired skins, which was too unpleasant and had severe impact on the health of workers. During bating, the hide is softened by partial degradation of the interfibrillar matrix proteins (elastin & keratin). Therefore, enzyme preparations with low levels of elastase and keratinase activity are particularly applicable for this process. Although proteases have been used for bating for about century, their use for soaking and dehairing is more recent. The ultimate aim of soaking is to swell the skin structure and can be facilitated by addition of small amount of protease to soaking liquor and reduces duration of soaking. For soaking and dehairing, generally crude protease preparation is preferred. However, it is important that it should not have collagenase activity, as the major constituent of leather is collagen. Proteases used for dehairing are mostly produced by bacteria and to some extent by fungi. (Zambare *et al.,* 2007; Sivasubramanym *et al.,* 2008; Sundararajan *et al.,* 2011; Laxman *et al.,* 2007).

Degumming of silk

Though proteases are exploited for diverse applications, one of the least explored areas of their use is in silk industry (Laxman, 2012). Raw silk is mainly composed of fibroin, a basic fibrous part of the silk and account for about 78-80 % of the total silk whereas sericin, a gluey material that gives harsh and stiff feeling to the silk fiber,

contribute 22 – 25 %. Fibroin is highly insoluble protein due to its characteristic amino acid composition, including non polar amino acids glycine, alanine and valine. In sericin polar amino acids like serine and aspartic acid are present predominantly and make it tend to be soluble in hot and soap water. (Chopra and Gulrajani, 1994). Removal of sericin from the raw silk is a preliminary and important step of silk processing thereby obtaining an ideal fiber for manufacturing of silk fabric, and is known as 'Degumming'. In the conventional method of degumming, silk fibers are treated with alkali and soap at 98-100°C or are boiled at elevated temperature and pressure for long duration to solubilize sericin and its subsequent removal from fibroin. Due to the harsh nature of these treatments, fiber may lose strength and degummed unevenly, therefore a precise control become necessary. Conventional degumming methods are high resources consuming processes with respect to water and energy. Moreover, the effluent generated by these methods has several environmental impacts. Therefore, an alternative method suggested is the use of enzyme preparations for degumming of silk. For enzymatic degumming, proteases, predominantly alkaline proteases are used. Being a milder process with negligible input of hazardous chemical and recovering of valuable byproducts like sericin proteins, enzymatic degumming process is gaining lot of attention. Although the first report on degumming dates back to more than ninety years, the use of proteases in silk industry is relatively new and has generated a lot of interest only in last twenty years (Laxman, 2012). One of the major advantage of enzymatic degumming is the easier recovery of sericin form degumming liquid as compared to alkali soap degumming method. Sericin protein and its hydrolysates has numerous applications in cosmetics, medicine and for preparation of medical biomaterials, compound polymer and hydrogels (Padamwar and Pawar, 2004: Laxman, 2012).

Pharmaceutical and therapeutic

Acidic proteases from *Aspergillus oryaze* are used in digestive aids. Elastase from *B. subtilis* 316M immobilized on bandage is used for healing wounds, abscesses, carbuncle and burns (Gupta *et al.,* 2002). Proteases are also used as potential bacteriocidal agents and for removal of protein contaminants from antibiotic preparation. An asparginase isolated from *E. coli* is used to eliminate aspargine from the bloodstream in the various forms of lymphocytic leukemia (Rao *et al*., 1998). An alkaline protease with fibrionolytic activity is used as a thrombolytic agent (Kumar

and Takagi, 1999).In addition, being targets for inhibitors, several proteases found useful in numerous medical applications and approved by FDA (Table 1.3).

Table 1.3: Various therapeutic uses of proteases (Craik *et al.,* 2011)

Usage	Protease	Indications	Source of protein	Target protein or pathway	Type of protease
Thrombolysis	Urokinase (u-PA)	Thrombus, catheter clearing	Extracted from urine or from primary kidney cell culture	Converts plasminogen into plasmin	Serine
	t-PA (alteplase, Activase®)	AMI, stroke, catheter clearing	Recombinant in CHO cells	Plasminogen activator	Serine
	Reteplase (Retevase)	AMI	Recombinant in *E. coli*	Plasminogen activator	Serine
	TNK-tPA (tenecteplase, Metalyse®)	Myocardial infarction	Recombinant in CHO cells	Plasminogen activator	Serine
Procoagulant	FIX	Haemophilia B	Human plasma	FX activator	Serine
	FIX (BeneFIX®)	Haemophilia B	Recombinant in CHO cells	FX activator	Serine
	FVIIa (NovoSeven®)	Haemophilia A and B	Recombinant in BHK cells	FX and FIX activator	Serine
	Topical thrombin in bandages	Bleeding	Bovine	Fibrinogen activator	Serine
	Thrombin (Recothrom®)	Bleeding	Recombinant in CHO cells	Fibrinogen activator	Serine
Sepsis	Activated protein C, (drotrecogin alfa, Xigris®)	Sepsis, septic shock	Recombinant in human cell line	Plasminogen activator	Serine
Neuromuscular	Botulinum toxin A (Botox®)	Various muscle spasms	Bacterial (*C. botulinum*)	Syntaxin and SNAP-25 deactivator	Zinc
	Botulinum toxin B (Myobloc)	Cervical dystonia	Bacterial (*C. botulinum*)	Synaptobrevin deactivator	Zinc
Digestion	Zenpep® (pancrelipase)	Exocrine Pancreatic Insufficiency	Porcine pancreatic extract	Aids digestion of protein	Serine

Waste management

Alkaline proteases group has emerged as a potential factor for the management of industrial and household waste, generated by various food-processing industries like poultry and meat. Mejor constituent of feather is a fibrous and insoluble keratin protein. Worldwide poultry-processing industries generated several million tons of feather annually as waste. Considering its high protein content, this waste could have a great potential as a source of protein and amino acids for animal feed, biofertilizer and for many other applications (Jeong *et al.,* 2010). Pretreatment of feather with NaOH, mechanical disintegration, and enzymatic hydrolysis results in total solubilization. The product is a heavy, grayish powder with a very high protein content, which could be used as a feed additive (Dalve, 1994: Kumar and Takagi, 1999). Considering its high protein content, this waste could have a great potential as a source of protein and amino acids for animal feed as well as for many other applications. In some countries, feather is used as animal feed supplement in the form of feather meal. Hydrolysis of feather to soluble proteins and amino acids by microbial and enzymatic method is extremely attractive, as it offers a cheap and mild reaction condition for the production of valuable products. Feather may also find important application in the fermentation industry for the production of commercial

enzymes (Gessesse *et al.,* 2003). A formulation containing proteolytic enzymes from *B. substilis*, *B.amyloliquefaciens* and *Streptomyces* sp and a disulfide reducing agent (thioglycolate) that enhances hair degradation, helps in clearing pipes clogged with hair-containing deposits and is currently available in the market (Gupta *et al.,* 200a). Leather sector is considered one of the environmental polluting causing industries, which generates huge amount of liquid and solid waste. Major solid wastes such as raw trimming, fleshings and crome shavings are generated during several stages of tanning operations and resulted in high TDS of effluent. Crispim and Mota (2003), treated chrome shavings with acidic protease pepsin instead of grinding, the resultant paste formed was subjected to gluteraldehyde treatment to introduce crosslinking and the paste was pressed and dried. Agglomerate thus formed was found useful in shoemaking. Kanagaraj *et al.* (2006) proposed several uses of chrome shaving hydrolysates produced by alkaline proteases. Alkaline protease digested wet blue shavings into a smaller material, which is used for casein formulation in leather finishing. Chrome shaving treated with alkaline proteases such as pepsin-trypsin and pepsin have yielded gelatin, chrome cake and hydrolysates. Application of collagen hydrolysates in retanning process gave good quality leather.

Other applications

Apart from above stated applications, proteases emerged as important tools in many scientific research, especially in biotechnological studies and are being used routinely. 'Proteinase K', is routinely used in DNA extraction procedure. In animal cell culture, cells are dissociated from monolayer by giving trypsin treatment. Alkaline protease from *Conidiobolus coronatus* was successively used for cell dissociation in animal cell culture (Chiplonkar *et al.,* 1985). Sutar *et al.,* 1983 used *Conidiobolus coronatus* protease to resolve the racemic mixtures of DL-phenylalanine and DL-phenylglycine. A neutral protease, isolated from *Bacillus sp* was used for cleaning contact lenses (Pawar *et al.,* 2009). Protease from *Conidiobolus coronatus* was used for silver recovery from waste photographic films (Shankar *et al.,* 2010). Peptide mass finger printing of proteins has become an important identification tool in proteomics, in which protein is fragmented in several peptides using trypsin and the hydrolysates are subjected to matrix-assisted laser-desorption ionization (MALDI) to identify their masses. The peptide masses thus obtained are compared with known peptide masses from database using computational program.

Market scenario of proteases

In recent years, there is increased demand of enzymes as catalyst at industrial level. Most of the commercial enzymes being used today are hydrolases and proteases constitute about 65 % of the total enzyme market (Rao *et al.,* 1998). The global market for industrial enzymes is forecast to reach US$3.74 billion by the year 2015 (http://www.strategyr.com/Industrial_Enzymes_Market_Report.asp) and India is an attractive market with high growth rates in the past years. Enzyme use is still in its infancy with growing awareness of enzyme potential and benefits providing attractive growth perspectives. The industrial enzymes segment, has an estimated worth of $75 million, and is a quickly growing market in India. In 2006–2007, the bio-industrial sector made an impressive turnover of $130 million, with a growth rate of 5.33 per cent (http://www.marketintelligences.com/the-sis-asia-business-journal/2008). Key factors driving market growth include new enzyme technologies endeavoring to enhance cost efficiencies and productivity, and growing interest among consumers in substituting chemical products with other organic compounds such as enzymes. Other factor boosting market growth includes surging demand from textile industry, animal feed producers, detergent manufacturers, pharmaceutical companies and cosmetics vendors, their application range is gradually increasing with newer applications being explored, and there is expected to be an upward trend in the use of proteases. The major producers of proteases are Novo Industries (Denmark), Gist – Brocades (Netherlands), Genecor International and Miles Laboratories (United States). In last few years several new companies has been arise in Indian Bioindustrial Scenario and their production and export is succeeding day by day. M/s Biocon, M/s Advance Biochemicals and M/s Maps India are some of the leading producers of proteases in India. Proteases (commercially available) being used for detergent and tannery applications are predominantly produced by bacterial stains, while to best of our knowledge there is no fungal protease available commercially for these purpose. Majority of the commercial fungal proteases are used in food, pharmaceutical, brewing and related processes. Various commercial fungal proteases, their optimum conditions and applications are listed in Table 1.4.

Table 1.4: Various commercial proteases from fungal sources and their applications (Nirmal *et al.*, 2011)

Name	Source	pH	Temp (^{o}C)	Application
Amano Pharmaceutical Co.				
Prozyme P 'Amano' 6	*Aspergillus* sp.	8	45	Food
Acid protease 'Amano' II	*Rhizopus* sp.	3	50	Food
Acid protease 'Amano' A	*Aspergillus* sp.	3	55	Food
Protease A 'Amano' 2	*Aspergillus* sp.	7	50	Food
Protease B 'Amano'	*Penicillium* sp.	6	45	Food
Protease M 'Amano'	*Aspergillus* sp	4.5	50	Food
Biocatalysts Ltd				
Promod 25P	*Aspergillus* sp	7.5	50	Food
FlavourPro192P	*Aspergillus* sp	4.5	50	Food, flavor
Promod 215P	*Aspergillus* sp	6	50	Food, flavor
Promod 194P	*Aspergillus* sp	7	50	Food, flavor
Promod 280P	*Aspergillus* sp	5 -7.5	55	Food, flavor
Dafa SA				
Dafazyme PR-F	*Aspergillus oryzae*	6 – 9	55	Baking
Enzyme Development corporation				
Enzeco Fungal Protease 180	*Aspergillus oryzae*	6 – 10.5	45 –60	Food
Enzeco Fungal Proteinase Conc.	*Aspergillus oryzae*	5 – 6	45 – 55	Food
Enzeco Fungal Acid Protease	*Aspergillus niger*	2.5 – 3.5	40 – 55	Food

Meiji Seika kaisha Ltd				
Proctase	*Aspergillus niger*	1.8 – 3	---	Feed, food, pharmaceutical
Nagase Biochemicals				
Denapsin AP	*Aspergillus oryzae*	7	50	Food
Denapsin	*Aspergillus niger*	3	50	Food
XP-415	*Rhizopus delemar*	3	55	Food
Novo Nordisk AS				
Flavourzyme	*Aspergillus oryza*e	5 – 7	45 – 50	Food
Rohm GmbH				
Corolase PN	*Aspergillus sojae*	5 – 6	45	Food
Veron PS	*Aspergillus oryzae*	5 – 6	45 –50	Food
Shin Nihon Chemical Co.				
Sumizyme AP	*Aspergillus niger*	3	60	Food
Sumizyme RP	*Rhizopus delemar*	4	55	Food
Sumizyme LP	*Aspergillus oryzae*	7 – 11	50	Food
Sumizyme LPL	*Aspergillus oryzae*	3	50	Food
Sumizyme FP	*Aspergillus sojae*	6 – 10	50	Food
Sumizyme MP	*Aspergillus melleus*	8	50	Food
Solvay Enzymes GmbH				
Fungal protease	*Aspergillus oryzae*	4.9 – 9	45 – 55	Alcohol, baking. Brewing, feed fermentation, waste

Stern-Enzym GmbH				
Sternzyme B2000	Fungus	4 – 6	45 – 55	Baking
Sternzyme B2010	Fungus	4 – 6	45 – 55	Specialty baking
Sternzyme B2050	Fungus	4 – 6	45 – 55	Biscuits and crackers
Yakult Biochemical Co.				
Pancidase NP-2	*Aspergillus oryzae*	---	---	Food
Protease YP-SS	*Aspergillus niger*	---	---	Food

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CHAPTER 2

ISOLATION AND IDENTIFICATION OF *Conidiobolus brefeldianus* AND OPTIMIZATION AND SCALE UP OF PROTEASE PRODUCTION

SECTION 1

ISOLATION AND IDENTIFICATION OF NEW STRAIN OF *Conidiobolus brefeldianus*

Abstract

The newly isolated fungal strain was identified as a new strain of *Conidiobolus* sp. on the basis of morphological features. The organism grows rapidly and forcibly discharged large whitish conidia with basal papillae. Mycelium is coenocytic which becomes septate in later stages. Conidiophores are indistinguishable from mycelium. The organism is zygosporic and forms round, smooth, thick walled zygospores with two distinct wall layers and granular contents inside. The 18S rDNA sequence homology studies revealed that the new strain showed 99% homology with *Conidiobolus brefeldianus* AF 368506.1 and 98% homology with various strains of *Conidiobolus coronatus.* The new strain of *Conidiobolus brefeldianus* was deposited in Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India with accession number MTCC 5185. The nucleotide sequences of 18S rDNA have been deposited in the NCBI GenBank database and assigned accession numbers: FJ895304.

Introduction

Isolation of microorganisms from environment is the preliminary and important step in screening for natural products such as secondary metabolites and enzymes. In order to design a suitable and commercially viable fermentation process for the production of enzymes, it is essential to isolate and identify an overproducing microbial strain. Fungi are ubiquitous heterotrophic organisms that inhabit nearly every possible environmental niche. Nutritional requirements of fungal types are diverse and can be used as a basis for isolation, consequently changing the media constituents can favor the isolation of one fungus over another. Generally, fungi are isolated from soil, water, dung, plant detritus and decaying vegetative matter. Fungi which shoot spores can be isolated and purified due to the typical nature of the organism where the mature spores are violently discharged which fall at some distance and germinate to form new fungal colonies. This character is very useful and distinctive to few genera. Examples of fungi showing violent discharge of spores are those belonging to the genera *Conidiobolus*, *Basidiobolus*, *Sporobolomyces* etc.

Once the organism is isolated, the correct identification of fungi is of great practical importance not only in the industrial studies but also in clinical, pathology, biotechnology and environmental studies. The age old classification system of fungi is widely based on the particular life cycle involved. The morphology of the reproductive structures and the way spores are produced are important features to classify the strain. They may have unique colours, which give the fungi characteristic appearance. The morphology of asexual spores is also helpful for identification. They may be small, single celled 'micro conidia' or large, single or nucleated or multi celled 'micro conidia'. The type of spore formation is distinctive for different fungi. ''Blastospores' are formed by budding, as in yeast. 'Arthrospores' are formed along the mycelium by segmentation and condensation of hyphae. Chalamydospores are thick walled resting spores formed by rounding up and thickening of hypal segments. A zygospore is a special type of chlamydospore arising from sexual conjugation. In addition to being thick walled and darkly colored, a zygospore is usually heavily ornamented with many spines or ridges. Kim *et al.* (2010) isolated several entomopathogenic fungal isolates from insects and identified them on the basis of morphological features. Samson *et al.* (2007) **r**eported diagnostic tools to identify black aspergilli based on colony morphology, conidial size and ornamentation of the

ex type cultures. Numerous alternative approaches have been developed, including nutritional and physiological studies, serologic tests, secondary metabolites, ubiquinone systems, and fatty acids. Although some of these are very useful for identifying poorly differentiated fungi such as yeasts and black yeasts, they are only complementary tools of morphological data in most cases (Guarro *et al.,* 1999). Fatty acid composition and cell wall composition have been used in classification and also in identification. Fungi can also be distinguished by their mycelial protein composition as the electrophoretic pattern of mycelial protein can discriminates the fungi at the species level.

Strains belonging to the genus *Conidiobolus* are generally found in soil and decaying plant debris and mostly distributed in tropical areas of Central America, equatorial Africa and India (http://www.doctorfungus.org). The important characteristic of *Conidiobolus* species is the forcible discharge of spores from sporangiophores which spread over the lid of inoculated plates or sides of tubes. This feature is a key determinant not only in identification of the genus but also found useful in isolation. Similar property of forcible spores discharge from fungal colonies is also exhibited by *Basidiobolus* sp. of *Entomopthorales* (Ingale *et al.,* 2002). However, *Conidiobolus* differs from *Basidiobolus* by having sporangiophores that are not swollen at their apices, by having primary spores with papillae and by the absence of beaks associated with zygospores. Srinivasan and Thirumalachar (1967) described morphological and taxonomic characteristics of genus *Conidiobolus* and presented a key for the separation of species based on conidial and mycelial diameter, presence or absence of micro conidia and zygospore. In addition, they also mentioned the distinguishing feature of *Conidiobolus* and *Entomophthora* with respect to conidiophore arrangement on the hyphae. The conidiophore is usually undifferentiated from the normal hyphae in *Conidiobolus*, while in *Entomophthora* it is well differentiated by septa. The conventional methods for identification of fungi are based on culture isolation and subsequent observations of morphological characteristics such as spore morphology, arrangement, color etc. In the order Entomophthorales, there are only three species occasionally pathogenic to humans: *Conidiobolus incongruous*, *Basidiobolus ranarum*, and *Delacroixia coronata* (*Conidiobolus coronatus*).

Molecular techniques of identification

The conventional methods based on the above mentioned information are time consuming, laborious and some times require several days. Some fungal cultures may not have developed reproductive structures or may be non sporulating types. In addition, not all the fungal species are culturable on a given medium which leads to analysis that may not accurately reflect the true fungal community in a sample. In contrast to conventional methods, molecular techniques are universally applicable. The aim of molecular studies in biodiversity is of phylogenetic and taxonomic interest. Use of molecular techniques also becomes essential to identify those fungal isolates which are non-sporulating in nature. The most frequently targeted gene for phylogenetic studies is the one that codes for rRNA. Introns of several protein-encoding genes, such as the β-tubulin, actin, chitin synthase, glyceraldehyde-3-phosphate dehydrogenase, lignin peroxidase or orotidine 5'-monophosphate decarboxylase genes, can also be applied and can provide important information **(**Guarro *et al.,* 1999**)**. Nucleic acid based techniques rely on the detection of genotypic differences in organism and are intrinsically more specific and more precise than those based on phenotypic features. Many studies have shown that polymerase chain reaction (PCR) amplification, restriction fragment length polymorphism (RFLP), the sequencing of the internal transcribed spacer (ITS) and intergenic spacer (IGS) of the nuclear rDNA are among the most dominant tools used to analyze the inter- and intra-specific variation in fungi (Sanon *et al.,* 2009). The main reason for the popularity of rDNA are that it is a multiple-copy, non-protein coding gene, whose repeated copies in tandem are homogenized by intensive evolution, and thus treated as a single-locus gene. For phylogeny of filamentous fungi, the 18S sequence is mostly used completely or in subunits of over 600 bp. In the 18S rDNA gene, the variable domains mostly provide insufficient information for diagnostic purposes and thus large parts of the molecule must be sequenced to obtain the resolution required (Guarroo *et al.,* 1999**).** In recent years, the methods most widely used for taxonomy at the species level have been sequencing and electrophoretic methods. Among the electrophoretic methods, restriction fragment length polymorphism (RFLP) emerged as remarkable technique for taxonomical studies. This involves digestion of DNA samples with a range of restriction enzymes. The digested DNA fragments create unique elecrophoretic pattern, which can be tabulated and compared or phenetic trees can be constructed. Most commonly, the RFLP of PCR-amplified rDNA is used

(Vaneechoutte *et al.,* 1992; Bunyard *et al.,* 1996; Edel *et al.,* 1996). This technique is also known as amplified rDNA restriction analysis and provides a quick insight into relationships between moderately distant fungi.

Materials and methods

Materials

Malt extract, yeast extract, peptone were obtained from M/s HiMedia Chemicals, India. Enzymes and chemicals for PCR and sequencing were obtained from Bangalore Genie (India). All other chemicals were of analytical grade.

Methods

Isolation and maintenance of fungal strain

The fungal culture was isolated from decomposing plant detritus collected from Pune, Maharashtra, India. Fine particles of plant detritus were superimposed on MGYP agar (malt extract-0.3%; yeast extract-0.3%; peptone-0.5%, gluscose-1%, agar-2%) blocks attached to the inner surface of the petri plate lid and plates were incubated at 28°C for a week. Isolated single colonies were picked up and transferred to MGYP plates.

Identification of the strain based on 18S rDNA sequence

PCR amplification and sequencing of 18S rDNA gene

Isolation of genomic DNA: The genomic DNA from freeze-dried mycelia was isolated according to the method described by Lodhi *et al.,* (1994) with slight modifications. For the isolation of DNA, the fungus was grown in 500 ml flask containing 100 ml MGYP medium. The growth was initiated by inoculating spores from 3 days old MGYP slant. The flasks were incubated on a rotary shaker (180-200 rpm) at 28°C for 48 h. The contents were centrifuged at 8000 rpm for 15 min, washed repeatedly to remove the media constituents. Three to five grams of wet mycelium was freeze-dried and ground using mortal and pestel in liquid nitrogen, followed by addition of 8-10 ml of cetyl trimethyl ammonium bromide (CTAB) extraction buffer, pH 8 containing 0.2% β-mercaptoethanol. The composition of 2X CTAB extraction buffer was -Tris-Base (100 mM; pH 8.0); CTAB (2% w/v); NaCl (1.4 M); EDTA (20 mM); PVP-40 (1-2%); β-mercaptoethanol (0.2-2%). After that 20 µl of proteinase K (20 mg/ml) was added and incubated at 65°C for 1 h. This was followed by addition of 20 µl RNase A (10 mg/ml) and further incubation at 65°C for 15 min. To the supernatant collected after centrifugation (8000 rpm, 10 min), 10 ml chloroform: isoamylalcohol (24:1) was added. The mixture was shaken for 5 min and centrifuged at 10,000 rpm at 4°C for 15 min. Two volumes of CTAB precipitation buffer (1 %

CTAB; 50 mM Tris (pH 8.0); 10 mM EDTA), was added to the supernatant and kept at room temperature for 1 h. The pellet collected after centrifugation was dissolved in 5 ml of 1.2 M NaCl and 5 ml of chloroform: isoamylalcohol (24:1) was added. Two volumes of absolute alcohol were added to the aqueous phase to precipitate the DNA. DNA was spooled out and washed with 70% ethanol and suspended in 5 ml of 0.1 M Tris EDTA buffer pH 8 and stored. The quantification of DNA was done by measuring the absorbance of the sample at 260 nm on spectrophotometer and purity was checked on 0.8 % agarose gel electrophoresis.

PCR amplification of 18S rDNA gene

PCR amplification of 18S ribosomal DNA was performed using commercially available primers. The primers used for the identification of fungal species were universal fungal 18S ribosomal DNA (rDNA) primers NS1-F (GTA GTC ATA TGC TTG TCT C), NS8-R (TCC GCA GGT TCA CCT ACG GA). The polymerase chain reaction (25µl) was set to amplify the 18S rDNA gene by using the genomic DNA. The reaction mixture typically contained genomic DNA-0.70 µl, 10X PCR Buffer-2.50 µl, 0.2 mM dNTPs-2.5 µl, forward and reverse primers 10-20 pmoles-1.25µl each, distilled water-16.60µl, and 1unit of *Taq* DNA polymerse-0.20µl. All the additions were done on ice and the PCR reaction was performed on Gene Amplifier PCR System 9700 (Perkin Elmer, USA). The PCR conditions for 18S rDNA gene amplification were: initial denaturation- 95°C for 3min; followed by 35 cycles of 94°C for 1min, 57°C for 30sec, 72°C for 2min and final extension at 72°C for 10 min. 5µl of the above PCR amplified product was used to check the amplification on 1.0% agarose gel in 1X TBE buffer (Working solution: 0.5 X; Stock: 5 X, 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA, pH 8). The gel was run at 80 Volt for 90 min using 1 X TBE as running buffer. The gel was stained in 1% ethidium bromide for 45 min and was observed under UV illumination.

Purification of PCR amplified product

To 20 µl, PCR amplified product, 12 µl of 20% PEG-NaCl (Polyethylene glycol - NaCl) solution was added and incubated at 37°C for 30 min. It was then centrifuged at 12,000 rpm for 20 min. The supernatant was discarded and the pellet was washed

twice with 70 % ethanol and separated by centrifuging at 12,000 rpm for 20 min. The pellet was dried and dissolved in 10 µl of double distilled water and stored at - 20°C.

Sequencing of the purified PCR product

The sequencing reactions of PCR product were carried out using *Taq* DNA polymerase dye terminator cycle applying automated DNA sequencing method based on dideoxynucleotide chain termination method (Sanger *et al.,* 1977). The sequencing reactions were carried out using the 'ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit' (Perkin Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's protocol. This Kit contains the four ddNTPs with different fluorescence labels termed as BigDye Terminators. 2 µl PCR product and 3 pmol of the sequencing primer were used in a 20 µl sequencing reaction. The sequencing primers were NS1 (GTA GTC ATA TGC TTG TCT C), NS2 (GGC TGC TGG CAC CAG ACT TGC), NS3 (GCA AGT CTG GTG CCA GCA GCC), NS4 (CTT CCG TCA ATT CCT TTA AG), NS5 (AAC TTA AAG GAA TTG ACG GAA G), NS6 (GCA TCA CAG ACC TGT TAT TGC CTC), NS7 (GAG GCA ATA ACA GGT CTG TGA TGC), NS8 (TCC GCA GGT TCA CCT ACG GA) for sequencing **(**White *et al.,* 1990**)**. The sequencing reaction mixer was subjected to 25 cycles in a Perkin Elmer thermal cycler 9700. Each cycle consisted of 95°C for 10 min, 50°C for 5 min and 60°C for 4 min. DNA sequencing was carried out on ABI 1500 Automated Sequencer at the DNA sequencing facility in National Centre for Cell Science (NCCS), Pune.

Sequence alignment and BLAST search

The sequences obtained by sequencer were in graphic form and hence it was converted to the word format by BioEdit (computer programme). The complementary reverse sequence was obtained by using computer software programme GeneRunner. The sequences obtained were in small fragments and hence it was aligned properly by overlapping the sequences. The nucleotide sequence was analyzed with the GenBank database using BLAST program (Altschul *et al.,* 1997).

Results and discussion

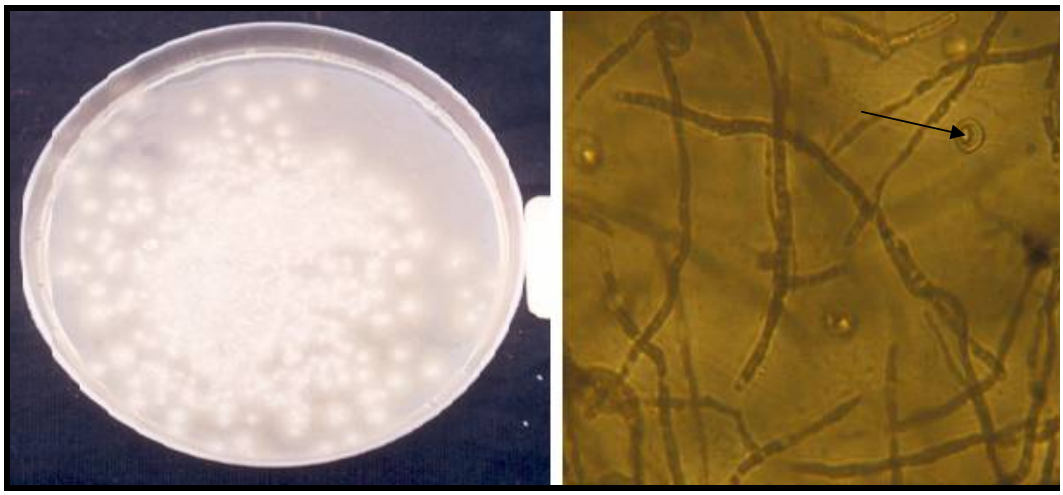
Within four days of incubation, only one colony which looked typical to *Conidiobolus* was seen along with other bacterial and fungal colonies originating from small particles of soil/plant detritus which spilled on the plate during isolation. The colony was placed on a small MGYP agar piece which was placed on the lid and was incubated at 28°C. Only 5-6 spores which were shot on to the MGYP agar medium were seen germinating and rapidly growing. Single colonies developing from forcibly discharged conidia were picked up and transferred to MGYP agar plates and allowed to grow in to colonies and sporulate. This process was repeated several times to obtain pure culture of the organism. The culture was routinely sub cultured and maintained on MGYP and PDA (potato dextrose) agar slants. The culture after sporulation (3-4 days) was preserved at 4 and 15°C.

Morphological characteristics of the isolate

The organism grows rapidly and was identified as a strain belonging to the genus *Conidiobolus* on the basis of the morphology of the forcibly discharged large globose conidia with basal papillae. Mycelium is coenocytic but becomes septate in later stages. Conidiophores are micronemous and indistinguishable from mycelium. At the tip of the conidiophore, large globose conidia are formed which are discharged forcibly. The size of conidia varies between 35-45 microns. Conidia either germinate to give rise to mycelium or to microconidia on radial sterigmata or to succession of secondary conidia. The discharged conidia are visible as whitish/creamish deposit on the glass above the growing culture (Figure 2.1.1a). The organism is zygosporic and the zygospores (resting spores) are round, smooth and thick walled (indicated by arrow in Figure 2.1.1b) with two distinct wall layers and granular contents inside.

Isolation of Genomic DNA

The genomic DNA when run in 0.8% agarose gel was found to be intact and of high molecular weight. The spectrophotometric analysis of the DNA showed that the ratio of absorbance at 260nm vs 280nm was 2 indicating that the preparation was pure and free from protein contamination.



(a): White deposit of discharged spores on the plate and the lid **(b) light microscopic picture showing fungal mycelium and zygospores**

Figure 2.1.1: Morphology of *Conidiobolus brefeldianus*

PCR amplification and sequencing of 18 S rDNA

The 18S rDNA gene was amplified as a single band using PCR method. The PCR products were purified and sequenced in automated sequencer using universal primers. The raw sequence data was converted to word form by BioEdit and the complementary sequence was determined by GeneRunner computer software programme. The nucleotide sequence of 18S rDNA of new isolate of *Conidiobolus* sp MTCC 5185 is deposited in NCBI GenBank database and assigned accession numbers FJ895304.

Sequence of 18S rDNA Gene

The 18S rDNA sequence of *Conidiobolus* sp was obtained by sequencing with the primers NS1 to NS8. The sequences so obtained by each set of primer were then overlapped to get 18S rDNA sequence. The complete sequence of isolated 18s rDNA gene has been given in Table 2.1.1.

Table 2.1.1: Sequence of 18S rDNA gene

5′CAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTTATACAGTGAAACTGCGA
ATGGCTCATTAAATCAGTTATAATTTCAGTGAAAGTTTACCAAATGGATAACCGTGG
TAATTCTAGAGCTAATACATGCAATTGAGTCTCGCTAATTTATTAGGAGATGCATTT
ATTAGATGAAACCAACAAATTGGTGAATCATAGTAACTTTGCTGATCGCATGGCCTT
GTGCTGGCGATAATTCATTCAAATTTCTGCCCTATCAACTATCGATGGTAGGATAGA
GGCCTACCATGGTGATAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAAGGCG
CCTGAGAAATGGCGACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCC
TGACACAGGGAGGTAGTGACAAAAAATAACAATACAGGGCCACTGGTCTTGTAATTG
GAATGAGTACAATGTAAACACCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCC
AGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAC
GCCCGTAGTCGAATTTCAGGAAGGAAAAACATTAGTTTTTCCAACCTTTAAATCTAC
CTGGTCTAGGTTATTAATTTAATTTAGGCCATTAAAGGTAGAACGTTTACTTTGAAA
AAATTAGAGTGTTCAAAGCAATTTTTTATGAATACATTAGCATGGAATAATGGAATA
AGTCAGCAGCTTATTTCGTTGGTTTAGAGTTGCAAACATGATGAATAGGGATGGTTG
GGGTCATTAGTATTTAATAGTCAGAGGTGAAATTCTTGGATTTATTAAAGACTAACT
TATGCGAAAGCATTTGACAAATCCATTCCCATTGATCAAGGACGAAAGTTGGGGGAT
CGAAGATGATTAGATACCGTCGTAGTCTCAACTATAAACTATGCCGACCAGGGATTG
GATTAAATAATAATAATTCAGCACCTTGAGTGAAAACAGAGTCTTTAGGTTTTGGGG
GGAGTATATTCGCAAGTATGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAG
TGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAATCTCACCAGGTCCAGACATAA
GAAGGATTGACAGTTTGATAGTGCTTTCTTGATTTTATGGGTGGTGGTGCATGGCCG
TTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAACGAGACCTTAAC
CTTTAAATAGTCTTTTATATTACGATATAAAAAACTTCTTAAAGGGACTGTTGATGT
TTAATCAACGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGG
CCGCACGCGCGCTACACTGATGAAGGCAGCGAGTTAAATGATTCTTTTAATCAGTTT
CCTGCTCCGGAAGGAGTGGGTAATCTTGTAAACTTCATCGTGCTGGGGATAATCCAT
TGCAATTATTGGATTTGAACGAGGAATTCCTAGTAAGTACGTGTCATCAGCACGTGC
TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGTT
TAGTGAGGCCTCTGGATTCGAAGTTTGGAGTTGGCAACGATTCTAAACTTTGGAGAA
ATTGGTCAAACTTGGTCA 3′

BLAST search and phylogenetic analysis and construction of phylogenetic tree

The sequences obtained were in small fragments and hence it was aligned properly by overlapping the sequences. Percentage homology and phylogenetic relationship of new strain of *Conidibolus* sp. using obtained sequences of 18S rDNA were done in NCBI database. The nucleotide sequence was analyzed with the GenBank database using BLAST program (www.ncbi.ncm.gov/blast). Table 2.1.2 shows the first 10 BLAST hit of 18S rDNA sequence in NCBI database. 18S rDNA showed maximum (99%) sequence homology with one strain of *Conidiobolus brefeldianus* AF 368506.1 and 98% homology with various strains of *Conidiobolus coronatus.*

Table 2.1.2: First 10 BLAST hit of 18S rDNA

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF368506.1	*Conidiobolus brefeldianus* small subunit ribosomal RNA gene, partial sequence	3000	3000	100%	0.0	99%
AF113417.1	*Conidiobolus coronatus* strain NRRL1912 18S ribosomal RNA gene, partial sequence	2922	2922	100%	0.0	98%
D29947.1	*Conidiobolus coronatus* gene for 18S rRNA	2922	2922	100%	0.0	98%
AF296753.1	*Conidiobolus coronatus* small subunit ribosomal RNA gene, partial sequence	2905	2905	100%	0.0	98%
AF368507.1	*Conidiobolus firmipilleus* small subunit ribosomal RNA gene, partial sequence	2900	2900	100%	0.0	98%
AF113418.1	*Conidiobolus coronatus* strain NRRL28638 18S ribosomal RNA gene, partial sequence	2900	2900	100%	0.0	98%
EF392543.1	*Conidiobolus coronatus* strain ARSEF 206 18S ribosomal RNA gene, partial sequence	1724	1724	58%	0.0	98%
AF113419.1	*Conidiobolus incongruus* 18S ribosomal RNA gene, partial sequence	2776	2776	100%	0.0	96%
AF368512.1	*Conidiobolus rhysosporus* small subunit ribosomal RNA gene, partial sequence	1387	1387	96%	0.0	82%
AF368511.1	*Conidiobolus pumilus* small subunit ribosomal RNA gene, partial sequence	1343	1343	96%	0.0	82%

Figure 2.1.2 shows phylogenetic relationship of the new isolate of *Conidiobolus* sp. MTCC 5185 with various strains of *Conidiobolus*. It can be seen

that strain *Conidiobolus brefeldianus* MTCC 5185 does not fall in the *Conidiobolus coronatus* cluster.

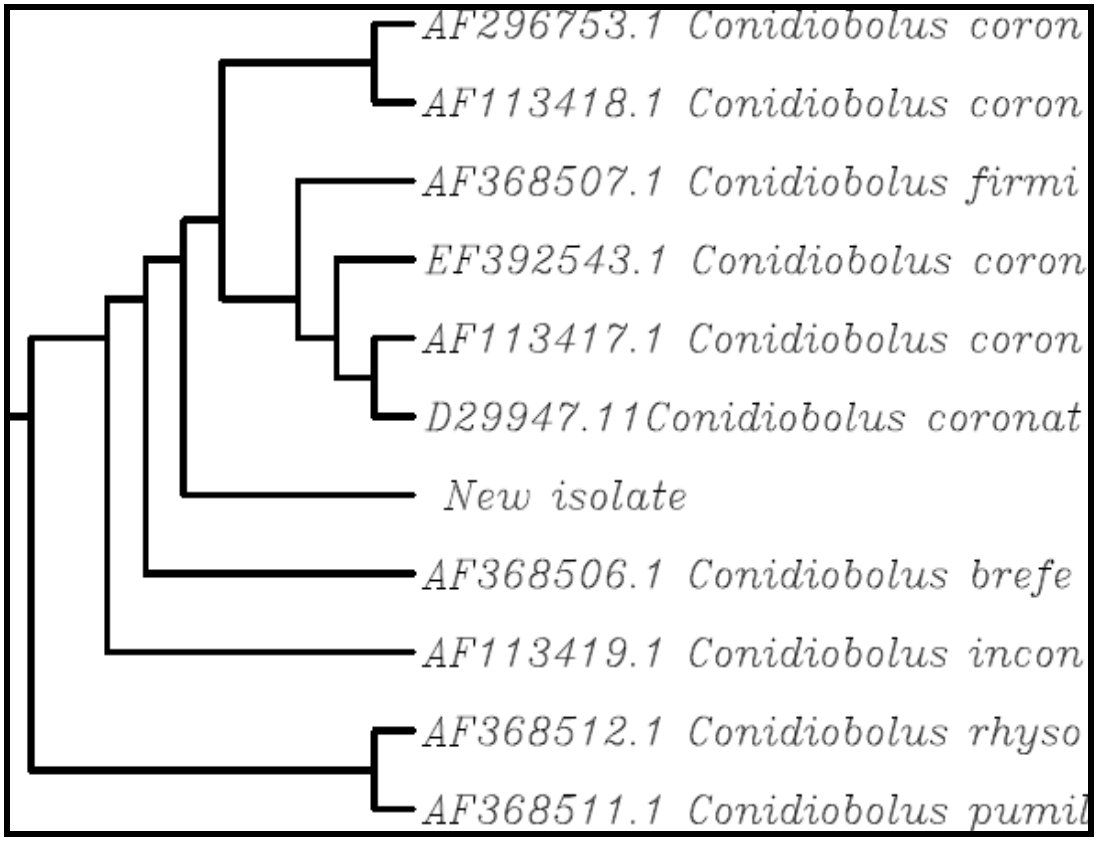


Figure 2.1.2: Phylogenetic relationship of the 18S rDNA sequence of new fungal isolate with different *Conidiobolus* strains.

Conclusions

The fungal strain was isolated from plant detritus collected from NCL campus on MGYP agar plate where spores discharged by the fungus were allowed to germinate and form single colonies which is the distinctive property of *Conidiobolus* sp. The culture grows rapidly and produces zygospores in addition to conidia. The 18S rDNA sequences showed maximum (99%) homology with *Conidiobolus brefeldianus* AF 368506.1 strain.

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SECTION 2

OPTIMIZATION OF MEDIA FOR PROTEASE PRODUCTION IN SHAKE FLASKS

Abstract

Optimization of protease production was initially studied in shake flasks where effects of various physicochemical and nutritional factors such as pH, temperature, carbon & nitrogen sources, inducers, additives, their concentrations, age of stock etc. were investigated. Protease production was highest in GYE medium with soyabean meal (SBM) or skim milk. Among various agricultural residues, SBM was found to be the best inducer. Additional nitrogen source did not show beneficial effect on production. Protease production was highest in the medium containing 2% glucose, 0.3% yeast extract and 3% SBM. Yeast extract in the medium was replaced by di-ammonium hydrogen phosphate (DAP) with 20% enhancement in activities. Similar activities were also obtained with commercial grade glucose and manure grade DAP. Protease production was observed in the pH range 5 to 10 and temperature range of 20 to 37°C, with optimal activities at pH 5.5-6 and 28°C. Optimum stock and inoculum age were found to be 2-3 days and 18-24 h respectively. Inoculum size of 10-15% gave maximum activities. Optimization resulted in more than four-fold increase in protease activities

Introduction

Protease market has experienced notable growth, owing to their characteristic degradative and synthetic functions and growing steadily due to heavy demand from various industries such as food, leather, detergent, pharmaceutical, diagnostics and waste management. Bacteria are the most dominant group of alkaline protease producers and genus *Bacillus* being the most prominent source exploited for industrially important proteases (Gupta *et al.,* 2002). However, in recent years there is interest in fungal proteases as seen from the large number of publications related to fungal proteases. Fungi elaborate a wider variety of enzymes, for example, *A. oryzae* produces acid, neutral and alkaline proteases. Moreover, enzymes of fungal origin are advantageous due to the ease of cell removal during downstream processing. In addition, fungi can effectively secrete various hydrolytic enzymes in submerged as well as solid state fermentation. The hyphal nature of fungi enables them to penetrate and colonize the substrate and thus suited better for solid-state fermentation. Variety of solid supports mainly agricultural residue such as wheat bran, rice bran, soyabean cake or combination of two or more substrates are used for production of fungal proteases by SSF (Haq and Mukhtar, 2004 ; Chutmanop *et al.,* 2008; Kranthi *et al*., 2012). Besides solid-state fermentation, fungal proteases are also produced by submerged fermentation in shake flasks (Sandya *et al*., 2005; Tremacoldi and Carmona, 2005; Hajji, *et al*., 2007; Ire *et al.*, 2011) as well as in fermentor (Sutar *et al*., 1992; Laxman *et al*., 2005; Rao *et al*., 2006; Kamath *et al*., 2010; M'hir *et al*., 2012).

A fermentation system is a complicated multi-phase, multi-component system. Growth and production are affected by a wide range of parameters, including cultivation medium, inoculum, pH, temperature, aeration agitation, shear stress, etc. The secretion of an enzyme depends on the growth phase of microorganism. The production of extracellular protease during the stationary phase of growth is characteristic of many microorganisms (Gupta *et al.,* 2002). Therefore, production is generally regulated by growth and fermentation conditions. Major factors influencing the production are nutrients like carbon and nitrogen sources, inducers, vitamins and metal ions, environmental and physiological factors such as pH and temperature, agitation/aeration, inoculum age, size etc. In commercial practice, media optimization is done to maintain a balance between the various medium components, thus

minimizing the amount of unutilized components at the end of fermentation (Kumar and Takagi, 1999). Some of the major factors influencing protease production are discussed below.

Carbon sources

Carbon sources fulfill the energy requirement of microorganisms for metabolism. In general, various sugars are used as carbon source for protease production such as glucose, fructose, sucrose, starch, lactose, maltose and sugar alcohols (Kumar and Takagi, 1999; Gupta *et al.,* 2002; Nirmal *et al*., 2011). However, protease activities vary from organism to organism depending upon the sugar used. Studies have also indicated that a high carbohydrate concentration repressed enzyme production. Therefore, carbohydrates can be added either continuously (continuous) or in aliquots (fed batch) throughout the fermentation to supplement the exhausted component (Kumar & Takagi 1999). Glucose is the most common carbon source used for protease production (Laxman *et al*., 2005; Chellapandi, 2010; Ire *et al*., 2011). Sucrose and fructose were found to be equally good as carbon sources for protease production by *C. coronatus* NCIM 1238 (Sutar *et al*., 1992) while sucrose was better than glucose for protease production by *C. coronatus* NCL 86.8.20 (Phadatare *et al.,* 1993). Although glucose is the most preferred carbon source used for protease production by various microorganisms, repression of protease production in presence of glucose is also reported for *Halobacterium* sp. (Vijayanand *et al.,* 2010), and alkaliphilic *Bacillus* sp. (Johnvesly and Naik, 2001).

Starch was found to be a better carbon source for protease production by *B. cereus* and *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004: Nilegaonkar *et al.,* 2007). Complex nitrogenous substances used as inducers can also provide certain amount of carbon in addition to nitrogen for the growth of microorganisms. Apart from sugars, various organic acids, such as acetic acid, methyl acetate, citric acid and sodium citrate have been demonstrated to increase production of proteases at alkaline pH (Kumar and Takagi, 1999; Johnvesly and Naik, 2001). Use of non-carbohydrates like kerosene, n-paraffinic hydrocarbons is also reported for protease production by *Pseudomonas* and *Fusarium* sp. (Morihara, 1965; Nakao *et al.,* 1973).

Nitrogen sources

Both organic as well as inorganic nitrogen sources have been used for protease production. Organic nitrogen sources generally include protein rich substances such as skim milk, casein, peptone, fishmeal, soyabean meal, corn gluten and variety of oil seed cakes. Among inorganic nitrogen sources, nitrates, ammonium salts, amino acids etc are used. Media is also supplemented with phosphate and metal salts. Organic compounds like protein rich substances and amino acids serve as efficient nitrogen sources for protease production by many organisms. Generally complex nitrogen source is preferred as it is slowly degraded resulting in the availability of low levels of amino acids/peptides in the medium which act as inducers of protease production. Enzyme production was found to be repressed by using rapidly hydrolysed synthetic media (Larcher *et al.,* 1996). Although researchers have investigated effect of amino acids on protease production, it becomes impractical to use amino acids for large-scale production. Moreover, some microorganisms prefer proteins and peptides to amino acids as nitrogen sources for growth as well as protease production (Phadatare, 1991**)**. Organic nitrogen sources used for protease production include peptone, yeast extract, tryptone, meat extract, corn steep liquor, casein, casein hydrolysate, and soyabean meal etc. Many of these organic nitrogen sources such as casein and soyabean meal also act as inducers (Roberta *et al.,* 2006; Shikha *et al*., 2007; Nilegaonkar *et al.,* 2007; Srinubabu *et al.,* (2007; Vijayanand *et al.,* 2010; Ire *et al.,* 2011; Vadlamani and Parcha, 2012). Among inorganic nitrogen sources, nitrates and ammonium salts are generally used for protease production (Kumar and Takagi, 1999). Srinubabu *et al.* (2007) reported that addition of di-ammonium hydrogen phosphate increased protease production by *A. oryzae*. Substitution of sodium nitrate in the basal medium with ammonium nitrate increased enzyme production by *C. coronatus* NCIM 86.8.20 (Phadatare *et al.,* 1993). In contrast, repression of protease production was reported in presence of inorganic salts (Ire *et al.,* 2011). Sodium nitrate strongly inhibited protease production by *Botrytis ceneria*, while there was no growth as well as protease production in presence of urea (Abidi *et al.,* 2008).

Inducers

Protease production is an intrinsic property of all organisms and these enzymes are generally constitutive and produced in very small amounts and many times they are partially inducible (Gupta *et al*., 2002). In industrial point of view, it is necessary to

attain high protease yields and therefore production medium generally supplied with protein rich materials as inducers in addition to nitrogen source. In the course of designing a medium for protease production, it is essential that the medium should contain a suitable inducer that favors substantial protease production. The choice of specific inducer is entirely depends on the microbial strain. An inducer for large scale protease production in submerged fermentation should follow certain criterion such as easy availability, good storage stability, low cost and forms homogenous mixture in the medium etc. In addition, it is advantageous to have an inducer which suitable/ stable during fermentation operations like sterilization, mixing and downstream processing.

The literature survey revealed that variety of protein rich substrates are used for protease production by diverse microbial strains. Commonly used proteinaceous materials as nitrogen sources and/or inducers include: skimmed milk (Vijayanand *et al.,* 2010), casein (Sutar *et al*., 1992; Phadatare *et al*., 1993; Roberta *et al.,* 2006; Nilegaonkar *et al.,* 2007; Abidi *et al.,* 2008; Namasivayam *et al.,* 2010; Vijayanand *et al.,* 2010), peptone (Roberta *et al.,* 2006; Vijayanand *et al.,* 2010; Ire *et al.,* 2011; Vadlamani and Parcha, 2012), soyabean meal (Phadatare *et al.,* 1993; Laxman *et al.,* 2005; Roberta *et al.,* 2006; Nilegaonkar *et al.,* 2007; Srinubabu *et al.,* 2007; Abidi *et al.,* 2008) and variety of oil seed cakes (Shikha *et al*., 2007; Ramachandran *et al.,* 2007; Shankar *et al.,* 2010; Vijayanand *et al.,* 2010). Other inducers used for protease production include wheat bran, rice bran, chick pea flour, black gram, horse gram (Shikha *et al*., 2007; Chutmanop *et al*., 2008; Vijayanand *et al.,* 2010; Namasivayam *et al.,* 2010; Kranthi *et al*., 2012). Use of feather meal (Roberta *et al.,* 2006), gelatin (Boer and Peralta, 2000; Abidi *et al.,* 2008; Vijayanand *et al.,* 2010), Spirulina powder (Abidi *et al.,* 2008) and fishmeal (Ellouze *et al.,* 2001; Abidi *et al.,* 2008; Namasivayam *et al.,* 2010; Ire *et al.,* 2011) are also reported as inducers for protease production.

Salts and metal ions

In addition to carbon and nitrogen, microorganisms require other elements like potassium, magnesium, phosphorous, sulphur and metal ions for growth and protease production. Phosphate and magnesium salts are usually added in most of the fermentation media designed for protease production (Sindhu *et al.,* 2009; Kamath *et*

al., 2010; Ire *et al.,* 2011). Manganese and calcium stimulated protease production by *B. cereus* (Shafee *et al.,* 2005; Nilegaonkar *et al.,* 2007), while maximum protease production was reported by *A. carbonarius* in presence of $FeSO_4$ (Ire *et al.,* 2011).

Effect of other factors on protease production

In general protease production is carried out with initial pH of the medium adjusted to neutral pH range of 6.5 to 7.5 (Phadatare *et al.,* 1993; Tremacoldi and Carmona, 2005; Rao *et al*., 2006); Younis *et al*., 2009). However, for protease production by alkalophilic organisms, the pH of the medium must be maintained in alkaline range above 7.5 throughout the fermentation period (Kumar and Takagi, 1999).

Temperature is another critical parameter that has to be controlled and varied from organism to organism. Majority fungi were found to produce protease in the temperature range of 28- 30ºC (Sutar *et al*., 1992; Laxman *et al*., 2005; Srinubabu *et al*., 2007; Nirmal *et al*., 2011; Ire *et al*., 2011). With the exception of few thermophilic organisms which need higher temperatures, microbial stains are known produce proteases in the temperature range of 28-32ºC (Kumar and Takagi, 1999).

Inoculum

It is essential to establish an effective inoculum for the fermentation process, which is influenced by variety of factors such as age of stock, number of pre cultures, composition of medium etc. It should be noted that the formation of product in seed inoculum is not the objective during inoculum development. The inoculum medium may vary from that of protease production medium and mostly devoid of inducers as found in many reports (Sutar *et al*., 1992; Laxman *et al*., 2005; Rao *et al*., 2006; Shankar *et al*., 2010). However in some instances pre-induced inoculum (inoculum medium supplied with some amount of inducer) has been tested for protease production, however there was no additional benefit on the activities was reported (Laxman *et al*., 2005; Srinubabu *et al*., 2007). In contrast, Phadatare (1991) reported significant increase in protease production by *C. coronatus* NCL 86.8.20 with pre-induced inoculum.

Sufficiently grown inoculum in appropriate volume is required for better growth and product formation in fermentation. Therefore, the age of inoculum is an important

factor and it varies from organism to organism depending on the growth rate. Commonly 12-48 h grown vegetative inoculum was used in several of the protease production studies (Phadatare, 1991; Sutar *et al*., 1992; Laxman *et al*., 2005; Srinubabu *et al*., 2007). In general, the amount of inoculum used for microbial fermentation is 3 and 10%, but it may change with the microorganism used (Stanbury *et al.,* 1995). Inoculum volume in the range of 3-5% for bacteria (Chauhan and Gupta, 2004; Shafee *et al*., 2005; Genckal and Tari, 2006) and 5-15% for fungi (Phadtare, 1991; Laxman *et al*., 2005; Anandan *et al*., 2007) has been reported to be optimum for protease production.

Aeration and agitation

The mass transfer rate between phases (e.g. oxygen transfer from gas phase to liquid) is one the most critical operational parameters for any aerobic fermentation since the maximum mass transfer will determine the cell mass. Aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be achieved by: changing aeration rate, variation in agitation speed and using oxygen-rich gas phase as oxygen source. In shake flasks, the rate of diffusion of air depends on the diameter of flask opening, volume of medium and shaker speed. Hence, it becomes necessary to optimize theses factors in shake flask experiments. Production of enzymes, especially in fermentors and bioreactors is greatly influenced by aeration and agitation. The variation in agitation speed influences the extent of mixing and hence nutrient availability in the bioreactor or in shake flasks. In addition, agitation rate was found to be influencing the morphological forms of many fungal stains, which determines the level of product formation (Stanbury *et al*., 1995; Wang *et al*., 2005).

Protease production by *C. coronatus* NCIM 1238 in 14 L laboratory fermentor was carried out with aeration and agitation rates of 0.5 vvm and 200 rpm respectively (Sutar *et al*., 1992). Another strain of *C coronatus* ATCC 4132 produces protease in 14 L laboratory fermentor with optimal rates of aeration and agitation being 0.5 vvm and 300-400 rpm respectively. Maximum protease activity by *B. bassiana* was attained with aeration rate of 0.6 vvm and agitation speed of 150 rpm in 5 L fermentor (Rao *et al*., 2006). Protease production by *R. oryzae* was carried out in a 20 L fermentor with agitation speed of 97.5 rpm and at a constant aeration rate of 1 vvm

(M'hir *et al*., 2012). Table 1.2.1 lists composition of some of the media used for protease production by various fungal strains.

In spite of the extensive research carried out on protease production by variety of microorganisms, no defined medium has been prescribed due to the diverse nutritional requirements of microbial strains. Consequently, the optimization of medium for individual strain becomes a primary and important step in the development of effective fermentation process. Present section deals with the optimization of nutritional and physical factors for the production of protease by newly isolated strain of *C. brefeldianus* in shake flasks.

Table 2.2.1: **Composition of media used for protease production by various fungal strains by submerged fermentation**

Fungal strain	Composition of medium (g/L)	Maximum activity	Substrate used for assay	One unit corresponds to	Reference
Entomopthora coronata	Difco Liver- 8; Glucose-30; $CaCO_3$-5; KH_2PO_4-1; $MgSO_4$-0.3	5 U/L (15 days)	Casein	Release of 1 meq Tyr/min	Jonsson, 1968
Conidiobolus coronatus NICM 1238	Peptone-7.5; Glucose-10; NaCl-3; K_2HPO_4-1; $MgSO_4$-1; $MnCl_2$-0.008; $CuSO_4$-0.007; $ZnSO_4$-0.002; $FeSO_4$-0.001; Casein-20	20 U/ml (40 h)	Casein	Release of 1 µM of Tyr/min	Sutar *et al.,* 1992
Conidiobolus coronatus NCL 86.8.20	Tryptone-20; Sucrose- 30; NH_4NO_3-3.8; KH_2PO_4 -1; KCl-2; $ZnSO_4$.-0.01; $CaCl_2$- 0.01; Casein-20; pH- 7.0-7.5	30 U/ml (48 h)	Casein	Increase in absorbance at 280 nm by 1.0/min	Phadatare *et al.,* 1993
Conidiobolus macrosporus NCIM 1298	Malt extract-3; Glucose-10; Yeast extract-3; Peptone-5; SBM-20; pH-7	25 U/ml (48 h)	Casein	Increase in absorbance at 280 nm by 1.0/min	Tanksale, 2001
Conidiobolus coronatus PTA-4132	Malt extract-3; Glucose-10; Yeast extract-3; Peptone-5; SBM-20; pH-7	72.46 U/ml (72 h)	Casein	Release of 1 µM of Tyr/min	Laxman *et al.,* 2005
Conidiobolus coronatus	$(NH_4)_2SO_4$-1; KH_2PO_4-4.5; K_2HPO_4-10.5; $MgSO_4$-0.25; Sodium citrate-0.5; Glucose- 2; pH-7.	82 U/ml (10 days)	N-Suc-Ala – Ala-Pro-Phe-p -Na	Increase in absorbance at 412 nm by 0.1/min	Bania *et al.,* 2006
Basidiobolus sp. (N.C.L. 97.1.1)	Malt extract-3; Glucose-10; Yeast extract-5; Soyabean meal-30	2780 U/ml	Casein	Increase in absorbance at 280 nm by 0.001/min	Ingale *et al.,* 2002

Continued

Ophiostoma piceae	Starch -30; Unsweetened soya drink- 280 ml; $CaCl_2$-0.4; KH_2PO_4-1; Na_2HPO_4-0.8; $MgSO_4$-0.5; K_2HPO_4-3; potassium hydrogen phthalate-3; Vogel micronutrients and vitamins (filter sterilized); pH–5.8	2.5 U/ml (9 days)	Azocall	Increase in absorbance at 520 nm at a rate of 0.1 OD/min	Abraham and Breuil, 1996
Beauveria sp.	Glucose- 10; Yeast extract- 3; Mustard seed cake- 20	10-12 IU/ml (3-4 days)	Casein	Release of 1 µM of Tyr/min	Shankar, 2010
Beauveria bassiana	Casein media with 10% horse gram extract	2.24 U/ml (3 days)	Casein	An increase in absorbance at 420 nm by 1.0 in 1 h	Namasivayam *et al.,* 2010
Aspergillus clavatus	Vogel medium with Glucose-10 and Casein-10	38 U/ ml (6 days)	Casein	Increase in the absorbance at 280 nm by 0.1/h	Tremacoldi and Carmona, 2005
Rhizopus oryzae CH4	Gluten-22.5; Starch-30; KH_2PO_4-2; KCl-5; $FeSO_4$-0.18; NaH_2PO_4-1.1; Na_2HPO_4-8; pH controlled at 6	266.5 U/ml	Azocasein	An increase in absorbance at 440 nm by 0.01 in 2 h	M'hir *et al.,* 2011

Materials and methods

Materials

Malt extract, yeast extract powder and yeast extract paste and peptone were procured from M/s Hi Media Chemicals, India. Hammerstein casein was obtained from M/s Sisco Research Laboratories, India. Soyatose, soyapeptone were purchased from M/s Warkem Biotech Pvt. Ltd, India. Tryptone and casamino acids were procured from Difco Laboratories, USA. Commercial grade glucose and fertilizer grade di-ammonium hydrogen phosphate were used for some of the experiments. Soybean meal (SBM) was purchased from M/s Ruchi Soya Industries Ltd, India. Mustard seed cake (MSC), cotton seed cake (CSC), groundnut seed cake (GNC), mung flour (MF), gram flour (GF), skim milk (SM) and baker's yeast were obtained from local market. All other chemicals used were of analytical grade.

Composition of media routinely used in the present study is given below in Table 2.2.2.

Table 2.2.2: Composition of media used in the present study

Media	Composition (g/L)
1%GYE	Glucose-10; yeast extact-3.0
2%GYE	Glucose-20; yeast extact-3.0
MGYP	Glucose-10; yeast extact-3; peptone-5; malt extract-3
GYEP	Glucose-10; yeast extact-3; peptone-5
Mikami	Glucose-1.5; yeast extract-1.5; peptone-5.0; beef extract-5.0

Microorganism

C. brefeldianus MTCC 5185 was maintained on MGYP agar slants and routinely sub-cultured once in 15 days and preserved at 15°C/4°C after growth and sporulation (3-4 days).

Methods

Inoculum preparation

Agar pieces (2x2 cm) with spores from 2-3 days old MGYP plate were inoculated in 100 ml of 1%GYE medium and incubated at 28°C and 180 rpm for 24 h on a rotary shaker.

Protease production

Twenty four hour old vegetative inoculum (10% v/v) was used to inoculate experimental flasks unless otherwise mentioned. Enzyme production was carried out in 500 ml Erlenmeyer flasks containing 100 ml medium and incubated at 28°C on a rotary shaker with shaking speed of 180-200 rpm. Flasks were incubated up to 48 to 96 h. Samples were drawn after regular time intervals, microscopically checked for purity and centrifuged at 5000 rpm for 5 minutes, biomass free clear supernatant was used as source of protease. The results presented are mean of minimum two experiments carried out in duplicate.

Determination of protease activity

Protease was estimated as caseinolytic activity. One milliliter of suitably diluted enzyme and 1 ml of 1% Hammerstein casein in 0.1 M sodium carbonate buffer, pH 9.0 was incubated at 50ºC, pH 9 for 10 min. The reaction was terminated by the addition of 3 ml of 5% acidified trichloroacetic acid (TCA) and the precipitate formed was filtered through Whatman No.1 filter paper after standing at room temperature for 30 min. The absorbance of TCA soluble fraction was measured at 280 nm. Tyrosine released was calculated from a pre-calibrated graph of absorbance at 280 nm against tyrosine concentration. One unit of protease activity is defined as the amount of enzyme required to release one micromole of tyrosine/min.

Results and discussion

Screening for protease production by plate assay

Since *Conidiobolus* cultures are known to secrete hydrolytic enzymes including proteases, secretion of protease by *C. brefeldianus* was investigated by plate assay. A loop full of 24 h old growth from MGYP liquid medium was placed in the center of the MGYP agar plate containing 1% skimmed milk and incubated at 28°C for 48 h. A clearance zone was visible around the fungal colony indicating the secretion of protease by the culture (Figure 2.1.1).

Figure 2.2.1: Clearance zone on MGYP+SM Plate

Extra cellular protease production is strongly influenced by media components, additives, metal ions and variation in C/N ratio (Brar *et al.,* 2007). Besides, different microorganisms have diverse nutritional requirements for growth and enzyme synthesis, consequently it is necessary to design most favorable nutritional base for individual strain. Therefore, protease production by the fungus was initially carried out in few media routinely used in our laboratory and later optimization was carried out in the medium where higher activities were obtained.

Protease production in different media

Production of protease was initially carried out in three different media, varied in their carbon and nitrogen contents. As preliminary screening for protease was carried out on agar media containing skim milk and soyabean meal is a commonly used inducer rich in nitrogen source, these two inducers were used in the media for protease production. Among the media tested, Mikami medium gave poor activity with both the inducers (Table 2.2.3).

Table 2.2.3: Protease production in different media

Time (h)	Protease Activity (IU/ml)					
	Skim milk (1%)			SBM (2%)		
	GYE-1	MGYP	Mikami	GYE-1	MGYP	Mikami
24	1.9182	Nil	0.1232	ND	0.3208	0.3378
48	4.0518	4.3538	1.6392	7.249	6.3996	2.062
72	5.0972	6.374	1.4672	9.7638	8.534	0.7756
96	3.2342	ND	0.672	ND	ND	0.6828

Nil- Not detected; ND- Not estimated.

Activities varied slightly in MGYP and 1%GYE media depending on the inducer used. Slightly higher activities were obtained with SBM as inducer in 1%GYE medium while higher activities were obtained in MGYP with 1% skim milk as inducer. Highest activities were obtained in 1%GYE + 2% SBM. Low activities in Mikami medium with both the inducers could be due to low glucose and nitrogen content of the medium. These findings suggest that glucose as carbon and yeast extract as organic nitrogen sources are important for protease production and peptone and malt extract in MGYP only have marginal influence on the enzyme production by *C. brefeldianus*. Among the four media tested for protease production by *C. coronatus* PTA-4132, MGYP was found to be best while comparable activities were also obtained in GYE medium with 3% SBM (Laxman *et al.,* 2005). Phadatare *et al.,* (1993) reported maximum protease production by *C. coronatus* NCL 86.8.20 in inorganic salts medium with 3% sucrose, 2% tryptone and 2% casein. Jonsson, (1968) studied protease production by *Entomophthora* species in various organic media and liver medium was found to be superior among them. Bore and Peralta, (2000) reported moderate to high levels of protease activities by *A. tamarii* in Vogel medium

containing glucose supplemented with casein (63.4 U/ml) or gelatin (110.5 U/ml). Srinubabu *et al.,* (2007) investigated different media for protease production by *A. oryzae* with 1% cotton seed cake and 2% SBM as inducers. Maximum activities were obtained in a modified medium which contents (g/100 ml): malt extract-1; glucose-6; yeast extract-1; peptone-2; K_2HPO_4-0.5; $MgSO_4$-0.5 and $FeSO_4$-0.01. Nileganokar *et al.,* (2007) investigated various media for protease production by *B. cereus* (MCM B-326) viz. synthetic medium with 1% casein; nutrient broth with 1% casein (NBC); 2% starch and 1% soybean meal medium and 1% soybean meal + 1% tryptone medium and reported maximum activity in starch soybean meal medium. As protease production was better or comparable in 1%GYE medium, optimization was carried out in this medium with soluble as well as insoluble inducers viz. skim milk and milk based proteins and agricultural residues such as soyabean meal, groundnut seed cake etc.

Effect of milk based proteins as inducers

Protease production was studied in 1%GYE medium with skim milk, casein, casamino acids and tryptone as inducers at 1% concentration. Maximum activities were obtained with casamino acids followed by tryptone (Table 2.2.4).

Table 2.2.4: Effect of milk based proteins as inducers

Inducer (1%)	Nitrogen content (%)	Nitrogen added (mg/100ml)	Activity (IU/ml)	
			48 h	72 h
Skim Milk	3.39	33.9	5.481	5.436
Casein	14.4	144.0	6.413	8.546
Casamino acids	9.21	92.1	14.58	15.384
Tryptone	11.22	112.2	11.936	11.834

Activities with casamino acids, tryptone and casein were almost three, two and one and half times more than obtained with skim milk. The higher nitrogen content of casamino acids (9.21%), tryptone (11.22%) and casein (14.4%) than skim milk (3.39%) might be supporting better enzyme production. Though the nitrogen content in casein is highest, its molecular weight is in the range of 75,000 to 100,000 and hence not easily available for the organism. Casamino acids and tryptone are hydrolytic products of casein derived by enzyme and acid respectively. These

compounds contain mixture of amino acids and small peptides which provide readily available nutritional support for fungal growth and rigorously stimulated protease production. Protease production by *B. cereus* MCM B-326 was 30 fold higher with 1% tryptone than 1% casein (Nilegaonkar *et al.,* 2007). However, Sutar, (1987) reported protease production by *C. coronatus* NCL 82.1.1 to be superior with casein than with tryptone and casamino acids. Protease production by *B. horikoshii* in tryptic soy broth (TSB) was enhanced by 30% by addition of 1% casein (Joo *et al.,* 2002). Casein (1-2%) is also used as inducer for protease production by *C. coronatus* NCL 86.8.20, *Aspergillus* strains and *Thermoascus aurantiacus* (Phadatare *et al.,* 1993; Anandan *et al.,* 2007; Merheb *et al.,* 2007; Kamath *et al.,* 2010; Sharma and De, 2011), while skim milk is used for screening proteolytic cultures by plate assay as well as in submerged fermentation (Damare *et al.,* 2006; Hajji *et al.,* 2007). Casamino acids at 1% concentration was used for protease production by *B. mojavensis* (Beg and Gupta, 2003).

Effect of tryptone and casamino acids concentration

Since tryptone and casamino acids gave 2-3 fold increase in activities, fermentation was carried out in 1%GYE medium with varying concentrations of these inducers. Increasing tryptone and casamino acids concentration increased mycelial growth as determined by pack cell volume (PCV) as well as protease activities (Table 2.2.5).

Table 2.2.5: Effect of tryptone and casamino acids concentration

Inducer	Conc. (%)	48 h PCV (%)	Activity (IU/ml)		
			24 h	48 h	72 h
Tryptone	**0**	12.5	2.5538	4.2968	3.4812
	0.25	15.0	4.4984	7.7054	5.0334
	0.5	17.5	7.1172	9.5904	7.624
	1.0	20.0	9.5784	**11.665**	8.727
	1.5	25.0	2.664	9.287	7.485
Casamino acids	**0**	12.5	2.9872	1.2854	-
	0.25	17.5	4.056	10.6184	4.5004
	0.5	25.0	6.6732	11.603	8.1974
	1.0	25.0	11.167	**17.044**	14.484
	1.5	22.5	10.708	15.428	13.518

Maximum activities were obtained in 48 h with both the inducers at 1% concentration. Further increase in the inducer concentration did not increase the activity. Optimum concentrations of tryptone and casamino acids for protease production by *C. coronatus* NCIM 1238 were 3 and 2% respectively (Sutar, 1987). Phadatare, (1991) investigated the effect of tryptone concentration (1-5%) on protease production by *C. coronatus* NCL 86.8.20 and reported highest activities with 2% tryptone beyond which there was no increase. Protease production by *Haloalkaliphilic* bacterium marginally increased with increasing concentrations of casamino acids up to 1.5% (w/v) and markedly repressed at 2% concentration (Joshi *et al.,* 2008).

Effect of glucose concentration with 1% tryptone as inducer

Effect of glucose concentration on protease production was studied in 1% tryptone and 0.3% yeast extract medium where glucose concentration was varied from 0 to 2%. Low levels of activity (2.5 IU/ml) were produced even in absence of glucose and increased with increase in glucose concentration up to 1.5% (Figure 2.2.2).

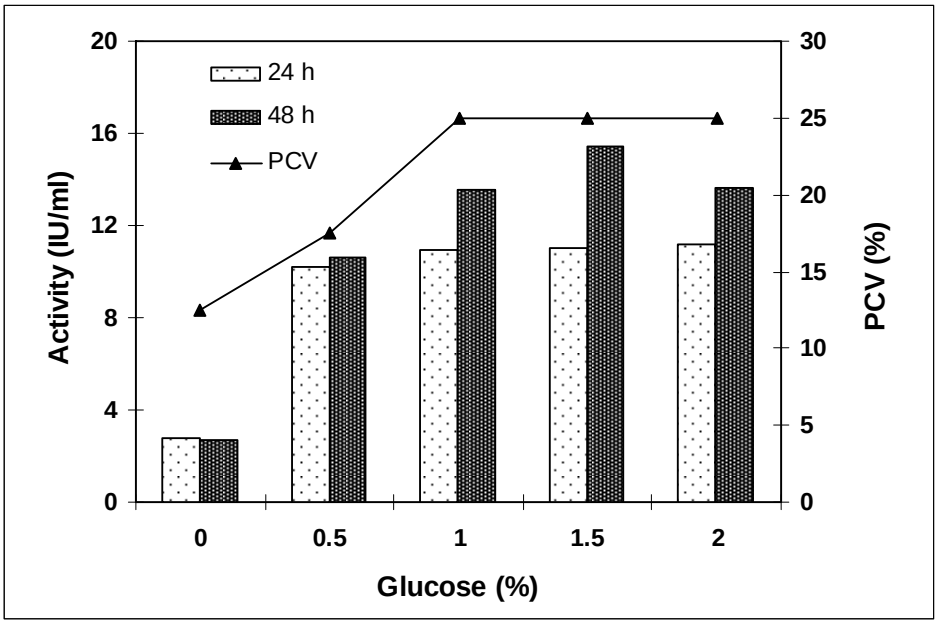
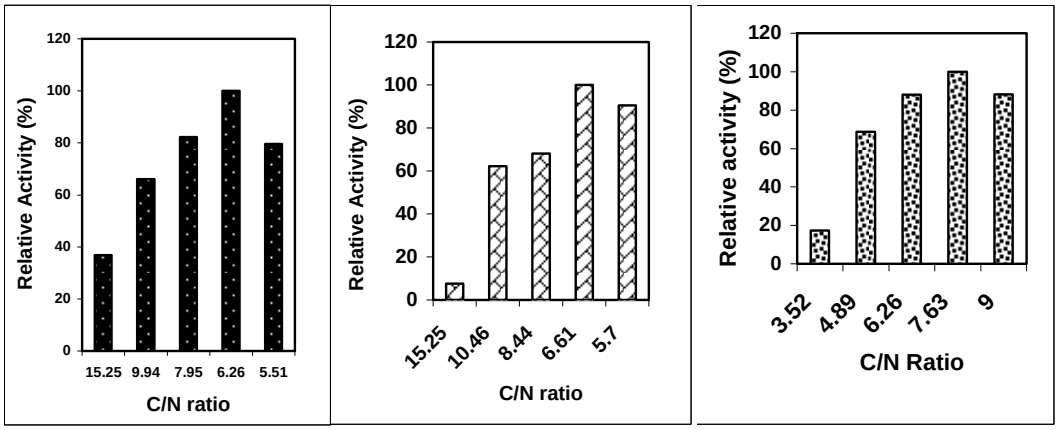


Figure 2.2.2: Effect of glucose concentration

Highest activities of 15.43 IU/ml were obtained in 48 h with 1.5% glucose. The PCV increased up to 1% glucose beyond which no further increase was observed. As already mentioned, extra cellular protease production is strongly influenced by C/N ratio. Effect of C/N ratio with the two inducers whose concentrations were varied

(keeping others constant) on protease production by *C. brefeldianus* is presented in Figure 2.2.3.



Tryptone **Casamino acids** **Glucose**

Figure 2.2.3: Effect of C/N ratio

It is observed that protease production increased with increase in C/N ratio from 5.51 to 6.26 for tryptone and 5.7 to 6.61 for casamino acids. This was checked again with the data obtained from the above experiment where glucose concentration was varied keeping tryptone concentration fixed at 1%. Highest activity was obtained when C/N ratio was 7.63. Therefore, the optimum C/N ratio seems to range from 6.26 to 7.63.

Highest activities were obtained with tryptone and casamino acids as inducers indicating the potential of the organism to secrete high levels of protease in 48-72 h. However, they increase the cost of enzyme production as they are expensive. For commercial viability of the process, it is necessary that cost of enzyme production is kept low. Hence, other inducers such as agricultural residues were investigated.

Effect of agricultural products and byproducts as inducers

Protease production was carried out in 1%GYE medium with 2% agricultural products as inducers which included soyabean meal (SBM), cotton seed cake (CSC), mustard seed cake (MSC) groundnut cake (GNC), mung (*Vigna radiata*) flour (MF), gram (*Cicer arietinum*) flour (GF). Except for groundnut cake, maximum activities were obtained in 48 h with all other inducers (Figure 2.2.4). SBM was found to be

best among them with highest activity of 9.5 IU/ml followed by groundnut seed cake (7.1 IU/ml).

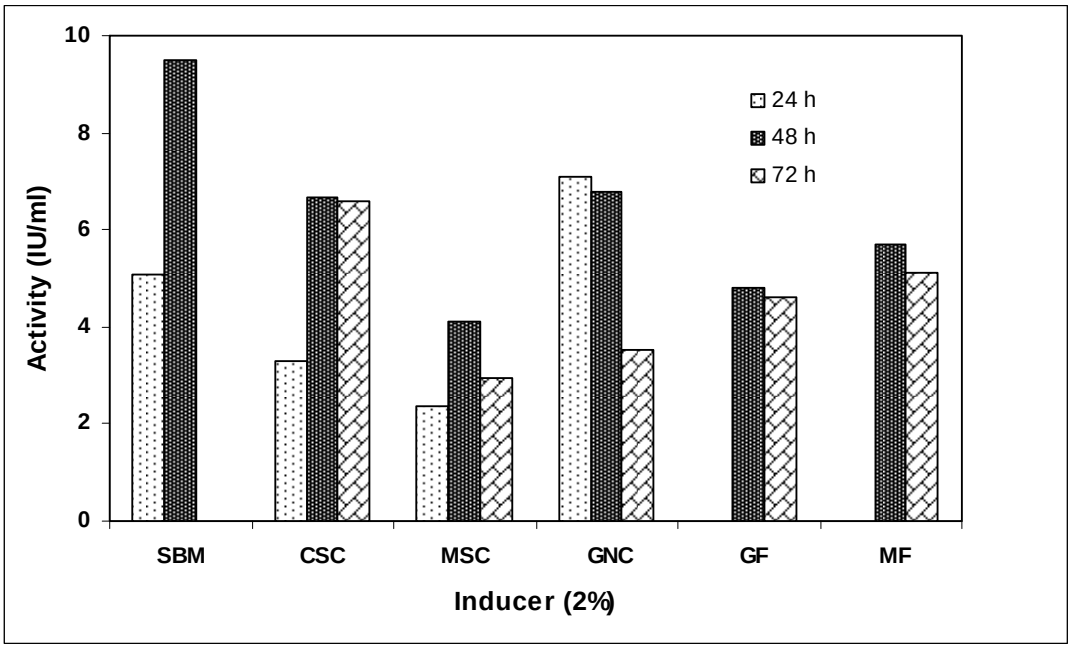


Figure 2.2.4: Effect of agricultural products and byproducts as inducers

The reason for activities being higher with SBM could be due to higher nitrogen content (8.56%). MSC which has nearly half the nitrogen content (4.49%) to that of SBM also gave half the activities. SBM is a cheap and nitrogen rich inducer and is routinely used in many fermentation media. SBM was also used for alkaline protease production by *C. coronatus* PTA-4132 (Laxman *et al.,* 2005), *A. oryzae,* (Lazim *et al.,* 2009) and *Fusarium* (Ueda *et al.,* 2007). Shankar *et al.,* (2010) used 2% mustard seed cake for protease production by *Beauveria* sp. *Botrytis cinerea* produced maximum protease when the medium was supplemented with marine *Spirulina* algae followed by 2% soybean protein (Abidi *et al.,* 2008). Fish flour and chicken feathers are also reported as inducers for protease production (Ellouz *et al.,* 2001; Gessesse *et al.,* 2003).

Effect of SBM concentration

Since SBM was found to be best inducer, all further studies were carried out using soyabean meal (SBM). In order to optimize the SBM concentration, protease production was studied in 1%GYE medium with SBM concentration varying from zero to 5%. Though low levels of activities were produced without SBM, increasing its concentration increased the activities and maximum activities were obtained with 4% SBM (Figure 2.2.5).

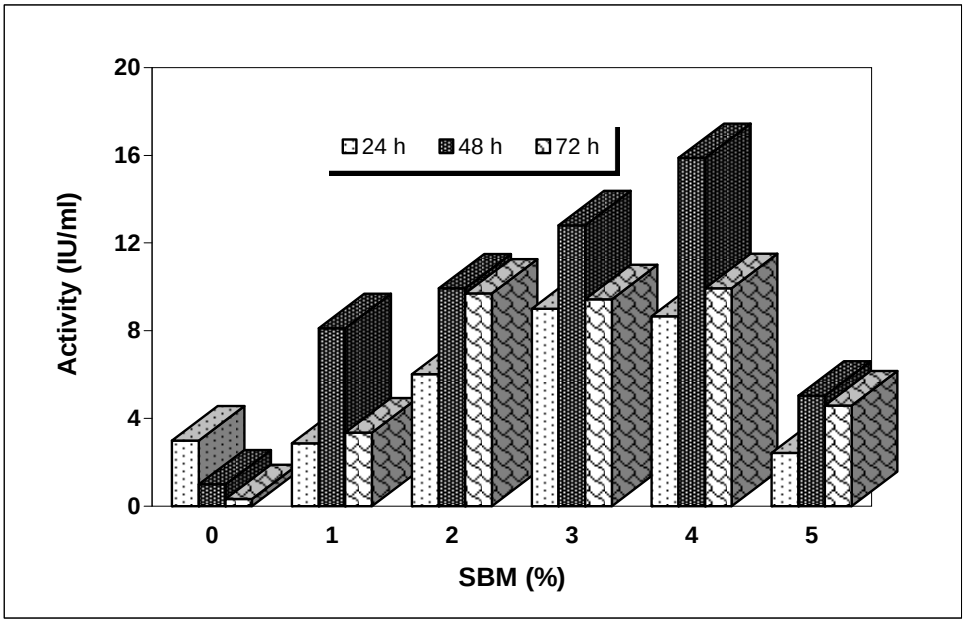


Figure 2.2.5: Effect of SBM concentration

There was drastic decrease in production (5.03 IU/ml) with 5% SBM which was almost one third of the activities produced by 4% SBM (15.88 IU/ml). This was due to poor mixing of the semi-solid medium. Several investigators have used soyabean meal at different concentrations for protease production. Sutar (1987) optimized the concentration of SBM for protease production by *C. coronatus* NCIM 1238 (NCL 82.1.1) and maximum activities were obtained with 4% SBM. Effect of SBM concentration (1 to 4%) in MGYP medium was investigated for protease production by *C. coronatus* PTA-4132 and 2% concentration was found to give maximum activity *(*Laxman *et al.,* 2005). Ingale *et al*., (2002) reported protease production by *Basidiobolus* strain (N.C.L. 97.1.1) in MGYP medium containing 3% SBM. Ire *et al.,* (2007) reported acid protease production by *A. carbonarius* in basal salts medium

containing 5% SBM. Moreira *et al.,* (2002) used 4% soya bean flour for protease production by *Nocardiopsis* sp. Joo *et al.,* (2002) investigated the effect of SBM concentrations (1-2.5%) on protease production by *B. horikoshii* in tryptic soy broth (TSB) and maximum activities were obtained with 1.5% SBM, while there was substantial decline in the production with 2.0 and 2.5% SBM.

Although activities were higher in 4% SBM, mixing was poor and sampling was difficult. Therefore, all further experiments were carried out with 3% SBM.

Effect of types of yeast extract and concentration

Effect of three different types of yeast extract viz. yeast extract powder, yeast extract paste and baker's yeast on protease production was investigated in 1%GYE medium containing 3% SBM. Both yeast extract paste as well as baker's yeast gave activities comparable to yeast extract powder in 48 h. Effect of yeast extract concentration was studied in the range 0.15 to 0.6% and 0.3% was optimal for protease production with no additional benefit on further increase (Figure 2.2.6).

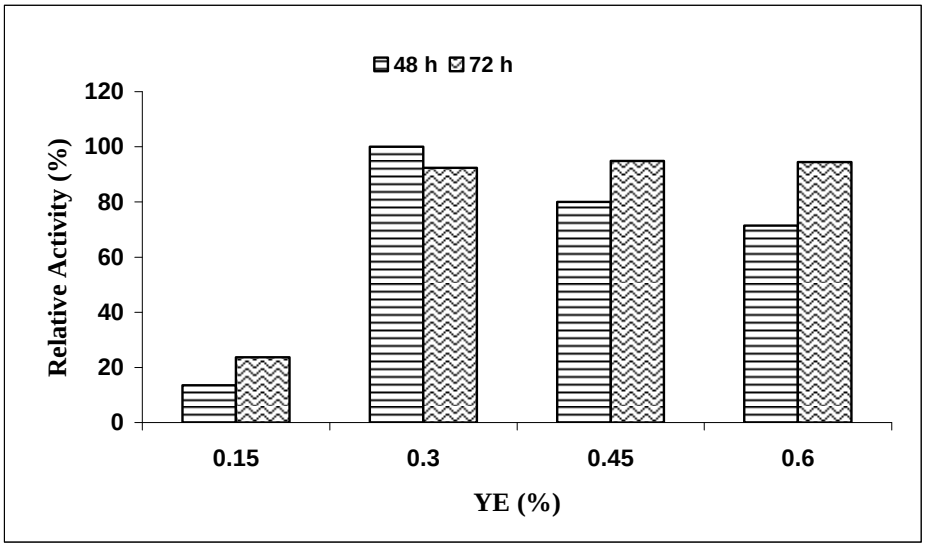


Figure 2.2.6: Effect of YE concentration

Yeast extract in the range of 0.3 to 0.5% was optimum for protease production by *C. coronatus* PTA-4132 (Laxman *et al.,* 2005). Growth and protease production by *B. cereus* BG1 were highest with 0.2% yeast extract (Frikha *et al.,* 2005). One percent

yeast extract was optimum for growth and enzyme production by alkalophilic actinomycete while further increase resulted in repression of the enzyme secretion (Mehta *et al.,* 2006).

Effect of carbon sources

Protease production varies greatly with the regulatory effects exerted by the carbon sources. Microorganisms have specific requirements and not all organisms can utilize and metabolize all the carbon sources. The need of specific carbon source for the growth of a particular microbial strain is its characteristic feature, which influences its metabolism and eventually enzyme production. Consequently, it becomes necessary to explore the most suitable carbon source for the growth and enzyme production.

Effect of carbon sources on protease production was studied in 1%GYE medium containing 3% SBM, where glucose was replaced with various sugars and sugar alcohols. The culture was able to utilize most of the sugars tested for its growth, however maximum protease production was obtained with glucose followed by fructose and lactose (Figure 2.2.7). Sugar alcohols and starch were poor carbon sources for protease production. In a similar study, among various sugars tested, glucose gave maximum protease yields by *C. coronatus* PTA-4132 (Laxman *et al.,* 2005).

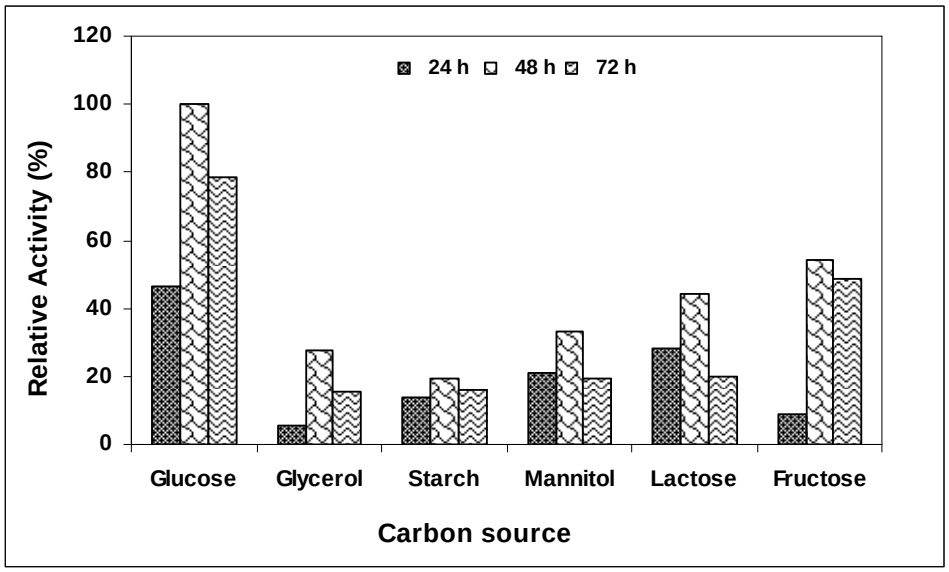


Figure 2.2.7: Effect of carbon sources

Phadatare *et al.,* (1993) reported highest protease production by *C. coronatus* NCL 86.8.20 with arabinose followed by sucrose, whereas Sutar *et al.,* (1992) found sucrose, glucose and fructose to be equally good while glycerol was found to be best carbon source at 3% concentration for protease production by *C. coronatus* NCIM 1238. Glucose was also found to be the best carbon source for protease production by *B. cereus* and *Penicillium godlewskii* (Shafee *et al.,* 2005; Sindhu *et al.,* 2009). On the contrary, extremophilic strain of *Halobacterium* sp. was unable to grow in the medium containing glucose (Vijayanand *et al.,* 2010). In some cases, sugars and sugar alcohols such as sucrose and mannitol were found to be better than glucose (Chellappan *et al.,* 2006; Devi *et al.,* 2008). Starch and molasses are also used as carbon sources for protease production (Shikha *et al.,* 2006; Chi *et al.,* 2007). Raffinose, arabinose, fructose and lactose were also used as carbon sources for protease production (Johnvesly and Naik, 2001). However, for commercial production, sugars like raffinose, arabinose or maltose are not suitable due to their cost. Most commercial processes use starch and glucose as carbon source.

Effect of glucose concentration

As glucose was found to be the best carbon source for protease production, effect of glucose concentration in the range 0 to 2.5% was investigated. Though low levels of activities were produced without glucose, increasing its concentration increased the production and maximum activities were obtained with 2% glucose (Figure 2.2.8).

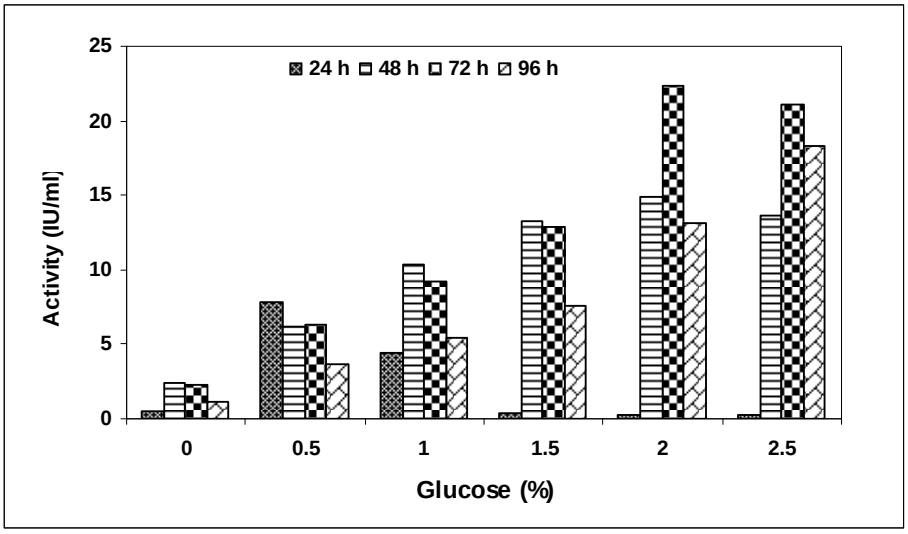


Figure 2.2.8: Effect of glucose concentration

These findings suggest that the concentration of glucose had marked influence on protease production. Protease production reached maximum in shorter time where glucose concentration was low and longer incubation time was required to reach maximum when concentration was above 1%. Thus peaks for 0.5, 1, 1.5. 2 and 2.5% glucose were 24, 48, 48, 72 and 72 h respectively. Compared to 1% glucose, there was 1.4 and 2.4 fold increase in activities with 2% glucose in 48 and 72 h respectively. Hence, all further experiments were carried out using 2% glucose and 0.3% yeast extract (2%GYE) medium. It is important to note that the activities significantly increased after the optimization studies compared to the initial activities of 9.76 IU/ml.

Protease activities by *C. coronatus* NCIM 1238 were nearly similar in media containing 0.5 to 2% glucose beyond which there was almost 50 to 70% decline in the activities (Sutar, 1987). Glucose at 1% concentration was optimum for alkaline protease production by *A. tamarii* and further increase (1.5–5.0%) appeared to inhibit the production (Anandan *et al.,* 2007). In contrast, the optimum concentration of glucose was found to be 5% for acidic protease production by *A. carbonarius* (Ire *et al.,* 2011). Glucose at 4% concentration was optimum for protease production by *P. godlewskii* by solid state fermentation, while higher concentrations (above 6%) were inhibitory (Sindhu *et al.,* 2009). On the contrary, protease production by alkaliphilic actinomycete was optimal with 0.5% glucose and activities decreased from 35 to 10 U/ml with 2% glucose (Mehta *et al.,* 2006). Protease production by *Bacillus* sp. was strongly repressed from 120 to 4 U/ml with increase in glucose concentration from 0.5 to 2% (Patel *et al.,* 2005).

Effect of additional nitrogen

As nitrogen concentration is known to influence enzyme secretion, effect of additional nitrogen (as organic and inorganic sources) on protease production in 2%GYE+3% SBM was investigated. Addition of inorganic nitrogen equivalent to 0.3% yeast extract had no beneficial effect on protease production (Table 2.2.6). All of them gave more than 88% activities compared to yeast extract in 48 h.

Table 2.2.6: Effect of additional inorganic N- sources

Additional N-source	Amount of added (g/L)	Relative Activity (%)	
		48 h	72 h
Nil	0	100.0	81.54
Urea	0.64	87.95	61.33
$(NH_4)_2SO_4$	1.4	91.46	65.67
DAP	1.6	88.02	80.62
$NaNO_3$	1.82	102.13	78.87
KNO_3	2.16	99.24	82.53
NH_4Cl	1.14	88.79	65.67

Majority of organic nitrogen sources inhibited protease production, except soyatose, which slightly enhanced the activities than control in 72 h (Table 2.2.7). It is interesting to note that tryptone and casamino acids in absence of SBM induced protease production and gave very high activities but repressed the production in presence of SBM. This observation is similar to Ingale *et al.,* (2002) who reported 46% decline in protease production by *Basidiobolus* strain (N.C.L. 97.1.1), when the production medium containing 3% SBM was supplemented with 0.5% casamino acids as compared to the medium devoid of it.

Table 2.2.7: Effect of additional organic N-sources

Additional N-Source (1%)	Nitrogen added (mg/100ml)	Relative Activity (%)		
		48 h	72 h	96 h
Nil	-	100.00	78.39	40.99
Soyapeptone	104.0	82.20	75.66	51.74
Soyatose	99.5	107.74	123.22	57.01
Tryptone	112.2	61.40	75.58	53.90
Casamino acids	92.1	61.10	71.83	52.40

Protease production by bacteria and fungi is reported in media containing organic nitrogen sources such as peptone, yeast extract, tryptone, casamino acids in addition to inducers such as soyabean meal and casein (Sutar *et al.*,1992; Phadatare *et al.*,1993; Rao and Narasu, (2007); Srinubabu *et al.,* (2007); Qadar *et al.,* (2009); Ire *et al.,* (2011); Sevinc and Demirkan, (2011).

Apart from carbon and nitrogen sources and inducers, there are reports of incorporation of inorganic salts in medium such as K_2HPO_4, KH_2PO_4, $MgSO_4$, and

$FeSO_4$ for protease production (Ghadage, 1986; Phadatare, 1991; Srinubabu *et al.,* 2007; Bhunia *et al.,* 2011; Lazim *et al.,* 2009). Phosphate salts are generally used to provide necessary phosphate for microbial growth and protease production. In addition, phosphate ions are also responsible for buffering action in the medium (Kumar and Takagi, 1999).

Effect of metal ions on protease production

Effect of various metal ions on protease production was studied where metal salts in the form of chlorides, nitrates and sulphates were added in the medium. Ca, Cu, Mn and Pb had no significant effect on protease production at 1mM and activities in 48 h were nearly similar to control where no metal was added (Table 2.2.8).

Table 2.2.8: Effect of metal ions

Metal salt	Relative Activity (%)			
	(1 mM)		(5 mM)	
	48 h	72 h	48 h	72 h
Nil	100.00	83.61	100	93.56
$CaCl_2$	114.84	87.84	91.56	87.10
$CoCl_2$	25.48	65.04	23.84	64.93
$CuCl_2$	105.73	65.20	ND	ND
Hg Cl_2	0	0	ND	ND
$FeSO_4$	ND	ND	77.82	58.30
$MnCl_2$	102.11	80.53	95.25	75.67
Ni $(NO_3)_2$	69.80	53.59	ND	ND
Pb $(NO_3)_2$	109.02	92.72	ND	ND
$ZnSO_4$	ND	ND	87.69	89.24

ND= not done.

Co, Ni and Hg inhibited the production by 75, 30 and 100% respectively. Hg inhibited growth as well as protease production. Activities decreased in 72 h for all samples including control except Co where there was more than two fold increase in activities indicating that the organism has partially recovered from the inhibitory effect of Co. Similar trend was also observed at 5 mM concentration. Fe inhibited the production by less than 25%. In general, calcium is known to enhance protease synthesis in many *Bacillus* strains (Frikha *et al.,* 2005; Nilegoankar *et al.,* 2007; Qadar *et al.,* 2009;

Vijayanand *et al.,* 2010). However, the effect of specific metal ion on enzyme synthesis is solely dependent on the microbial strain. Phadatare (1991) studied effect of 0.001% Ca, Co, Cu, Fe, Mn and Zn on protease production by *C. coronatus* NCL 86.8.20 and found no significant effect. Ire *et al.,* (2011) studied effect of replacing Mg in the medium with 0.01% Ba, Ca, Co, Fe, Mn, Ni and Zn on protease production by *A. carbonarius*. It was found that the highest protease secretion was obtained in the medium containing Fe while other metals showed only marginal increase.

Effect of surfactant

Non-ionic surfactants like Tweens were found to have stimulatory effect on enzyme production by various fungi (Reese and Maguire, 1969; Pardo, 1996; Mandviwala and Khire, 2000). They are known to modify the cell membrane and facilitate secretion of proteins in to the medium. Non-ionic detergents are generally used to extract enzyme from fungal biomass (Silva *et al.,* 2005). Influence of various surfactants on protease production was investigated in 2%GYE +3% SBM medium supplemented with 0.1% surfactant. Control without addition of any surfactant was included for comparison. Tween 20 and Triton X-100 inhibited growth as well as protease production while Tween 40 and Tween 60 slightly inhibited protease production (Table 2.2.9).

Table 2.2.9: Effect of surfactants

Surfactant (0.1%)	Relative Activity (%)		
	24 h	48 h	72 h
Nil	23.24	100	83.75
Tween 20	6.87	0	0
Tween 40	16.78	67.75	90.09
Tween 60	17.08	63.80	84.92
Tween 80	39.34	91.37	79.40
Triton X-100	4.91	0	0

This could be due the fact that the *C. brefeldianus* mycelium undergoes lysis immediately after depletion of nutrients and protease is released in to the medium and does not seem to require action of surfactants. Contrary to this, Tween 80 was found to stimulate protease production by *A. carbonarius* (Ire *et al.,* 2011). Effect of surfactants on protease production by *C. coronatus* NCL 86.8.20 showed no inhibition

with Tween 80 at 0.1 and 0.3% concentrations and gave activities similar to control without surfactant indicating that surfactant had no stimulatory effect (Phadatare, 1991). Though Tween 20 showed no inhibiton at 0.1%, there was 63% reduction in activity when increased to 0.3%

Optimization of* glucose, yeast extract and SBM *concentrations in the medium

During the course of the investigation, it was found that glucose, yeast extract and SBM were the main carbon, organic nitrogen sources and inducer respectively for protease production by *C. brefeldianus*. In order to design an effective fermentation medium, to study their mutual effects and to optimize the medium further, fermentation was carried out by varying the concentration of one of the components at a time while keeping the concentration of other two ingredients constant as shown in the Table 2.2.10.

Table 2.2.10: Media composition

Set	Glucose (%)	Yeast extract (%)	Soyabean meal (%)
1	2	0.6	4
2	2	0.6	3
3	2	0.3	4
4	2	0.3	3
5	1	0.6	4
6	1	0.6	3
7	1	0.3	4
8	1	0.3	3

The results obtained revealed that, combined effect of concentrations of all three ingredients influenced the level of protease production and concentration of glucose was found to be the key determinant. Among the combinations tested, glucose at both the concentrations with 0.3% yeast extract and 3.0% SBM (Set 4 & Set 8) resulted in higher protease production. However, activities with 2% glucose (Set 4) were 37% more compared to 1% glucose (Set 8). Interestingly, activities were lower when the concentrations of all three ingredients were highest (Set 1), compared to the activities obtained with all the ingredients at lower concentrations (Set 8) indicating that higher SBM and yeast extract concentrations were not beneficiary (Figure 2.2.9).

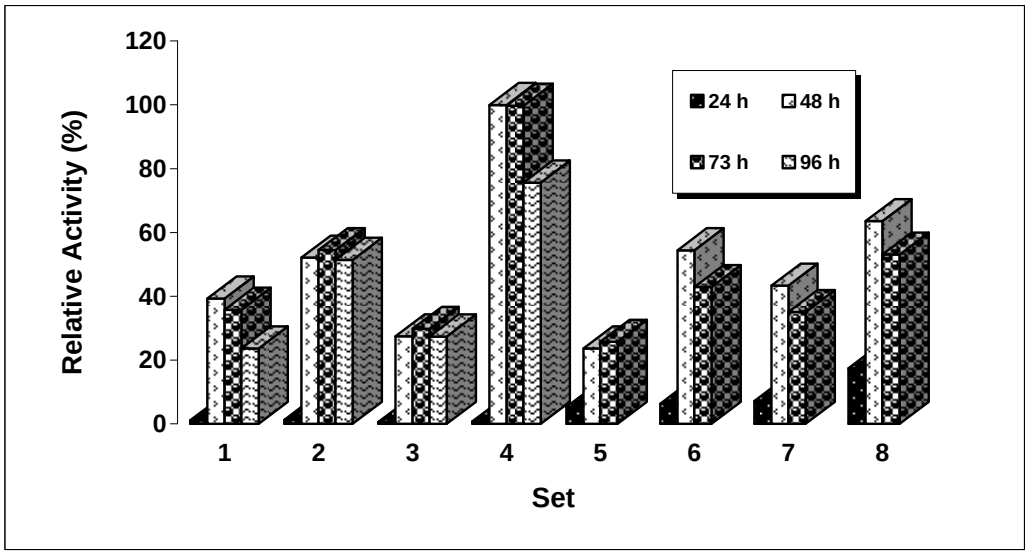


Figure 2.2.9: Effect of concentrations of media constituents

Only increasing yeast extract and SBM concentrations with 1% glucose gave lowest activities among all the combinations. In this experiment, highest activities were obtained with 2% glucose+0.3% yeast extract and 3% SBM with C/N ratio of 7.56. This value is nearly similar to C/N ratio of 7.63 where highest activities were obtained in earlier experiment with glucose concentration being varied with fixed tryptone concentration (Figure 2.2.3). Laxman *et al.,* (2005) optimized the concentrations of ingredients of MGYP medium for protease production by *C. coronatus* PTA-4132 and found sharp enhancement in the activities when glucose concentration was increased from 0.5 to 1%. In contrast, repression of protease production was observed in presence of 1 to 2% glucose by alkalophilic actinomycete (Mehta *et al.,* 2006).

Effect of inorganic nitrogen sources

Earlier we have studied the effect of additional organic as well as inorganic nitrogen sources in 2%GYE + 3% SBM on protease production and found that there was slight inhibition in activity. However, in absence of SBM, organic nitrogen sources tryptone, soyatose and casamino acids increased the activities several folds. One of the reasons for this could be due to imbalance in C/N ratio in the medium. Therefore, it was decided to replace the yeast extract a costly media component with cheap inorganic nitrogen source and see the effect. Accordingly, we studied effect of various inorganic nitrogen sources on protease production. Yeast extract in the medium

(2%GYE + 3% SBM) was replaced with one of the inorganic nitrogen salts (at nitrogen concentration equivalent to that of 0.3% YE).

All the inorganic nitrogen sources were effective and slightly higher activities were obtained compared to yeast extract. Around 15-20% more activities were obtained with di-ammonium hydrogen phosphate (DAP) as nitrogen source, while most of the other inorganic salts gave more than 10% protease production compared to yeast extract (Figure 2.2.10).

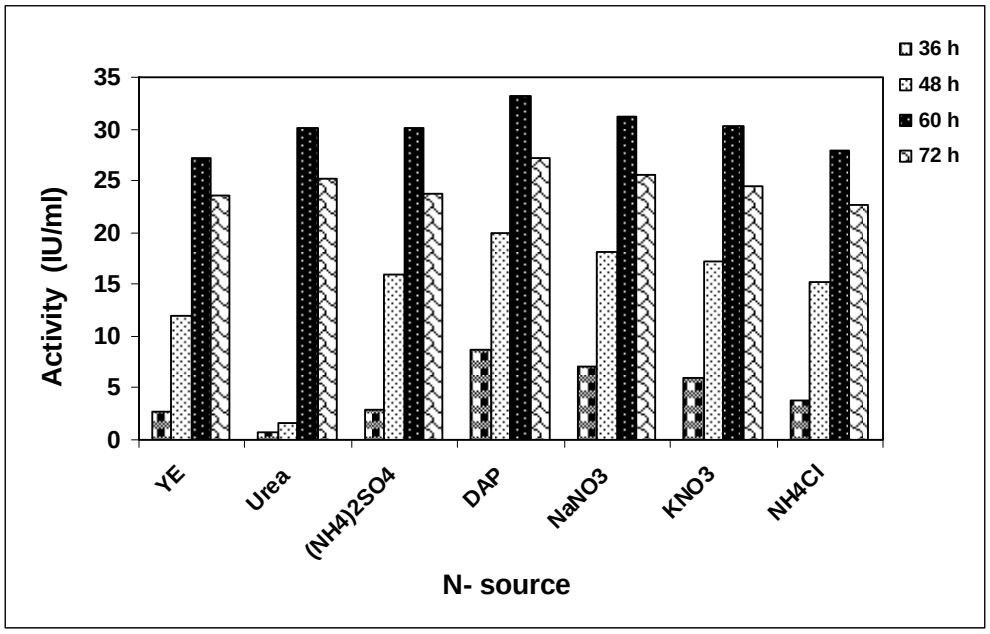


Figure 2.2.10: Effect of inorganic nitrogen source

The advantage of di-ammonium hydrogen phosphate is that it can simultaneously serve a good source of nitrogen as well as provide phosphate source for fungal growth. These findings are in agreement with the results reported by Laxman *et al.,* (2005) for protease production by *C. coronatus* PTA-4132. However, Sutar (1987) reported very poor growth and low protease activities by *C. coronatus* NCIM 1238 in 1% glucose and synthetic medium containing 0.3% inorganic N-sources. Phadatare (1991) found ammonium nitrate to be the best inorganic N-source for protease production by *C. coronatus* NCL 86.8.20. Literature survey revealed that type of inorganic nitrogen source greatly influences the level of protease production and activities vary from organism to organism. Sodium nitrate was stimulatory for

alkaline protease production by marine yeast *A. pullulans* and its substitution showed lower activities (Chi *et al.,* 2007). Kamath *et al.* (2010) found that potassium nitrate leads to maximum protease production by *A. niger*. Different ammonium salts showed diverse effect on protease production by *B. cereus* where ammonium phosphate was inhibitory while other ammonium salts supported protease production (Nilegonkar *et al.,* 2007). Inorganic nitrogen sources also were inhibitory for protease production by many *Bacillus* stains and *Halobacterum* sp. (Kaur *et al.,* 2001; Chauhan & Gupta, 2004; Patel *et al.,* 2005; Shikha *et al.,* 2006; Vijayanand *et al.,* 2010).

Effect of initial pH of the medium

Effect of initial pH on protease production was studied in 2%GYE + 3% SBM medium in the pH range 5 to 10. Medium was adjusted to required pH with sterile 0.1 N HCl or 0.1 N NaOH before inoculation. The pH of the medium during the fermentation was not controlled. Irrespective of the initial pH of the medium, final pH at the end of the fermentation reached 7.5 to 8. The organism secreted protease over wide pH range of 5 to 10 (Figure 2.2.11). Maximum activities were obtained between pH 5 to 6.12 for all the samples tested (48-96 h). Though further increase in pH up to 10 decreased the production, nearly 50% of the activities compared to pH 5 to 6.12 were still produced at pH 9 to 9.5.

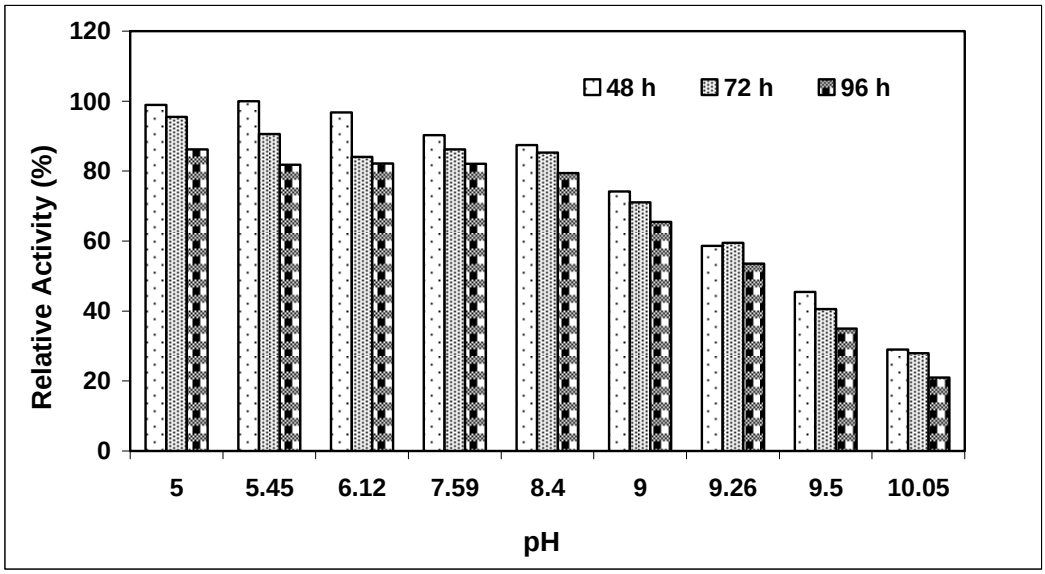


Figure 2.2.11: Effect of initial pH of the medium

These results are similar to those obtained by Phadatare *et al.* (1993) who reported maximum activities in the range of 6-8.5 beyond which the growth was comparatively poor resulting in the reduced enzyme production. Sutar *et al,* (1992) reported that pH range of 6.8 to 7 to be optimum for protease production by *C. coronatus* NCIM 1238. Acidic protease by *A. niger* is optimally produced at pH 4 (Kamath *et. al*., 2010). Optimum pH for protease production by *Beauveria felina, A. pullulans* and *B. cinerea* was in the range of 6-7 (Agrawal *et al.*, 2005; Chi *et al*., 2007; Abidi *et al*., 2008), while optimum pH range for *Bacillus* sp. was 7-7.5 (Qadar *et al*., 2009; Younis *et al*., 2009). However, Srinubabu *et al.,* (2007) reported growth and protease production by *A. oryzae* in the pH range 7-11 with maximum activity at initial pH of 10.

Effect of temperature

Temperature is a key determinant in microbial fermentation and the optimum temperature for growth and production varies from organism to organism. The mechanism of temperature control of enzyme production is not well understood. However, a link between enzyme synthesis and energy metabolism in *Bacilli* controlled by temperature and oxygen uptake is reported (Kumar and Takagi, 1999). Most of the protease producing fungal cultures grow in the temperature range of 28 to 32°C except few thermophilic organisms which need higher temperature (45-60°C) for their growth and protease production (Kumar and Takagi, 1999. Effect of temperature on protease production was studied in the temperature range of 20–45°C. Maximum production was obtained at 28°C followed by lower temperature of 20°C which favored the growth and protease production with more than 70% of maximum activity while growth and production were significantly lower (25%) at 37°C (Table 2.2.11). No growth was observed at 45°C and hence no activity was detected.

Table 2.2.11: Effect of Temperature

Temp (°C)	Relative Activity (%)		
	24 h	**48 h**	**72h**
20	2.57	66.19	71.78
28	7.97	82.74	100
37	2.80	28.88	25.08
45	0.85	0.75	Nil

Nil- Not detected

Sutar *et al.* (1992) reported optimum temperature for protease production by *C. coronatus* NCIM 1238 to be 30°C. Phadatare *et al.* (1993) reported optimum temperature for protease production by *C. coronatus* NCL 86.8.20 to be 28°C while growth and enzyme production were adversely affected at 30°C and above.

Effect of ratio of volume of medium to capacity of flask

The volume of medium in the flaks makes considerable effect on mixing and ultimately on the rate of oxygen distribution in liquid phase. Medium volume at both low and high level may affect the growth of microorganism, consequently influence product formation. Effect of ratio of volume of medium to capacity of flask on protease production was studied. Protease activity was highest when the ratio was 10-20% and further increase in the ratio greatly reduced the level of protease production which may be due to onset of oxygen deficiency/insufficient mass transfer (Table 2.2.12). Protease production increased with incubation time and reached maximum after 72 h.

Table 2.2.12: Effect of ratio of volume of medium to capacity of flask

Ratio of volume of medium to capacity of the flask (%)	Relative Activity (%)		
	24 h	48 h	72 h
10	59.19	80.41	100.00
15	34.27	69.23	94.36
20	6.74	49.14	83.97
25	4.11	23.00	41.64
30	3.46	3.55	8.08

The culture used in fermentation must fulfill following criteria for optimum results i.e. maximum yield in shorter time i) must be in healthy and active form to minimize the lag phase ii) should be in sufficient large volume to provide optimum size inoculum iii) must be in suitable morphological form. It is a general practice to use a spore suspension as seed for inoculum development for majority of industrially important fungi (Stanbury *et al.,* 1995). However use of vegetative growth as inoculum is advantageous in terms of simplicity in development, monitor the purity of culture at different stages of inoculum, as it reduces lag phase and easier to operate aseptically. Considering these aspects, optimization of conditions related to inoculum development like effect of age of stock, age and size of inoculum on protease

production was investigated. The present strain grows rapidly (2-3 days of incubation) on solid media with formation of numerous spores. Such sporulated culture was directly used to develop vegetative inoculum in liquid media.

Effect of age of stock

As mentioned earlier, it is essential that the culture should be in actively growing state. Either an overgrown and old culture which might have lost viability or young culture with no sporulation or immature spores are not useful as they may delay the inoculum development. Therefore, effect of age of the stock used for inoculum development on protease production was investigated. Spores from 2 to 30 days old slant were transferred to inoculum (1%GYE liquid medium) and incubated at 28°C, 180-200 rpm for 24 h. Visibly gradation in growth was seen with age. Maximum growth with 2 days old stock while lowest growth was seen in 30 days old stock (2&5 days: ++++; 10&15 days: +++; 20&25: ++ and 30 days: +). Ten percent of this vegetative inoculum was used to inoculate the experimental flasks. It was observed that protease production decreased with increasing age of the stock Figure 2.2.12). Highest activities were obtained with 2-5 days old stock while more than 80% of the activities were obtained with 10-20 days old stocks. There was drastic decrease in activity beyond this, which could be due to lower number of viable spores inoculated in to the inoculum medium.

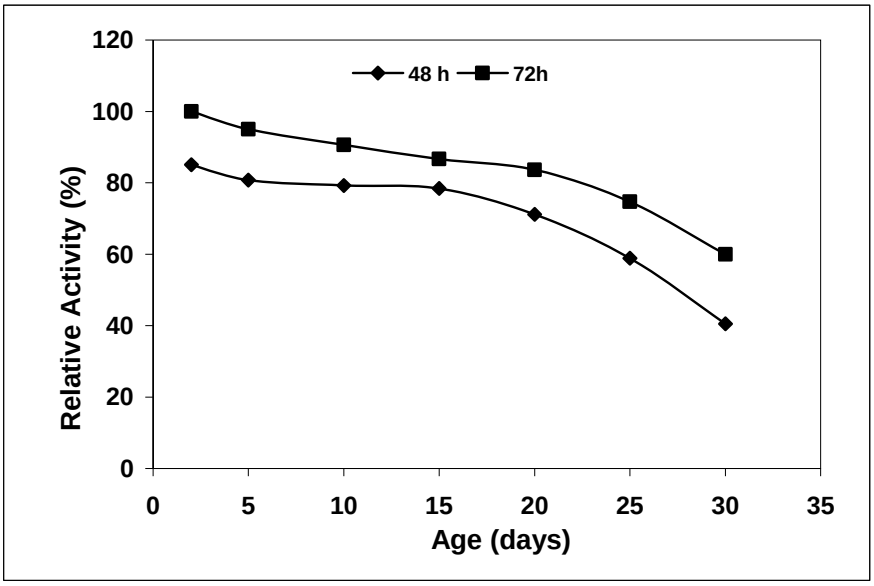


Figure 2.2.12: Effect of age of stock

Effect of inoculum age

Effect of inoculum age (12 to 48 h) on protease production was studied. Spores from 2-3 days MGYP slant were used for inoculum development (1%GYE) and 10% (v/v) inoculum was used. Highest protease production was obtained with 18 to 24 h old inoculum while activities with 12 and 36 h old inoculum were nearly 56 and 87% respectively (Figure 2.2.13). No protease activity was seen with 48 h old inoculum as mycelial lysis was observed in the inoculum.

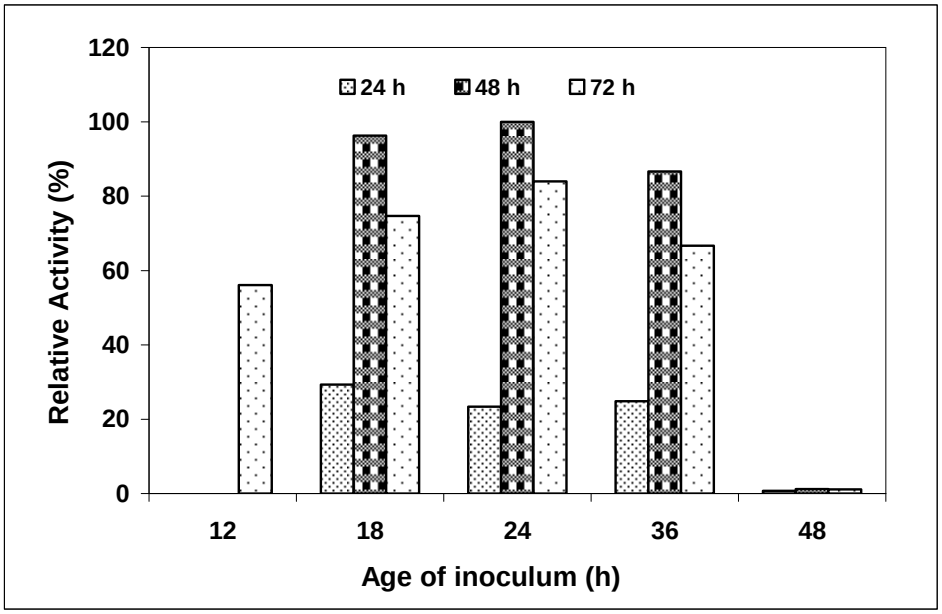


Figure 2.2.13: Effect inoculum age

Effect of inoculum size

Inoculum volume determines the level of biomass and product formation and plays vital role in microbial fermentation. It is obvious that the inoculum size shows diverse impact on biomass formation and enzyme synthesis depending on the characteristics of microbial strains (Genckal and Tari, 2006). Therefore, effect of inoculum size ranging from 5 to 20% (v/v) on protease production was investigated in 1%GYE + 2% SBM medium. Protease activities after 48 h increased gradually with increase in inoculum size from 5 to 10% (Figure 2.2.14) and there was decline in activity with 20% inoculum. The decline in protease level with larger inoculum size could be due to the shortage of nutrients available for the larger biomass and aster growth of culture.

The quantity of inoculum for microbial fermentation normally used is between 3 and 10% but can vary with culture to culture (Stanbury *et al.,* 1995**)**. In general, 2-5% inoculum volume is reported to be optimum for protease production by bacterial cultures. Phadatare (1991**)** reported 10-15% inoculum to be optimum for protease production by *C. coronatus* NCL 86.8.20. Anandan *et al*. (2007) investigated the effect of inoculum size on protease production by *A. tamarii* and reported maximum activities with 5% inoculum in submerged as well as solid state fermentation. Genckal and Tari (2006) found 5% (v/v) inoculum to be optimum for protease production by different *Bacillus* strains while Shafee *et al* (2005) found 4% to be optimum inoculum size for protease production by *B. cereus*.

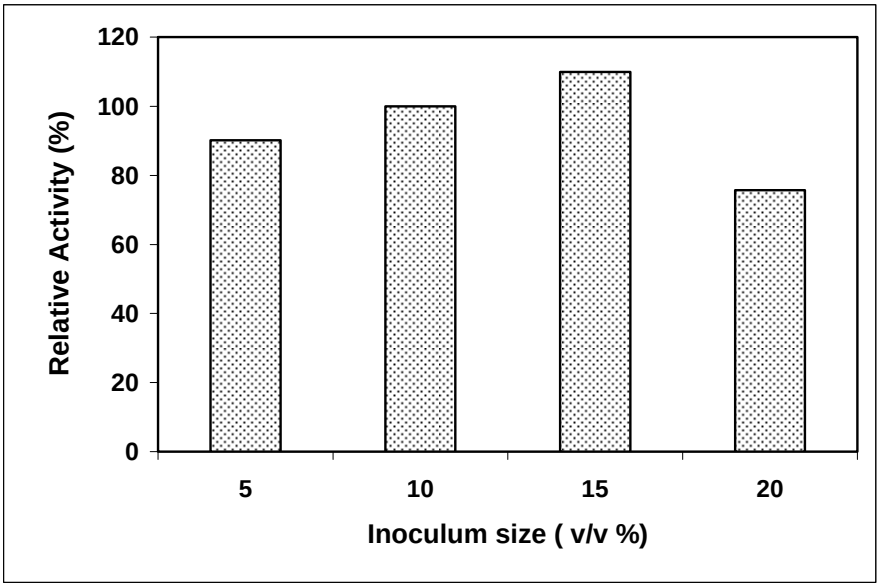


Figure 2.2.14: Effect inoculum size

Protease production using commercial media ingredients

As stated earlier, it is estimated that the cost of the growth medium accounts nearly 30-40% of enzyme production. Therefore cost of production can be brought down by reducing the fermentation time, cost of media ingredients or by increasing the activities among other factors. Optimization of media for protease production by *C. brefeldianus* has resulted in identifying a simple medium with glucose as carbon source, yeast extract or DAP as nitrogen source and soyabean meal as inducer. For production at commercial scale, commercial grade media ingredients are generally used to cut down the cost of production. Hence, the protease production by the fungus

was investigated in media prepared using commercial grade media constituents. Analytical grade glucose and analytical grade DAP were replaced with commercial grade glucose and manure grade DAP respectively. Hi Media yeast extract was replaced by baker's yeast in the medium. The various combinations of the media constituents tested and their effect on protease activities are presented in Table 2.2.13). It was observed that the commercial media ingredients did not affect the protease production and activities comparable to those of analytical grade chemicals were obtained. The cost of the medium was reduced by nearly 8 fold. Eventually this medium comprised of commercial grade glucose and manure grade DAP was used for pilot scale production.

Table 2.2.13: Effect of commercial media ingredients

Medium	Glucose (2%)	Yeast Extract (0.3%)	DAP (0.16%)	Relative Activity (%)	
				48 h	72 h
QG+HY	Qualigens	HiMedia	-	100.00	93.94
QG+BY	Qualigens	Baker's yeast	-	95.12	89.81
QG+SD	Qualigens	-	SD'fine	122.96	105.2
QG+CD	Qualigens	-	commercial	111.17	100.47
CG+HY	commercial	HiMedia	-	102.11	90.12
CG+BY	commercial	Baker's yeast	-	96.3	87.12
CG+SD	commercial	-	SD'fine	120.12	110.34
CG+CD	commercial	-	commercial	127.38	108.29

Conclusions

The present strain of *C. brefeldianus* was able to utilize variety of carbon and nitrogen sources for protease production. Among the soluble milk based inducers in glucose and yeast extract medium, maximum activities were obtained with casamino acids and tryptone at 1% concentration. Among the agrobased inducers tested, groundnut cake and SBM gave very good activities SBM being the best. Glucose followed by fructose were the best carbon sources for production. Optimization studies revealed that 2% glucose, 0.3% yeast extract and 3% soyabean meal gave highest activities. The optimal C/N ratio for production was in the range 6.6 to 7.63. Additional inorganic or organic nitrogen sources slightly repressed the production. Mercury inhibited growth as well as production while Co, Cu and Ni were slightly inhibitory. Surfactants at 0.1% had no beneficiary effect. Yeast extract could be replaced with di ammonium hydrogen phosphate with 20% enhancement in the activities. Protease production was optimum at 28°C and pH range of 5.45 to 6.12. Two to three days old stock and 10-15% (v/v) of 18-24 h old inoculum gave highest activities. Analytical grade media constituents were replaced with commercial grade/manure grade ingredients with slightly higher activities which is very important for scale up and pilot scale production of enzyme for commercialization. The optimization studies resulted in nearly 4-5 fold increase in activities and 8 fold reduction in medium cost compared initial activities and cost.

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SECTION 3

SCALE UP OF PROTEASE PRODUCTION IN INSTRUMENTED FERMENTORS

Abstract

Optimization of process parameters for protease production by *C. brefeldianus* was initially studied in 7.5 L bioreactor with 5 L working volume. Agitation greatly influenced protease production both in terms of activity as well as fermentation time. Activities were low when the agitation was kept at fixed rate of 200 and 300 rpm irrespective of aeration rates. Further increase in agitation (400 and 500 rpm) resulted in increased protease activities. Glucose consumption was directly proportional to the rate of agitation and increased during the progress of fermentation. Similarly, biomass produced was more with higher agitation rates of 400 and 500 rpm compared to 200 and 300 rpm. Highest activities were obtained when the agitation rate was slowly increased from 250 to 450 rpm during the course of fermentation at both the aeration rates. Protease yields of 30 and 32 IU/ml were obtained at 0.5 and 1.0 vvm aeration respectively under these conditions. Production of protease was successfully scaled up in 75 and 700 L fermentor using commercial grade glucose and DAP. Maximum protease yield (40 IU/ml) was obtained within a period of 45 h in 700 L fermentor. Productivities increased from 700 IU/L/h for 7.5 L fermentor to 900 IU/L/h for 700 L fermentor. Crude enzyme was concentrated by various methods viz. ammonium sulphate precipitation, membrane concentration, lyophilization and spray drying with 70-90% recovery depending on the method applied. Ammonium sulphate precipitated and spray dried proteases were stable at ambient temperature for more than one and half years. The studies demonstrated that the alkaline protease production could be scaled up successfully in cost effective medium with yields similar to shake flasks and the crude protease has very good shelf life indicating the potential of the enzyme for its commercialization.

Introduction

It is essential for an enzyme having industrial value with bulk quantity requirements, to be produced on large-scale. Development of bioprocess for enzyme production generally needs screening of microorganisms for potent producers and media optimization in shake flasks followed by scale up in laboratory scale bioreactor and subsequently in pilot plant fermentor. The fundamental idea of scale up is to translate the fermentation conditions which are optimized in shake flasks to the large scale production in fermentors. There are large differences in terms of growth conditions which need to be controlled to get yields similar to or higher than those obtained in shake flasks. These include not only physical (mass transfer ability, mixing ability, shear field) and chemical factors (medium composition and concentration) but also process factors (number of pre-cultures, sterilization conditions, etc.). Several physical and chemical parameters that influence the behavior of cultured organisms change as the process is being carried out at a large scale.

Most of the reports on production of protease by variety of fungal isolates relate to shake flask studies (Laxman *et al.*, 2005; Sandhya *et al.,* 2005; Chi *et al.,* 2007; M'hir *et al.*, 2012). Though few reports on protease production in instrumented fermentors (mostly at 3 to 15 L) are available in literature (Sutar *et al.*, 1992; Singh *et al.,* 2004; Laxman *et al*., 2005; Rao *et al.,* 2006; Potumarthi *et al.,* 2007), reports on production on large scale/pilot scale are meager (Jonsson, 1967; Laxman *et al.,* 2005).

Commercially, approximately 90% of the industrial enzymes are produced by submerged fermentation. Production in bioreactors is generally carried out either by batch, continuous, fed batch or by airlift systems. In batch fermentation, sterilized nutrient is inoculated with microorganisms and incubation is allowed to proceed. In the course of entire fermentation, there is no addition of nutrients except oxygen (in aerobic fermentation), antifoam and acid or base to control the pH. There is constant change in the composition of medium, biomass and metabolite concentration due to metabolism of microorganism.

Majority of reports on protease production studied in controlled fermentors are batch fermentations. Protease production by *B. cereus* in 2 L glass bottle (800 ml working volume) and fibrinolytic alkaline protease production by *Fusarium* sp. in a jar fermentor (with 5 L working volume) were reported by Nilegaonkar *et al.* (2007) and Ueda *et al.*

(2007). Protease production by *Pseudomonas putida* SKG-1 in 3 L bioreactor was reported by Singh *et al.* (2011). Loc *et al.* (2012) optimized fermentation parameters for neutral protease production by recombinant *E. coli* strain in 14 L fermentor with 10 L working volume. Protease production by *E. coronata* in 10 L fermentor with 6 L working volume was reported by Jonsson (1968). Sutar *et al*. (1992) reported protease production by *C. coronatus* in 14 L fermentor. Laxman *et al.* (2005) optimized protease production by *C. coronatus* PTA-4132 in 14 L fermentor which was later scaled up in 100 L batch fermentor with excellent productivity. Protease production by *Alternaria tenuissima* in 100 L fermentor with 60 L working volume was reported by Jonsson (1967).

In fed batch fermentation, the critical elements of the medium are added in small concentration at the beginning of the fermentation and these nutrients continue to be added in small doses during the production phase. Singh *et al.* (2004) reported protease activity and its productivity in a fed-batch process to have increased by 44% over batch process which they felt was due to the longer maintenance of increased rates of growth and enzyme production by providing continuous and controlled supply of additional substrate and nutrients.

In continuous fermentation system, sterilized liquid nutrients are fed into the fermentor at the same flow rate as the fermentation broth leaving the system. This achieves a steady state condition wherein the specific growth rate is controlled by the dilution rate. Raninger and Steiner (2003) carried out protease production by *B. licheniformis* using batch (3 and 15 L), fed batch (15 L) and continuous system of fermentation (pilot scale) and maximum production obtained with fed batch fermentation (10 times those found during regular batch culture).

In recent years, airlift fermentors have been investigated as an alternative to stirred tank bioreactors. In airlift bioreactor, mixing is accomplished without mechanical agitation. There are various forms of airlift bioreactor. In usual form, air is fed into the bottom of a central draught tube through a sparger ring. The air flow passes up through the drought tube to the head space of the bioreactor, where excess air and the by-product, CO_2 release. Rao *et al.* (2006) stated that in airlift fermentor, the flow pattern with high liquid velocities in line with efficient mixing and low uniformly distributed shear stresses can create an optimal environment for many productive microorganisms. A special feature of

airlift fermentor is liquid circulation loop formed by the interconnected aerating and re-circulating sections. Duration for protease production by *B. bassiana* was considerably shorter than the corresponding duration needed for the shake flask and stirred tank fermentor (Rao *et al.,* 2006). However there are some disadvantages associated with airlift fermentors viz. high capital cost for large vessel, high energy cost due to requirement of pressurized air for large volumes, maintenance of nutrient and oxygen homogeneity is difficult, the separation of gas from the liquid is not efficient in case of foaming. Table 2.3.1 shows details of protease production by microbial cultures in fermentors along with fermentor parameters. As there is no standard method for fermentation scale up process, which is related to the characteristics of the microorganism, the scale up has to be optimized for individual culture.

For commercialization and technology development, the production of enzyme has to be scaled up to a reasonably larger scale before cost calculations become meaningful. This section deals with the optimization of protease production on a bench scale in 7.5 L bioreactor with 5 L working volume. This section also includes optimization of various process parameters such agitation and aeration rates etc. Later production was scaled up under optimized conditions in 75 L with 50 L working volume and finally to pilot scale in 700 L fermentor with 500 L working volume. The section also describes various methods adopted in downstream processing of the enzyme.

Table 2.3.1: Details of Protease production by microbial cultures in fermentors

Microorganism	Media Composition (g/L)	Working Volume (L)	Agitation (rpm)	Aeration (vvm)	Activity peak in (h)	Maximum activity (U/ml)	Reference
B. sphaericus	Glucose-10; Biopeptone-5; Yeast extract-5; KH_2PO_4-1; $MgSO_4$-0.2; Na_2CO_3-10	5	300	1	30	680[#]	Singh *et al.*, 2004
***B. licheniformis* NCIM-2042**	Casein-10; Malt extract-10; Polypeptone-10; Na_2CO_3-10	1	300	2	72	340[♣]	Potumarthi *et al.*, 2007
P. putida	Glucose-12.5; Yeast extract-5; $MgSO_4$-0.1	3	150	0.8	48	882[♦]	Singh *et al.*, 2011
Recombinant *E.coli*	HSG medium; Lactose-5	10	500	0.4	48	103[♦]	Loc *et al.*, 2012
***Streptomyces* sp 594**	Casitone-3; Molasses-10	3	200	1.0	120	99[¤]	Azeredo *et al.*, 2004
Alternaria tenuissima	Liver (Difco)-4; Glucose-30; $CaCO_3$-5; KH_2PO_4-1; $MgSO_4$-0.3	60	260	0.5	60	$1.5x10^{-3}$[§]	Jonsson, 1967
A. pullulans	Starch-25; $NaNO_3$-20 in sea water	2	150	4	30	7.2[♦]	Chi *et al.*, 2007

Continued …

Table 2.3.1: Details of Protease production by microbial cultures in fermentors

..... Continued

Microorganism	Media Composition (g/L)	Working Volume (L)	Agitation (rpm)	Aeration (vvm)	Activity peak in (h)	Maximum activity (U/ml)	Reference
E. coronata	Liver (Difco)-4; Glucose-30; $CaCO_3$-5; KH_2PO_4-1; $MgSO_4$.-0.3	6	260	1.0	33	5.0×10^{-3} §	Jonsson, 1968
***C. coronatus* NCIM 1238**	Glucose -10; Bacto peptone-7.5; Casein-20; NaCl-3; KH_2PO_4-1; $MgSO_4$.-1; $MnCl_2$ -0.008; $CuSO_4$-0.008; $ZnSO_4$-0.002; $FeSO_4$-0.001	6	200	0.5	40	19-20 ♣	Sutar *et al.*, 1992
***C. coronatus* PTA 4132**	Malt extract-3; Yeast extract-3; Glucose-10; peptone-5; SBM-30	10	300-400	0.5	40-48	72-90 ♣	Laxman *et al.*, 2005
***C. coronatus* PTA 4132**	Glucose-10; Yeast extract-3; SBM-40	100	100-200	0.5	40	45-50 ♣	Laxman *et al.*, 2005
B. bassiana	Shrimp shell powder-2; Soya powder-6; Sucrose-6.8; Yeast extract-1.9	5	150	0.6	144	238.77 ♦	Rao *et al.*, 2006
***Beauveria* sp.**	Glucose-5; Yeast extract-3; MSC-20; Tween 80-1	80	350-400	0.5-0.6	84	12-15 ♣	Laxman *et al.*, 2011

azocasein units/h; ♦ µg Tyr/min; ¤ 1U= absorbance of 0.01 at 440 nm/10 min; § meq Tyr/min; ♣ micromoles Tyr/min.

Material and methods

Materials

Glucose and di-ammonium hydrogen orthophosphate (DAP) used in 5 L fermenter was procured from M/s Qualigens Chemicals, India. Emulsified silicon oil, yeast extract, peptone, malt extract and maltodextrin were procured from M/s HiMedia Chemicals, India. Di nitro salicylic acid (DNSA) was procured from M/s Sigma Chemicals, USA. Air filters (0.2 micron) were purchased from M/s Pall Corporation (USA). Soyabean meal (SBM) was purchased from M/s Ruchi Soya Industries, Pvt. Ltd, India. Glucose and DAP used for protease production at 50 and 500 L scale were of commercial and manure grade respectively. Ammonium sulphate used for precipitation of enzyme was of fertilizer grade. Hammerstein casein was obtained from M/s Sisco Research Laboratories, India. All other chemicals were of analytical grade.

Methods

Media sterilization and fermentor operation

The fermentation medium comprised (g/L) of glucose-20; DAP-1.6 and SBM-30. Sterilization time for large medium volume especially with insoluble solid contents like SBM is generally carried out for longer time which often causes caramelization of glucose if all the media constituents are sterilized together. Hence separate sterilization of individual media ingredients becomes necessary. The medium was prepared and sterilized in two stages. In the first stage, only SBM was sterilized in fermentor vessel for 30-35 min at 121°C. In the second stage of sterilization remaining ingredients i.e. glucose and DAP were added and re-sterilized for 30 min in order to minimize the chance of glucose charring and provide appropriate sterilization time to SBM. Two-stage sterilization process is also advantageous to prevent over exposure of pH and DO probes to high temperature for longer time to prolong life span of these probes. pH and DO probes were calibrated as per manufacturer's instructions. The fermentation was carried out at constant temperature of 28±2°C. pH and dissolved oxygen (DO) concentration were monitored during the fermentation. Constant supply of sterile air through air filter was provided to the fermentor by oil less air compressor.

Protease production in fermentors

Optimization of fermentation with respect to agitation/aeration etc. was carried out in 7.5 L New Brunswick fermentor with 5 L working volume. Minimum 3 batches for each variable condition viz. agitation and aeration were performed. Inoculum for the fermentor was developed in 1 L conical flasks. Protease production in 75 and 750 L fermentors was carried out more than five times in different geological locations of India (Pirangut near Pune and Chennai). Inoculum for 75 L fermentor was developed in 7.5 L fermentor while for 750 L it was developed in 75 L fermentor. All the fermentation experiments were performed at 28°C±2°C. Fermentation was continued till the activity started declining and/or onset of cell lysis. Contents of the fermentor were immediately subjected to downstream processing and concentrated and stored at room temperature until use. Schematic flow chart of fermentations is illustrated in Figure 2.3.1.

Sampling

Samples were drawn at regular intervals from the fermentor through sampling port in sterile condition. Purity of the sample was checked by microscopic observation as well as by streaking on sterile MGYP plate.

Wet weight determination

During the optimization of protease production in 7.5 L fermentor, fungal growth was monitored during the course of the fermentation as wet weight of biomass. A known volume of sample from the fermentor was transferred in a pre-weighed centrifuge tube and centrifuged at 10000 rpm for 5 min, the supernatant obtained was used as source of enzyme. Residual biomass in the tube was washed twice with deionized water and re-centrifuged to remove the adhering media salts. Weight of wet biomass and the tube was noted and the biomass was suspended in deionized water to its initial volume. Wet weight of fungal biomass was noted and expressed in mg/ml. All the values expressed are mean of duplicate samples of minimum 3-4 batches for each condition.

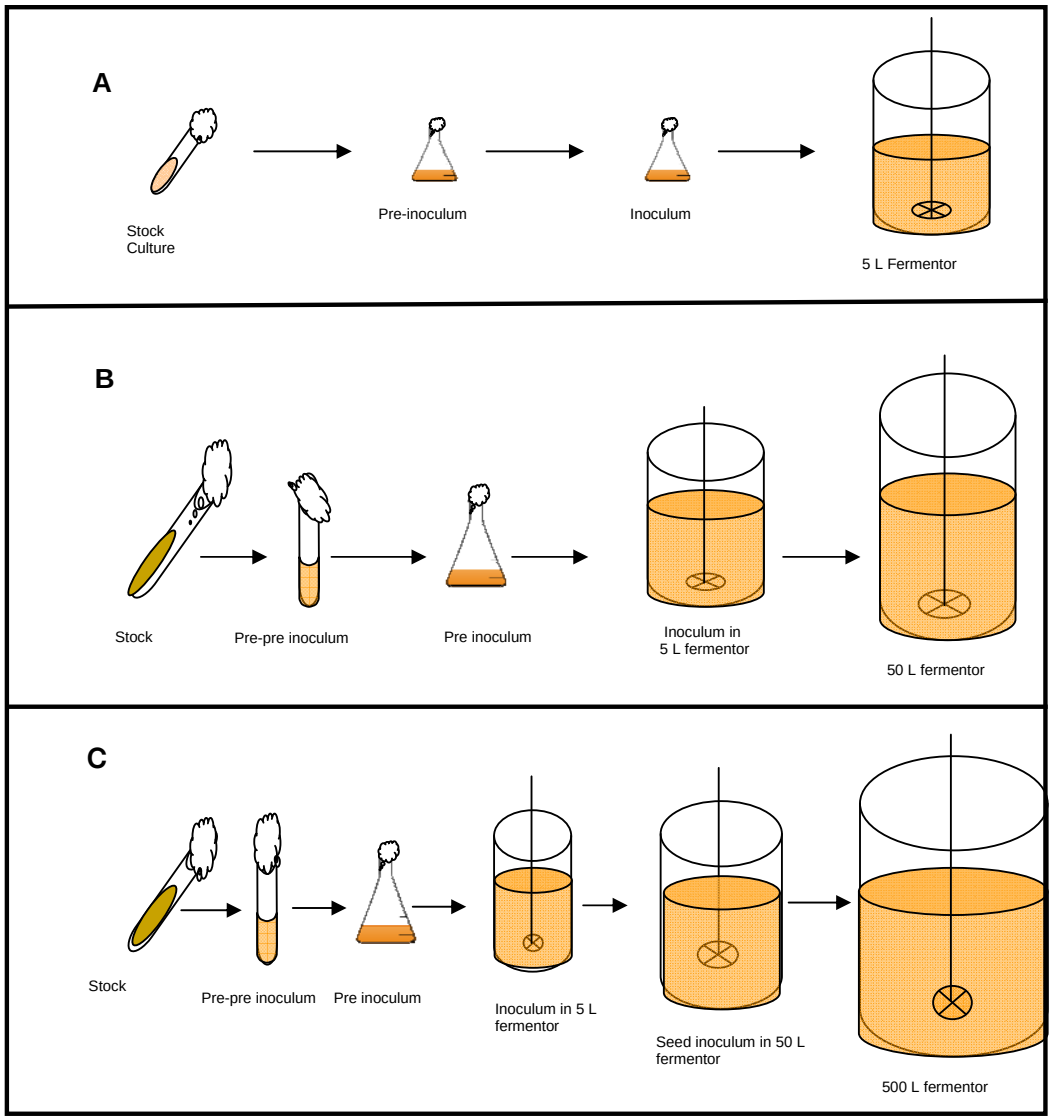

Figure 2.3.1: Flow chart of protease production in fermentors (A- 7.5 L; B- 75 L; C- 700 L)

Determination of residual glucose and protease activity

During the course of fermentation, residual glucose in the culture filtrate was estimated by reducing sugar method using DNSA **(**Bernfeld, 1955). Protease activity in the culture filtrate was estimated as described earlier.

Downstream processing

Fermentor was terminated when activity started declining and/or onset of lysis was observed. Mycelial biomass was separated from the broth either by simple filtration

on cheese cloth or by centrifugation in bucket centrifuges. The protease in the cell free broth was concentrated by one of the following methods: ammonium sulphate precipitation, membrane filtration, lyophilization and spray drying.

Results and discussion

Optimization of protease production in 7.5 L fermentor

Protease production was optimized in 7.5 L New Brunswick Fermentor Model Bio Flow 110 having operational volume of 5 L. Production was studied in glucose-di-ammonium hydrogen phosphate with 3% SBM as inducer. Fermentor was inoculated with 24 h vegetative inoculum (10% v/v) grown in 1%GYE medium. In aerobic fermentation, constant and adequate supply of air is necessary for oxidation of sugar by microbial metabolism, as the necessary oxygen can not be supplied in one addition due to poor solubility in the medium. Therefore, optimal agitation along with appropriate aeration rate is needed to establish adequate and constant supply of oxygen for microbial growth and product formation. The effect of agitation varying from 200 to 500 rpm on protease production was investigated at two fixed aeration rates viz. 0.5 and 1.0 vvm.

Effect of agitation rate at 0.5 vvm aeration

Protease production was very low (0.72 IU/ml) at 200 rpm even after 84 h and slowly increased with increase in agitation and reached maximum of 16.39 IU/ml at 500 rpm in 60 h (Table 2.3.2, Figure 2.3.2a). The reason for low activities could be due to the fact that lower agitation resulted in poor oxygen mass transfer and inadequate mixing of nutrients especially SBM which resulted in poor growth. Higher agitation rate of 400 and 500 rpm facilitated better mixing of SBM with good growth (Figure 2.3.2b).

As glucose was a key determinant for protease production, residual glucose concentration during the fermentation was monitored to examine the correlation of glucose consumption with protease production. There was direct correlation between glucose consumption and agitation rate which increased with increase in agitation rate (Table 2.3.2; Figure 2.3.2c). Glucose consumption at 200 and 300 rpm was very slow and was 14.22 and 60.74% respectively at the end of 84 h. There was sharp increase in glucose consumption at 400 and 500 rpm (80 to 95%) with corresponding increase in protease production and biomass formation. There was no significant change in pH at 200-300 rpm but there was sharp increase in pH to reach around 8 when agitation was 500 rpm indicating the degradation of protein rich substrates to peptides/amino acids and formation of ammonia **(**Figure 2.3.2d).

Table 2.3.2: Effect of agitation rates on protease production at 0.5 vvm aeration

Agitation (RPM)	Time (h)	pH	Growth as wet biomass (mg/ml)	Glucose consumed (%)	Activity (IU/ml)
200	0	6.49	108.0	0.00	-
	24	6.53	216.4	0.63	0.2232
	36	6.51	201.5	10.00	0.3984
	48	6.45	200.9	11.59	0.5818
	60	6.36	205.8	11.93	0.6806
	72	6.45	201.1	13.14	0.7038
	84	6.53	202.1	14.22	**0.7233**
300	0	6.55	122.8	0.00	-
	24	6.42	230.3	7.71	0.0984
	36	6.23	262.2	40.00	0.3164
	48	5.82	273.9	39.77	0.3798
	60	5.96	278.0	58.66	0.4470
	72	6.27	283.5	60.14	0.9300
	84	6.42	287.0	60.74	**2.3740**
400	0	6.68	118.0	0.00	-
	24	6.63	201.9	26.31	0.0350
	36	5.76	233.3	53.38	0.1542
	48	6.43	251.4	71.59	3.9424
	60	6.73	302.5	76.33	6.2780
	72	7.18	309.4	79.11	**14.482**
	84	8.11	310.5	79.49	14.282
500	0	6.69	104.5	0.00	-
	24	6.58	268.4	42.51	0.1310
	36	6.96	364.9	73.13	7.9200
	48	7.81	378.3	89.60	14.296
	60	8.11	387.3	94.48	**16.386**
	72	8.34	370.8	94.97	14.618
	84	8.45	367.8	95.15	14.522

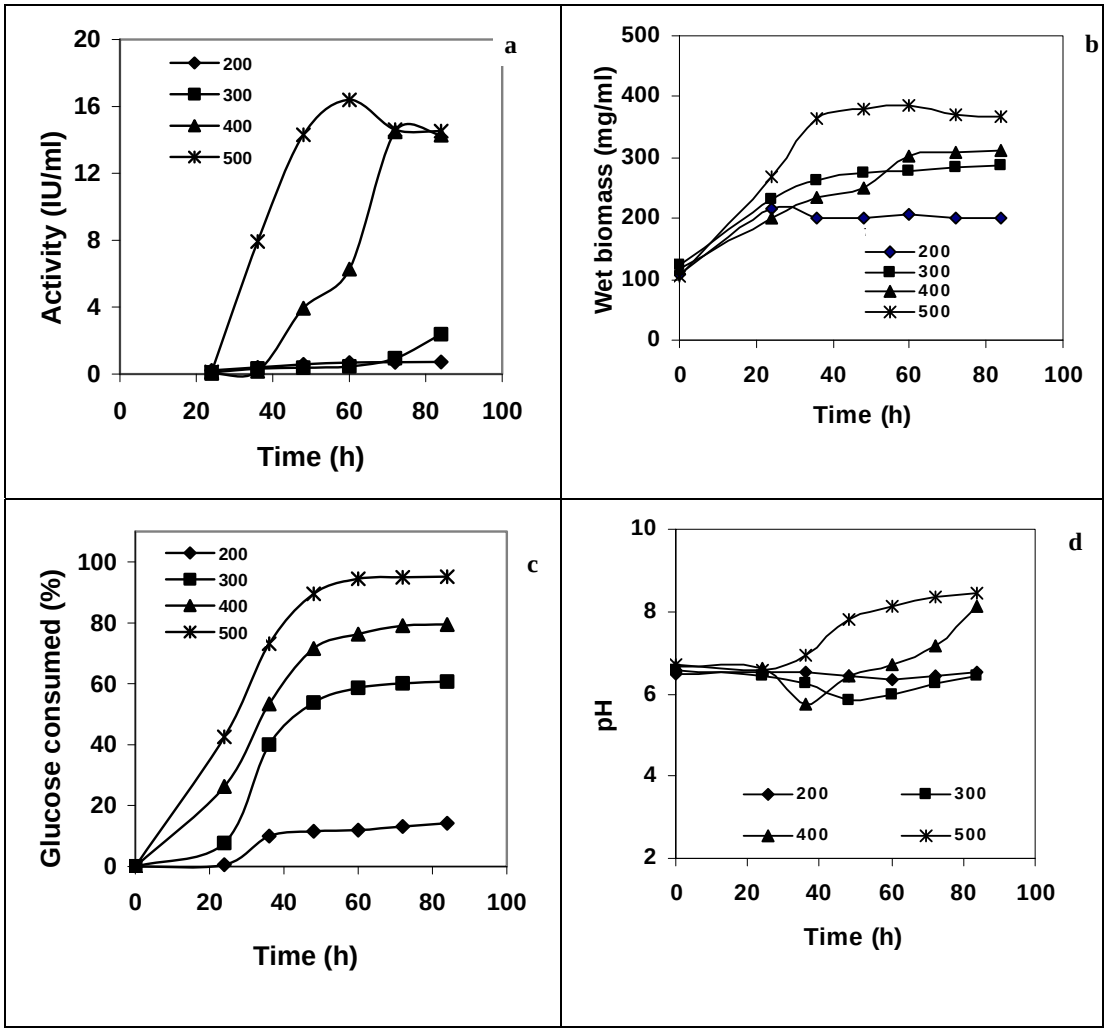

Figure 2.3.2: Effect of agitation rates on protease production and growth at 0.5 vvm aeration

Effect of agitation rate at 1 vvm aeration

The trend was similar to that of 0.5 vvm and increase in agitation increased activities **(**Table 2.3.3, Figure 2.3.3a). It can be noted that apart from giving higher activities, maximum activities were reached in shorter time when agitation was increased. In general except for 200 rpm, activities were higher at 1 vvm compared to 0.5 vvm under identical agitation rates. Thus, maximum activities of 2.37, 14.48 and 16.39 IU/ml were reached in 84, 72 and 60 h with 0.5 vvm at 300, 400 and 500 rpm respectively. The corresponding activities with 1 vvm at 300, 400 and 500 rpm were 6.32 (84 h), 16.75 (60 h) and 18.64 IU/ml (60 h) respectively.

Table 2.3.3: Effect of agitation rates on protease production at 1 vvm aeration

Agitation (RPM)	Time (h)	pH	Growth as wet biomass (mg/ml)	Glucose consumed (%)	Activity (IU/ml)
200	0	6.68	104.4	0.00	-
	24	6.58	221.0	7.52	0.1604
	36	6.56	224.1	8.21	0.1940
	48	6.33	240.9	18.15	0.2070
	53	6.23	242.3	19.23	0.2087
	60	6.11	243.7	20.38	0.2138
	65	6.08	244.1	20.78	0.2766
	72	6.01	244.4	21.51	0.3584
	84	5.87	245.4	22.34	**0.3977**
300	0	6.65	111.3	0.00	-
	24	6.56	227.9	11.04	0.0038
	36	5.85	286.9	44.70	0.0638
	48	5.89	287.0	86.56	0.0546
	53	5.93	291.2	87.54	0.8779
	60	6.85	294.2	89.30	1.1288
	65	6.96	298.7	91.23	2.6788
	72	7.49	308.3	92.84	5.7240
	84	7.68	311.4	93.47	**6.3170**
	96	7.89	292.7	93.72	5.9480
400	0	6.68	107.7	0.00	-
	24	6.19	201.5	32.88	0.1106
	36	6.50	227.0	89.48	0.6780
	48	7.32	269.2	92.56	11.882
	53	7.57	276.6	93.55	14.233
	60	7.99	289.9	94.18	**16.748**
	65	8.23	287.7	94.37	16.466
	72	8.34	276.6	95.73	16.365
500	0	6.62	105.3	0.00	-
	24	6.55	313.2	49.57	0.1322
	36	7.48	402.3	94.96	15.576
	48	7.81	407.8	96.39	16.506
	53	7.89	405.9	96.53	18.492
	60	8.11	372.9	96.58	**18.644**
	65	8.52	371.1	96.64	16.567
	72	8.52	359.0	96.72	14.758

The production peak was delayed by 12 h at 0.5 vvm when compared with 1.0 vvm aeration at 400 rpm.

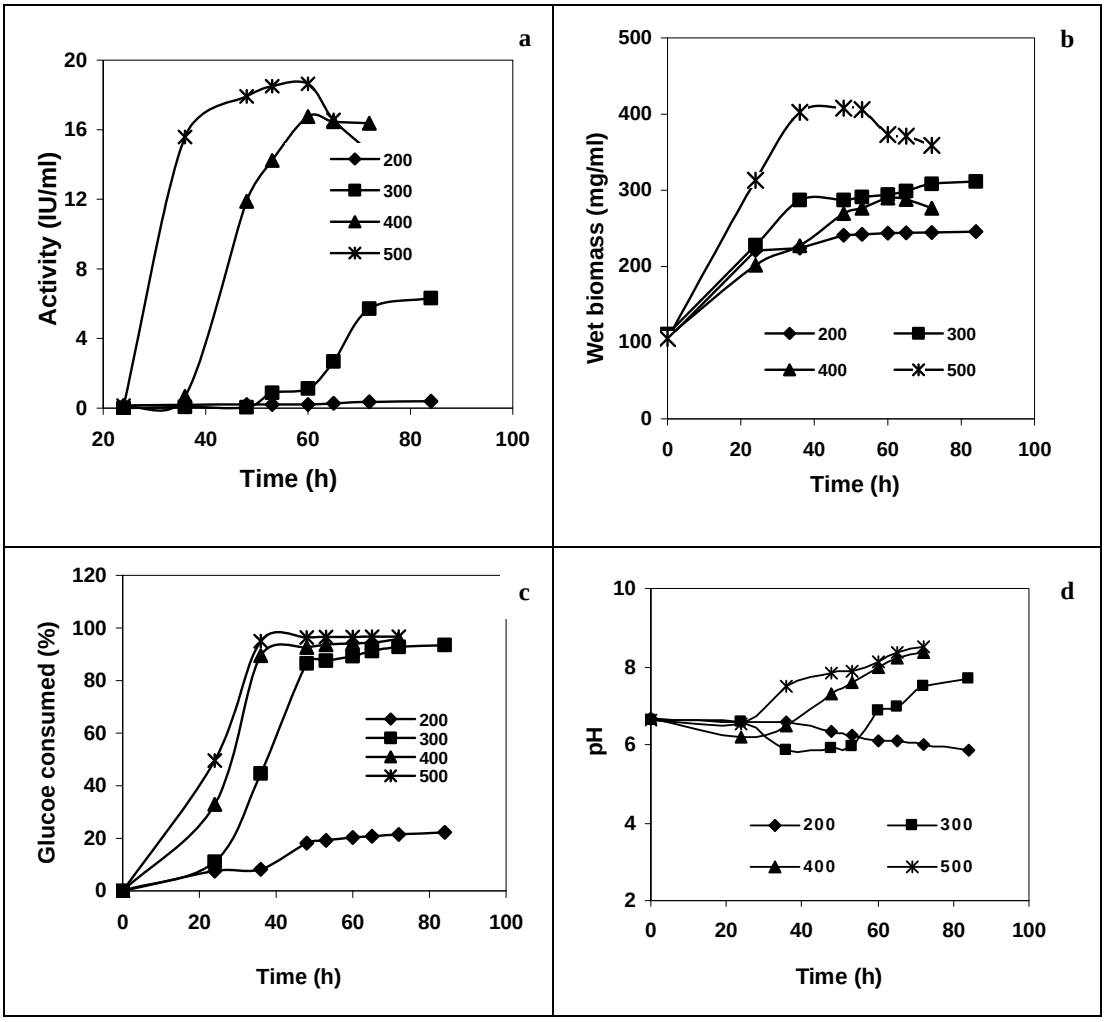

Figure 2.3.3: Effect of agitation rates on protease production and growth at 1 vvm aeration

Unlike in 0.5 vvm, except with 200 rpm, glucose was almost completely utilized, where time taken being longer with lower agitation rates **(**Figure 2.3.3c). Thus the sugar consumption after 24 h with 500, 400 and 300 rpm was 50, 33 and 11% respectively. Except in 200 rpm, glucose was almost completely consumed at the end of fermentation (72-84 h). Although more than 90% glucose was consumed at 300 rpm and 1.0 vvm aeration after 72 h, activities obtained were nearly 2.5 and three times lower than those obtained at 400 and 500 rpm. Growth and pH increased with time when agitation was maintained above 200 rpm (Figures 2.3.3b & 2.3.3d). Mycelial wet weight was more or less similar at both the aeration rates but increased with agitation rate. At 200 rpm, mycelial wet weight was 210 to 220 mg/ml in 24 h

which did not increase but at 500 rpm it reached maximum mycelial wet weight of 370 to 400 mg/ml in 48 h.

Though, aeration and agitation were found to influence protease production, agitation plays a crucial role compared to aeration in enzyme secretion and agitation of 400-500 rpm gave high levels of activities. *C. coronatus* PTA-4132 gave maximum protease production when agitation was adjusted between 400-500 rpm and aeration rate of 0.5 vvm in 10 L fermentor (Laxman *et al.*, 2005**)**. However, Sutar *et al.* (1992) reported optimum agitation and aeration for protease production by *C. coronatus* NCIM 1238 in 14 L bioreactor with working volume of 6 L to be 200 rpm and 0.5 vvm respectively. Protease production by *P. putida* SKG-1 in 3 L bioreactor was optimum at 0.8 vvm and 150 rpm **(**Singh *et al.,* 2011). Optimum agitation and aeration for protease production by *A. tenuissima* and *E. coronata* were 260 rpm; 0.5 vvm and 260 rpm; 1vvm respectively (Jonsson, 1967; 1968).

Another important point to be noted is that protease secretion starts in stationary or late exponential phase and just before the onset of cell lysis. Rao *et al.* (2006) reported sharp decrease in residual sugar concentration during protease production by *B. bassiana* in bioreactor and stated that the depletion in sugar concentration might be triggering protease synthesis.

The dissolved oxygen concentration (DO) profile of fermentation at 0.5 vvm aeration revealed that, oxygen concentration started going down sharply and dropped to around 50% within 5-6 h immediately at 200 and 300 rpm but more slowly when the agitation was 400 to 500 rpm (Figure 2.3.4). In case of 200 and 300 rpm, the DO dropped to below 10% in 24 to 30 h and remained constant till the end of fermentation. In both these cases, activity was negligible. At higher agitation rates of 400 and 500 rpm, there was an initial drop of DO to around 50% in 12 h after which the drop was slower and reached less than 10% in 20 h. After remaining below 10% for some time, it started increasing and reached around 25-35% at the end of the fermentation. There was simultaneous increase in protease secretion and reached maximum in 60-72 h. However, these values were still low and were nearly 20-25% of the activities obtained in shake flasks.

When the aeration was increased to 1 vvm, DO dropped to around 50% in 12 h except in case of 500 rpm which dropped to 50% in less than 12 h. The reason for this could be that the higher agitation is facilitating better mixing which favours faster growth leading to depletion in dissolved oxygen. Similar to 0.5 vvm, the DO remained below 10% for almost 28-30 h and never increased while at higher agitation rates of 400 and 500 rpm the DO increased to reach maximum of 16% (400 rpm) and 42% (500 rpm) after 65 and 72 h respectively (Figure 2.3.4). Protease activities were nearly 16-18 IU/ml which is 40% of those obtained in shake flasks indicating that the best conditions for production being 1 vvm aeration and agitation of 500 rpm. The results obtained during optimization of conditions for protease production in fermentor revealed that the *C. brefeldianus* required adequate oxygen supply and nutritional homogeny during fermentation for growth and enzyme production, which is governed by appropriate agitation speed.

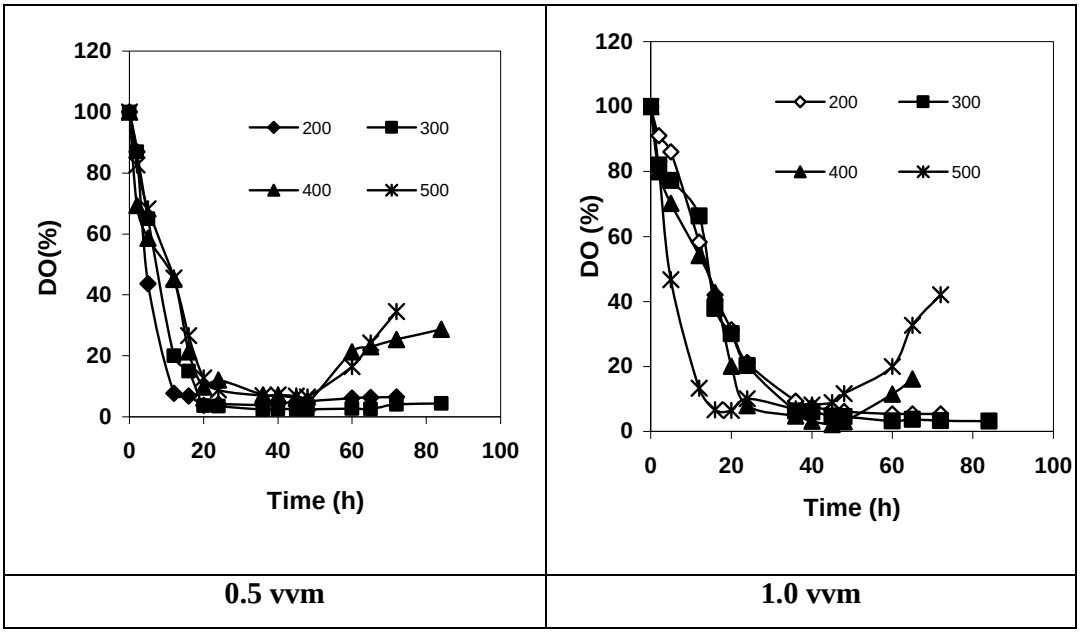

Figure 2.3.4: Dissolved oxygen (DO) profile

Effect of progressive increase in agitation rate during fermentation

In the next step in optimization process in 7.5 L fermentor, production was carried out in batch system where agitation was increased from 250 to 450 rpm during the progress of fermentation under two aeration rates of 0.5 or 1.0 vvm. Gradual increase of agitation speed enhanced protease production at both the aeration rates when compared to the fixed agitation rate of 500 rpm studied earlier with 0.5 or 1 vvm aeration (Figures 2.3.5 & 2.3.6).

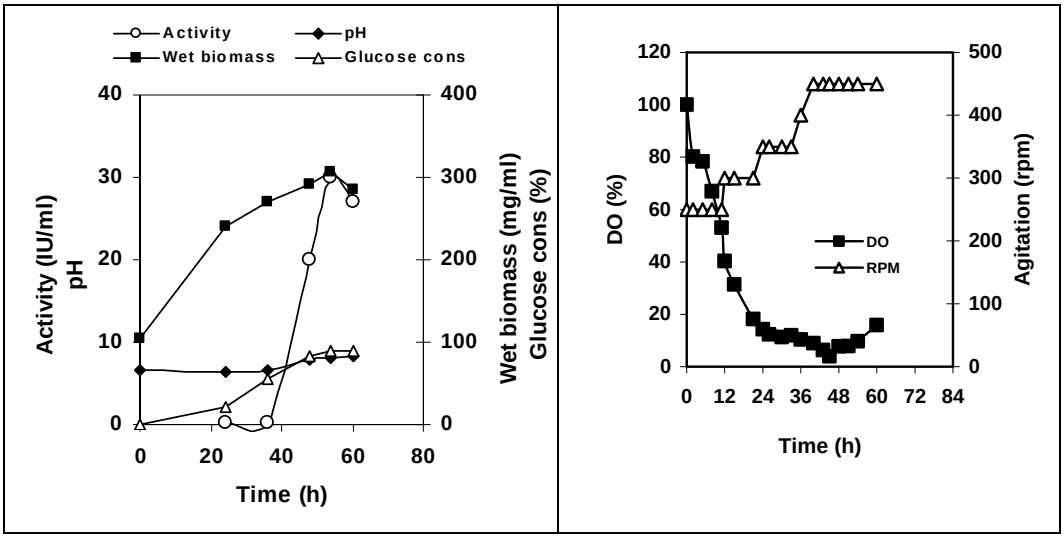

Figure 2.3.5: Effect of progressive increase in agitation (0.5 vvm aeration)

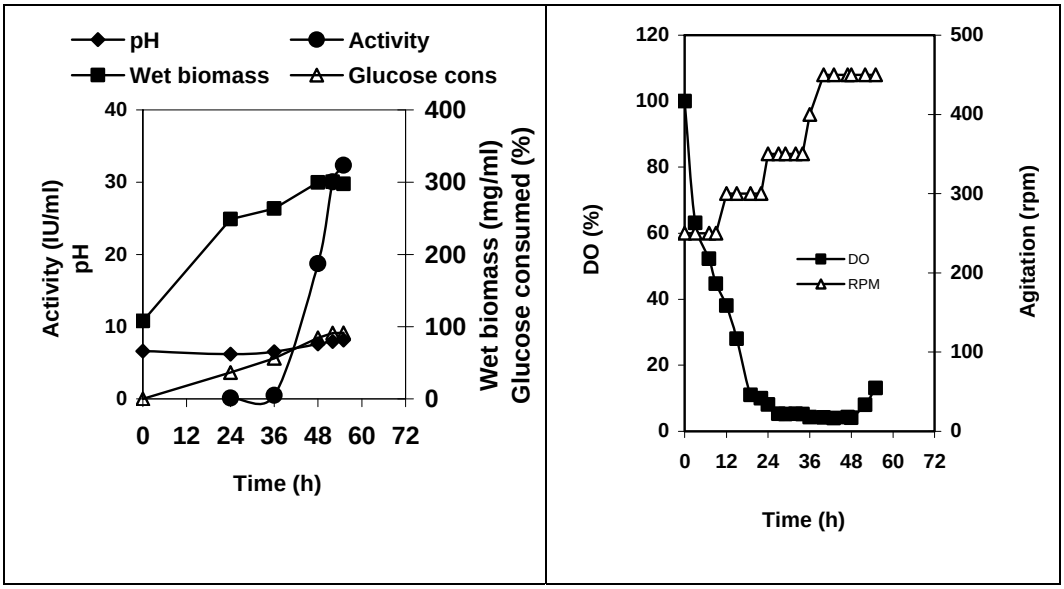

Figure 2.3.6: Effect of progressive increase in agitation (1 vvm aeration)

Fermentation profiles at both the aeration rates followed almost identical trend with respect to protease production, pH, biomass formation and glucose consumption. In both the cases, no protease activities were detected up to 36 h and there was steep increase in activity from 36 to 48 h and reached to maximum value of 29.95 IU/ml and 32.33 IU/ml in 54-55 h at 0.5 and 1.0 vvm respectively. Activities obtained with increasing agitation rates were almost two times more than that obtained with fixed agitation rate of 500 rpm.

Around 55 to 56% glucose was consumed within first 36 h and utilized more than 90% at the end of 55 h. Dissolved oxygen concentration (DO) dropped to about 40% in first 12 h and reached minimum of 4.0% between 43 to 45 h (Figure 2.3.5 & Figure 2.3.6). After 45 h, DO started increasing and reached to the maximum values of 16% and 13% at 0.5 and 1.0 vvm respectively at the end of fermentation. pH profile of fermentation is an indicative feature and reflects on the level of product formation. There was slight drop in the pH in the first 24 h of fermentation and protease production was initiated when the pH reached to near neutral and reached at maximum level when pH of the medium reached near or above pH 8. Similar trend of pH profile during protease production was reported for other *Conidiobolus* strains **(**Sutar *et al.,* 1992: Laxman *et al.,* 2005).

Though the activities were higher with agitation increasing progressively, growth in terms of wet biomass produced under these conditions was lower (around 300 mg/ml) compared with fixed agitation rate of 500 rpm (around 400 mg/ml). One of the possible reasons for higher growth could be utilization of major portion of nutrients towards biomass formation. Secondly, the fungal growth was denser and was in the form of large pellets during fixed agitation rate of 500 rpm whereas growth was in the form of loose mycelia when agitation was slowly increased. This type of growth was observed in shake flasks where maximum activities reached 35-40 IU/ml in 48 to 72 h.

The morphological forms of filamentous microorganism were found to have marked effect on enzyme production (Stanbury *et al.,* 1995; Teng *et al.,* 2009). Agitation is one among many parameters, which influences pellet formation (Wang *et al.,* 2005). The scheme of varying agitation rate during fermentation facilitated the culture to

grow in loose mycelial form rather than pellety growth. The loose mycelial growth is more homogenous which may facilitate better mass transfer and secretion of enzyme. The extracellular protease activity of recombinant *A. niger* AB4.1 varied with the pellet size and decreased dramatically when the morphology was changed from free mycelia to pellets indicating the relationship between protease production and morphology (Xu *et al.*, 2000). Laxman *et al.* (2005**)** reported the relationship between protease activity and biomass formation in different media for *C. coronatus*. They found that the medium containing (g/L) beef extract-20; glucose–30 and SBM-20 gave lower activities of 38.17 IU/ml but gave higher biomass of 4.95 ml (expressed as pack cell volume). However, activities obtained in MGYP + 2% SBM were much higher (72.46 IU/ml), even though the corresponding biomass produced was 2.10 ml. Shafee *et al.* (2005) reported similar results of lower protease activities by *B. cereus* with higher biomass/growth with 5% inoculum compared to 4% where growth was poor but activities were higher.

Protease production in 75 L and 700 L pilot scale fermentors

After optimizing the process of protease production in 7.5 L fermentor where activities were obtained similar to those in shake flasks, further production was carried in 75 L fermentor and 700 L fermentors. The production medium used for both the fermentors comprised of (g/L) commercial glucose-20; manure grade DAP-1.6; and SBM-30.

Production in 75 L fermentor

Sterilization of medium in 75 L fermentor with working volume of 50 L was performed in two stages. SBM was sterilized initially, followed by subsequent sterilization of glucose and DAP in the same pre-sterilized SBM medium to avoid charring effect due to excess cooking. After sterilization, the medium was cooled and inoculated with seed inoculum as described in materials and methods. The method followed for inoculum development is shown in Table 2.3.4.

Table 2.3.4: Inoculum development for 75 L fermentor

	Medium used	Incubation time (h)	Growth conditions
Stock used	MGYP slant	48-72	28°C, stationary
Pre-pre inoculum	MGYP	24	28°C, 500 ml conical flask, rotary shaker, 180 rpm
Pre-inoculum	GYEP	24-28	28°C, 1 L conical flask, rotary shaker, 180 rpm
Inoculum	GYE	16-18	28°C, 5 L in 7.5 L NBS fermentor, agitation, initially 200 and increased to 350 rpm, aeration 0.8 vvm

In 75 L fermentor, aeration was kept initially at 70 rpm and slowly increased to reach 120 rpm at 51 h and was kept constant till the end of fermentation. Very low levels of activity were detected after 24 h and steeply increased after 42 h to reach maximum of 39.20 IU/ml after 56 h of fermentation (Figure 2.3.7).

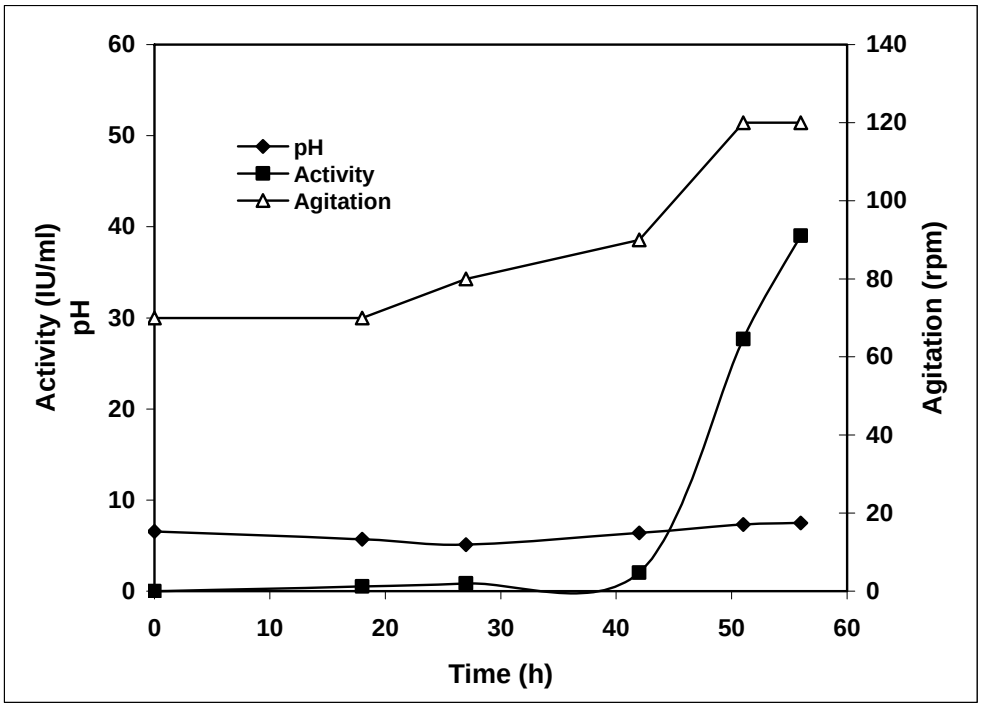

Figure 2.3.7: Protease production in 75 L fermentor

Production in pilot scale bioreactor

Sterilization of medium in 700 L fermentor with working volume of 500 L was carried out in two stages. In situ sterilization of SBM (3%) was carried out initially, followed by subsequent sterilization of glucose and DAP. After sterilization, the medium was cooled and inoculated with seed inoculum as shown in Table 2.3.5.

Table 2.3.5: Inoculum development for 700 L fermentor

	Medium used	Incubation time (h)	Growth conditions
Stock used	MGYP slant	48-72	28°C, stationary
Pre-pre inoculum	MGYP	12-16	28°C, 500 ml conical flask, rotary shaker, 180 rpm
Pre-inoculum	GYEP	12-16	28°C, 1 L conical flask, rotary shaker, 180 rpm
Inoculum	GYEP	16-20	28°C, 5 L in 7.5 L NBS fermentor, agitation, initially 200 and increased tto 350 rpm, aeration 0.8 vvm
Seed	GYE	18-24	28°C, 50 L in 75 L fermentor, agitation, initially 80 and increased to 120 rpm, aeration 0.5-0.6 vvm

Aeration was kept initially at 70 rpm and slowly increased to reach 100 rpm at 45 h and was kept constant till the end of fermentation (Figure 2.3.8).

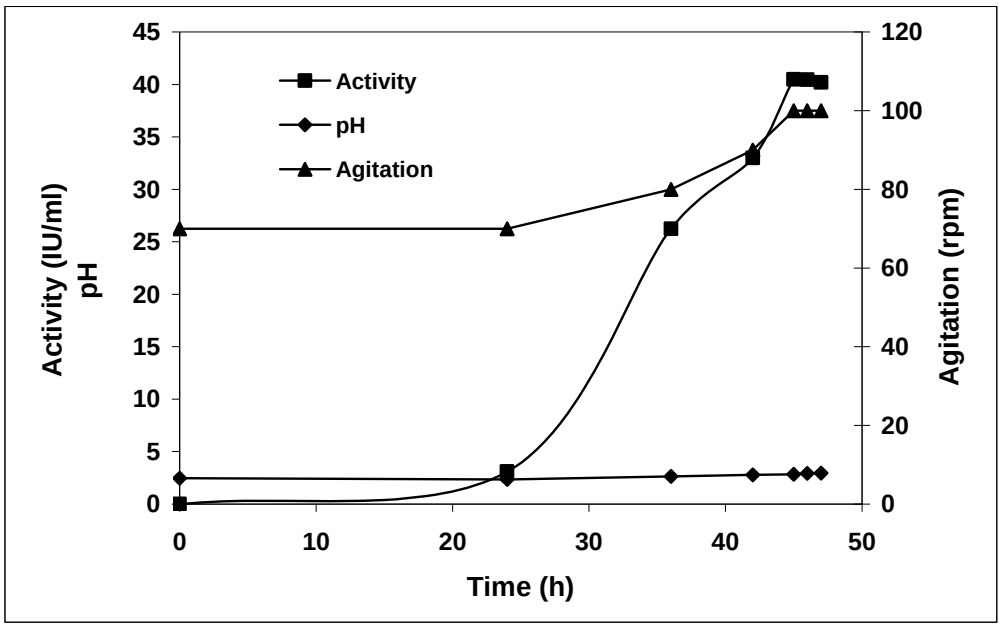

Figure 2.3.8: Protease production in 700 L fermentor

In 700 L fermentor, although onset of protease production took place after 24 h, there was a sharp increase and activities of 26.25 IU/ml were obtained after 36 h and reached a maximum 40.45 IU/ml in 45 h (Figure 2.3.8). These activities are significantly or slightly higher than those obtained in 7.5 L fermentor and shake flasks respectively. Moreover, peak of protease activity in 700 L fermentor reached in shorter time compared to 7.5 and 75 L fermentors. In addition, enzyme productivity was considerably higher in 500 L fermentor compared to 7.5 and 75 L fermentors (Table 2.3.6).

Nilegoankar *et al.* (2007) found that protease production by *B. cereus* was comparably higher in fermentor than shake flasks. The time required for maximum protease production in 700 L fermentor is considerably less than that reported earlier (Azeredo *et al.*, 2004; Rao *et al.*, 2006; Potumarthi *et al.*, 2007). This is an added advantage in terms of energy and man hour saving at industrial level. Sutar *et al.* (1992), reported protease productivity of 600 U/L/h for *C. coronatus* NCIM 1238 while Laxman *et al.* (2005) reported that protease productivity of *C. coronatus* PTA-4132 was around 1200–1500 IU/L/h. Protease productivity for *B. bassiana* was reported to be 1658 U/L/h (Rao *et al.*, 2006**)**.

Table 2.3.6: Protease productivities by different microbial cultures in fermentors

Organism	Working volume (L)	Productivity (U/L/h)	Unit definition	Reference
B. sphaericus	5	27917	Increase in abs by 1.0 at 420 nm/h with azocasein	Singh *et al.*, 2004
B. licheniformis NCIM- 2042	1	4722	Micromole/ Tyr/min	Potumarthi *et al.*, 2007
P. putida	3	18375	µg Tyr/min	Singh *et al.*, 2011
Recombinant *E.coli*	10	2146	µg Tyr/min	Loc *et al.*, 2012
Streptomyces sp. 594	3	825	Increase in abs by 0.01/10 min at 440 nm with azocasein	Azeredo *et al.*, 2004
A. tenuissima	60	0.025	Meq Tyr/min	Jonsson, 1967
A. pullulans	2	240	µg Tyr/min	Chi *et al.*, 2007
B. bassiana	5	1658	µg Tyr/min	Rao *et al.*, 2006
Beauveria sp.	80	143-179	micromole Tyr/min	Laxman *et al*., 2011
Entomopthora sp.	6	0.15	Meq Tyr/min	Jonsson, 1968
C. coronatus NCIM 1238	6	600	micromole Tyr/min	Sutar *et al.*, 1992
C. coronatus PTA 4132	10	1500-1875	micromole Tyr/min	Laxman *et al.*, 2005
C. coronatus PTA 4132	100	1200-1500	micromole Tyr/min	Laxman *et al.*, 2005
C. brefeldianus MTCC 5185	5	588	micromole Tyr/min	Present study
	50	697		
	500	899		

Downstream processing and enzyme concentration

As mentioned earlier, fungal mycelium offers a distinct advantage in separating the culture filtrate from fungal biomass by simple filtration methods. Various methods of downstream processing were explored in order to find a suitable method which give better recoveries/stabilities. Once the protease activity reached maximum, harvesting was done by passing the contents through cheesecloth to separate fungal biomass from the culture filtrate. The filtrate thus obtained, was used as source of alkaline protease and concentrated using one of the following methods.

- Precipitation with ammonium sulphate
- Freeze drying
- Spray drying
- Membrane filtration

- ***Ammonium sulphate precipitation***

  Inorganic salts are often utilized for the precipitation of proteins in crude culture filtrate, not only because of its importance in purification step but also offer an ideal method of protein concentration. Ammonium sulfate is the most common salt, being used for this purpose. Deutscher (1990) mentioned several advantages of ammonium sulphate for protein precipitation, which includes high solubility, precipitation of most proteins at saturation, low heat generation during precipitation, easy sedimentation of precipitated proteins during centrifugation, concentrated solutions are generally bacteriostatic and stable to denaturation.

  The culture filtrate obtained from 75 and 700 L fermentor was transferred back to the fermentor. Precipitation was carried out in cold by slowly adding ammonium sulphate (600 g/L) with gentle stirring to get 90% saturation and allowed to settle. The clear supernatant was decanted and the volume of the suspension was reduced to get 4-fold concentration of protease. Protease activity of the precipitated enzyme was estimated. This method offered an effective process to concentrate the protease with very good recoveries (Table 2.3.7). The precipitated enzyme was stable for several months at room temperature (28 to 35°C).

- ***Freeze drying***

  Crude culture filtrate obtained after harvesting was frozen in a round bottom flask using liquid nitrogen to facilitate the adhesion of culture filtrate on the inner wall of the flask to provide more surface area. Lyphilization was carried out at minus 55° C for six hours. The lyophilized enzyme thus obtained was dissolved in deionized water and activity of the lyophilized sample was estimated and compared with the initial activity to calculate the recoveries. It was found that this method was also suitable which gave more than 95% recoveries (Table 2.3.7).

- ***Membrane concentration***

  The culture filtrate was subjected to ultra filtration unit using 10 kDa cut off membrane. The process was carried out at 4°C to avoid local heating. The retentate showed 70-75% activity and around 20-30% of activity was recovered in the filtrate (Table 2.3.7).

- ***Spray drying***

  In another method of enzyme concentration, culture filtrate was spray dried with 10% and 15% maltodextrin as additives with inlet temperatures of 150-160°C and outlet temperatures of 70-80°C. Recoveries ranged from 70-80% depending on the maltodextrin concentration (Table 2.3.7).

Table 2.3.7: Methods employed for downstream processing of protease

Method	Protease Recovery (%)
Ammonium sulphate precipitation	94
Freeze drying	96
Membrane concentration	74
Spray drying (15% maltodextrin)	80
Spray drying (10% maltodextrin)	70

Shelf life of ammonium sulphate precipitated protease

Commercially available enzymes are generally formulated using specific stabilizers to achieve maximum shelf life of the product. There are number of compounds such as sodium chloride, polyethylene glycol (PEG), sorbitol, glycerol which are used as additives for stabilization and preservation of enzymes. However, ammonium sulphate concentrated enzyme formulation is advantageous, because the salt itself acts as preservative and additional preservative dose not required **(**Laxman *et al.,* 2005**)**. In order to determine the shelf life of ammonium sulphate precipitated protease, enzyme was incubated at 28°C. Protease activity was estimated after regular time intervals. The salt precipitated enzyme was highly stable and retained almost 90% activity even after 570 days **(**Figure 2.3.9). Ammonium sulphate concentrated protease from *C. coronatus* PTA 4132 was found to be stable for two years at room temperature (Laxman *et al.*, 2005).

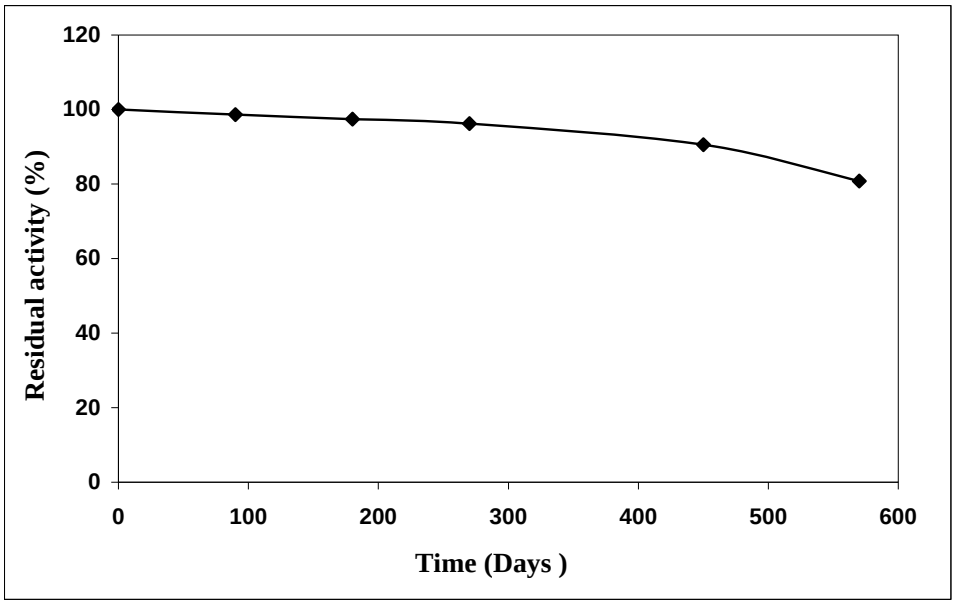

Figure 2.3.9: Shelf life of ammonium sulphate precipitated enzyme

Shelf life of spray dried protease

It is prime concern to protect stability and improve storage of commercial proteins and enzymes, as the water present in the solution facilitates or mediates degradation of protein during handling. Consequently, dry solid formulations are often developed to provide an acceptable protein shelf life. Spray drying is the most widely use

industrial process for removal of water content from the product of interest. Although dehydration itself may introduce a stress to proteins due to protein unfolding, it is advantageous for improving the shelf life, ease storage and transport, reduce transportation cost, and protect the biological activity. Spray drying is also associated with main problem of stickiness of dried product. To eliminate this problem, spray drying is perform by adding drying and protective aids such as high molecular weight carbohydrates and polymers (Namaldi *et al.,* 2006; Kanpairo *et al.,* 2012).

Crude protease produced at 500 L fermentor was spray dried with 10 and 15% maltodextrin as additives. The stability of spray dried protease was studied at temperatures ranging from 4 to 37°C. Samples were distributed in vials and stored at 4, 28 and 37°C. One vial incubated at each temperature was removed at regular intervals and residual activity was measured. Spray dried protease was stable up to 26 months (780 days) at all the temperatures tested and retained 85-90% activity (Figure 2.3.10).

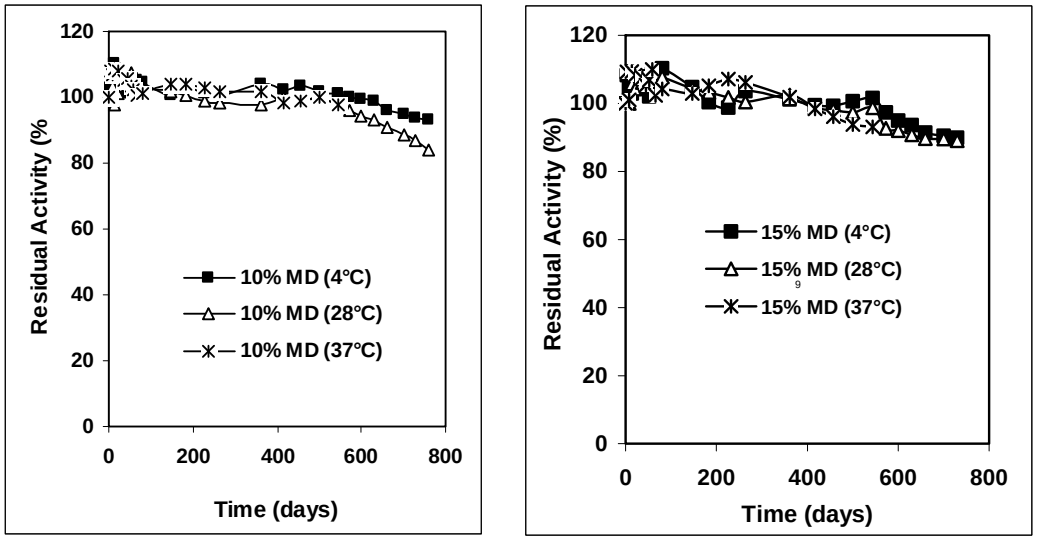

Figure 2.3.10: Shelf life of spray dried alkaline protease

Namaldi *et al.* (2006) investigated effect of different inlet temperatures ranging from 70-130°C and concentration of glucose and maltodextrin on the spray drying of alkaline protease from *B. subtilis.* Drying of enzyme with 1% (w/v) glucose at 110°C offered highest enzyme recovery and the resultant spray dried protease was stable for 6 months at 4°C.

Conclusions

The newly isolated *C. brefeldianus* (MTCC 5185) strain secretes high levels of alkaline protease in simple low cost medium. The production was initially optimized in 7.5 L bioreactor in medium containing 2% glucose, 0.16% DAP and 3% SBM. Preliminary studies were carried out with fixed agitation rates with aeration rates of 0.5 and 1 vvm. With both the aeration rates, very low activities (<5%) were detected with agitation rates between 200 to 330 rpm. It was noticed, that increasing agitation to 400 to 500 rpm, increased activities as well as glucose consumption and biomass formation. However, they were still 40% of the maximum activities normally obtained in shake flasks. Hence, effect of gradual increase in agitation from 250 to 500 rpm was investigated which resulted in significant increase (similar to those obtained in shake flasks) in shorter fermentation cycles. These results were successfully translated at higher scale in 75 and 700 L fermentors. Highest activities of around 40 IU/ml were obtained in 75 and 700 L fermentors in 56 and 45 h respectively. Productivities in 700L fermentor reached nearly 900 IU/L/h. Crude culture filtrate was concentrated by ammonium sulphate precipitation, membrane concentration, lyophilization and spray drying with recoveries ranging from 70 to 96%. Shelf life studies of ammonium sulphate precipitated and spray dried protease showed that the enzyme was stable at temperatures ranging from 28 to 37°C for more than 18 months. Thus, the process production at pilot scale level in a low cost medium and in short fermentation cycles including down stream processing and shelf life studies demonstrate that the present alkaline protease is a suitable candidate for its commercial exploitation.

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SECTION 4

STUDIES ON CRUDE ALKALINE PROTEASE AND OTHER ASSOCIATED ENZYMES

Abstract

The crude protease preparation of *C. brefeldianus* is active in the temperature range 30 to 60°C and pH range of 6-11 with optimum at 50ºC and pH 9. the enzyme was active in presence of Ca^{+2}, Cd^{+2}, Co^{+2}, K^{+}, Mg^{+2} and Mn^{+2} while Ni^{+2} and Zn^{+2} resulted in 35-40% inhibition. Cu^{+2} and Hg^{+2} totally inhibited the activity. The crude protease was active towards keratin azure, azocall, elastin-orcin, azocasein but did hydrolyze synthetic chromogenic collagen substrate. In addition, the crude enzyme exhibited various enzyme activities such as chondroitinase, laminarase and chitinase. It was fairly stable up to 12 h in presence of most of the organic solvents tested at 28°C, while at 37°C, it retained about 80, 70 and 60% of activity in presence of acetone, DMSO and methanol respectively. Protease is stable in presence of 1% non-ionic detergents, β-mercaptoethanol (BME), EDTA, 4 M urea and retained more than 30-40% activity in presence of 1% SDS. The protease retained 50-70% activity at 40ºC in presence of commercial detergents up to 1 h and effectively removed blood stains from cloth when applied in combination with commercial detergent. The culture was able to produce extra cellular lipase using various vegetable oils as inducers. The lipase was optimally active at pH 7 and 50°C.

Introduction

Enzymes are currently used in several different industrial products and processes and new areas of application are constantly being added. The world enzyme market is currently at $5.1 billion and is expected to rise by 6.3% annually by 2013 (Singhal *et al.*, 2012**)**. With the growing concern about environmental issues, microbial enzymes have emerged as alternative biocatalyst in several industrial processes and their utility is expanding day by day, as the newer enzymes with novel properties are being explored.

Most of the currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Majority of processes in food, pharmaceutical, leather, textile and detergent industries exploit crude enzyme preparations in bulk quantities whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Rao *et al*, 1998; Kumar and Takagi, 1999).

Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries. Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent and baking industries, represent the second largest group. The technical industries, dominated by the detergent, starch, textile and fuel alcohol industries, account for the major consumption of industrial enzymes (Kirk *et al.*, 2002).

The overall potential of alkaline proteases in industrial process is yet to be exploited fully. The inherent disadvantages in the use of proteases, in particular, are related to thermal, operational and storage problems as they are easily prone to inactivation by self-digestion (autolysis), whereas a good industrial catalyst should be stable under the toughest operating conditions and for long durations. Increased attention has been focussed on the stabilization of enzymes in soluble forms, and many efforts have been made in search of new and different methods to obtain soluble, but stabilized, enzymes (Anwar and Saleemuddin, 1998), as it is impossible to use insoluble enzymes in several biotechnological applications including detergent, food, cosmetic and textile industries.

Microorganisms account for a two-thirds share of commercial protease production worldwide. Natural microorganisms have over the years been a great source of enzyme diversity.

In addition, presence of various associated activities in the crude protease is useful in many instances. For example in leather manufacturing, protease preparation containing activities like elastase, chondroitinase and keratinase may have added advantage in dehairing and fiber opening of skin (Edmonds, 2008; Sivasubramanian *et al.*, 2008; Archana and Pillai, 2012). However, to retain intact grain structure for high quality leather it is essential that the protease preparation is collagenase free as the main constituent of leather comprised of collagen.

The detergent industry has now emerged as the single major consumer of several hydrolytic enzymes. Detergents containing different enzymes; proteases, amylases and lipases are available in the international markets under several brand names. The use of different enzymes as detergent additives arises from the fact that proteases can hydrolyse proteinaceous stains e.g. blood, milk, egg, cocoa and sauces, amylases are effective against starch and other carbohydrate stains while lipases are effective against oily or fat stains. Detergent industries are the primary consumers of enzymes for detergent formulation, as it enhances the ability to remove tough oil stains and making the detergent environmentally safe. In recent years, many laundry-detergent products contain cocktails of enzymes including proteases, amylases, cellulases, and lipases (Cherif *et al.*, 2011). In addition to detergent industry, lipases have potential applications in various industries such as food, dairy, pharmaceutical, leather processing, textile, bio-diesel, and cosmetic industries and in synthesis of fine chemicals, agrochemicals, and new polymeric materials.

An ideal detergent enzyme should be stable at high pH and temperatures up to 40°C, stable in presence of surfactants, withstand oxidizing and chelating agents, and be effective at low enzyme levels in detergent solutions (Gupta *et al.*, 2002). Moreover, it should also have broad substrate specificity. Subtilisins have emerged as major detergent proteases over the past years. However, search for newer proteases other than subtilisins with diverse properties of enzymes such as low temperature performance has gained lot of interest as well as complexity of patent situation (Maurer, 2004). Although bacterial proteases have long been used in detergents, the

main drawback in their use is that they require cost-intensive filtration methodologies to obtain a microbe-free enzyme preparation. However, proteases from fungal origin offer an advantage that mycelium can be easily removed by filtration techniques. The alkaline protease from *C. coronatus* NCL 86.8.20 showed a high level of stability up to pH 8.5 and up to 40°C, also compatible with most of the detergents tested, was also able to hydrolyse various protein substrates tested implying that the protease has all the potential to be used as a detergent enzyme (Phadatare *et al.,* 1993).

In recent years, proteases have received great attention due to its application in production of certain oligo-peptides as an alternative to chemical synthesis (Okazaki *et al*., 2000; Wang *et al*., 2008). Normally, proteases bring about hydrolysis of protein in aqueous solution, however opposing to this, it can catalyze peptide synthesis in organic solvents or in solvents with low water activity. Peptide synthesis using proteases has numerous advantages over chemical synthesis, e.g. regio and stereo selectivity, absence of racemization, non-requirement of side-chain protection and milder non-hazardous reaction conditions However, due to low specificity and instability of proteases in organic solvents, their use in peptide synthesis is limited (Ghorbel *et al*., 2003). Due to the advantages of solvent stable proteases, attempts were made to improve their activity and stability, based on physical, chemical and protein engineering techniques. The stability of the modified enzymes in the organic media, however, has not yet been fully satisfied (Rachadech *et al*., 2010).Therefore, search for proteases, having natural stability in presence of organic solvents may be more convenient approach for their use in synthetic reactions.

C. brefeldianus secretes high levels of alkaline protease in short periods on simple and inexpensive medium. The protease production is successfully scaled up to 500L in 700 L fermentor with very good productivities. Various methods of down stream processing and concentration/stabilization proved useful. The protease finds application in leather processing for dehairing. Strains belonging to genera *Conidiobolus* and *Basidiobolus* are known by secrete singly or in association with each other various hydrolytic enzymes like chitinase, lipases and proteases (Okafor and Gugnani, 1990; Laxman *et al*., 2005; Mishra *et al.,* 2011). Therefore, it was of interest to analyze the crude protease preparation for presence or absence of enzymes such as collagenase, elastase, keratinase, chondroitinase etc which is essential and

critical for production of high quality leather. Secondly, characterization the crude preparation with respect to optimum conditions for activity, substrate specificity and stability to pH, temperature, surfactants, detergents, chelators, compatibility to commercial detergents and its performance etc. is useful in assess its potential for other applications.

This section discusses the properties and few applications of crude protease preparation as well as production of other hydrolytic enzymes like lipases, chitinases etc by the new strain of *C. brefeldianus.*

Materials and methods

Materials

Various substrates viz. bovine serum albumin (BSA), hemoglobin, elastin-orcin, azocasein, azocoll, laminarin, chondroitin sulphate-A, chitin, gum arabic, di-nitro salicylic acid (DNSA) and sodium dodecyl sulphate (SDS) were procured from M/s Sigma Chemicals, USA. Non-ionic detergents (Tween 20, Tween 40, Tween 60, Tween 80 and Triton X-100), tributyrin, beef extract, malt extract, peptone, yeast extract were procured from M/s HiMedia Chemicals, India. Commercial detergents were purchased from local market. All other chemicals used in the study were of analytical grade. Acetone, acetonitrile, 1-butanol, dimethylsulphoxide (DMSO) isopropanol, methanol, (EDTA) and agar agar were obtained from Qualigens, India. Sunflower, soyabean and olive oils and Ranipal used for chitinase plate assay were obtained from local market.

Methods

Effect of temperature on protease activity and stability

Optimum temperature of the protease was determined by estimating the protease activity at pH 9 and temperatures ranging from 30 to 70ºC for 10 min. Thermal stability of protease was examined by incubating the crude enzyme at 40 and 50°C up to 2 h. Samples were withdrawn at regular time intervals and residual protease activity was estimated at 50°C, pH 9 and expressed as percent of initial activity taken as 100%.

Effect of pH on protease activity and stability

Optimum pH was determined by estimating the protease activity at 50°C and pH values ranging from 5 to 12 (dilutions with 0.1 M buffer of respective pH). Stability of protease was examined by incubating the enzyme at 28°C in buffers at pH values ranging from 4 to 12 (pH adjusted with 1M buffers) for 2 h. Following buffers were used: (acetate pH 4 & 5; phosphate pH 6 &7; Tris-HCl pH 8; carbonate bicarbonate pH 9 & 10; glycine-NaOH pH 11 and KCl-NaOH pH 12). Samples were withdrawn at regular time intervals and residual protease activity was estimated at 50°C and pH 9.0 and expressed as percentage of the initial activity taken as 100%.

Effect of metal ions on protease activity

Effect of metal ions on protease activity was determined by estimating the activity in absence or in presence of 5 mM metal ions at 50°C, pH 9. Stock metals (100mM) were prepared and added to the reaction mixture at a final concentration of 5 mM. Activity with metal (relative activity) was expressed as percentage of activity without metal taken as 100%.

Effect of non-ionic detergents, denaturants and chelator on protease

The enzyme was incubated with non-ionic detergents (Tween-20, Tween 40, Tween 60, Tween 80, Triton-X-100), denaturants (Urea, mercaptoethanol, SDS) and EDTA at 28°C for 1 h and residual protease activity was estimated at 50°C, pH 9 and expressed as percentage of without detergents or denaturants taken as 100%..

Compatibility of protease with commercial detergents and wash performance

Crude protease was incubated with various commercial detergents at final concentration of 0.7 mg/ml at 40°C for 1 h. Detergents were heated at 100°C for 10 min before use. Samples were removed at intervals of 15 min and residual activity was measured and expressed as percentage of initial activity with respective detergents taken as 100%. Wash performance of the protease with and without added detergent on blood stained cloth was investigated.

Effect of organic solvents on protease activity and stability

Culture filtrate was incubated with various water miscible and water immiscible solvents at the final concentration of 20% at 28°C and 37°C. Following organic solvents were used: acetone, acetonitrile, 1-butanol dimethylsulphoxide, isopropanol and methanol. Samples were removed at different time intervals and residual activity was estimated. Sample without organic solvent served as control. Effect of organic solvent on activity was expressed as relative activity without organic solvent taken as 100%. For stability, initial activity with respective solvents was taken as 100%.

Screening for other enzyme activities by plate assay

- ***Screening for chitinase***

  Plate assay for chitinase was performed according to Anil *et al.* (2007) with slight modifications. Two percent agar containing 0.01% acid swollen chitin was poured

in to plates and holes were drilled on the agar medium. Fifty micro litters of crude enzyme was added in the wells made on the plate and incubated at 37°C for 1 h. For detection of chitinase activity, the plate was flooded with 0.1% Ranipal (cloth whitener) for 15 min followed by washing with DW twice for 30 min each. The plate was then observed under UV light for visualizing the clearance zone.

- ***Screening for chondroitinase***

Screening for chondroitinase activity was performed as described by Seddon *et al*. (1990) with some modification. Chondroitin sulphate-A and BSA were filter sterilized and added to sterile MGYP agar to a final concentrations of 0.4% (chondroitin sulphate-A) and 1% (BSA) and the plates were poured. The culture was spot inoculated and incubated at room temperature. At the end of incubation, the plate was flooded with 2 N acetic acid and allowed to stand for 15 min to visualize the clearance zone.

- ***Screening for lipase by plate assay***

Lipase secretion by *C. brefeldianus* was initially screened by plate assay method using tributyrin as substrate. Mikami agar containing 1% emulsified tributyrin plates were poured. The culture was spot inoculated and incubated at 28°C and checked for clearance zone around the growing colonies of the fungus.

Estimation of other enzyme activities in crude protease preparation

- ***Activity towards natural substrates***

The crude protease was incubated with natural substrates like casein, hemoglobin and bovine serum albumin (BSA). The reaction mixture contained an aliquot of suitably diluted protease enzyme and 10 mg substrate in 0.1 M sodium carbonate buffer, pH 9 in a total volume of 2 ml. After incubation at 50°C for 10 min, the reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid (acidified with concentrated hydrochloric acid). The precipitate formed was filtered through Whatman No.1 filter paper after standing at room temperature for 30 min. The absorbance of trichloroacetic acid soluble fraction was measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to produce one micromole of tyrosine/min.

- ***Azocaseinase***

Azocaseinase activity was estimated with azocasein as substrate at 50ºC, pH 9 by the method described by Singh *et al.* (1999). The reaction mixture contained an aliquot of suitably diluted protease enzyme and 1 mg azocasein in 0.05 M carbonate bicarbonate buffer, pH 9 in a total volume of 500 µl. Heat inactivated enzyme (by boiling for 15 min) was taken as blank. After incubation at 50°C for 30 min, reaction was terminated by addition of 500 µl of 10% (w/v) trichloroacetic acid. After cooling on ice for 15 min, contents were centrifuged at 10,000 rpm for 10 min. To 800 µl of supernatant 200 µl of 1.8N NaOH and the absorbance was measured at 420 nm. One unit of azocaseinase activity is defined as the amount of enzyme required to increase the absorbance by one unit at 420 nm in one min.

- ***Elastase***

Elastase activity was estimated with elastin-orcin as substrate at 50ºC, pH 9. The reaction mixture contained an aliquot of suitably diluted protease enzyme and 20 mg elastin-orcin in 0.1 M carbonate bicarbonate buffer, pH 9 in a total volume of 3 ml. Heat inactivated enzyme (by boiling for 15 min) was taken as blank. After incubation at 50°C for 30 min, the reaction was terminated by addition of 2 ml 0.7 M phosphate buffer, pH 6. Contents were centrifuged and absorbance of the supernatant was measured at 578 nm. One unit of elastase activity is defined as the amount of enzyme required to cause an increase in absorbance by one unit at 578 nm in one minute.

- ***Keratinase***

Keratinase activity was estimated with keratin-azure as substrate at 50ºC, pH 8 by the method described by Bressollier *et al.* (1999). The reaction mixture contained an aliquot of suitably diluted enzyme and 4 mg of keratin azure in 0.05 M Tris HCl buffer, pH 8 in a total volume of 1 ml. Heat inactivated enzyme (by boiling for 15 min) was used in blank. The reaction mixture was incubated at 50°C for 1 h with intermittent shaking. Reaction was terminated by centrifuging the mixture and absorbance of the supernatant was measured at 595 nm. One unit of keratinase activity is defined as the amount of enzyme required to cause an increase in absorbance by 0.01 at 595 nm in one hour.

- ***Azocollagenase***

Azocollagenase activity was estimated with azocoll as substrate at 37ºC, pH 8. The reaction mixture contained an aliquot of suitably diluted protease and 10 mg azocoll in 0.05 M Tris HCl buffer, pH 8 in a total volume of 2.5 ml. Heat inactivated enzyme (by boiling for 15 min) was taken as blank. After incubation at 37°C for 10 min, the reaction was terminated by filtering through Whatman No.1 filter paper. The absorbance of filtrate was measured at 580 nm. One unit of enzyme activity was defined as the amount of enzyme required to cause an increase in absorbance by one unit at 580 nm per min.

- ***Collagenase with synthetic substrate***

The non-collagenase nature of the protease from *C. brefeldianus* MTCC 5185 is demonstrated by fluorescence studies using two synthetic substrates (NMITLI-l for protease activity and Collagenase-1 for collagenase activity). The assay was carried out in two steps. In the first step where the protease nature of the sample was detected, the assay contained 290 µl of 0.1M TEA buffer, pH 8 having NMITLI-l substrate concentration of 1.8 µM (10 µl of 112 µM stock solution). The reaction was initiated by adding 5 µl of enzyme samples. The excitation wavelength was 340 nm and the emission was scanned from 425-625 nm, at different intervals of time. Increase in the fluorescence of AEDANS chromophore indicates proteolytic nature of the sample. In the second step, collagenase activity in the sample was screened at room temperature with Collagenase substrate-I. Typically, the assay contained 290 µl of 0.1 M TEA buffer pH 8, having substrate (Collagenase substrate-I) concentration of 1. 6 µM (5 µl of 0.08 M stock solution). The reaction was initiated by adding 5 µl of enzyme samples. The excitation wavelength was 340 nm and the emission was scanned from 425-625 nm, at different intervals of time. Increase in the fluorescence of AEDANS chromophore indicates collagenolytic nature of the sample. These assays were carried out by Prof. P. Balaram's group at IISc, Bangalore.

- ***Chondroitinase***

Chondroitinase assay was performed as per manufacture's protocol at 37ºC, pH 8 (Sigma Chemicals, USA). To 0.8 ml of suitably diluted enzyme (pre-equilibrated

at 37°C for 2 min), 0.2 ml 0.5% chondroitin sulphate-A in 0.05% BSA prepared in 0.025 M Tri-HCl buffer, pH 8 containing 0.3 M sodium acetate was added and mixed. The assay mixture was incubated at 50°C up to 21 min. Aliquots of 0.1 ml each were withdrawn at 3 min intervals and transferred to tubes containing 0.9 ml 0.05 M KCl adjusted to pH 1.8 which were incubated for another 10 min at 37°C. Contents were then centrifuged and absorbance was measured at 232 nm. Absorbance at zero minute served as blank. Activity was calculated from the slope (increase in absorbance/min) of the linear portion of the graph of absorbance against time as shown in the equation below.

$$U/ml = \frac{\text{Slope x 1 (ml) x dilution factor}}{\text{5.1 (EmM) x 0.1 x 0.8}}$$

where, 5.1 is milimolar extinction coefficient of unsaturated disaccharides for chondroitin sulphate-A, 0.1 represents the aliquot taken at an interval of 3 minutes for measuring the absorbance, 0.8 ml represents the amount of enzyme used for assay.

One unit of chondroitinase activity is defined as the amount of enzyme required to liberate one micromole of 2–acetamido -2- deoxy-3- O–(ß–D-gluc-4-ene-pyranosyluronic acid)–4-O-sulfo–D-galactose from chondroitin sulphate-A per min under assay conditions.

- ***Chitinase***

Chitinase activity in the culture filtrate was estimated with acid swollen chitin as substrate as described by Chavan (2009). The reaction mixture contained an aliquot of suitably diluted protease enzyme and 1 ml of 0.7% acid swollen chitin in 0.05 M acetate buffer, pH 5 in a total volume of 3 ml. Assay was performed by incubating the reaction mixture at 50°C for 1 h. The N-acetyl glucosamine liberated was estimated by measuring the absorbance at 585 nm with p–dimethyl amino benzaldehyde. One unit of chitinase activity is defined as the amount of enzyme required to liberate one micromole of N–acetyl glucosamine per min.

- ***Laminarinase***

The reaction mixture contained 0.5 ml of 1% laminarin prepared in 0.05 M phosphate buffer, pH 7 and 0.5 ml of suitably diluted enzyme. The reaction mixture was incubated at 50°C for 30 min and the reducing sugar liberated was measured by di-nitro salicylic acid method (Bernfield, 1955). One unit of laminarinase activity is defined as the amount of enzyme required to release one micromole of reducing sugar as glucose equivalents per min under assay conditions.

Production of lipase

Lipase production was carried out in Mikami medium containing oils and tributyrin as inducers. Culture grown in GYE medium for 24 h was used for inoculating the experimental flasks. Duplicate flasks were run for each inducer. Samples were removed at regular intervals and lipase activity was estimated.

Estimation of lipase activity

Lipase activity was determined by titrimetric method. The substrate was prepared by mixing 20 ml of olive oil, 165 ml of 10% gum arabic and 15g ice in grinder mixer for 10 minutes and filtered on glass wool and stored at 4°C. The reaction mixture contained 2 ml 0.05 M phosphate buffer, pH 7, 5 ml substrate and 1 ml crude culture broth and incubated at 50°C for 1 h with shaking at 50 rpm. The reaction was terminated by addition of 4 ml of acetone: ethanol (1: 1). In blank the enzyme was added after the termination of reaction by acetone: ethanol. Free fatty acids released were titrated with 10 mM NaOH. The lipase activity is expressed as μ moles of free fatty acids released per min under the assay conditions.

Results and discussion

For successful exploitation of alkaline proteases in various processes and for its commercialization, it is important to determine the optimal conditions for their activity. In addition their stability against solvents, surfactants, detergents, metal ions etc. also reflects on the potential of the enzyme. Theses properties are known to vary from organism to organism. If the crude enzyme preparation also exhibits other enzyme activities, the preparation will find more industrial applications. This study is directed towards exploring the potential of the organism in secreting diverse enzyme activities along with alkaline protease and their properties.

Properties of alkaline proteases and their applications

Effect of temperature on activity stability

In general, fungal alkaline proteases are active in the temperature range of 35 to 60°C while those from bacteria have higher temperature optima (Shankar, 2010; Nirmal *et al.,* 2011). The present enzyme was active in the temperature range of 30-60°C with an optimum at 50°C (Figure 2.4.1). This is slightly higher than the proteases reported from other strains of *Conidiobolus* (Sutar *et al.,* 1991; Phadatare, 1991; Tanksale, 2001; Laxman *et al.,* 2005). Optimum temperature of these proteases was falling in the range of 37 to 45°C.

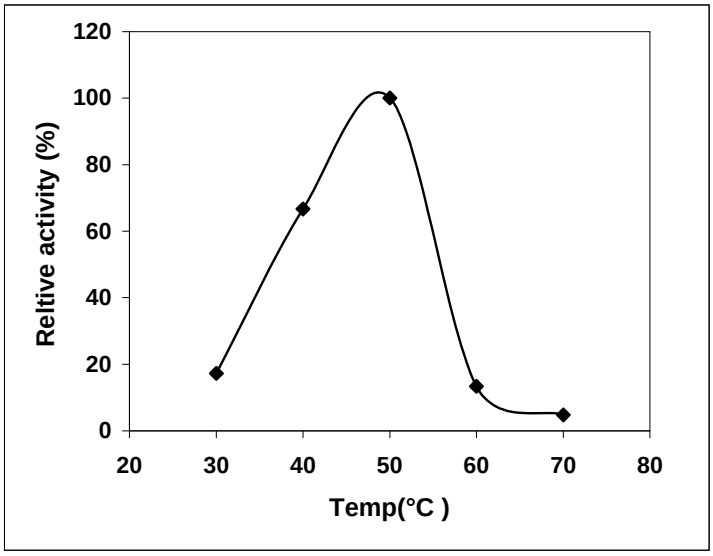

Figure 2.4.1: Optimum temperature of protease

Optimum temperature for proteases from *A. clavatus ES1; A. clavatus* and *A. niger* were 50°, 40 and 45°C respectively (Hajji *et al.,* 2007; Tremacoldi and Carmona, 2005; Devi *et al.,* 2008). Crude proteases from *Penicillium* sp. and *Fusarium* sp. had optimum temperatures of 45 and 50°C respectively (Germano *et al.,* 2003; Ueda *et al.,* 2007).

C. brefeldianus protease was more stable at 40°C than 50°C and retained around 90 and 60% of the activities after 1 and 2 h respectively. However at 50°C, only 45% residual activity was present after 1 h which dropped to 25% residual activity after 2 h Figure 2.4.2).

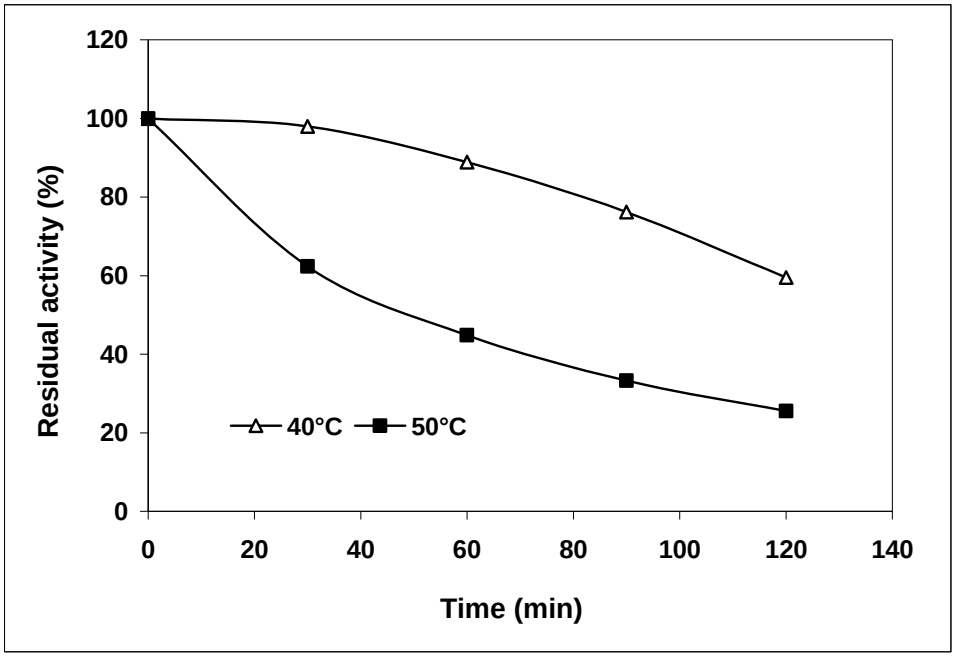

Figure 2.4.2: Temperature stability of protease

Phadatare *et al.* (1993) reported *C. coronatus* NCL 86.8.20 alkaline protease to be stable up to 40°C. *A. tamari* protease was stable for more than ten hours up to 45°C (Boer and Peralta, 2000). Alkaline protease from *A. terreus* retained 60% activity at 60°C (Chakrabarti *et al.,* 2000) while *A. sydowi* was stable up to 45°C (Masato *et al.,* 1995). *A. niger* protease retained most of the activity up to 60 min at 40°C, but sharp decrease in activity was noticed on further incubation up to 90 min (Devi *et al.,* 2008). A protease which is active and stable in the temperature range of 40-50°C is

advantageous for its exploitation in detergent formulation, as the general soaking temperature of clothes falls in this range.

Optimum pH and pH stability

The protease was active in the pH range of 6-11 with an optimum at pH 9 (Figure 2.4.3). It showed more than 60% activity at pH 8 and 10. Optimum pH of proteases from other strains of *C. coronatus* was reported in the range of 8.0 to 10 (Sutar *et al*., 1991; Phadatare *et al*., 1993; Tanksale, 2001; Bania *et al*, 2006).

Optimum pH for alkaline protease from *A. niger* was found to be 10 (Devi *et al*., 2008). However acidic protease from *A. niger* active at pH 4 has also been reported (Kamath *et al*., 2010). Protease from *Trichophyton vanbreuseghemii* showed optimum pH to be 8.0 (Moallaei *et al.,* 2006). Shankar *et al*. (2010a) reported that, protease from *Beauveria* sp. was optimally active at pH 9.0, while *B. bassiana* protease was found to be optimally active at pH 8 (Namasivayam *et al*., 2010).

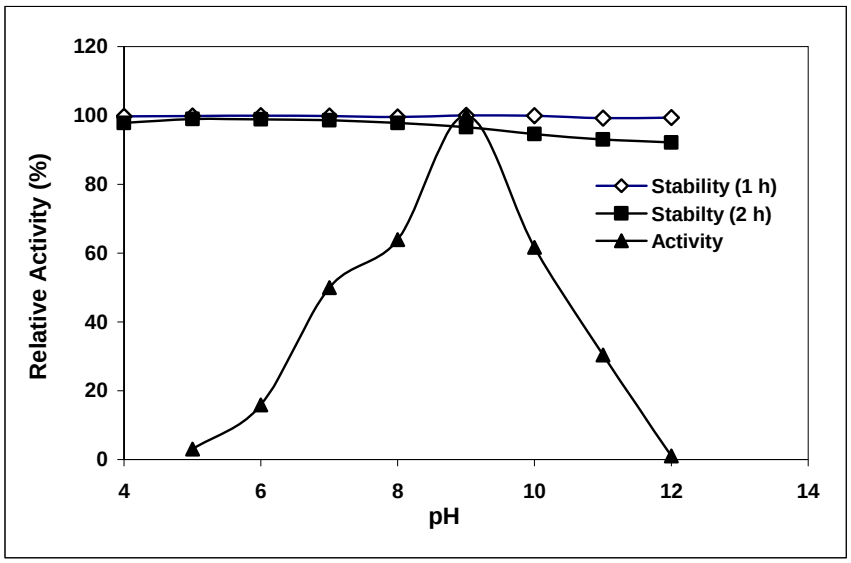

Figure 2.4.3: Effect of pH on activity and stability of protease

Alkaline proteases are generally stable between pH 6-10 **(**Nirmal *et al.*, 2011). *C. brefeldianus* protease was highly stable in broad pH range of 4-12 and retained almost complete activity after 1 h of incubation (Figure 2.4.3). The enzyme showed more than 95% initial activity even after 2 h with highest stability was observed between

pH 6 to 7 where 98-99% initial activity was retained. The excellent stability of present protease over broad pH range tends to make it attractive for detergent formulation. *C. coronatus* protease was stable in pH range 6 to 10 but there was considerable loss in the activity below pH 5 after 30 min (Bania *et al*., 2006). Alkaline protease from *C. coronatus* NCL 86.8.20 was stable between pH 6.0-8.5 (Phadatare *et al*., 1993). Alkaline protease from *C. macrosporus* was stable over a wide pH range of 5 to 12 with maximum stability at 7.5 (Tanksale *et al*., 2001). *C. coronatus* NCIM 1238 protease was stable at pH 7 and was completely inactivated below 5 and above pH 9.5 (Sutar, 1987).

Crude protease from *Penicillium* sp. was stable in the pH range of 6-9 (Germano *et al.*, 2003). *A. clavatus* protease was stable for 1 h at 4°C in the pH range 6 to 11 with highest stability between pH 8 to 9 (Hajji *et al.*, 2007). *Paecilomyces lilacinus* protease was stable in the pH range of 5 to 11.5 for 1 h (Kotlova *et al*., 2007). *A. tamari* protease was stable in the pH range of 5–9 and declined by about 20 and 60% at pH 9.5 and 10 after 1 h (Bore and Peralta, 2000). Ammonium sulphate precipitated alkaline protease from *A. clavatus* was stable in the pH range of 6-12 but was unstable below pH 5 (Tremacoldi and Carmona, 2005).

Effect of metal ions on activity

C. brefeldianus protease was active in presence of most of the metal ions tested (Table 2.4.1). Ca^{2+}, Cd^{2+}, Co^{2+}, K^{+}, Mg^{2+} and Mn^{2+} had stimulatory effect while Cu^{2+}, Hg^{2+}, Ni^{2+} and Zn^{2+} inhibited the protease by 87, 97, 35 and 40% respectively. Generally, serine proteases do not depend on metal ion for their catalytic activity (Islam, 2008). However, different metal ions have shown diverse effect on the activity, depending on the enzyme source. Inhibitory effect of heavy metal ions like Hg^{2+} is well known (Nascimento and Martins, 2004; Upadhyay *et al*., 2010; Yadav *et al*., 2011). It is known that the ions mercury, cadmium and lead react with the protein thiol groups (converting them to mercaptides), as well as with histidine and tryptophan residues. Upadhyay *et al*. (2010) reported 80 and 90% inhibition of *A. flavus* MTCC 277 protease activity by 50 and 100 mM Hg^{2+} respectively. On the contrary, alkaline proteases of *Rhizobium* strain R-986 and *Bradyrhizobium* strain R-993 were completely stable in presence of 1-5 mM Hg^{2+} (Oliveira *et al*., 2010). Similarly, Hg^{+} did not show any inhibitory effect on protease of *Bacillus* sp. Y (Mala

and Srividya, 2010). Proteases from *B. subtilis* PE-11 (Adinarayana *et al*., 2003), *A. Parasiticus* (Tunga *et al*., 2003) and *Halogeomatricum borinquense* TSS 101 (Vidyasagar *et al*., 2006) did not show significant inhibition by Hg^{2+}. Protease from *Nocardiopsis* sp. was activated in presence of K^{+}, Mg^{2+} and Na^{2+}, while Co^{2+} inhibited the same by 44% (Moreira *et al*., 2002). Strong inhibition of protease from thermophilic *Bacillus* sp. was observed in presence of K^{+}, Cu^{2+} and Zn^{2+} (Nascimento and Martins, 2004).

Table 2.4.1: Effect of metal ions

Metal ion (5 mM)	Relative activity (%)
None	100.00
$CaCl_2$	118.25
$CdCl_2$	114.3
$CoCl_2$	118.71
$CuCl_2$	12.78
$HgCl_2$	3.27
KCl	158.50
$MgCl_2$	132.24
$MnCl_2$	104.49
$NiCl_2$	65.60
$ZnCl_2$	60.21

Stability in presence of surfactants, denaturants and chelator

The crude enzyme was stable in presence of non-ionic detergents viz. all the Tweens and Triton X-100. EDTA had only 7% inhibitory effect indicating that the enzyme does not require metal ion for activity (Table 2.4.2). The stability of the enzyme in presence of EDTA is advantageous for use of enzyme as detergent additive. This is because detergents contain high amounts of chelating agents, which function as water softeners and also assist in stain removal. These agents specifically bind to and chelate metal ions making them unavailable in the detergent solution **(**Beg and Gupta, 2003). The protease retained nearly 80% activity in presence of denaturants like BME and 4 M urea. However, protease retained 43% of its activity in presence of 1% SDS, an anionic surfactant.

Table 2.4.2: Effect of surfactants and denaturants

Surfactants/denaturants	Concentration (%)	Residual Activity (%)
None	-	-
Tween-20	1	101.53
Tween-40	1	99.68
Tween-60	1	99.62
Tween-80	1	98.63
Triton X-100	1	98.43
BME	1	79.17
SDS	1	43.01
Urea	4 M	79.17
EDTA	1	92.71

Proteases from various fungal sources are reported to be stable in presence of surfactants and exhibited certain level of stability in presence of SDS. Protease from *Bacillus* sp. was stable in presence of 1% Tween 80, Triton X-100 and 0.2% SDS (Joo *et al.,* 2004). *A. clavatus* protease retained 100% activity in presence of 5% Tween 80 and Triton X-100. The enzyme retained 90% activity in presence of 0.1% SDS but when the concentration was increased to 0.5%, there was more than 60% loss in activity (Hajji *et al.,* 2007). Tanksale *et al*. (2001), observed that protease from *C. macrosporus* retained 25% of its original activity in presence of 0.3% SDS and attributed the loss due to the binding of negatively charged detergent to positively charged protease. Devi *et al.* (2008) reported that the protease from *A. niger* retained 11, 61 and 68% activity in presence of EDTA, BME and urea respectively at 5 mM concentration.

Effect of organic solvents on protease activity and stability

Proteases are being increasingly exploited for asymmetric synthetic transformations, fuelled by the growing demand for enantiopure pharmaceuticals. But as long as the use of enzymes is restricted to their natural, aqueous reaction media, the scope of industrial bioconversions, especially for the production of speciality chemicals and

polymers, is necessarily limited by a variety of considerations. In principle, most of these problems might be overcome by switching from water to organic solvents as the reaction media (Klibanov, 2001; Barberis *et al*., 2006). However, being protein in nature, enzymes generally get denatured and loose their catalytic activity in presence of solvent media due to loss of native confirmation/to disruption of hydrogen bonds and hydrophilic interactions. Over the years, the stabilization of enzymes in organic media has been attempted by chemical, immobilization, protein engineering and directed evolution (Gupta and Khare, 2009). In addition, it is also necessary to search for proteases, which are naturally stable in the presence of organic solvents used in the synthetic reaction. Few reports have appeared in recent years on the organic solvent from microorganisms, plants and animals.

Initially, effect of organic solvents on protease activity was investigated. *C. brefeldianus* protease was stable in presence of most of the solvents tested **(**Figure 2.4.4).

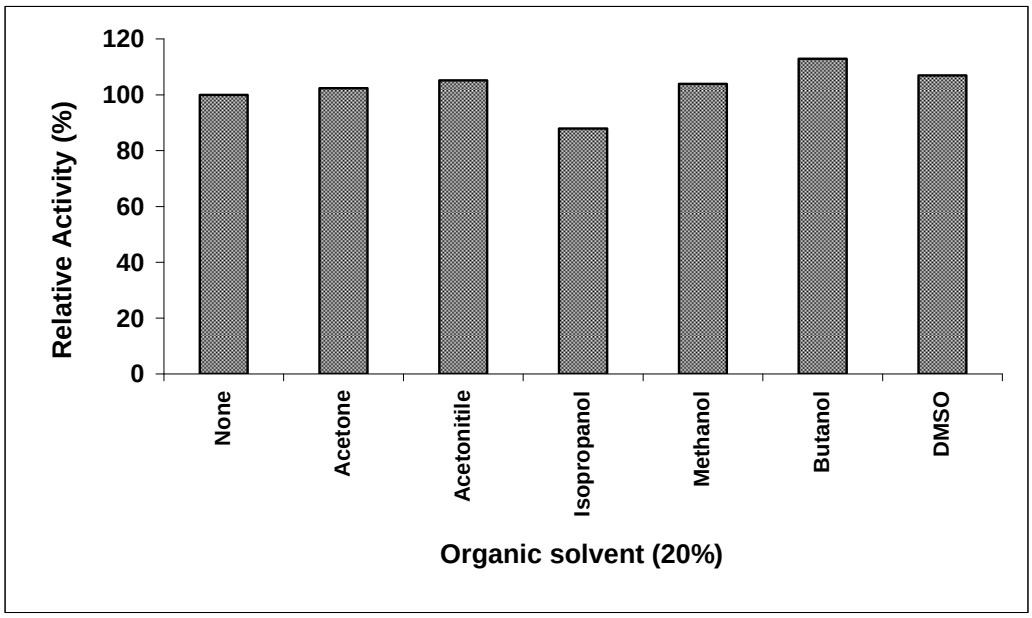

Figure 2.4.4: Effect of organic solvents on protease activity

Except isopropanol, activities were slightly higher in presence of 20% organic solvent. Stability of protease in presence of 20% organic solvent was investigated at 28 and 37°C. Except in presence of butanol, protease was stable and showed more

than 90% residual activity after 5 h (Table 2.4.3). In presence of butanol, residual activity was 75%. However, on longer incubation of 12 h, there was nearly 50% loss in activity which was slightly more than control (absence of solvent). Protease showed highest stability at 37°C for 1 h in presence of acetone (81.10%) followed by DMSO (68.94%) and methanol (59.45%), while more than 30% activity was retained in presence of rest of the solvents (Table 2.4.4). After 120 min, protease showed 60% stability in presence of acetone and 48% stability in presence of methanol and DMSO. These activities are much lower than control indicating inactivation of enzyme in presence of organic solvents.

Table 2.4.3: Stability of protease in presence of organic solvents at 28°C

Type of the solvent	Solvent	Relative polarity	Residual Activity (%)		
			0h	5 h	12 h
Control	**None**	-	100	97.70	76.55
Water miscible	**Acetone**	0.355	100	95.66	55.51
	Acetonitile	0.406	100	98.31	49.90
	Isopropanol	0.546	100	100.29	57.79
Water immiscible	**Methanol**	0.762	100	100.13	52.64
	Butanol	0.602	100	75.26	39.93
	DMSO	0.444	100	98.12	54.55

Wang and Yeh (2006) reported that *B. subtilis* TKU007 protease retained 90 to 100% activity in presence of toluene, DMF and acetonitrile at 37°C for 30 min. Gupta and Khare (2007) found that protease from *P. aeruginosa* PseA was more stable in hydrophobic organic solvents than hydrophilic solvent. In contrast, Ogino *et al*. (1999) reported protease from *P. aeruginosa* PST-01 to be stable for several days in water-soluble organic solvents when incubated at room temperature. *P. aeruginosa* strain K protease tolerated up to at least 50% (v/v) of benzene, *n*-hexane, 1-decanol, isooctane and *n*-hexadecane and was also stable in the presence of 25% (v/v) *n*-decane and *n*-dodecane (Goek *et al*., 2003).

Table 2.4.4: Stability of protease in presence of organic solvents at 37°C

Type of the solvent	Solvent	Residual Activity (%)				
		0h	30 min	60 min	120 min	240 min
Control	**None**	100	96.40	93.51	85.85	79.04
Water miscible	**Acetone**	100	91.66	81.10	60.57	29.53
	Acetonitile	100	37.19	36.40	17.52	13.45
	Isopropanol	100	46.06	34.35	18.73	17.11
Water immiscible	**Methanol**	100	59.43	59.45	48.36	16.60
	Butanol	100	47.57	30.60	33.00	13.53
	DMSO	100	69.48	68.94	48.70	28.53

The stability of *P. aeruginosa* PST-01 protease in solutions containing water-soluble organic solvents or alcohols was higher than that in the absence of organic solvent. Furthermore, in general, PST-01 protease was more stable than commercially available proteases, namely, subtilisin Carlsberg, thermolysin, and a-chymotrypsin, in the presence of water soluble organic solvents or alcohols (Ogino *et al.,* 1999). It can be concluded that diverse organic solvents might be influencing the active confirmation at varied extent, thereby varying the activity of protease in presence of different organic solvents.

Stability in presence of commercial detergents and wash performance

An ideal ingredient of detergents should have good stability in presence of commercial detergents apart from its stability over a wide pH range. The present protease showed stability and compatibility in presence all the detergents tested and retained 80-96% activity after 15 min and 50-70% activity even after 1 h depending on the detergent (Figure 2.4.5).

C. coronatus NCL 86.8.20 showed good stability in presence of various detergents at 40°C up to 1 h and retained activities ranging from 40 to 90% (Phadatare *et al*., 1993). Stability of *C. coronatus* NCL 86.8.20 protease in presence of detergents was also investigated at 50°C in presence of 25 mM $CaCl_2$ as stabilizer which showed 7 to 16% residual activity after 1 h (Bhosale *et al*., 1995). Among the detergents tested for stability and compatibility of *B. bassiana* protease, no loss in activity was observed

with Henko, Surf Excel and Rin while Arial showed complete loss in activity when incubated at 40°C for 1 h (Namasivayam *et al*., 2010). *A. niger* protease retained 80-92% of its original activity in various detergents except Arial which retained only 23% of enzyme activity when incubated at 35°C for 1 h (Devi *et al.*, 2008).

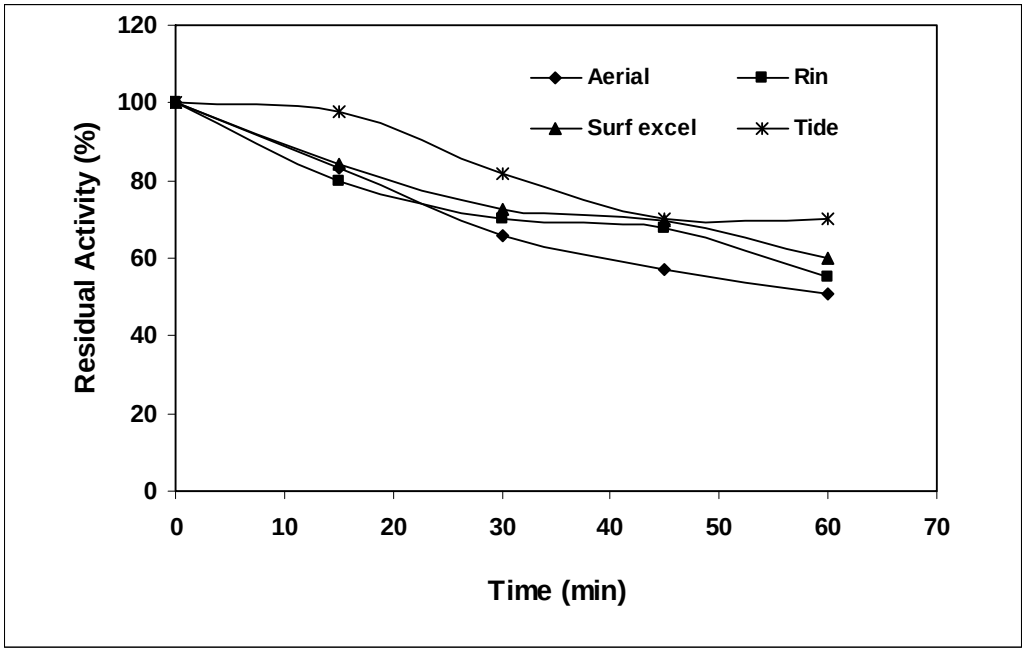

Figure 2.4.5: Stability in presence of commercial detergents

There was no effect of detergents on proteases from *Bacillus mojavensis* (Beg and Gupta 2003); *Bacillus clausii* I-52 (Joo and Chang, 2006) when incubated at room temperature. *C. macrosporus* protease retained 47 to 80% activity in presence of detergents at 30°C for 1 h (Tanksale *et al*., 2001). *Nocardiopsis* sp alkaline protease retained between 48 to 64% activity in presence of commercial detergents at 40°C and 1 h (Moriera *et al*., 2002).

In view of the compatibility and stability of the protease in presence of detergents, washing performance of the crude protease was tested for removal of bloodstain from cotton cloth as described ahead. A piece of white cloth was dipped in blood and air dried to stain it uniformly. It was cut into four equal parts and dipped in 2% formaldehyde solution for 2 min. cloth pieces were then washed with distilled water to remove excess formaldehyde. Cloth pieces were then soaked in 30 ml washing

solution and incubated at 28-30°C for 30 min. Washing performance was tested under 4 sets of conditions: A- water alone; B-detergent alone; C-protease alone and D-detergent + protease. Cloth pieces were rinsed under tap water. Wash performance was best where detergent was used in combination with protease. Blood clots could not remove completely with detergent only, while protease alone was able to remove blood clots while protease and detergent could completely remove blood clots as well as blood stains (Figure 2.4.6). These results are similar to those reported by Cavello *et al.* (2012) where *Paecilomyces lilacinus* protease in combination with detergent was able to remove blood stains completely in 60 min at 30 and 40°C.

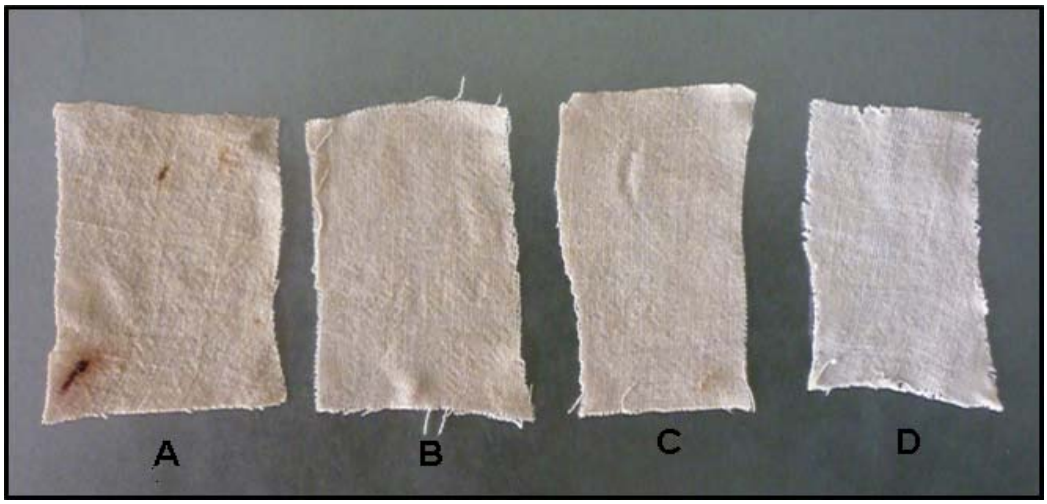

Figure 2.4.6: Removal of blood clots and blood stains

Adinarayana *et al.* (2003) investigated wash performance with *B. subtilis* at 60ºC and was observed that protease in combination with detergent could remove the blood stains more effectively than the detergent alone. Najafi *et al.* (2005) and Abou-Elela *et al.* (2011) performed removal of blood stains with bacterial proteases in the temperature range of 45-50ºC, however the extent of stain removal was inadequate as evidenced by red coloration on the washed cloth pieces.

Hydrolysis of gelatin from waste photographic film

Silver is one of the precious and noble metals used in large quantities in the photographic industry. The waste X-ray/ photographic films contain 1.5-2% (w/w) black metallic silver which is a good source for silver recovery compared to other types of film. Around 18-20% of the world's silver needs are supplied by recycling

photographic waste. Existing method of silver recovery is by burning which generates foul smell and is highly polluting and hence eco-friendly methods using proteases are being explored. Since silver is linked to gelatin in the emulsion layer, the potential of protease to hydrolyze the gelatin layer can be employed to break the same and release the silver (Nakiboglu *et al.*, 2001; Shankar *et al.*, 2010b). Microbial proteases are being explored as alternatives to the burning and oxidation methods of silver recovery from photographic/X-ray films (Singh *et al.*, 1999; Ingale *et al.*, 2002; Nakiboglu *et al.*, 2001*;* Masui *et al.*, 2004).

Used X-ray films were washed with deionized water and air dried. Five grams of film (cut into 1x1 cm pieces) was incubated with 10 IU of protease in 0.01 M carbonate bicarbonate buffer, pH 9 in a total volume of 50 ml in 250 m1 conical flask at 37°C and 180 rpm. A control without protease was also incubated under identical conditions. Stripping of gelatin layer from the film started within few minutes and was complete within 10 min (Figure 2.4.7B). The solution looked blackish due to the blackish silver and the film was clean and white (Figure 2.4.7C).

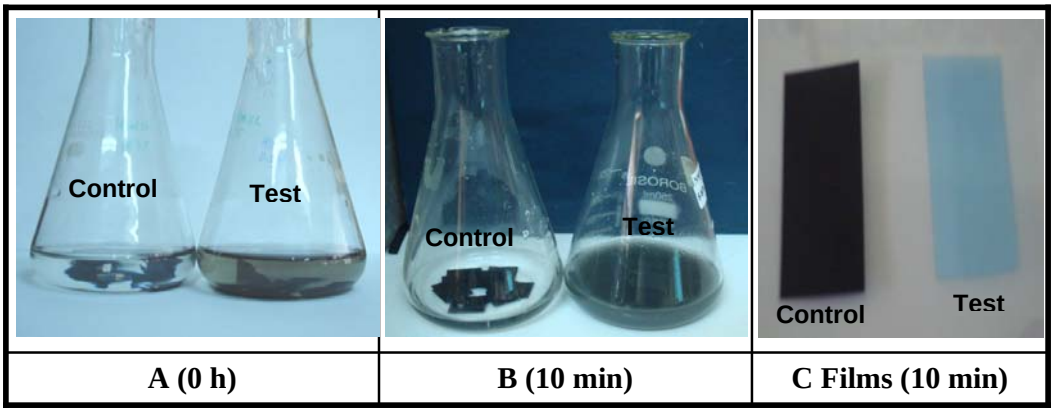

Fig: 2.4.7: Removal of silver from waste x-ray film by *C. brefeldianus* protease

Shankar *et al.* (2010b) studied influence of several parameters such as pH, temperature, enzyme concentration, time etc. on silver removal from the film using *C. coronatus* protease and found that Gelatin layer was stripped completely within 6 min with 1.35 U/ml of protease at 40˚C, pH 10. Ingale *et al.* (2002) reported 0.1% (w/w) of silver recovery and complete hydrolysis of gelatin from 1 g of photographic film using 1000 U of protease from *Basidiobolu*. Fujiwara *et al.* (1989) reported complete

breakdown of gelatin by an alkaline protease from alkalophilic *Bacillus* sp. B21-2 in 8 min at 40˚C, pH 10.5 and at enzyme concentration of 100 U ml- 1 while all the alkaline proteases from the neutrophile *Bacillus subtilis* took more than 20 min to act. Singh *et al*. (1999) reported gelatin hydrolysis of X-ray film by a bacterial protease within 8 min at 60˚**C**, pH 11-12. Masui *et al.* (2004) reported hydrolysis of gelatin layer from X- ray film at 40ºC with *Bacillus* sp. protease in 15 min. Chandan *et al*. (2011) performed hydrolysis of X-ray film with *B. subtilis* MTCC 9226 protease (100 U/g) at pH 9 and 45ºC and reported that complete gelatin removal was observed in 25 min. Ammonium sulphate precipitated and dialyzed proteases from *B. pumilus* and *S. auricularis* when incubated with waste X-ray photographic film at pH 8 and 40ºC hydrolyzed gelatin layers and 0.4013 and 0.3823 g of silver respectively was recovered from 1 g X-ray film (Bholay *et al*., 2012).

Screening for other enzyme activities by plate assay

Chondroitinase assay

Screening of chondroitinase activity was performed on MGYP agar medium containing BSA and chondroitin sulphate-A. One loopfull of 48 h grown culture was spot inoculated and plate was incubated at 28°C. After four days, a zone of clearance around the colony was visible when flooded with 2 N acetic acid indicating the secretion chondroitinase, while the undigested chondroitin-A BSA conjugate appeared opaque (Figure 2.4.8).

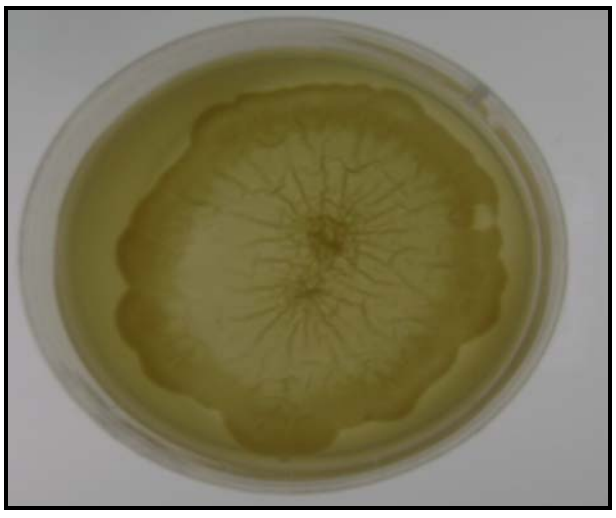

Figure 2.4.8: Plate assay for chondroitinase

Chitinase assay

Chitinase plate assay was performed on 2% agar containing 0.01% acid swollen chitin. Production of chitinase was carried out in 1%GYE + 0.1% chitin. Samples were removed at different time intervals and 50 µl of cell free broth was added in the wells made on the plate and incubated at 37°C for 1 h. For detection of chitinase activity, the plate was flooded with 0.1% Ranipal (cloth whitener), washed and observed under UV light. A light blue clear zone around the sample spot was observed due to chitin degradation, indicating the secretion of chitinase (Figure 2.4.9). There was no clearance zone around control well containing water while zone of clearance increased with fermentation time and maximum clearance was observed in 72 h sample.

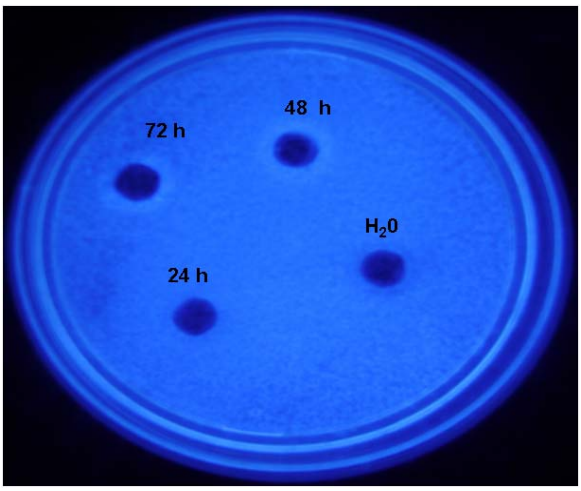

Figure 2.4.9: plate assay for chitinase

Various enzyme activities in the crude protease

Culture filtrate of *C. brefeldianus* exhibited various enzyme activities other than caseinolytic activity **(**Table 2.4.5). In general, alkaline proteases have broad substrate specificity and hydrolyze variety of natural as well as synthetic substrates. The activity of protease towards substrates like casein, BSA and haemoglobin was determined. The protease was able to degrade the above substrates to varying degrees. It was more active against casein compared to haemoglobin and BSA. These results are in agreement with other reports where the proteases from *Conidiobolus coronatus* strains are more active towards casein compared to BSA (Sutar *et al.,* 1991; Phadatare, 1991). In contrast, protease from *C. macrosporus* showed higher activity towards hemoglobin than casein (Tanksale, 2001). The crude enzyme preparation

exhibited azocaseinase, elastase, keratinase, azocollagenase, chondroitinase, chitinase, laminarinase activities.

Table 2.4.5: Various enzyme activities exhibited by crude culture filtrate

Enzyme activity	**U/ml**
Protease (with casein)	40.00
Protease (with hemoglobin)	32.98
Protease (with BSA)	21.48
Azocaseinase	40.00
Elastase	1.30
Keratinase	932.20
Azocollagenase	18.94
Chondroitinase	0.03
Chitinase	0.02
Laminarinase	1.46
True collagenase	Nil

Nil- Not detected

However, the preparation was devoid of true collagenase activity as evidenced by increased fluorescence of AEDANS chromophore of NMITLI-I substrate (Figure 2.4.10a) showing protease activity with unchanged fluorescence of AEDANS chromophore of collagen substrate (Figure 2.4.10b) indicating absence of collagenase.

Figures 2.4.10a: NMITLI substrate –I

Figures 2.4.10b: Collagenase substrate -I

This is very important for its application in leather processing because collagen forms the chief constituent of leather and its destruction has adverse impact on the leather quality (Choudhary *et al.*, 2004). On the other hand, activities like elastase, keratinase and chondroitinase are desirable in leather processing. Archana and Pillai (2012) summarized characteristics of several dehairing proteases from different sources and found that majority of them were showing keratinase activity but lacked collagenase activity. They further pointed that most of researchers have not paid attention to elastase activity in their dehairing preparations. Another such unattended activity in dehairing preparation is chondroitinase, probably because it does not directly involve in dehairing. In addition, majority of these enzymes also have several other applications. Elastase is used in food, pharmaceuticals and cosmetics industries (Qihe *et al*., 2007). Keratinase is useful in cosmetics, medicine, sewage cleaning aids and in the production of fertilizer and poultry food from feather meal. Keratinases are also finding applications in treatment of mad cow disease (degradation of prion) and biodegradable plastic (Gupta and Ramnani, 2006). Multiple chondroitinase (ABC) finds application in articular cartilage tissue engineering to increase construct tensile properties (Natoli *et al*., 2009) and successfully used for the treatment of spinal cord injury in rats (Alias *et al*., 2008). Although, chitinase in fungi is thought to have autolytic, nutritional and morphogenetic role, they can be used for the production of chitooligosaccharides, which have been found to function as antibacterial agents, elicitors of lysozyme inducers, and immunoenhancers (Dahiya *et al*., 2006). Enzymes like chitinase and ß-1, 3 glucanase (Laminarinase) are gaining lot of interest due to their potential in controlling various crop pests (Chavan, 2009). Gabriel (1968) screened several entomopthorous fungi for chitinase activity by plate assay method and found no chitinase production in presence of glucose while *C. brefeldianus* secretes chitinase in yeast extract medium containing acid swollen chitin with or without glucose. Mishra *et al*. (2011) studied chitinase production by three *Conidiobolus* and two *Basidiobolus* strains with colloidal chitin as a carbon source. All of them secreted chitinase which ranged from 0.19 to 0.26 IU/ml (expressed as μM of NAG liberated per hour).

Production of lipase by C. brefeldianus and its properties

Lipases catalyze the hydrolysis of esters, especially long chain triacylglycerols, to di- and mono-acylglycerols, glycerol and free fatty acids. They also catalyze the reverse reactions like esterification, trans-esterification (acidolysis, interesterification, alcoholysis), aminolysis, oximolysis and thio-transesterification in anhydrous organic solvents (Sharma *et al.*, 2011; Mahadik, 2007).

Lipases are not only physiologically important for human beings, but their versatile catalytic properties also make them ideal biocatalyst for potential applications in medicine, detergent, food, dairy, paper and pulp and leather industry (Singh and Mukhopadyay, 2012; Houde *et al*., 2004). Lipases from bacteria and fungi are extensively studied and commercially exploited. Fungi are considered to be best source of lipase and preferred for applications in food industry (Schmid and Verger, 1998).

Screening by plate assay method

Lipase secretion by *C. brefeldianus* was initially screened by plate assay on Mikami agar containing 1% emulsified tributyrin. Twenty-four hours grown culture in GYE medium was spot inoculated at the center of the plate and incubated at 28°C for 3 days. A zone of clearance around the fungal colonies was observed due to hydrolysis of tributyrin, indicating secretion of lipase by the culture (Figure 2.4.11).

Figure 2.4.11: Plate assay for lipase activity

Okafor *et al.* (1987) screened several isolates of *Basidiobolus* and *Conidiobolus* by plate assay for various hydrolytic activities including protease and lipase and reported that all the strains tested produced protease and lipase. However, they did not study production of lipase in submerged fermentation.

Production of lipase by submerged fermentation

Since the plate assay indicated that the fungus was capable of secreting lipase, its production by submerged fermentation was investigated. Lipase production was carried out in Mikami medium containing 1% soyabean oil, olive oil and tributyrin as inducers. Culture grown in GYE medium for 24 h was used for inoculating the experimental flasks. Lipase production was observed with all the inducers (Figure 2.4.12). Lipase activities in 72 h were highest in olive (0.75 IU/ml) followed by SBM oil (0.66 U/ml). However, tributyrin was found gave lower activities of 0.16 U/ml. There was sharp decline in lipase activity after 96 h for olive oil, while activity with SBM oil dropped to 0.5 U/ml.

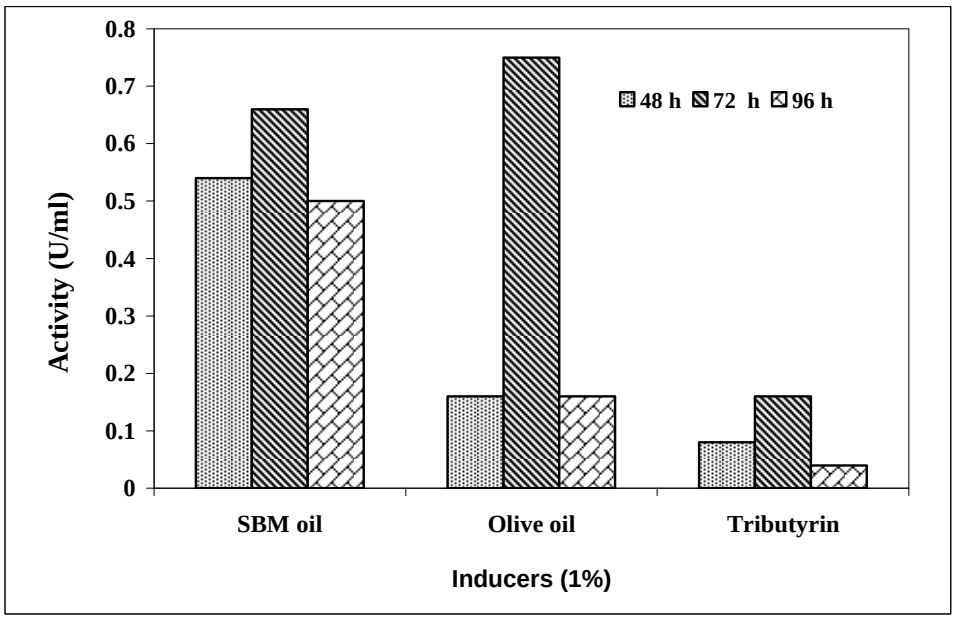

Figure 2.4.12: Lipase production with different inducers

Among fungi, *R. oryzae* (Minning *et al.*, 2001) *R. oligosporu* (Iftikhar *et al.*, 2010), *A. niger* (Kamini *et al.*, 1998) *A. nidulans* (Mayordomo *et al.*, 2000) and *P. aurantiogriseum* (Lima *et al.*, 2003) *P. notatum* (Rehman *et al.*, 2011) are the known

sources of lipase which have been studied in detail. However, reports on lipases from strains belonging to the genus *Conidiobolus* and *Basidiobolus* are limited and mostly confined to reports on screening by plate assay (Gabriel, 1968; Okafor *et al.*, 1987). Okafor and Gugnani (1990) reported lipase production by two isolates of *Basidiobolus, B. haptosporus* (ATCC 36600) and *B. ranarum* (MHB_1), and one isolate of *C. coronatus* (C_1) were grown in modified Sierra medium. They reported detectable lipase activites only after 96 h which reached maximum after 12 days. The present isolate secretes lipase in 2-4 days with a peak at 3rd day.

Effect of pH on lipase activity

For determination of optimum pH for lipase activity, assay was performed at 50°C and pH ranging from 4-9. Buffers used were acetate (pH 4, 5), phosphate (pH 6, 7), Tris-HCl (pH 8) and carbonate bicarbonate (pH 9). The lipase was active in the pH range 6 to 9 with an optimum at pH 7 (Figure 2.4.13). Fungal lipases are optimally active in the pH range of 4-8 (Sharma *et al.*, 2011).

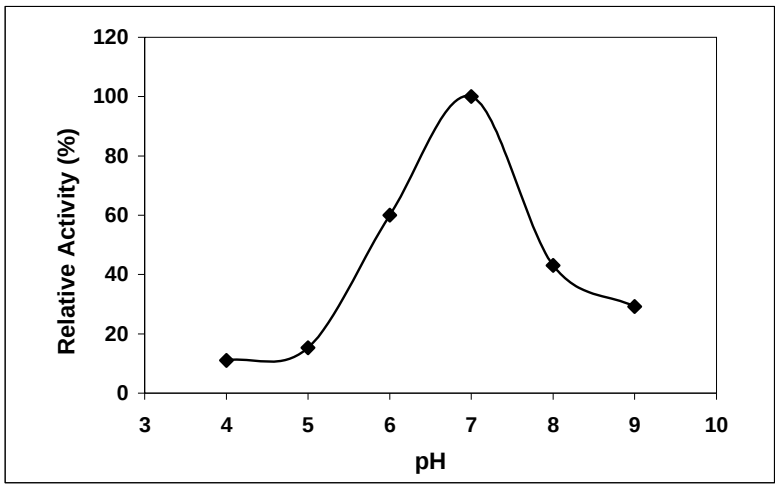

Figure 2.4.13: Optimum pH of lipase

Effect of temperature on lipase activity

For determination of optimum temperature for lipase activity, assay was performed at pH 7 and temperatures ranging from 30 to 60°C. The enzyme was active in the temperature range of 40 to 60°C with an optimum at 50°C Figure 2.4.14). Most fungal lipases are optimally active in the temperature range of 25-40°C while very few fungal lipases exhibit temperature optima above 40°C (Razak *et al*. (1997). However

lipases from some thermophilic fungi have temperature optima between 45-75°C (Sharma *et al*., 2011).

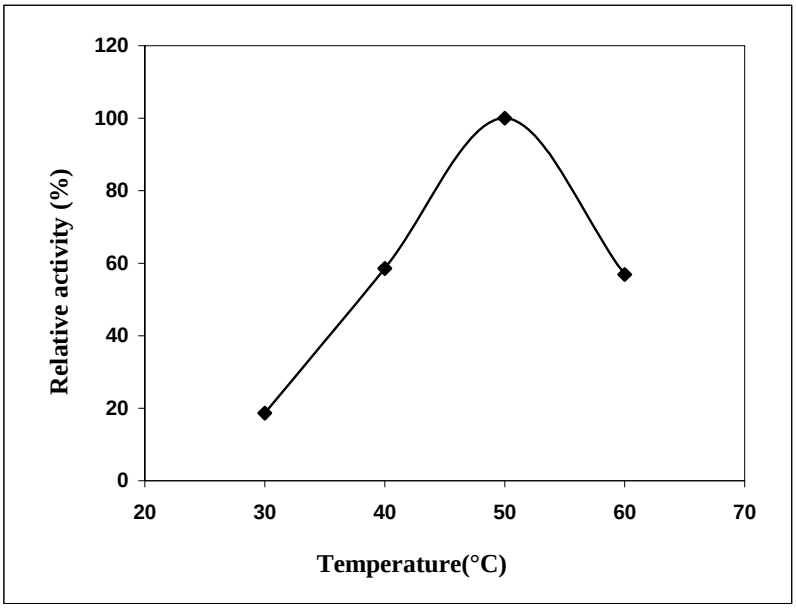

Figure 2.4.14: Optimum temperature for lipase

The present lipase differs from that of *C. coronatus* lipase reported earlier with respect to pH and temperature optima, which was optimally active at pH 5 and 35°C (Okafor and Gugnani, 1990). *C. brefeldianus* lipase active over a broader temperature range and neutral pH makes it useful for applications in leather industry apart from its applications where reactions at high temperature are carried out.

Conclusions

C. brefeldianus secretes an array of enzymes, alkaline protease being the major among them. *C. brefeldianus* alkaline protease is optimally active at pH 9 and 50°C. The protease exhibits stability over broad pH range of 4 to 12 and retained more than 60% activity even after 2 h at 40°C. Except Cu^{2+}, Hg^{2+}, Ni^{2+} and Zn^{2+} which inhibited the protease by 87, 97, 35 and 40% respectively, the enzyme was stable in presence of most of the metals. The protease was stable in presence of nonionic detergents and EDTA. The enzyme retained nearly 80% of its activity in presence of denaturants like BME and 4 M urea and 43% in 1% SDS. Except in presence of butanol, protease was stable and showed more than 90% residual activity at 28°C after 5 h. In presence of butanol, residual activity was 75%. Protease showed highest stability at 37°C for 1 h in presence of acetone (81.10%) followed by DMSO (68.94%) and methanol (59.45%), while more than 30% activity retained in presence of rest of the solvents. These activities are much lower than control indicating inactivation of enzyme in presence of organic solvents. *C. brefeldianus* protease showed stability and compatibility in presence of all the commercial detergents tested and retained 80-96% activity after 15 min and 50-70% activity even after 1 h depending on the detergent. The protease also effectively removed blood clots and stains from cotton cloth at ambient temperature. This property makes it suitable as a potential ingredient in detergent formulations. *C. brefeldianus* protease completely removed gelatin layer from the waste X- ray film within 10 min.

The crude protease preparation showed other useful enzyme activities viz. chondroitinase, chitinase, elastase, keratinase and laminarinase without true collagenase activity. Hence, this crude protease preparation with above enzyme activities is beneficial and makes it attractive for use in leather processing. The culture also produces extracellular lipase optimally active at pH 7 and 50°C within 48-72 h which may find applications in leather processing and detergent formulation.

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CHAPTER 3

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF PROTEASE

Abstract

C. brefeldianus secretes two proteases out of which the major protease was purified to homogeneity by salt precipitation and concentration, ion exchange and gel filtration chromatographic methods. There was 3.37 folds purification with a specific activity of 156 IU/mg and 54% yield. The molecular weight of the purified protease was found to be 30.19 kDa, 28.1 kDa and 27.8 kDa by SDS PAGE, gel filtration chromatography and MALDI-TOF respectively. Protease had an isoelectric point of 9.74. Protease was optimally active pH 9.0 and 50°C. The K_m and V_{max} of the protease were 2.5 mg/ml and 15.38 IU/ml respectively. Total inhibition of the protease was observed in presence of phenylmethylsulphonyl fluoride (PMSF), while L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) and *N*-tosyl-L-lysine chloromethyl ketone (TLCK) partially inhibited the enzyme. Protease was active in presence of ethylene diamine tetra acetic acid (EDTA), iodoacetate, benzamidine and soyabean trypsin inhibitor (SBTI). The protease was stable in most of the metal ions tested except Hg^{+2} and Cu^{2} which inhibited completely and by 31.8% respectively**.** The protease showed highest activity with casein followed by hemoglobin and BSA. The protease was highly active towards Succ-Ala-Ala-Pro-Leu-pNA, followed by Succ-Ala-Ala-Pro-Phe-pNA, N-Bez-Arg-pNA and Succ-Ala-Ala-Ala-pNA in the order of catalytic efficiencies while it was inactive against Gly-Phe-pNA. Protease also showed esterase activity and hydrolyzed BAEE and BTEE. MALDI-TOF analysis of peptides obtained after hydrolysis of oxidized insulin β chain by the protease showed five major and three minor sites of cleavage. The amino acid modifier, phenylmethylsulphonyl fluoride (PMSF), diethyl pyrocarbonate (DEPC), Woodward's reagent (WRK) and N-bromosuccinimide (NBS) inhibited the protease, indicating the involvement of serine, histidine and aspartic acids for the activity of protease. Incubation of protease with casein prior to addition of PMSF offered protection against inhibition indicating the presence serine residue in the active site. Successive addition of NBS to the protease brought about progressive decrease in absorbance at 280 nm and simultaneous loss in activity and the number of tryptophan residues oxidized per mole of the enzyme was found to be 2.7. The sites of attack were identical to those of subtilisins but differed from site of attack for trypsin indicating it to be a subtilisin like serine protease.

Introduction

Most of the commercially available proteases are crude preparations from diverse sources including animal (rennin), plant (papain) and microbial (Alcalase, Neu, Degumase) and are widely exploited in food, pharmaceutical, leather and detergent processes. Purification of alkaline protease is important for studying the properties and for better understanding of the functioning of the enzyme for exploitation in wider applications (Kumar and Takagi, 1999). Proteases like trypsin, chymotrypsin, protenase K etc. are available in purified form and utilized as biotechnological tools in molecular studies. Purified proteases are expensive due to requirements of high cost inputs in purification processes and low yield. Extracellular proteases have been purified from various microbial sources including bacteria, fungi and actinomycete using variety of methods (Gupta *et al.,* 2002a; Marco and Felix, 2002; Wang *et al.,* 2005; Tatineni *et al*., 2007; Balchandran *et al.,* 2012). Majority of the procedures involve concentration of the culture filtrate either using salt or solvent precipitation or by ultrafiltration. The concentrated enzyme is then subjected to variety of chromatographic procedures such as ion exchange chromatography (cationic or anionic using CM-cellulose, DEAE-cellulose, phenyl-sepaharose), affinity chromatography (using matrix bound to inhibitor or substrate or CNBr-activated sepharose) and gel filtration chromatography (using sephadex or Biogel matrix). Apart from these conventional procedures, others such as fast protein liquid chromatography (FPLC) (Wasko *et al.,* 2012), crystallization (Park *et al.,* 1997) and preparative PAGE (Tanksale, 2001) have been used for the purification of the proteases. Many times more than one method is required to obtain homogeneous preparion. Methods applied for the purification of fungal proteases and their biochemical characteristics are summarized in Table 3.1.

Alkaline proteases are also characterized by their substrate selectivity, and utility of these enzymes depend often on their substrate specificity. In general, alkaline proteases have broad substrate specificity and hydrolyze variety of natural as well as synthetic substrates. Natural substrates include keratin, gelatin, casein, albumin collagen, elastin etc. Specific types of alkaline proteases such as collagenase, elastase, keratinase and insect cuticle degrading proteases degrade collagen, elastin, keratin and insect cuticle respectively.

Table 3.1: Strategies adopted for purification of fungal alkaline proteases and properties of the proteases

Organism	MW (kDa)	pI	Purification steps	Opt pH	Opt Temp (°C)	Inhibitors	References
A. clavatus	32	-	Solvent precipitation, Sephadex G-100, CM Sepharose.	8.5	50	PMSF	Hajji *et al.* (2007)
A. clavatus	35	-	Salt precipitation (40-75%), Sephadex G-75,	9.5	40	PMSF, Chymostatin	Tremacoldi *et al.* (2007)
A. fumigatus TKU003	124	8.3	DEAE-Sepharose CL-6B chromatography	8	40	PMSF	Wang *et al*. (2005)
A. niger	38	-	Salt precipitation (60-75%), DEAE-cellulose chromatography.	10	50	EDTA	Devi *et al.* (2008)
B. bassiana	35	-	Salt precipitation (60-75%), Bio-Gel P-60 followed by Bio-Gel P-10 column.	8.5	37	PMSF	Bidochka and Khachatourians, (1987)
Beauveria sp.	29	9.3	Salt precipitation (40-70%), DEAE-cellulose chromatography.	9	50	PMSF	Shankar *et al*. (2011)
Dactylellina varietas	30	-	Cation exchange and hydrophobic interaction chromatography	8	60.5	PMSF partially inhibited by EDTA	Yang *et al.* (2007)
Ophiostoma piceae	33	5.6	Ammonium sulphate precipitation; preparative hydropbhobic interaction chromatography	7-9	40	PMSF, EDTA	Abraham and Breuil (1996)
T. harzianum	18.8	-	Ammonium sulphate precipitation, phenyl sepharose	8	37	-	Marko and Fleix (2002)

Table 3.1: Strategies adopted for purification of fungal alkaline proteases and properties of the proteases

continued…..

Organism	MW (kDa)	pI	Purification steps	Opt pH	Opt Temp (°C)	Inhibitors	References
C. coronatus NCL 86.8.20 (Protrease 1)	23.17	9.9	Salt precipitation (0-90%), preparative PAGE, CM cellulose chromatography	9.7	40	PMSF	Phadatare, 1991
C. coronatus NCL 86.8.20 (Protrease 2)	19.27	9.0	Salt precipitation (0-90%), preparative PAGE, CM cellulose chromatography	9.7	40	PMSF	Phadatare, 1991
C. coronatus NCIM 1238 (small)	6.8	8.5	Alcohol precipitation, DEAE-cellulose chromatography, ammonium sulphate precipitation and preparative PAGE	9.7	45	PMSF	Sutar *et al.* (1991)
C. coronatus NCIM 1238 (large)	28.5	9.2	Alcohol precipitation, DEAE-cellulose chromatography, salt precipitation, preparative PAGE, CM-cellulose chromatography, sephadex G-50 column	10	45	PMSF	Ghadge (1986)
C. macrosporus NCIM 1298	28.5	9.8	Ultrafiltration, preparative PAGE, DEAE-cellulose chromatography	10	40	PMSF	Tanksale (2001)
C. coronatus	30		Membrane concentration, Sephadex G-75, HPLC ion exchange	8.3		PMSF, Chymostatin, AMCI-1 SBTI	Bania *et al.* (2006)

Requirement of specific amino acid on substrate for catalytic activity of protease can be determined using synthetic substrates, a wide variety of synthetic substrates are now available for rapid and reliable in vitro assay. Cleavage specificity of proteases can also be investigated by using oxidized insulin β-chain as peptide substrate. The hydrolysates of insulin β-chain formed due to peptide bond cleavage are analyzed for their molecular masses using HPLC or LC-MS/MSD or MALDI TOF (Kobayashi *et al.,* 2007; Kervinen *et al.,* 2008; Donoghue *et al.,* 2008).

Active site directed chemical modification is an important tool for identifying the residues at the active site of an enzyme responsible for catalytic activity. Presently, site directed mutagenesis is widely employed for this purpose, which requires the knowledge of three-dimensional structure of protein. In contrast, the active-site directed chemical modification is simpler because it does not require the information of three dimensional structure of an enzyme.

Separation of microbial biomass from the fermented broth is prerequisite in downstream processing and further purification of the enzyme. Fungal proteases offer additional advantage of cost effective downstream processing required for large-scale production as fungal mycelia can be easily be separated by simple filtration to obtain culture free broth, (Andrade, 2002). The literature survey on purification of alkaline protease from fungal sources revealed that there are no set rules for the purification of proteases to homogeneity and methods have to be standardized for the individual fungal proteases.

The present chapter deals with the purification and biochemical characterization of *C. brefeldianus* protease. Chemical modification and substrate specificity studies of the protease are also described in this chapter.

Materials and methods

Materials

DEAE cellulose, Sephadex G-100, alcohol dehydrogenase, bovine serum albumin (BSA), ovalbumin, carbonic anhydrase, cytochrome C, Bradford reagent, SDS, N, N, N', N' Tetramethylethylenediamine (TEMED), Commassie Brilliant Blue R-250, synthetic substrates (Succ-Ala-Ala-Pro-Leu-pNA, Succ-Ala-Ala-Pro-Phe-pNA, Succ-Ala-Ala-Ala-pNA, Gly-Phe-pNA, N-Bez-Arg-pNA, BAEE, BTEE), oxidized insulin β-chain, trifluoroacetic acid (TFA), sinapinic acid, β-mercaptoethanol (BME), N,N,N'N'-Tetramethyl ethylene diamine (TEMED), PMSF, Benzamidine, TLCK, TPCK, WRK, phenylglyoxal, diethyl pyrocarbonate (DEPC), NBS, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), iodoacetate, SBTI, were obtained from M/s Sigma Chemical Co. USA. Molecular weight markers for SDS PAGE were procured from M/s BioRad, India. Ammonium sulphate, acrylamide, bis acrylamide, silver nitrate were purchased from M/s Qualigen Chemicals, India. Casein was purchased from M/s Sisco research Laboratories, India. All other chemicals used were of analytical grade.

Methods

Enzyme production

Protease used for purification was produced in GDAP + 3% SBM medium as described in Chapter 2. The mycelium was removed by filtration followed by centrifugation at 7000 rpm at 4°C to obtain a clear supernatant which was used as source of protease.

Protease assay

Protease activity using casein as substrates was estimated as described in Chapter 2.

Protein assay

Protein was estimated using commassie brilliant blue as described by Bradford (1976).

All the purification steps were carried out at 4°C unless otherwise mentioned.

Fractional ammonium sulphate precipitation

The clear supernatant obtained after centrifugation was subjected to fractional ammonium sulphate precipitation (20% increments) with constant stirring. After complete dissolution of ammonium sulphate, the suspension was allowed to stand in cold overnight for complete precipitation. The pellet from each fraction (10,000 rpm, 20 min) was suspended in deionized water and the supernatant was used for the next fractionation step. Protease activity in each fraction was estimated. The 50-80 ammonium sulphate precipitate was dissolved in minimum volume of deionized water and dialyzed (10 kDa cut off) overnight against deionized water with two changes. Increase in volume after dialysis was noted and necessary corrections were made. The dialyzed enzyme concentrate was stored at -20°C until further use.

Ion exchange chromatography

Dialyzed ammonium sulphate fraction (approximately 22 mg protein) was loaded on a DEAE-cellulose column (2.5 x 25 cm) previously equilibrated with 0.05M phosphate buffer, pH 7. The column was eluted with same buffer at a flow rate of 20 ml/h and 3 ml fractions were collected. Fractions showing protease activity were pooled and concentrated.

Gel filtration on Sephadex G-100 column

Pooled and concentrated sample from DEAE cellulose chromatography (2.5 mg protein) was loaded on Sephadex G-100 column (1.2 x 150 cm) pre-equilibrated with 0.05M phosphate buffer, pH 7.0. The column was eluted with 0.05M phosphate buffer, pH 7.0 at a flow rate of 12 ml/ h. Initially 30 ml fraction was eluted and collected separately after which 2 ml fractions were collected. Protease and protein in the fractions were estimated.

Native PAGE

Cationic PAGE was run with 10 % separating gel (pH 4.3) and 6% stacking gel (pH 6.8) according to Laemmli (1970). Electrophoresis was carried out at 100 V with 20 mA current with basic fuchsin as a tracking dye. After electrophoresis, gel was subjected to silver staining to visualize protein bands (Morrissey, 1981).

Gelatin zymography of protease

Gelatin zymography was performed as described above in native PAGE with few changes. Gelatin (5 mg/ml final concentration) was co-polymerized with 12% acrylamide in the separating gel. Spacer gel contained 5% acrylamide without gelatin. The gel was cast in a Tarson minigel unit. Protease samples were loaded into the wells. Electrophoresis was performed at 4°C to avoid heat inactivation of the enzyme during electrophoresis. After completion of electrophoresis, gel was carefully detached from the plates and immersed in adequate amount of 0.1M carbonate bicarbonate buffer pH, 9. Gel was incubated at 40°C for 20 min with intermittent shaking to activate the enzyme. The gel was stained overnight with 0.5% commassie brilliant blue stain and destained with destaining solution (glacial acetic acid: ethanol: distilled water in the ratio of 5:25:70) with repeated washes.

Molecular weight determination by SDS PAGE

Molecular weight of purified protease was estimated by their migration in SDS polyacrylamide gel as described by Laemmli (1970). The SDS treated protease was loaded on 12% SDS gel and electrphoresis carried out with 20 mA for 3 h. Low molecular weight markers for SDS PAGE were used as reference proteins. The protein bands were visualized by silver staining method and Rf values were calculated. A plot of log molecular weight versus relative mobility of standard proteins was used to estimate molecular weight of protease.

Molecular weight determination by gel filtration

Molecular weight of purified alkaline protease was determined by the molecular sieve chromatography method of Andrews (1964) with Sephadex G-100. The column was equlibated and eluted as described earlier with a flow rate of 12 ml/ h. Void volume (V_0) of the column was estimated with blue dextran (molecular weight approx 2,000,000). The column was calibrated with marker proteins viz. bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), soyabean trypsin inhibitor (20,300), cytochrome C (12,400). Two milligrams of each marker and purified protease (445 µg) were dissolved in 1 ml of 0.05M sodium phosphate buffer, pH 7 and applied to the column. Fractions of 2.0 ml each were collected and the proteins were detected in the effluent by measuring the absorbance at 280 nm. The elution volume (Ve) for each protein was calculated from mid point of peak absorbance value.

Matrix assisted laser desorption ionization–Time of flight (MALDI-TOF)

The molecular mass of purified protease was also determined by matrix-assisted laser desorption ionization-time of flight (MALDI–TOF) mass spectrometry using a Voyager DE-STR (Applied Biosystems) equipped with a 337 nm nitrogen laser. 5 µl of the purified enzyme (3 µg) was mixed with 20 µl of sinapinic acid. Two micro litter of the mixture was spotted on MALDI target plate and dried in oven at 37°C and analyzed.

Isoelectric focusing

Isoelectric point of the protease was determined by the modified native slab gel method as described in 'Protein Methods' using ampholines in the narrow pH range of 8-10. Gel slab was caste in Tarson mini gel unit by mixing 6% polyacrylamide gel according to Laemmli (1970). Purified enzyme (1.2 µg) was loaded in each well. One well was left empty as control for checking the pH gradient. Electrophoresis was carried out initially at 150 V for one and half-hour and then at 300 V for one and half-hour subsequently. Electrophoresis was terminated when current flow in the gel was stopped. Control gel was cut into 0.5 cm slices, each slice was crushed and suspended in 1.5 ml of deionized water and pH was measured. Similarly, one part of gel was cut into 0.5 cm slices and used for protease assay while the second part of gel was stained by silver staining to visualize the protein bands.

Determination of optimum pH

Optimum pH of purified protease was determined by estimating the protease activity at 50°C and pH values ranging from 5 to 12. Following buffers at 0.1 M concentration were used: sodium acetate (pH 5), sodium phosphate (pH 6 and 7), Tris-HCl (pH 8), carbonate bicarbonate (pH 9 and 10) and sodium phosphate- NaOH (pH 11 and 12) and expressed as percentage of maximum activity taken as 100%.

Determination of optimum temperature

Optimum temperature of purified protease was determined by estimating the protease activity at pH 9 and temperatures ranging from 30 to 70ºC for 10 min and expressed as percentage of maximum activity taken as 100%.

Determination of Michaelis -Menten constant (Km)

K_m and V_{max} values of the pure enzyme were determined by measuring the activity with casein concentrations ranging from 1 to 10 mg. Kinetic constants were calculated from Lineweaver-Burk plots.

Effect of protease inhibitors and chelators

To determine the nature and type of protease, purified enzyme (16-17.8 µg) was pre-incubated at 28°C for 30 min with following inhibitors: PMSF, TPCK, TLCK, EDTA, iodoacetic acid, benzamidine hydrochloride and SBTI and residual activity was estimated at 50°C, pH 9. Activity of control without inhibitor was taken as 100%.

Effect of metal ions

Purified protease (5 µg) was incubated with 0.01M Ca, Cd, Co, Cu, Fe, Hg, K, Zn, Ag (added as chlorides) at 28°C for 30 min and the residual protease activity was measured as described earlier and expressed as percentage of activity without metal taken as 100%.

Activity of protease towards natural substrates

Activity of purified protease was estimated by using various natural substrates including casein, hemoglobin and bovine serum albumin. The reaction mixture contained an aliquot of suitably diluted protease enzyme and 10 mg substrate in 0.1 M sodium carbonate buffer pH 9 in a total volume of 2 ml. After incubation at 50°C for 10 min, the reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid (acidified with concentrated hydrochloric acid). The precipitate formed was filtered through Whatman No.1 filter paper after standing at room temperature for 30 min. The absorbance of acid soluble fraction was measured at 280 nm. One unit of activity is defined as the amount of enzyme liberating one micromole of tyrosine per min.

Protease activity towards synthetic peptide substrates

Activity of the protease towards different p-nitroanilides was tested with the following substrates: N-Benz-DL Arg–pNA (Bz-Arg-pNA), N-succinyl-Ala-Ala-Ala-pNa (Succ-Ala-Ala-Ala-pNA), N-succinyl-Ala-Ala-Pro-Leu-pNA (Succ-Ala-Ala-Pro-Leu-pNA), N-succinyl-Ala-Ala-Pro-Phe-pNA (Succ-Ala-Ala-Pro-Phe-pNA) and Glycine-Phe-pNA (GF). The assay mixture contained 0.16 ml of 3 mM substrate, 0.5 ml of 0.05 M

carbonate bicarbonate buffer, pH 9, 0.1 ml of enzyme and incubated at 50°C for 30 min. Assay was terminated by addition of 1M Na_2CO_3. A suitable blank without enzyme was also carried out. Absorbance of the reaction mixture was measured at 410 nm. One unit of enzyme activity is defined as the amount of enzyme required to increase the absorbance by 0.01 in one min.

Protease activity towards esters

Esterase activity of protease with BAEE and BTEE was estimated spectrophotometrically. The assay mixture contained 1.5 ml of 0.05 M Tris-HCl buffer, pH-8 and 1.4 ml of 1 mM substrate. Blank and test samples were placed in reference and sample cuvettes respectively and set to zero. Hundred micro liters of deionized water and purified protease (6 µg) were added to blank and test cuvettes respectively and mixed well. Increase in absorbance at 247 nm (BAEE) and 256 nm (BTEE) at regular intervals of 10 seconds was recorded up to 5 minutes. A graph was plotted with absorbance against time and a slope was obtained from the linear portion of the graph. One unit of activity is defined as the amount of enzyme causing an increase in the absorbance of 0.001 per min.

Hydrolysis of oxidized insulin ß-chain and analysis of the products

The cleavage specificity of *C. brefeldianus* protease was determined by analysis of the peptide fragments obtained after incubation with the oxidized insulin ß-chain as substrate. Oxidized insulin ß-chain (1 mg) in 0.01 M carbonate bicarbonate buffer, pH 9 was incubated with 20µl (1µg) of purified protease in a total volume of 1ml at 50°C. Samples (100 µl) were withdrawn at regular time intervals and 5 µl of 0.1% tri-fluro acetic acid (TFA) was added immediately to terminate the reaction. The products of hydrolysis were analyzed by MALDI-TOF. Five micro liters of the digest was mixed with 20 µl of matrix containing 30% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and 1.0 % (w/v) sinapinic acid. One microlitre samples were spotted onto a stainless steel target MALDI plate and air-dried before analysis in the mass spectrometer. Identification of the cleavage products was performed on a (MALDI-TOF) mass spectrometer (AB SCIEX). The cleavage sites were defined by using a computer program, FindPept (http://ca.expasy.org/tools/findpept/), which enables peptide identification after protein cleavage based on the experimentally determined size of the products.

Chemical modification studies

Amino acids were modified with following chemicals: serine with PMSF, aspartic acid with WRK, arginine with phenylglyoxal, histidine with DEPC, tryptophan with NBS and cysteine with DTNB. WRK, NBS and DTNB were prepared in 0.05 M phosphate buffer, pH 7. PMSF, phenylglyoxal, DEPC were prepared in DMSO, methanol and absolute ethanol respectively. Purified protease in 0.05 M phosphate buffer, pH 7 was incubated with inhibitors in a final volume of 1 ml at 28°C, for 30 min. Residual activity was determined as described earlier. Protease incubated in absence of modifying reagents served as control.

Inhibition kinetics with PMSF

Purified protease (16 μg) was incubated with 2 to 8 μM PMSF at 28°C, for 20 min. Aliquots were removed at 5 min intervals and residual activity was determined. Protease sample incubated in the absence of PMSF served as the control.

Substrate protection studies

The protective effect of substrate on modification of serine was studied by pre-incubating enzyme (8.4 μg) with varying concentration of casein (1-10 mg), followed by addition of 4 μM PMSF and incubating at 28°C for 30 min. Residual activity was measured as described earlier. Protease without casein and without PMSF served as control. Another control where protease was incubated under identical conditions without casein but with PMSF was also included.

Quantitation of the tryptophan residues

Spectrophotometric titration of tryptophan by NBS was carried out in two cuvettes, one containing 1 ml protease (58 μg) in 0.05 M phosphate buffer, pH 7 and another containing only buffer. Five micro litters of 0.5 mM NBS (2.5 μM each time) was added to both the cuvettes in stepwise manner and decrease in absorbance at 280 nm was monitored. Similarly, one more protein sample was treated with NBS and residual protease activity was estimated. The number of tryptophan residues oxidized per mole of enzyme was calculated by the method of Spande and Witkop (1967).

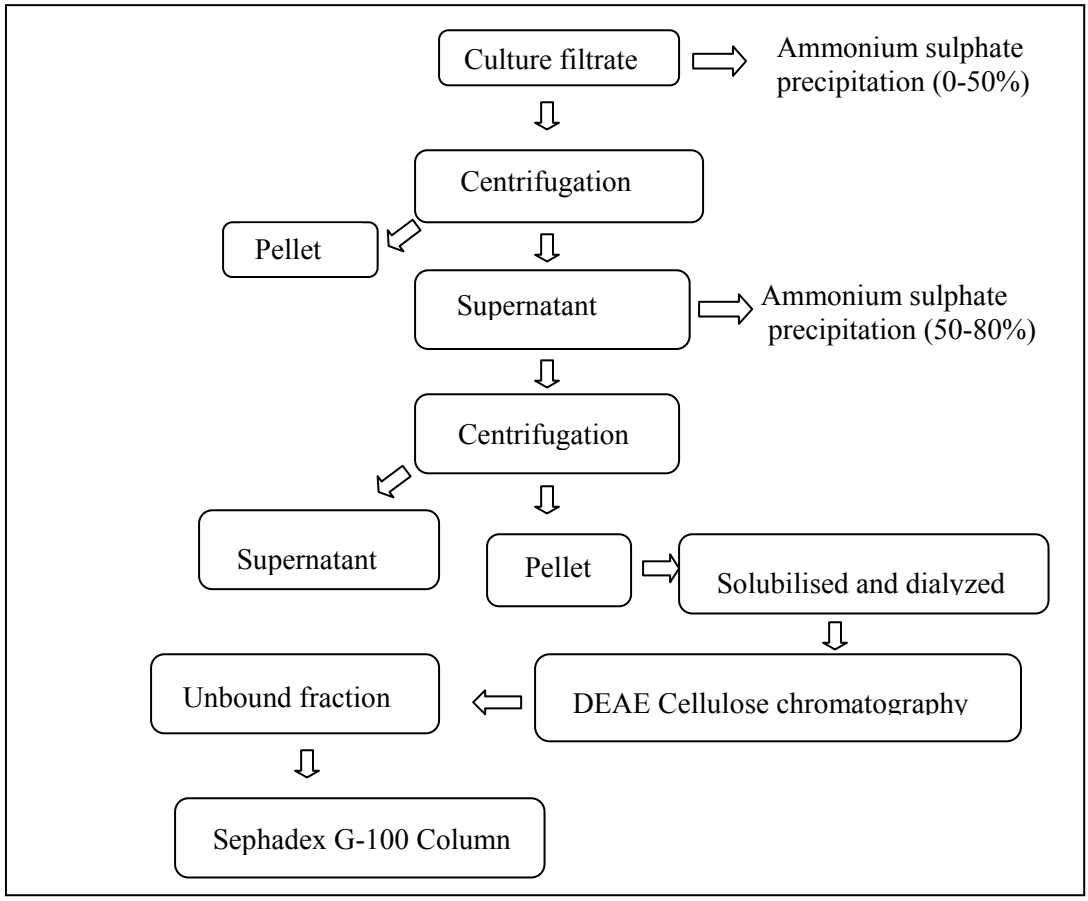
$$\Delta n = \frac{1.31 \times \Delta A280 \times M.W \times V}{5500 \times W}$$

Where ΔA280 is the decrease in absorbance at 280 nm, 1.31 is an empirical factor based upon oxidation of model tryptophan peptides by NBS (Patchornik *et al*., 1958), 5500 is the molar absorption extinction coefficient for tryptophan at 280 nm, M.W = Molecular weight of protein, V- Initial volume of titrated solution (ml), W- Weight of protein titrated (mg).

Results and discussion

Purification of C. brefeldianus protease

C. brefeldianus secretes two alkaline proteases in the culture filtrate. The steps involved in the purification of major alkaline protease from *C. brefeldianus* having higher mobility are shown in the flow chart below.



The clear supernatant obtained after centrifugation at 10000 rpm for 10 min, was initially subjected to fractional ammonium sulphate precipitation to precipitate the protease and remove other impurities. Protease activity in each fraction was measured and recovery calculated. More than 80%of protease activity was obtained in 60-80% fraction (Table 3.2). Therefore, in subsequent purification experiments, crude broth was precipitated at 0-50% to remove unwanted proteins and the supernatant was further precipitated at 50-80% to obtain partially purified protease.

Table 3.2: Protease recovery during fractional ammonium sulphate precipitation

Saturation (%)	Total units	Recovery (%)
0	1901.80	100
20-40	4.12	0.21
40-60	19.55	1.02
60-80	1662.80	87.43

The 50-80 ammonium sulphate precipitated fraction was dialyzed and further subjected to DEAE-cellulose column chromatography. The protease was not adsorbed on DEAE-cellulose and eluted out as unbound fractions. However some cationic proteins and coloured impurities were eliminated in this step. Unbound protease fractions from DEAE-cellulose column were pooled, concentrated, and further subjected to gel filtration chromatography on Sephadex G-100 matrix. Protein in the eluted fractions from Sephadex G-100 column was measured as absorbance at 280 nm. The elution profile on sephadex G-100 column revealed a non protease protein peak which eluted in initial fractions (Figure 3.1).

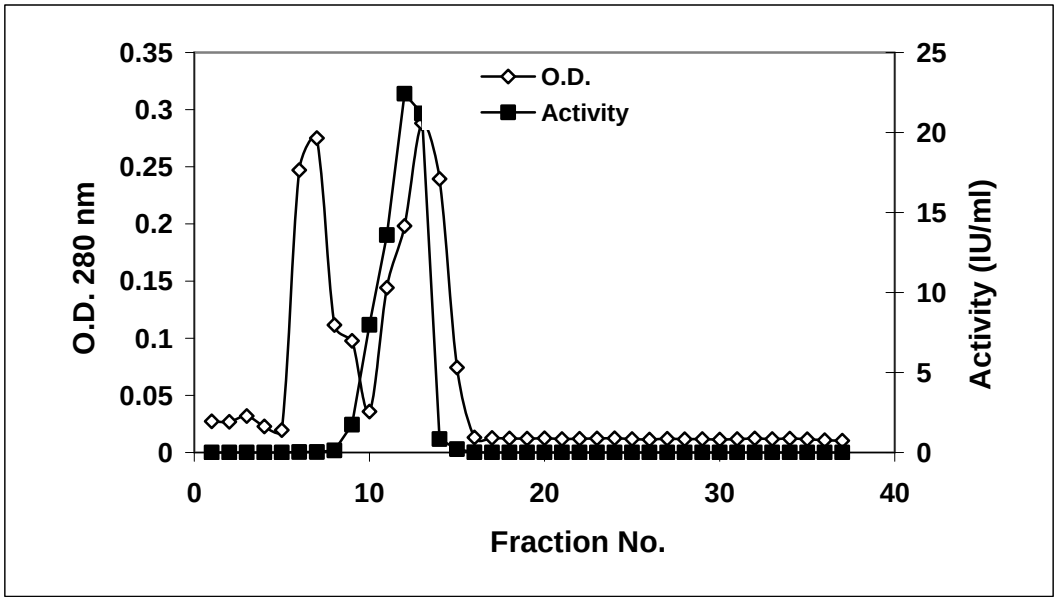


Figure 3.1: Elution profile of protease on Sephadex-G-100

Very minute protein was detected in the subsequent fraction before the onset of protease elution. Fractions showing higher absorbance were estimated for protease activity and protein content by Bradford method.

The results of the purification of *C. brefeldianus* protease are summarized in Table 3.3.

Table 3.3: Summary of purification of protease from *C. brefeldianus*

Step	Total Activity (IU)	Total Proteins (mg)	Specific activity (IU/mg)	Fold purification	Recovery (%)
Crude enzyme	3065.0	66.35	46.19	1	100
50-80% precipitation	2408.0	ND	ND	ND	78.0
50-80% precipitation (Dialyzed)	2151.5	32.04	67.15	1.45	70.2
DEAE-cellulose (Unbound fractions)	1812.0	20.35	89.04	1.93	59.1
Sephadex G-100	1654.1	10.63	156.0	3.37	54.0

ND: Not determined.

Specific activity (IU/mg protein) of the dialyzed 50-80% ammonium sulphate precipitated fraction increased from 46.19 to 67.15 (Table 3.3). There was 10-15% loss in activity on dialysis. Similar loss in activity was also observed during concentration of enzyme by membrane filtration (discussed in earlier chapter). After DEAE chromatographic step, specific activity and fold purification further increased to 89.04 and 1.93 respectively (Table 3.3). The protease was purified to homogeneity after gel filtration on Sephadex G-100 with 3.37 fold purification and specific activity of 156. Total yield was of 54% which is one of the highest reported for fungal proteases. It is interesting to note that though fold purification of *C. brefeldianus* protease is only 3.37, the yield and specific activity are considerably high, suggesting that the major protein portion of the culture filtrate is alkaline protease. Similar observations were made by Abraham and Breuil (1996) who reported purification of the

alkaline protease (which was the major protein in the culture filtrate) from sap staining fungus *Ophiostoma piceae* strain 387Np by hydrophobic interaction chromatography with 2.2 fold purification and 46% yield.

Proteases from *C. coronatus* NCL 86.8.20, NCIM 1238 and *C. macrosporus* were purifed by preparative polyacrylamide gel electrophoresis and chromatographic methods. Ghadge (1986) reported 11.4 fold purification of *C. coronatus* NCIM 1238 protease A with final yield of 9% in 5 step purification, while Sutar *et al.* (1991) achieved 12 fold purification of *C. coronatus* protease B with 3.8% yield in 6 step purification. Phadatare (1991) reported 17.3 &11.5 fold purification of Protease I and Protease II with 24 and 10.7% yield respectively in three step purification. *C. macrosporus* (NCIM 1298) was found to secrete five alkaline proteases as detected by the gel X-ray film contact print and one of them showing higher electrophoretic mobility was purified up to 7.3 fold with recovery of 1.92% and specific activity of 222 U/mg of protein in a 3 step purification (Tanksale 2001). Bania *et al.* (2006) achieved 28 fold purification of protease from *C. coronatus* using combination of ion exchange on HPLC and size exclusion chromatography with specific activity of 616 U/mg of protein (with synthetic substrate) and 45% yield. An alkaline protease from *A. clavatus* ES1 was purified up to 7.5 fold and with the yield of 29% by steps including solvent precipitation, size exclusion and ion exchange chromatography (Hajji *et al.,* 2007). Tremacoldi *et al.* (2007) reported 2 step purification of alkaline protease from *A. clavatus* with 4 fold purification and 22.1% recovery. Wang *et al*. (2005) reported 5.77 fold purification of *A. fumigatus* TKU003 protease with 25% recovery in a 2 step purification process. Devi *et al.* (2008) reported 34.4 fold purification with 32% recovery of *A. niger* protease in 2 step purification process. Bidochka and Khachatourians, (1987) reported 17.7 fold purification with 20.9% recovery of *B. bassiana* protease. In a 2 step purification method, Shankar *et al*. (2011) reported 10 fold purification and 38.6% yield of protease from *Beauveria* sp. Yang *et al.* (2007) reported purification of *Dactylellina varietas* protease by cation exchange and hydrophobic interaction chromatography and reported 26% yield and 2.3 fold purification. Marko and Fleix (2002) reported purification of *Trichoderma harzianum* 1051 protease with 27% yield and four folds purification.

Figure 3.2 shows the protein bands and zymography of protease samples at different stages of purification. The zymogram revealed presence of two proteases in the crude culture filtrate. Major and prominent one (brighter clearance band on the gel) having greater electrophoretic mobility was purified to homogeneity (Figure 3.2). In earlier studies *C. coronatus* strains also secreted two alkaline proteases (Sutar *et al*., 1991; Phadatare, 1991).

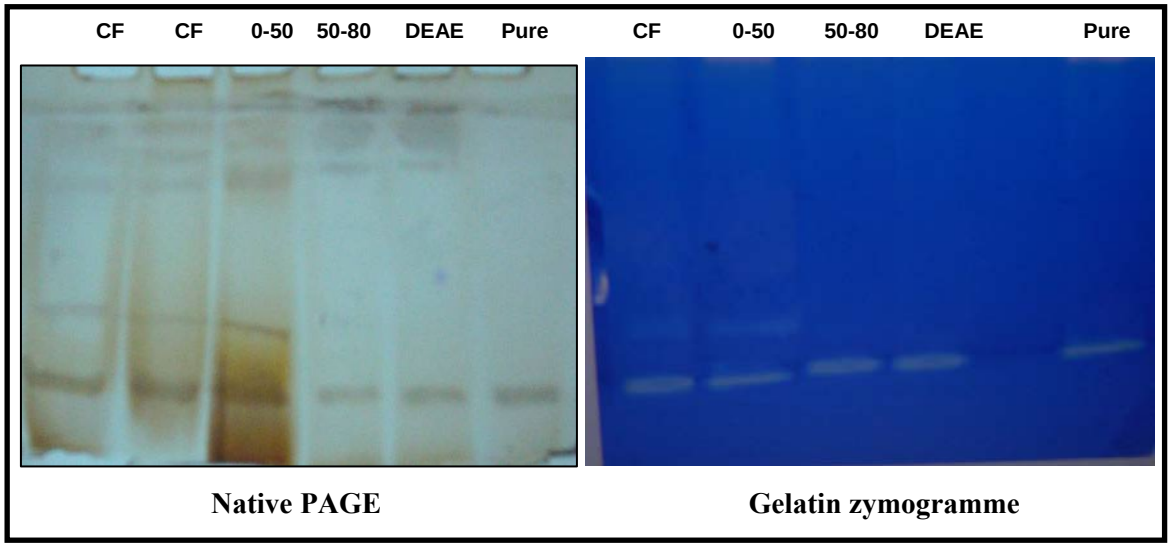


Figure 3.2: Electrophoretic and zymographic patterns of the samples during the course of purification

Molecular weight of protease on SDS PAGE

The molecular weight of purified protease by SDS-PAGE was found to be 30.19 kDa (Figure 3.3). This value is similar to the molecular weight of 30 to 32 kDa reported for the protease from *C. coronatus* (Bania *et al.,* 2006) and slightly higher than that of *C. macrosporus* protease which was 28.5 kDa on SDS PAGE (Tanksale, 2001). Molecular weights of protease I and protease II from *C. coronatus* NCL 86.8.20 by SDS PAGE were found to be 22.0 and 19.5 kDa respectively (Phadatare, 1991) while molecular weight of protease A from *C. coronatus* NCIM 1238 was 22.0 kDa (Sutar *et al*., 1991). An alkaline protease from *Penicillium expansum* was found to be 20.5 kDa (Dahot, 1994).

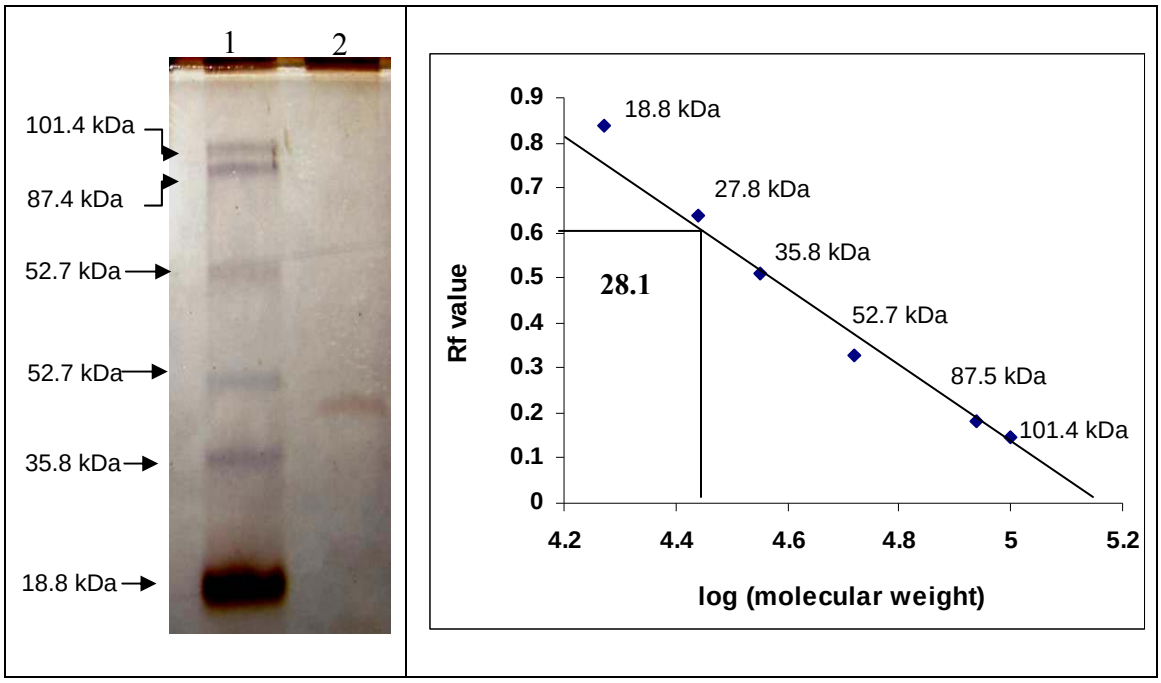


Figure 3.3: **SDS-PAGE of purified protease and the molecular weight markers**

Lane 1: molecular weight markers; lysozyme (18.8 kDa); soyabean trypsin inhibitor (27.9 kDa); carbonic anhydrase (35.9 kDa); ovalbumin (52.7 kDa); bovine serum albumin (87.5 kDa) and phosphorylase b (101.4 kDa). Lane 2: purified protease (4-5 µg).

The molecular mass range for serine alkaline protease generally falls in 18 to 35 kDa (Gupta *et al.*, 2002b). However, there are few exceptions where the molecular weights deviate from this range. Sutar *et al.* (1991) purified alkaline protease 'B' from *C. coronatus* NCIM 1238 strain having molecular mass of 6.8 kDa. Molecular weight of 63 kDa was reported for *A. tamarii* EF661565.1 alkaline protease (Sharma and De, 2011). The highest molecular weight of 124 kDa for fungal protease from *A. fumigates* TKU003 is reported by Wang *et al.* (2005).

Molecular weight determination by gel filtration chromatography

Molecular weight of purified protease was determined by the molecular sieve chromatography method on Sephadex G-100 column. The void volume (V_0) and the elution volume (V_e) for each protein was estimated as described in materials and methods. The plot

of V_e/V_0 against logarithm of molecular weight gave a straight line (Figure 3.4). The molecular weight of protease was found to be 28.1 kDa by this method.

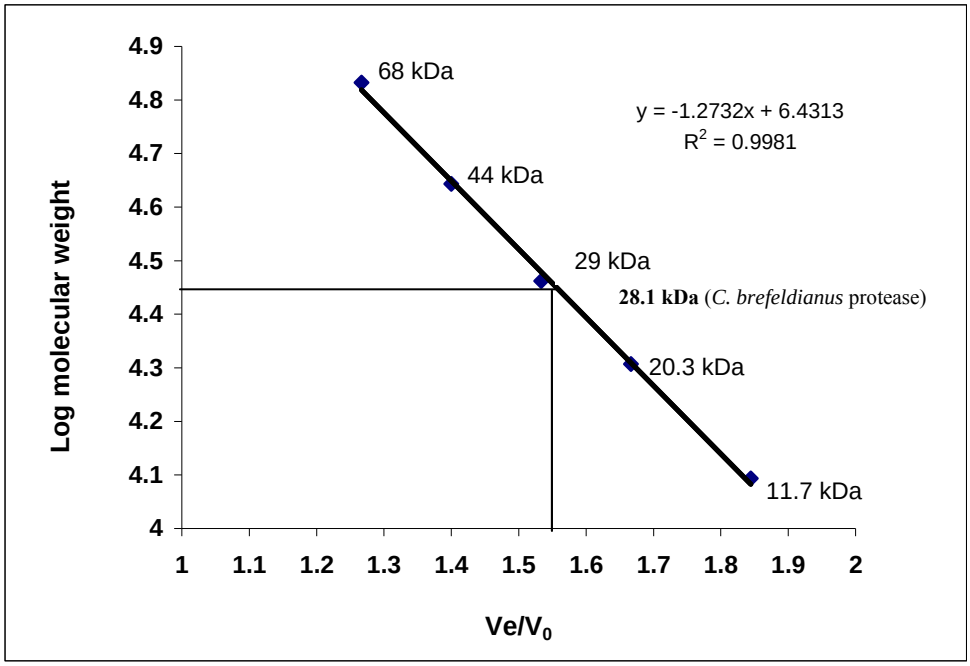


Figure 3.4: Molecular weight determination of protease by gel filtration

Molecular weight markers: bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), soyabean trypsin inhibitor (20300), cytochrome C (12,400).

The molecular weight of protein may slightly vary sometimes depending on the method used. This is more so when estimated by SDS PAGE due to minor differences in calculating exact Rf values. Molecular weights of *C. coronatus* NCL 82.1.1 protease A and B were 22 and 6.8 kDa from SDS PAGE, while by gel filtration chromatography they were 21.88 and 6.0 kDa respectively (Ghadge, 1986; Sutar, 1987). Similarly, molecular weights of *C. coronatus* NCL 86.8.20 protease I and II were 24 and 19.5 kDa by SDS PAGE respectively. But the molecular weights of these proteases by gel filtration were 23.17 and 19.27 kDa which are slightly lower than the values obtained by SDS PAGE (Phadatare, 1991). Alkaline protease from *C. macrosporus* showed molecular weight 28.5 and 27.0 kDa by SDS PAGE and gel filtration respectively (Tanksale, 2001).

Molecular weight determination by MALDI-TOF

MALDI-TOF analysis of purified protease showed a major peak corresponding to the molecular weight of 27.8 kDa (Figure 3.5). This value is nearly similar to that obtained by gel filtration method and slightly lower than SDS-PAGE.

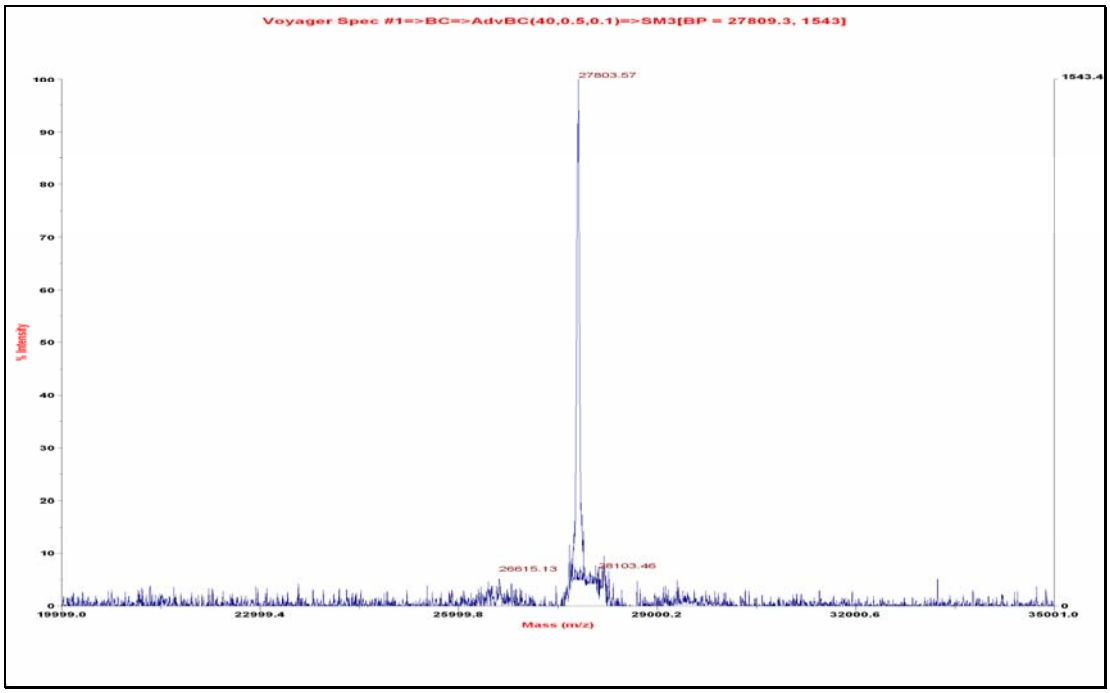


Figure 3.5: MALDI–TOF of purified protease

(Major peak with molecular weight of 27.8 KDa)

Hattori *et al*. (2005) reported identical molecular weight of 23.4 kDa by SDS PAGE and MALDI-TOF for purified protease P-1-1 from *Cordyceps militaris.* Molecular weights of protease PoSI from *Pleurotus ostreatus* by SDS PAGE, gel filtration and MALDI-MS were reported to be 75, 74 and 75.16 respectively (Palmeiri *et al.* (2001). Molecular weights of protease from *Fusarium culmorum* by SDS PAGE and MALDI-TOF were 26.8 and 28.7 kDa respectively (Pekkarinen *et al.*, 2002).

Determination of isoelectric point of protease

The pH and activity of the fractions and the silver stained gel are shown in (Figure 3.6a and b).

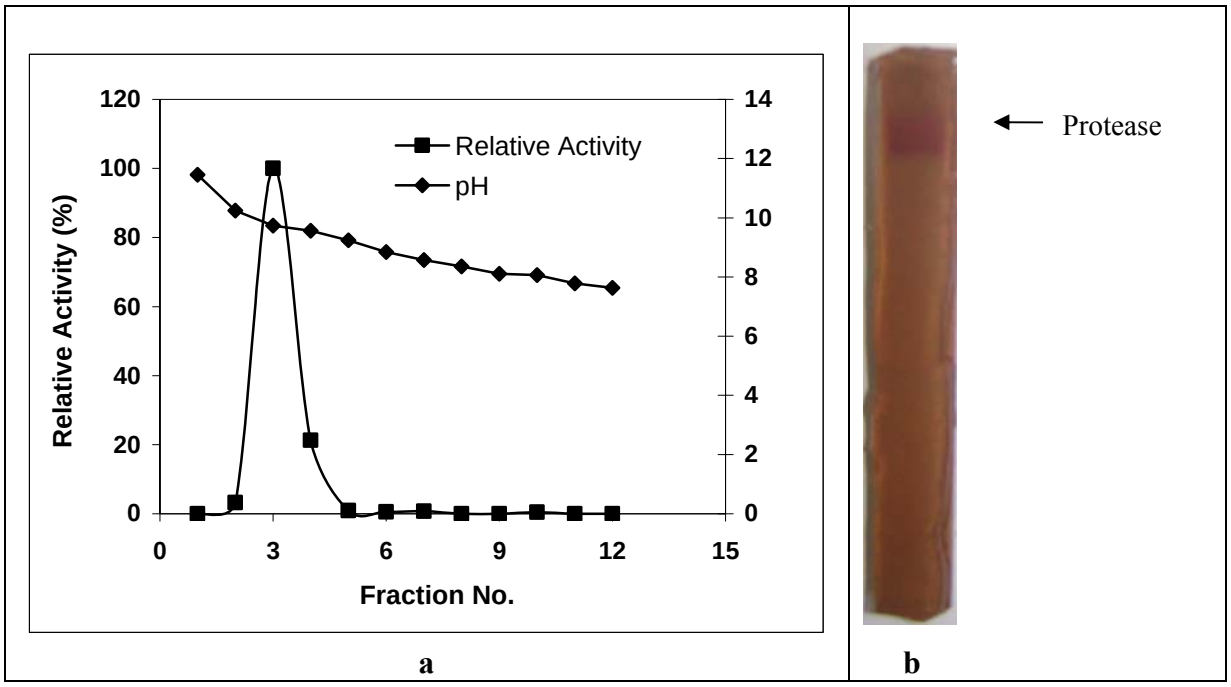


Figure 3.6: Isoelectric focusing of purified protease

The pI of purified protease was found to be 9.74. Isoelectric points of alkaline proteases generally fall in the range of 8-11. The optimum pH of alkaline proteases also coincides with these values. Most of the fungal alkaline proteases have their pI in alkaline range. The isoelectric points of all the proteases reported from *Conidibolus* strains fall in the range of 8.5 to 9.9 (Sutar *et al.,* 1991; Phadatare *et al.,* 1993; Tanksale, 2001). The isoelectric points of alkaline proteases from *Monoascus purpureus* CCRC31499, *Clonostachys rosea* and *Beauveria* sp were found to be 7.9, 10.5 and 9.31 respectively (Liang *et al.,* 2006; Zhao *et al.,* 2005; Shankar *et al.,* 2011). The pI value of protease in alkaline range is advantageous for its use in detergent formulation. Abraham and Breuil (1996) reported pI of 5.6 for alkaline protease from *Ophiostoma piceae* where as Dienes *et al.* (2007) purified a trypsin like protease from *Trichoderma reesei* QM9414 with pI value of 7.3.

Determination of optimum pH

The optimum pH of the protease was determined by measuring its activity at 50°C over a range of pH values.The purified enzyme was active in a pH range of 7 to 11 with pH optima at 9 (Figure 3.7). The enzyme showed 58 and 26% of its maximum activity at pH 7 and pH 11 respectively. This pattern was similar to that of the crude protease. Most of the alkaline proteases from fungal source have their pH optima in the range of 8 to 11 (Nirmal *et al.,* 2011), while optimum pH of alkaline proteases from *Conidiobolus* strains was fall in the range of 8.3 to 10 (Table 3.1). Proteases active in alkaline pH have great industrial importance especially in formulation of detergents (Gupta *et al.,* 2002a).

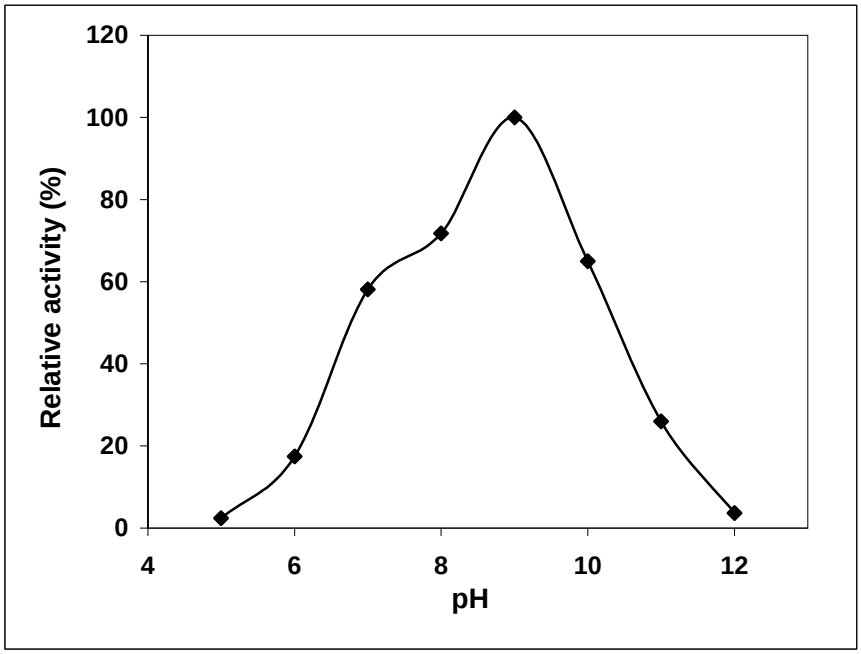


Figure 3.7: Optimum pH of purified protease

Determination of optimum temperature

Optimum temperature for the protease was determined by measuring the activity at pH 9 over a temperature range of 30 to 70°C. The protease was active in the temperature range of 30 to 60°C with an optimum value at 50°C which was similar to that of the crude enzyme preparation (Figure 3.8). The decline in activity beyond 50°C is possibly due to the alteration in enzyme structure and denturation at higher temperatures. Proteases from *Conidiobolus* strains are active in temperature range of 40 to 45°C (Table 3.1). Generally,

proteases from mesophilic fungi have their optimum temperature in the range of 37 to 60°C. However as an exception, Namasivayam *et al.* (2010) reported an alkaline protease from *B. bassiana* strain whose optimum temperature is 70°C. Proteases active in the temperature range of 30-50°C are very important where the operations have to be performed at ambient to slightly higher temperatures such as dehairing, degumming and laundry applications.

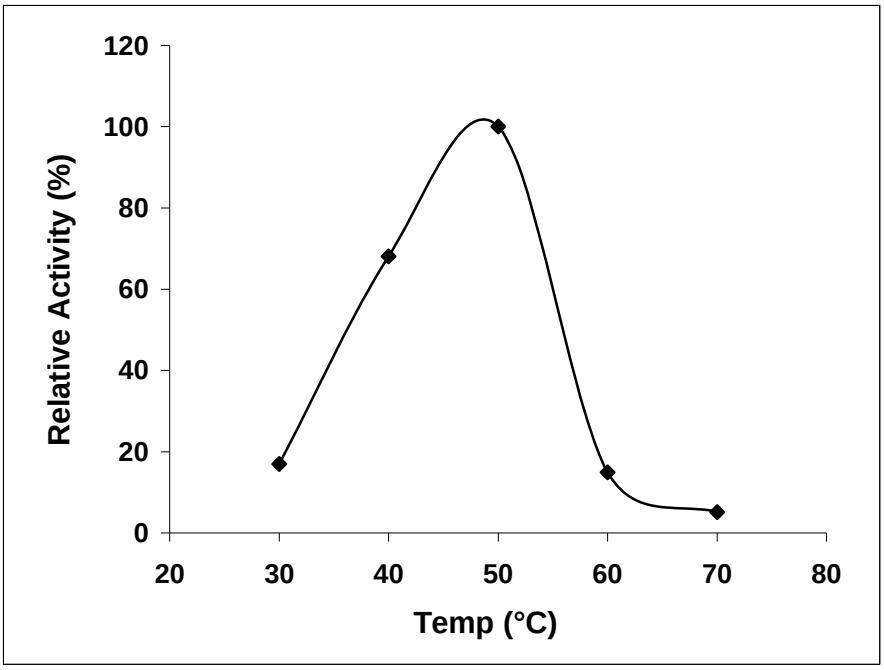


Figure 3.8: Optimum temperature of protease

Effect of substrate concentration and determination of K_m and V_{max}

Kinetic constant for purified *C. brefeldianus* protease was obtained using standard assay condition and varying the casein concentration (1-10 mg) in the reaction mixture. The enzyme activity increased with increase in casein concentration and reached to its maximum level when the casein concentration was 5 mg, beyond this concentration there was slight decline in the activity (Figure 3.9).

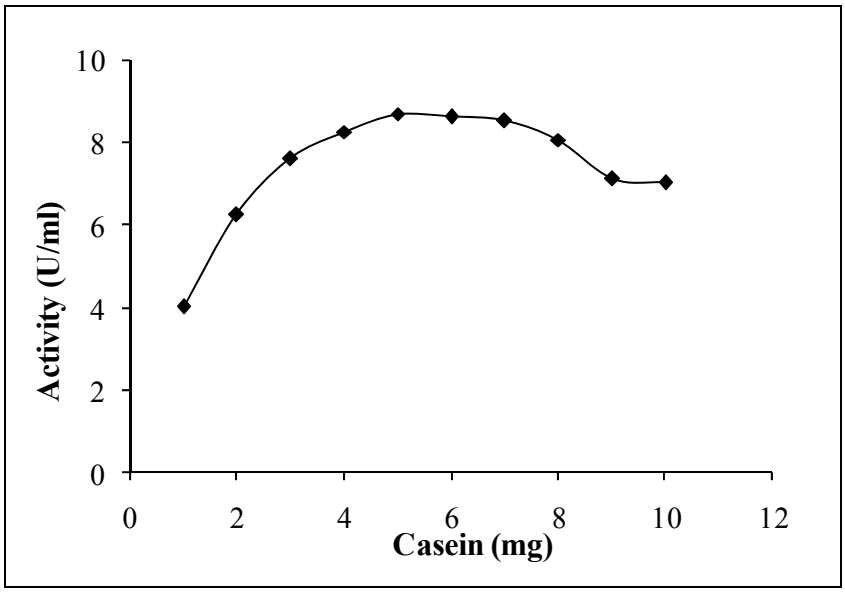


Figure 3.9: Effect of substrate concentration

Michaelis constant (*Km*) and maximum velocity (*Vmax*) were calculated from the double reciprocal plot of Lineweaver-Burk (Figure 3.10). The *Km* and *Vmax* values of the enzyme were found to be 2.5 mg/ml and 15.38 IU/ml. Tanksale (2001) reported the *Km* value of 2.9 mg/ml with casein as substrate for *C. macrosporus* protease. Dodia *et al.* (2008) reported similar *Km* value of 2.5 mg/ml with casein as substrate for serine alkaline protease from *Halloalkalophilic* bacterium. Shankar and Laxman (2011) reported *Km* and *Vmax* for crude protease from *C. coronatus* PTA-4132 with casein to be 2.56 mg/ml and 40 U/ml respectively. The *Km* and *Vmax* of purified protease from *Beauveria* sp. were reported to be 5.1 mg and 29.67 U/ml respectively (Shankar, 2010). *Vmax* and *Km* for *A. niger* alkaline protease with casein is reported to be 0.8mg/ml and 85 U/mg protein (Devi *et al.,* 2008). *Km* value of 2.9 mg/ml was reported for alkaline protease of *A. clavatus* with casein as substrate (Tremacoldi *et al.,* 2007).

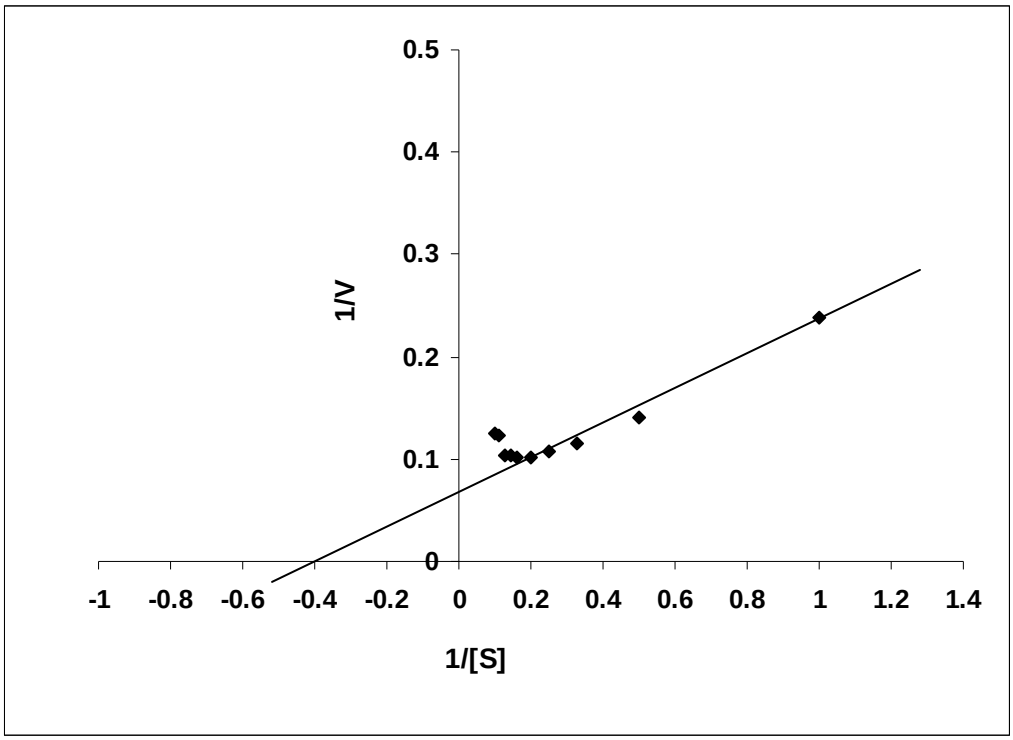


Figure 3.10: Lineweaver-Burk plot of purified protease

Effect of protease inhibitors and chelators

Effect of inhibitors is an important aspect in the study of proteolytic enzymes, because it facilitates to identify and ascertain the type and nature of the protease. The influence of various inhibitors on enzyme activity was studied under standard assay conditions. The purified enzyme was completely inhibited by 0.1 mM PMSF, indicating its serine nature, while the enzyme remained active in presence of all other inhibitors and chelator (Table 3.4). Effect of two synthetic inhibitors, TLCK and TPCK, known to interact irreversibly with serine proteinases exhibiting chymotrypsin-like and trypsin-like specificities, respectively showed only 7.66 and 13.9% inhibition. Lack of inhibition by trypsin-specific TLCK was not surprising, although it was striking that TPCK also appeared to be ineffective. SBTI, which is another inhibitor known to inhibit chymotrypsin-like enzymes also did not show any inhibition. EDTA and idoacetamide had no inhibitory effect on protease indicating that the present enzyme is neither metallo nor cystine type of protease. The enzyme remained active in presence of benzamidine and SBTI which are known to inhibit trypsin, chymotrypsin and subtilisin like proteases. Theses findings are in accordance

to those reported for other *Conidiobolus* proteases where PMSF completely inhibited the enzyme but did not show inhibition with TLCK, TPCK and EDTA (Ghadge, 1986; Sutar, 1987; Phadatare, 1991). In contrast, Bania *et al*. (2006) reported 60% inhibition of *C. coronatus* protease in presence of SBTI at 10 mM concentration.

Table 3.4: Effect of inhibitors and chelator

Inhibitor	Inhibits	Concentration (mM)	Inhibition (%)
Control	-	-	0.00
TPCK	Trypsin like	5	13.90
TLCK	Chymotrypsin like	5	7.66
Benzamidine	Trypsin like	5	0.16
Iodoacetate	Cystein	10	0.30
PMSF	Serine	0.1	100.00
EDTA	Metallo	20	0
SBTI	Trypsin like	0.5	1.00

Effect of metal ions on protease stability

Effect of various metal ions on purified protease is presented in Table 3.5. The enzyme was stable in presence of all the metals tested except Hg^{2+} and Cu^{2+} which showed total and 31.8% inhibition respectively. Cadmium and nickel showed negligible inhibition. Calcium did not show any stimulatory effect. Mercury also inhibited other *C. coronatus* proteases. Zn showed 43-58% inhibition of *C. coronatus* NCL 86.8.20 proteases while most of the other metal ions tested did not inhibit (Ghadage, 1986; Sutar, 1987; Phadatare, 1991). Cu^{2+} Hg^{2+} and Fe^{2+} ions completely inhibited *C. macrosporus* protease (Tanksale, 2001). Mercury ions react with thiol groups and amino acids like tryptophan and histidine of proteins and alter its active confirmation (Vallee and Ulmer, 1972; Ryzhakov *et al.,* 2010). The effect of metal ions on microbial proteases varies greatly from source to source, consequently a specific metal ion may inhibit one kind of protease, and on the other hand, it may activate the other.

Table 3.5: Effect of metal ions on protease stability

Metal salt (10 mM)	Residual activity (%)
None	100.00
$CaCl_2$	101.84
$CdCl_2$	96.84
$CoCl_2$	101.08
$CuCl_2$	68.21
$HgCl_2$	0.00
KCl	105.18
$MgCl_2$	102.17
$MnCl_2$	100.00
$NiCl_2$	94.71
$ZnCl_2$	100.59

Serine alkaline protease from *A. clavatus* ES1 was slightly activated by Ca and Mg while Co^{2+}, Cu^{2+} and Zn^{2+} inhibited activity by 100, 38 and 84% respectively (Hajji *et al.,* 2007). Alkaline protease from *B. clausii* GMBAE 42 was activated by Cu^{2+} and Mn^{2+} ions (Kazan *et al.,* 2005). Activity of alkaline protease from *A. niger* was inhibited by 98, 36, 54 and 80% in presence of 5 mM Co^{2+}, Cu^{2+}, Hg^{2+} and Zn^{2+} respectively (Devi *et al*., 2008). Zn^{2+} and Mg^{2+} enhanced the activity of *A. tamari* protease (Sharma and De, 2011). Fe^{2+} stimulated the activity of the crude alkaline protease from *A. flavus* while Cu^{2+} and Hg^{2+}completely inhibited the same (Yadav *et al.,* 2011). Calcium was found to enhance the activity and stability of proteases from several *Bacillus* strains (Beg and Gupta, 2003; Deng *et al.,* 2010; Ahmed *et al.,* 2011). Activity of *C. coronatus* proteases A and B increased by 55 and 45% respectively in presence of Ca^{2+} (Ghadge, 1986; Sutar, 1987), while *C. coronatus* NCL 86.8.20 protease and *Beauveria* sp. protease did not show any activation in presence of calcium (Phadatare, 1991; Shankar, 2010). Stability of *C. brefeldianus* protease in presence of wide range of metals is an added advantage for their applications where these metals may be present.

Activity of protease towards natural Substrates

Serine alkaline proteases generally show broad substrate specificity and react with native protein materials at varied degrees. *C. brefeldianus* protease was able to hydrolyze natural substrates like casein, haemoglobin and BSA to varying degrees (Table 3.6).

Table 3.6: Activity of protease towards natural substrates

Substrate	Relative Activity (%)
Casein	100.00
Hemoglobin	82.31
BSA	53.71

Activity of the enzyme was more against casein compared to haemoglobin and bovine serum albumin. Several microbial alkaline proteases were more active against casein than other substrates like haemoglobin or bovine serum albumin (Ghadge, 1986; Sutar *et al.,* 1991; Phadatare *et al.,* 1993; Kobayashi *et al.,* 2007; Shankar *et al*., 2011. In addition, casein is a choice of substrate for spectrophotometric assay of alkaline proteases, in view of its reliability, ease of performance and cost effectiveness. However, alkaline protease from *C. macrosporus* showed 1.85 times more activity with haemoglobin than casein and gave 22.6% of activity with BSA as compared to casein (Tanksale, 2001). Protease from *Beauveria* sp. showed only 5% activity with BSA compared to casein (Shankar, 2010). Present protease exhibited very good activity towards natural substrates tested compared to many of the reported proteases. Proteases active against hemoglobin and albumin may found useful in detergent formulation, as these proteins are present in body secretions.

Activity of protease towards synthetic substrates

A wide variety of synthetic substrates are available for rapid and reliable in vitro assay of different proteases or peptidases. In addition these substrates are useful for characterization of the substrate specificities of the proteases. They are made up of either single amino acid or short peptides in which the $–NH_2$ and /or the –COOH groups are substituted with a blocking group usually through either amide or ester linkages. Both groups are blocked in those substrates, utilized for proteases (endopeptidases). The acyl group is usually

conjugated to a chromogenic or fluorogenic group. On bond cleavage, these groups are liberated and are conveniently monitored by the associated changes in absorbance or fluorescence. Assays using paranitroanilide (p-NA) acyl derivatives as substrates which leaves p-NA as a chromogenic group are the most common and convenient spectroscopic method for activity measurement for a large array of peptidases. Several such substrates are available for different serine and cysteine proteases. Substrates used in the present study are specified for serine proteases, including subtilisins and for esterases (Brue and Walde, 1991; Hua *et al.,* 2008; Salamin *et al*., 2010). The activity of purified protease from *C. brefeldianus* was estimated with various synthetic substrates (Table 3.7).

Table 3.7: Protease activity with different synthetic substrates

Substrate	Recommended for	Activity
Succ-Ala-Ala-Pro-Leu-pNA	Elastase	339.1*
Succ-Ala-Ala-Pro-Phe-pNA	Chymotrypsiin Cathepsin G	327.3*
Succ-Ala-Ala-Ala-pNA	Trypsin	39.55*
Gly-Phe-pNA		0.00
N-Bez-Arg-pNA	Trypsin	109.0*
N-Benzoyl-arginine Ethyl Ester (BAEE)	Trypsin	780.00♦
N-Benzoyltyrosine Ethyl Ester (BTEE)	Chymotrypsin	270.00♦

*One unit - one μM of p-NA released /ml/min
♦One unit - increase in absorbance by 0.001/ml/min

Amino acids present in the P1 position of substrate have strong influence on the catalytic action of proteases. The results obtained suggest that the present protease preferred leucine and phenylalanine followed by arginine at P1 position and exhibited the highest activity towards Succ-Ala-Ala-Pro-Leu-pNA (339.1 U/ml) and Succ-Ala-Ala-Pro-Phe-pNA (327.3 U/ml). However, activity with N-Bez-Arg-pNA where P1 position is occupied by arginine was considerably lower (109.0 U/ml) compared to former two synthetic substrates. The protease showed lowest activity (39.55 U/ml) when alanine is present at P1 position as seen with Succ-Ala-Ala-Ala-pNA and no activity was detected with Gly-Phe-pNA, even though phenylalanine is present at P1 position, probably due to the very weak or no binding of this

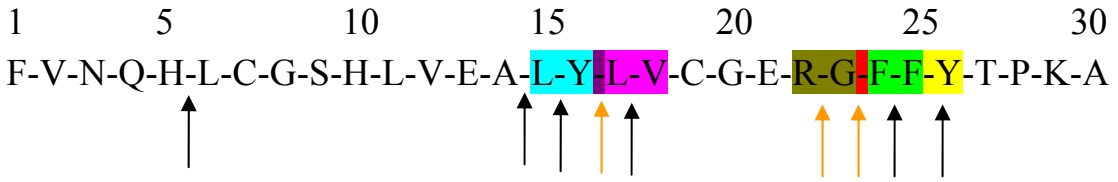
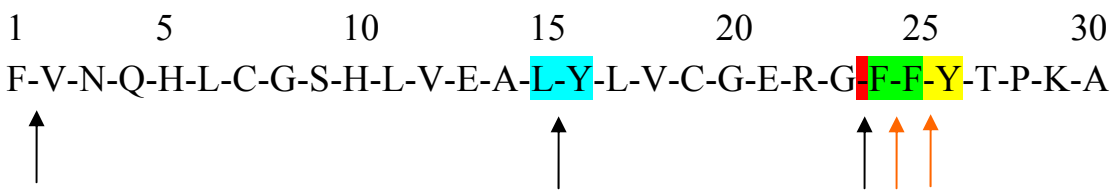
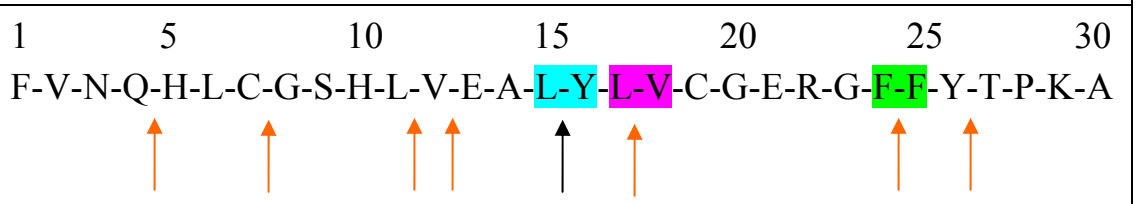
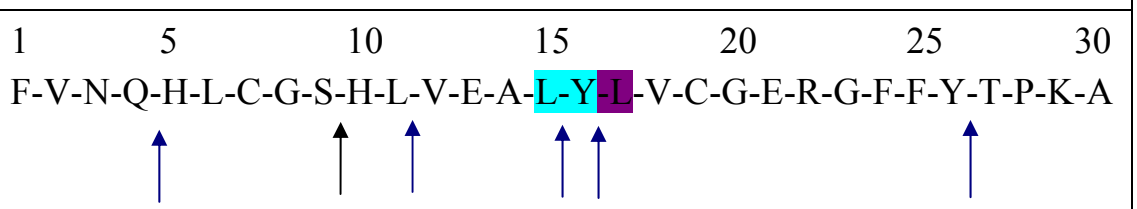
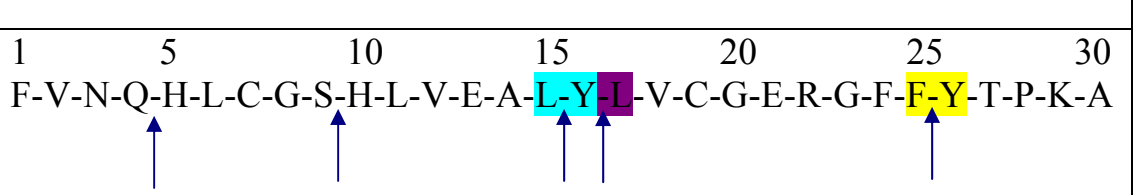
short peptide substrate with enzyme. Similar trend of hydrolysis was also reported for keratinase from *Bacillus pumilus* KS12 where hydrolysis of N-Suc-Ala-Ala-Pro-Phe-pNA > N-Ala-Ala-Pro-Leu-pNA > N-Suc-Ala-Ala-Ala-pNA (Rajput *et al.,* 2010).

Alkaline protease A from *C. coronatus* NCL 82.1.1 and alkaline proteases I and II from *C. coronatus* NCL 86.8.20 cleaved ester bonds of BAEE and BTEE. However, they could not hydrolyze N-Bez-Arg-pNA substrate (Ghadge, 1986; Phadatare, 1991). *C. macrosporus* protease was active towards Succ-Ala-Ala-Pro-Phe-pNA, TAME and BAEE. However it did not show any activity towards N-Bez-Arg-pNA (Tanksale, 2001). *Beauveria* sp. showed very little activity with BAPNA (Shankar, 2010). *Conidiobolus* proteinase hydrolyzed N-Suc-Ala-Ala-Pro-Phe-pNa and N-Suc-Ala-Ala-Pro-Leu-pNa while specific substrates for other serine proteinases, such as N-Bez-Arg-pNA i.e. BApNa (trypsin), N-Suc-Ala-Ala-Pro-Val-pNa (human leukocyte elastase), and N-Suc-Ala-Ala-Ala-pNa (porcine pancreatic elastase) were not cleaved by *Conidiobolus* proteinase (Bania *et al.,* 2006). These findings differentiate present protease from the reported *Conidiobolus* proteases. Benzoyl –L-arginine ethyl ester (BAEE) and benzoyl-L-tyrosine ethyl ester (BTEE) are used for assaying trypsin and chymotrypsin activity respectively (Krzyzosiak, 1997). The enzyme was found to be active towards both the substrates. However the degree of hydrolysis of BAEE was higher than BTEE.

Hydrolysis of insulin β chain

After estimating the activity of *C. brefeldianus* protease with various synthetic substrates, substrate preference for cleavage sites was further investigated with oxidized insulin β chain. This is a 30 amino acid peptide containing different amino acids resembling natural substrate. The resultant data obtained from MALDI TOF was further processed to obtain the cleavage sites by using a computer program, FindPept (http://ca.expasy.org/tools/findpept/), which enables peptide identification after protein cleavage based on the experimentally determined size of the products. From the peptides obtained after the hydrolysis of the oxidized insulin β-chain, it is clear that the enzyme showed endopeptidase activity and a broad specificity, nine peptide bonds in the β-chain of insulin were hydrolysed, out of which six cleavages were observed after 15 min of incubation. The presence of aromatic residue

(Phe) and (Tyr) and an aliphatic amino acid (Leu) at P1 position favored bond cleavage by the protease. This is also supported by the catalytic efficiency of protease towards synthetic substrates containing Phe and Leu at P1 position. The cleavage sites of the *C. brefeldianus* protease on the oxidized insulin β chain resembled the cleavage sites of subtilisins BPN, Carlsberg and Novo (Figure 3.11). Leu^{15}-Tyr^{16} is a common preferable cleavage site among bacterial serine proteases. The cleavage site at Phe^{25}-Tyr^{26} has also been reported earlier for subtilisin BPN while cleavage at Tyr^{16}-Leu^{17} and Tyr^{26}-Thr^{27} were identical with those observed for subtilisin Carlsberg and Novo respectively (Toyokava *et al.,* 2010; Bakhtiar *et al.,* 2005). The cleavage pattern of *C. brefeldianus* protease seemed to differ from the protease of *C. coronatus*, which cleaved five peptide bonds (Bania *et al.,* 2006), out of which four cleavages are common with both the proteases, while the bond cleavage obtained at Phe^{1}-Val^{2} is absent with present enzyme.

C. brefeldianus protease (15 min- Black; 60 min-Orange)	
C. coronatus protease (15 min- Black; 35 min-Orange) Bania *et al.*, 2006	
B. licheniformis RKK04 protease (5 min- Black; 60 min-Orange) Toyokava *et al.*, 2010	
Subtilisin Carlsberg Bakhtiar *et al.*, 2005	
Subtilisin BPN' **(**Bakhtiar *et al.*, 2005**)**	

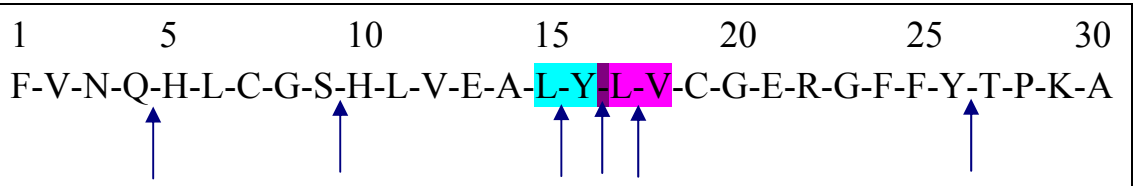
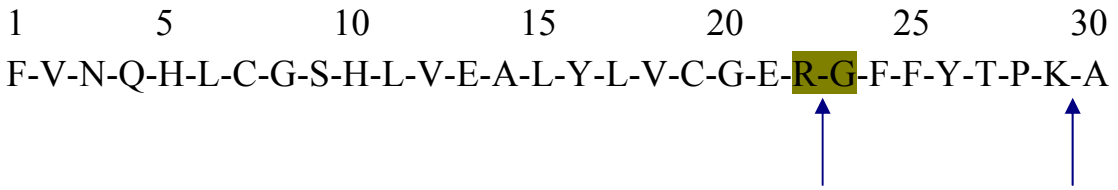
Subtilisin Novo (Bakhtiar *et al.*, 2005)	
Trypsin (Gradisar *et al.*, 2005)	

Figure 3.11: **Cleavage sites by different proteases on insulin β chain. Blue arrow indicates major cleavage sites after 15 min of incubation, while orange arrow indicates minor cleavage site after 60 min of incubation with *C. brefeldianus* protease.**

Chemical modification

For studying structure function relationship of enzymes and other proteins, active site directed chemical modification is important. The involvement of specific amino acid functional groups for the activity of protease can be determined by chemical modification studies using chemical agents with restricted amino acid specificity. Purified protease (15 to 28 µg) was incubated with varying concentrations of inhibitors. The inhibitors used and their concentrations are follows: PMSF (5 to 50 µM); WRK (1 to 10 mM); freshly prepared DEPC (1-10 mM); NBS (0.01-0.1 mM); DTNB (0.1-1.0 mM) and phenylglyoxal (1 to 10 mM). Results of inactivation with the highest concentrations of the inhibitor tested are presented in Table 3.8.

Table: 3.8: Effect of modification agent on protease activity

Modifying Agent	Highest concentration of inhibitor tested	Possible amino acid modification	Residual activity (%)
PMSF	50 µM	Ser	0.0
WRK	10 mM	Asp	17.9
DEPC	20 mM	His	7.9
NBS	0.1 mM	Trp	6.5
DTNB	1 mM	Cys	97.0
Phenylglyoxal	10 mM	Arg	99.0

The enzyme was not inactivated by DTNB and phenylglyoxal suggesting the non-involment of cysteine and arginine residues in catalysis. Strong inhibition of enzyme by PMSF, WRK, DEPC and NBS was observed which indicated the involvement of serine, aspartic acid, histidine and tryptophan residues for its activity as these amino acids are essential for the activity of protease. The central catalytic machinery of the serine proteases consists of an Asp-His-Ser triad (Iengar and Ramakrishnan, 1999). This triad in the folded structure forms an essential element of the active site in all proteases (Phadatare *et al.,* 1997). The catalytic mechanism of serine proteases involve the nucleophilic attack by the serine hydroxyl group on the carbonyl carbon atom of the substrate which is catalyzed by a histidine imidazole group as a general base. This leads to formation of tetrahedral intermediate which breaks

down to an acyl-enzyme, an imidazole base and amine product (elimination reaction). The acylation step is followed by deacylation through reverse reaction pathway in which water molecule act as nucleophile. Few other serine proteases such as peptidase A from *E. coli* and the repressor Lex A show distinctly different mechanism of action without involvement of this classic Ser-His-Asp triad (Rao *et al.,* 1998). Besides the characteristic residues of the catalytic triad (Asp, His, Ser), involvement of tryptophan and cystine residues were reported to be present in the subtilisins. An essential Trp and thiol residue were detected in the active site of *C. macrosporus* alkaline protease (Tanksale, 2001). However, the present protease retained its activity in presence of 1mM DTNB. This indicates the non-intervention of thiol residue for the activity. Protease A and B from *C. coronatus* NCIM 1238 showed complete inhibition in presence of 0.1 mM PMSF, while phenylglyoxal at 10 mM concentration did not show any inhibitory effect (Ghadge, 1986; Sutar, 1987). Further kinetics of inhibition of protease with PMSF was studied. The plots of residual activity versus time of inactivation for various PMSF concentrations were found to be linear (Figure 3.12).

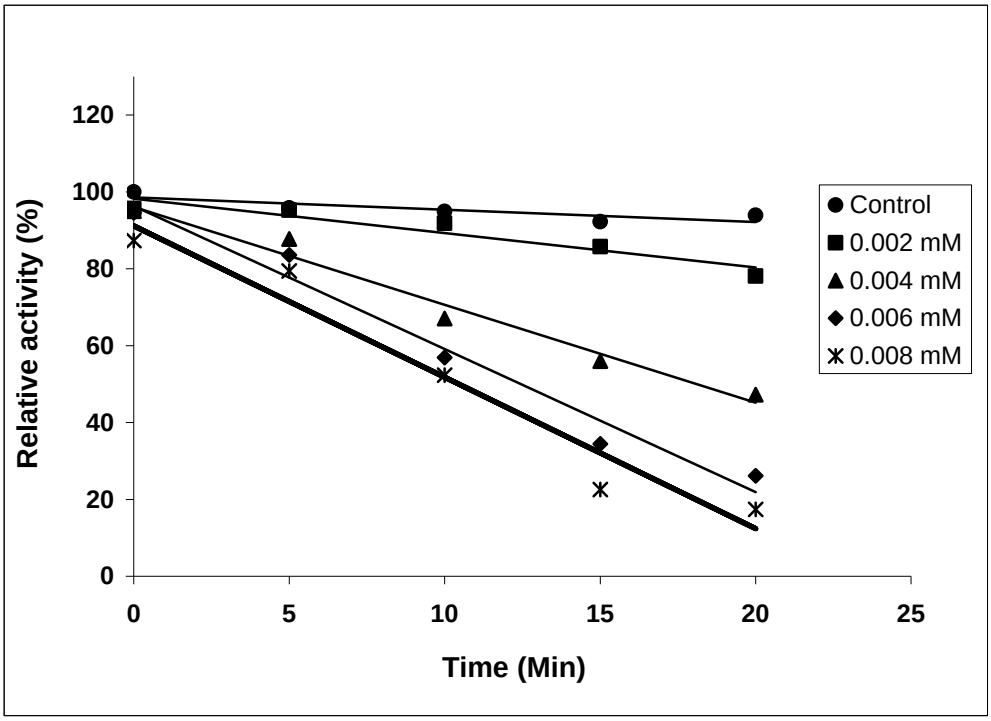


Figure 3.12: Inhibition kinetics of protease with PMSF

Substrate protection studies

To ascertain whether serine is present at the active site of protease, substrate protection assay was carried out. Pre incubation of protease with casein resulted in activity retaintion to varying levels. Inhibitory effect decreased with increasing concentration of casein (Table 3.9). Without casein, there was 80% inhibition which reduced to 53% (2.3 fold increase in residual activity) in presence of 10 mg casein.

Table 3.9: Ligand protection assay

Sample	Inhibition (%)
Enzyme without inhibitor	0.00
Enzyme without substrate	80.00
Enzyme + 1 mg casein	76.17
Enzyme + 2 mg casein	75.15
Enzyme + 3 mg casein	74.95
Enzyme + 4 mg casein	75.51
Enzyme + 5 mg casein	67.55
Enzyme + 6 mg casein	60.64
Enzyme + 7 mg casein	57.51
Enzyme + 8 mg casein	56.47
Enzyme + 9 mg casein	55.31
Enzyme + 10 mg casein	53.26

2mM PMSF was used as inhibitor.

Determination of tryptophan residues

Successive addition of NBS to the protease brought about progressive decrease in absorbance at 280 nm and simultaneous loss in activity (Figure 3.13). The number of tryptophan residues oxidized per mole of the enzyme was found to be 2.7. Tanksale (2001) reported the value 3.15 for *C. macrosporus* protease.

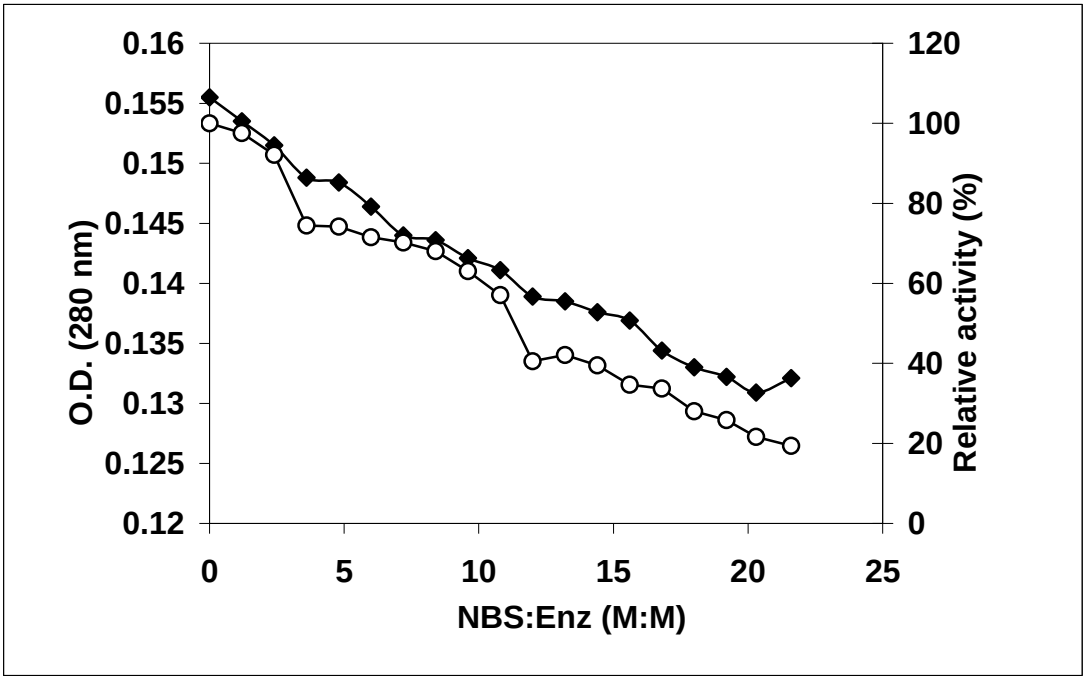


Figure 3.13: Titration of protease with NBS

Aliquot (5µl) of NBS (5 x 10-4 M) were added successively to the enzyme (50 µg). After each addition, residual activity (o) and absorption (■) at 280 nm were monitored.

Conclusions

A major protease from *C. brefeldianus* was purified to homogeneity with 3.37 fold purification and 156 IU/mg specific activity. The final yield of purified protease was 54%, which is one of the highest reported for fungal protease. The molecular weight of purified protease by SDS PAGE, gel filtration and MALDI-TOF was 30.19, 28.1 and 27.8 kDa respectively. *Km, Vmax* and pI of the protease were 2.5 mg/ml, 15.38 IU/ml and 9.74 respectively. The protease was optimally active at 50°C and pH 9. The protease was completely inhibited by PMSF and partially inhibited by TPCK and TLCK. However, EDTA and SBTI did not inhibit the protease indicating it to be a serine type protease. Hg^{2+} and Cu^{2+} inhibited the protease completely and partially. Protease hydrolyzed synthetic substrates Succ-Ala-Ala-Pro-Leu-pNA, Succ-Ala-Ala-Pro-Phe-pNA, N-Bez-Arg-pNA and Succ-Ala-Ala-Ala-pNA in the order of catalytic efficiencies but was totally inactive against Gly-Phe-pNA. Protease also showed some esterase and peptidase activities. Chemical modification studies revealed that serine, histidine and aspartic acids to be essential for protease activity. Titration studies with NBS showed that there were 2.7 moles of tryptophan residues per mole of enzyme. Analysis of hydrolytic peptides from oxidized insulin β chain by *C. brefeldianus* protease by MADLI-TOF indicated that the cleavage sites of protease to be similar to subtilisin cleavage sites.

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CHAPTER 4

APPLICATIONS OF CRUDE ALKALINE PROTEASE FROM *C. brefeldianus*

SECTION 1

APPLICATION OF CRUDE ALKALINE PROTEASE IN LEATHER PROCESSING

Abstract

Application of alkaline protease from *C. brefeldianus* was tested as a substitute for conventional lime and sulfide dehairing in leather processing. Alkaline protease from *C. brefeldianus* was efficient in unhairing various types of skins and hides. The crude protease preparation was active towards keratin azure, elastin-orcin, azocasein and azocoll but did not show true collagenase activity. In addition, the crude enzyme exhibited other enzyme activities such as chondroitinase, laminarinase and chitinase. Complete hair removal of skin/hide by the protease was achieved in 16-18 h. The dehaired pelt showed smooth and white appearance due to hair removal along with epidermal layer. In addition, the grain was clean and without damage in enzymatically dehaired pelts. The microscopic observation of the cross section of dehaired goat skin and cow hide showed absence of epidermis and hair shaft with empty follicles. Enzymatic dehairing resulted in complete and uniform fiber opening in the dermis and corium region. SEM pictures of the dehaired pelt and dyed crust revealed grain structure to be clean with more visible fiber separation compared to those obtained by conventional method. Physical properties viz. tensile strength, elongation and tear strength of dyed crust of enzymatically and conventionally dehaired pelts were comparable. Results were also validated on large scale with goat skins and cow hides.

Introduction

Indian leather sector

Leather industry holds a prominent position in the Indian economy with an annual turnover US$ 7.5 billion. The export of leather and leather goods increased several folds over the past few decades and touched US$ 4.86 billion in 2011-2012 (http://www.leatherindia.org/about-council/industryatGlance.asp). As per the estimates of Planning Commission, the exports from this sector are expected to touch $8.5 billion by the financial year 2016-17. In an attempt to make the Indian leather industry globally competitive, the government has approved Rs. 600-crore mega leather cluster development scheme for the 12th Five Year Plan (2012-2017) under the Indian Leather Development Programme (SME news, August 22, 2012/ http://news.indiamart.com). The major markets of Indian leather and leather products are Germany, Italy, USA, UK and other European countries. Region wise export share and percent share of various leather products exported is given in Figures 4.1.1 and 4.1.2 respectively (http://www.leatherindia.org/about-council/industryatGlance.asp).

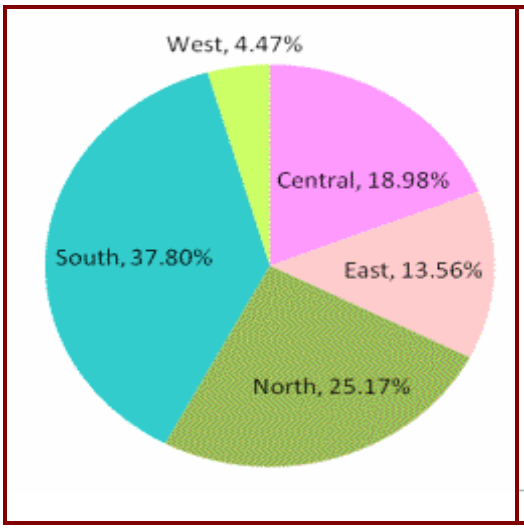

Figure 4.1.1: Region-wise export of leather & leather products (2011-12)

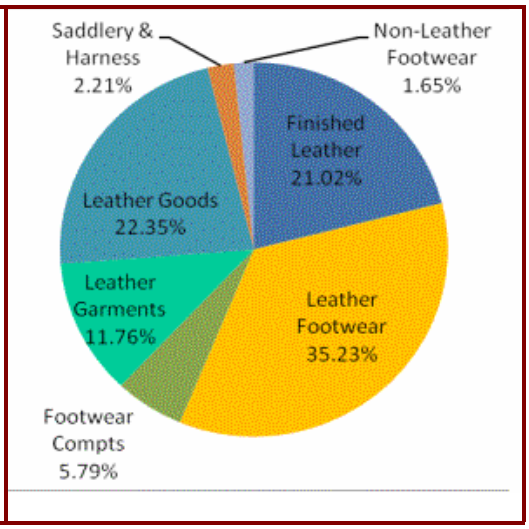

Figure 4.1.2: Percentage share of leather products in export performance (2011-12)

Global trade in leather and leather goods has grown several folds during past few decades and constructively affected the socio-economic status of several developing countries like India. The reason behind this was large shift of leather industries from developed nations to developing countries in past years due to labour costs and stringent environmental regulations in developed nations. In addition, India is

endowed with large population of livestock, which is most essential for regular supply of raw materials for leather industry. Besides, Indian leather sector holds several potential factors like trained manpower, availability of cheap labour, world class institutional support for research and development, availability of supporting industries like chemicals and finishing auxiliaries. Leather sector is a large employment generator providing jobs to about 2.5 million people and most of them are from weaker sections of society.

Ancient methods of tanning

Leather sector of India has seen a long journey of growth from an obnoxious trade, which involved very primitive methods of tanning to a modernized diligence. However, leather manufacturing by age-old methods still exists in many parts of India. Processing of hides and skins to convert them into leather has long been an important industrial activity and generally termed as 'Tanning'. Traditionally, tanning involved use of tannin, an acidic chemical compound from which the tanning process draws its name (tannin is an old German word for oak or fir trees). Tanning or leather manufacturing has very ancient history. Tanning was considered a noxious or "odiferous trade" and relegate to the outskirts of town due to the foul smell generated by ancient tanning methods. The ancient tanners used to soak the skins in water to clean and soften them followed by pounding and scouring to remove flesh and fat. Hair removal or dehairing was generally carried out by soaking the skin in urine, painting it with an alkaline lime mixture, or simply letting the skin putrefy for several months then dipping it in a salt solution. Once the hair is removed, tanners used to bate the material by pounding dung in to it for softening the skins (The term 'bating' in leather manufacturing has been derived from this process). Among the kinds of dung commonly used was that of dogs or pigeons. Sometimes they used to mix dung with water in a large vat, and the prepared skins were kneaded in the dung water until they became supple (http://www.leathernet.com/tanning.htm; http://goodleathergroup.com/tannery.php).

These types of activities were common in leather making processes of ancient rural India. Modern methods of leather tanning were introduced in India by the British in 1857 and the first leather factory was set up in Kanpur (Kumar, 1997). Indian leather industry had seen persistent development in the forthcoming years. However, the

industry had bitter reminiscence of foreclosing of several tanneries for violating pollution control norms. Certainly, leather industry contributes to one of the major industrial pollution problems facing the country. The generation of pollution is significantly high in pre-tanning and tanning operations of leather processing and contributes to around 80-90% of the total pollution load. It generates solid waste, volatile organic compounds, heavy metals especially chromium, and carcinogenic arylamines and obnoxious gas like ammonia and hydrogen sulfide which can have severe health impact such as dermatitis, ulcers perforation of septum and respiratory and increased risk of lung and nasal cancers on tannery workers (Thanikaivelan *et al.*, 2005; Rastogi *et al.*, 2008; Ramos and Liu, 2010).

Tanning– an art to convert perishable to precious

Skin and hair structure

Skin or hide can be divided in to two main layers from leather making point of view. The outermost thin keratinous epidermal layer is comprised of epidermis and its appendages (hair, hair root, sheaths etc.). Just bellow epidermis, corium or dermis is present (Figure 4.1.3). These two layers differ from each other on structural and functional basis.

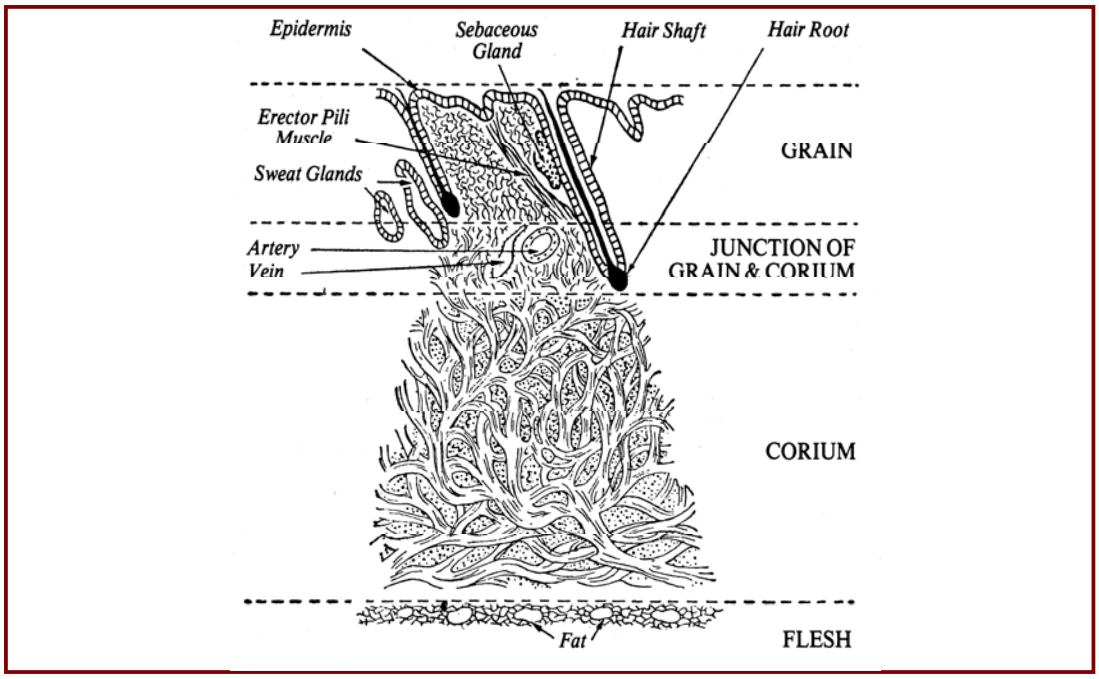

Figure 4.1.3 Structural features of skin (Edmonds, 2008)

Corium or dermis constitutes about 98% of thickness and mainly composed of weave of collagen fibers. Apart from collagen, dermis is comprised of various glycoproteins and proteoglycans (protein-carbohydrate compounds) and cementing materials like dermatan sulphate, hyaluronic acid and elastic fibers. These structures interlinked in a close network to form collagen fibers woven together to form fibril bundles. The unique structure of fibril bundles tends to make the corium stretchable and strong enough to withstand stress. Hair emerges from the hair follicle embedded in corium region of skin where germination center is formed by matrix cells that build layers of hair shaft, including cuticle, cortex and medulla. Cortex is the major part of hair shaft and located immediately beneath cuticle (Figure 4.1.4).

For making different types of leathers except fur and wool leathers, removal of hair from the skin is very important because even minute presence of hair can affect the final quality. In addition, it is impractical to perform various tanning operations such as bating, chrome tanning, dyeing etc. without hair removal.

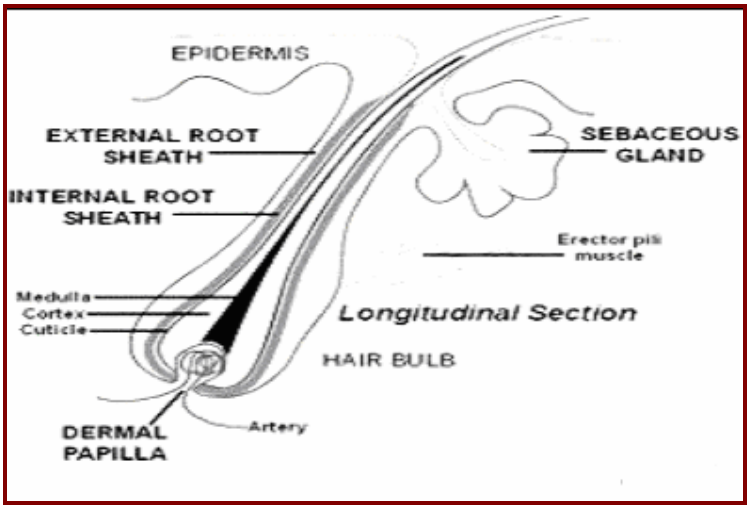

Figure 4.1.4: Structure of hair

Hair growth occurs in cyclic manner in three distinct phases: anagen which is the active growth phase, catagen the breakdown change phase and telogen, the resting stage (Oro and Higgins, 2003). Majority of hair follicles remain in anagen phase and hence dehairing is mainly concerned with removal of anagen hair, which is firmly attached to the follicle (Archana and Pillai, 2012).

For making different types of leathers except fur and wool leathers, removal of hair from the skin is very important because even minute presence of hair can affect the final quality. In addition, it is impractical to perform various tanning operations such as bating, chrome tanning, dyeing etc. without hair removal.

Leather processing

The ultimate aim of tanning operation is the removal of the unwanted materials like hair, flesh, globular proteins and transformation of the fibrous corium layer into leather where the collagen fibrils are packed in such a manner that it can resist microbial attack. Leather making process involves series of operations (Figure 4.1.5). Various steps involved in conventional tanning process and associated effluent contents are listed in Table 4.1.1**.**

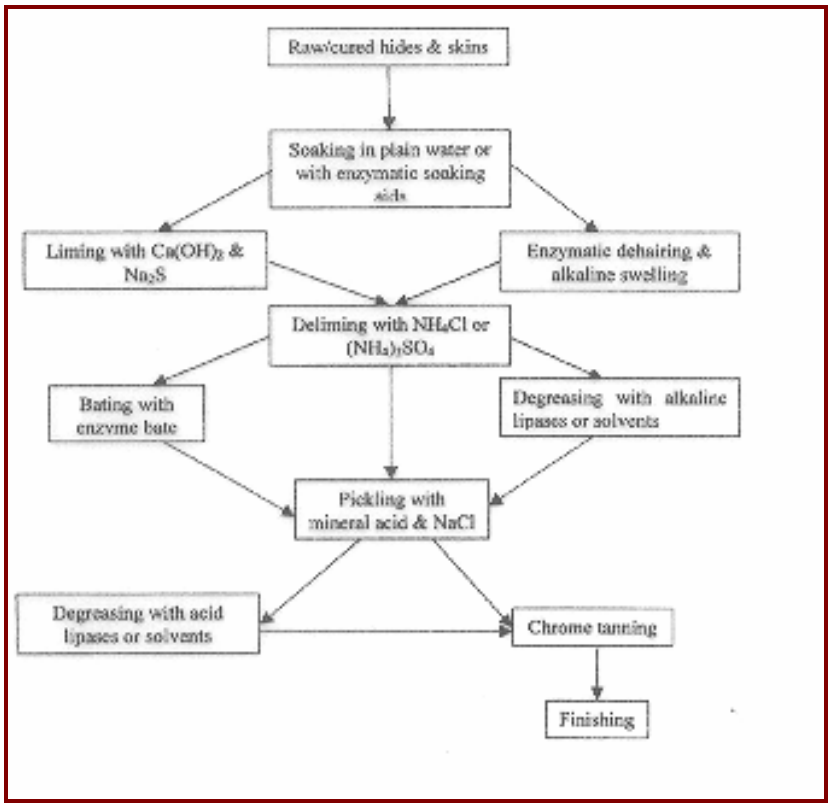

Figure 4.1.5: Steps of the pre-tanning processes of leather manufacture
(Kamini *et al.*, 1999)

Soaking

Soaking is the first operation in the tannery wherein the hides and skins are cleaned and softened with water. Soaking is necessary for solubilization and elimination of salts and globular proteins contained within the fibrous structure of hides and skins. It is carried out at ambient temperature and in presence of preservatives and detergents under alkaline conditions. Enzymes such as proteases, amylases, lipases are added to loosen the scud, initiate the opening of the fiber structure and reduce the processing time.

Table 4.1.1: Various steps in conventional tanning and their effluent composition

Tanning step	Method	Function	Effluent content
Soaking	Dipping the skins in excessive water with small amount of wetting agent and antimicrobial compounds	Re-hydration of skins/ hides, removal of dirt, blood, solubilization of globular proteins	Salt, dirt and blood
Dehairing or depilation	Treatment with lime and sulfide	Removes hair or wool, epidermis and soluble proteins	Highly alkaline, hair pulp, sulfide and solid wastes
Liming	Treatment with lime	It gives the desired swelling to the skin and opens up the fiber bundles	Lime and hair
De-liming	Treatment with ammonium sulphate or ammonium chloride	Removal of alkali from pelt, deswelling of fibers and lowering the pH of liquor for subsequent bating step	Ammonia nitrogen discharge
Fleshing	Mechanical removal of flesh from de-haired skin or hide	Prepare the skin for chrome tanning	Flesh pieces
Re-liming	Soaking of de-haired skin or hide in lime solution	It gives the desired swelling to the skin and open up the fiber bundles	Lime
Bating	Treatment with alkaline /acid bates (proteases)	Removal of non-leather proteins	Alkaline, protein rich wastes
Pickling	Acid treatment	Bring down the pH of hide or skin to nearly 2-3 to prepare the skin for chrome tanning	Acids
Chrome tanning	Treatment with basic chrome sulphate, sodium bicarbonate	Convert the skin into stable form resistant to microbial attack	Non exhausted chromium and other salts

Dehairing

Dehairing is the major step of tanning operation wherein the hair, epidermis, some portion of non-collagenous proteins and other cementing materials are removed from the skin (Sivasubramanian *et al*., 2008a). Conventionally, hair is removed using lime and sodium sulfide. The main objective of liming is removal of hair, flesh, and splitting up of fiber bundles by chemical and physical means. Lime increases pH of the pelt to 12–13 and also causes osmotic swelling. Liming is followed by deliming, where the elevated pH of dehaired skin is brought down gradually to neutral range using ammonium sulphate to avoid sudden changes in pH.

Bating

Bating is essential step in the manufacturing of fine textured leathers like glace, glove, softy nappa etc. The main object of bating is to remove some of the non-leather forming proteinaceous materials like albumins, globulins and mucoids from skins and hides. Bating is normally performed with alkaline proteases in the pH range of 8-9 at 37-40°C after de-liming. This leads to further splitting up of collagen fibers, which facilitates the penetration of tanning agents, dyes and fat liquor in the subsequent steps. The efficiency of process depends on enzyme concentration, pH, temperature and duration of treatment. Acid bating is performed after pickling or chrome tanning step in acidic range (pH 4 to 5).

Conventional dehairing

Conventional method of dehairing involves the use 2-4% sodium sulfide and 10% lime. Dehairing by this method is due to hair loosening, which takes place due to the chemical reaction of lime and sulfide on hair root (Choudhary *et al.,* 2004). The major constituent of hair root is a cystine rich fibrous protein known as keratin, which is prone to alkaline hydrolysis (Horvath, 2009). The major stabilizing factor of keratin structure is the disulphide linkage (S-S) present in cysteine amino acids, which undergoes nucleophilic attack by sulfide ions leading to breakdown of disulphide linkage of polypeptide (Sivasubramanian *et al*., 2008a). Sulfide brings about degradation of hair from the terminal ends towards the hair follicles and often leaves behind remnants of un-degraded hair in follicles. If not removed by scudding they are visible in finished leather and have adverse effect on the quality of leather (Archana and Pillai, 2012). Conventional dehairing is considered to be one the most polluting

processes and has severe impact on water sources and soil. It contributes around 40% of biochemical oxygen demand (BOD) and 50% of chemical oxygen demand (COD) (Dayanandan *et al.,* 2003).

There are number of negative factors associated with conventional dehairing process which include

- Damage of skin structure due to over exposure to sulfide
- Difficulty in precise process control
- Loss of hair and wool due to damage by sulfide
- Heavy pollution load making effluent treatment costly

Among the factors that are likely to affect the growth of the leather industry, the most important are environmental related concerns. Apart from health hazards, pollution of ground water as well as surface water and soil, leather exports will be affected as importing countries are constantly raising the bar on quality parameters thereby impacting the entry of Indian leather and leather goods into international market (Natesh, 2009**)**. Considering these aspects coupled with the pressure to comply the environmental regulations of pollution and discharge legislation, leather sector is seeking cleaner options to find environment friendly alternatives to chemical processing of skins/hides for leather manufacture.

Enzymatic dehairing

Enzymes as alternatives to some of the chemical based methods in leather processing are gaining attention in recent years due to the stringent environmental pollution norms. First enzyme application in tannery was made by Rohm in 1908 who introduced Oropon (comprised of pancreatic enzymes) as bating material. This resulted in the elimination of dung bates used earlier in tanning operation. Later he applied this knowledge for the first successful enzymatic unhairing called 'Arazym process' where skins were initially soaked in dilute lime solution followed by neutralization with sodium bicarbonate after which they were treated with pancreatic enzyme (Taylor *et al.,* 1987). Thereafter, several attempts were made where enzymes were used for dehairing in presence of chemicals such as lime, sulfide or other agents (enzyme-assisted dehairing). In most of these methods, skins were first treated with alkali and neutralized before enzyme application (Cordon *et al.*, 1958).

Patents filed on various applications of enzymes and their roles in leather processing are presented in Table 4.1.2. A patent is filed on production and application of *C. brefeldianu*s alkaline protease for dehairing as well as other applications bearing PCT/IB2011/000516 is also included in the Table.

Table 4.1.2: Patents filed on applications of enzymes in leather processing (Dehairing)

Patent	Enzyme	Function	Treatment involved	References
Enzymatic **dehairing** of hides and skins (2988488)	Microbial keratinase	Hair removal, softening of pelts, reduce pollution load	pH adjusted with lime or buffer **(without sulfide)**	Robison *et al.*, 1961
Method for **dehairing** of hides or skins by means of enzyme (US005834299)	Commercial protease	Hair removal, softening of pelts, reduce pollution load	Addition of 2.5 % lime **(without sulfide)**	Andersen, 1998
Process for the preparation of alkaline protease (US Patent No. 7186546)	Fungal protease	Soaking, dehairing and bating	**Without lime** but with **small amount of sulfide**	Laxman *et al.*, 2007
Enzymes from *Beauveria* sp. and process for preparation thereof (PCT/IB2011/000178)	Fungal protease	Hair removal, softening of pelts, reduce pollution load	Dehairing with only protease **(Without lime or sulfide)**	Laxman *et al.*, 2011b
Novel protease for industrial applications (US 2008/020499)	Bacterial protease	Hair removal, softening of pelts, reduce pollution load	Dehairing with protease only **(Without lime or sulfide)**	Nilegaonkar *et al.*, 2007
Process for lime and sulfide free unhairing of skins or hides using animal and/or plant enzymes (US00719864)	Animal and plant proteases	Hair removal, softening of pelts, reduce pollution load	Dehairing with protease only **(Without lime or sulfide)**	Rose *et al.* ,2007
Eco-friendly bio-process for leather processing (US006708532)	Protease	Hair removal, softening of pelts, reduce pollution load	Dehairing with protease with dehairing enhancing compound	Thanikaivelan *et al.*, 2004
Dehairing and fiber opening process for complete elimination of lime and sodium sulfide (US006957554)	Protease	Hair removal, softening of pelts, reduce pollution load	Dehairing with protease only **(Without lime or sulfide)**	Saravanabhavan *et al.*, 2005a
Enzymes from *Conidiobolus brefeldianus* and process for preparation thereof (PCT/IB2011/000516)	Fungal protease	Hair removal, softening of pelts, reduce pollution load	Dehairing with only protease **(Without lime or sulfide)**	Laxman *et al.*, 2011a

Currently many commercial enzyme preparations as well as most of the reported proteases used for dehairing require presence of small amount of sulfide with or without lime and termed as enzyme assisted dehairing (Thanikaivelan *et al.,* 2005; Sivasubramanian *et al.,* 2008b; Kandasamy *et al.,* 2012). However, few reports have appeared recently on lime and sulfide free dehairing with alkaline proteases (Rajkumar *et al.,* 2011; Sundararajan *et al.,* 2011; Verma *et al.,* 2011). Majority of the proteases studied for dehairing are of bacterial origin and are produced by *Bacillus* strains while reports on fungal proteases used for dehairing are limited (Archana and Pillai, 2012; Madhavi *et al*., 2011). Dehairing proteases generally belong to serine type and are active near neutral or alkaline pH range thereby necessitating dehairing to be performed at alkaline pH (Archana and Pillai, 2012). Enzymatic dehairing is carried out with crude enzyme preparations. If the crude protease preparations contain collagenase activity, it attacks the collagen of the grain layer leading to damage of grain structure and its destruction which has a major impact on the final quality of leather (Choudhary *et al.,* 2004; Macedo *et al.,* 2005). Therefore, it is important that the crude protease preparation is devoid of collagenase activity.

Dehairing process by means of microbial proteases offers following advantages.

- Elimination of toxic chemicals
- Reduces the pollution load of effluent
- Milder process conditions
- Recovery of good quality hair and wool
- Safer atmosphere for workers
- Resultant leather acquires better smoothness and strength properties
- Elimination of bating step in pre tanning process

Mechanism of enzymatic dehairing

In conventional method of lime-sulfide dehairing, hair removal is brought about by hair burning with the action of sulfide in alkaline condition, which leaves back remnants of hair shaft in the pelts. In contrast, enzymatic method removes intact hair from the root. The detailed mechanism of dehairing with enzyme is not fully understood due lack of specific substrates. However, visual and microscopic observations of enzymatically dehaired skin indicate that the enzyme acts upon the

protein part and immature keratin of hair root, which are more prone to proteolytic attack than hair itself (Archana and Pillai, 2012). It involves digestion of the basal cells of hair bulb and cells of the malphigian layer, followed by attack on the outermost sheath and subsequent swelling and breakdown of inner root sheath thereby degrading unkeratinized hair parts and finally dislodging of intact hair from hair bulb (Kamini *et al.,* 1999). Archana and Pillai, (2012**)** suggested that the term 'epilation' for enzymatic dehairing due to the characteristic feature of intact hair removal in enzymatic dehairing instead of 'depilation' which is, commonly used term for hair removal by conventional method.

Though there is no direct correlation between the elastase, keratinase, chondroitinase, esterase, hyaluronidase or collagenase activities in the crude enzyme preparation used for depilation, broad-spectrum endopeptidase activity was found to be more important for successive depilation (Edmonds, 2008). Foroughi *et al.,* (2006) conducted systematic study of different dehairing preparations from commercially available sources as well as those produced from newly isolated cultures. Most of the efficient dehairing preparations showed activity towards azocoll and the action of these enzymes did not affect the grain structure. Sivasubramanian *et al.,* (2008a) observed that there was marked reduction in proteoglycan constituents of skin as compared to lime and sulfide dehaired skin while collagen content remained unchanged with both the treatments. They also concluded that removal of proteoglycan aggregates play important role in opening up of collagen fiber during enzymatic dehairing. Archana and Pillai, (2012**)** stated that the enzyme preparation having activity against cementing substances might help the enzyme to reach up to the hair bulb to mediate its action. Based on the above observations, it can be predicted that protease preparation rich in chondroitinase activity may facilitate the degradation of glycosaminoglycans of proteoglycans thereby improving dehairing and fiber opening of the skins/hides.

Although, enzyme mediated dehairing has been known to be technology feasible, exclusive use of enzymes for dehairing, defleshing and removal of other matrix components of skin without using any chemical has not been achieved yet. To address various challenges related to present Indian leather sector, a network of different institutions was formed under the program New millennium Indian Technology

Leadership Initiative (NMITLI) of the Council of Scientific and Industrial Research (CSIR). The present protease was developed under this programme and is extensively evaluated for dehairing of skins and hides.

The present section deals with application of alkaline protease produced by a strain of *Conidiobolus brefeldianus* for dehairing of skins and hides. Process parameters with respect to enzyme concentration and treatment time were optimized for skins and hides.

Materials and methods

Materials

Wet salted skins (goat and sheep) and hides (cow and buffalo) were purchased from local market. For enzymatic dehairing experiments, crude concentrated *C. brefeldianus* protease obtained by ammonium sulphate precipitation (90% saturation) was used. Amylase and lipase used were purchased from M/s Sisco Research Laboratories, India. Commercial grade lime, sodium sulfide, ammonium sulphate, NaCl, H_2SO_4, basic chromium sulphate ($Cr(OH)SO_4$), sodium formate, sodium bicarbonate, dyes and fat liquors etc. were used for conventional dehairing and tanning operations.

Methods

Soaking of skins

Prior to dehairing, skins/hides were soaked with three changes of water containing wetting agent and preservative (300% v/w of salted skin/hide) until they were free from dirt, dung and blood stains. After soaking, the skins/hides were piled for about one hour before application to drain excess water and the soaked weight of the skins/hides was noted. Lime and sulfide or enzyme applied was based on the soaked weight of skin/hide.

Dehairing of skins/hides

Dehairing was performed by paint method. A paste of lime (10%) and sulfide (3%) or protease with 10% water was applied uniformly on the flesh or grain side of the skin/hide and left at ambient temperature (28-32°C) for different periods. Hair removal was performed either by gently scraping the hair with scalpel or by blunt knife depending on the skin type. Dehairing was expressed as percentage removal where complete hair removal was treated as 100%. Enzymatic dehairing was performed with 0.2 to 3% crude concentrated protease. The dehaired pelts were processed further to dyed crusts by conventional methods.

Physical properties of dyed crust

The dyed crusts of enzyme and lime sulfide treated leathers were tested for physical strength properties. After conditioning the crust leather at room temperature over a

period of 48 hours, the properties such as tensile strength, elongation at break and tear strength were assessed in comparison with control samples using standard methods.

Histological analysis

A small piece (2 x 2 cm) of dehaired skin and hide was cut and washed with water and fixed in 10% formaldehyde solution. Sample pieces were dehydrated by series of ethanol treatment and embedded in paraffin blocks. Sections of three micron thickness of the embedded samples were cut using ASCO make microtome, stained with hemotoxylin and eosin and the sections were examined under light microscope and photomicrographs were taken.

SEM studies

Morphological characterization of dehaired pelts and dyed crust leather of goat skin was performed by means of scanning electron microscope. The surface of dehaired pelts and cross sections of dyed crust leather were scanned on scanning electron microscope Model Sterioscan- 440 from LEICA– Cambridge UK.

Results and discussion

The Leather industry is bestowed with an affluence of raw materials as India is endowed with 21% of world cattle & buffalo and 11% of world goat & sheep population. Normally hides and skins of domestic live stock mainly cow and buffalo, goat and sheep are used in tanning industry. For broad utility of the *C. brefeldianus* protease and its commercialization, it is necessary that the enzyme is useful in dehairing wide range of skins and hides varying in fat content and thickness etc. Therefore, dehairing efficiency of *C. brefeldianus* protease was evaluated with goat/sheep skins and cow/buffalo hides. Dehairing was initially carried out for 16 h with fixed enzyme concentrations of 2 and 3% for skins and hides respectively. Since hides are thicker than skins, higher enzyme concentrations were used. The thickness of cow hide is 3-15 mm compared to 1-2 and 1-3 mm for goat and sheep skins respectively.

Dehairing of goat and sheep skins

The dehairing potential of crude protease from *C. brefeldianus* towards goat and sheep skins was initially investigated on small pieces (12x15 to 12x21cm) weighing approximately 50-90g. Two percent protease was applied on the flesh side and skin pieces were piled. After 16 h, hair was removed manually by gentle scraping with scalpel. Dehairing by conventional method was also performed in similar manner for comparison. Hair could be easily removed by both conventional and enzymatic method from goat as well as sheep skins (Figures 4.1.6 to 4.1.10). The dehairing efficacy of the enzyme was assessed in comparison with that of the lime and sulfide method. In case of the enzymatic dehairing, hair was removed with the epidermis while in chemical method, short hairs in some places and incomplete removal of epidermis were seen. Enzymatically dehaired pelts were cleaner, more whitish with smooth feel due to hair removal along with epidermis and hair obtained was intact (Figure 4.1.8). In contrast, dehaired pelt obtained by conventional method showed presence of epidermis layer and hair obtained had damaged ends.

Figure 4.1.6: Dehairing of goat skin with lime and sulfide

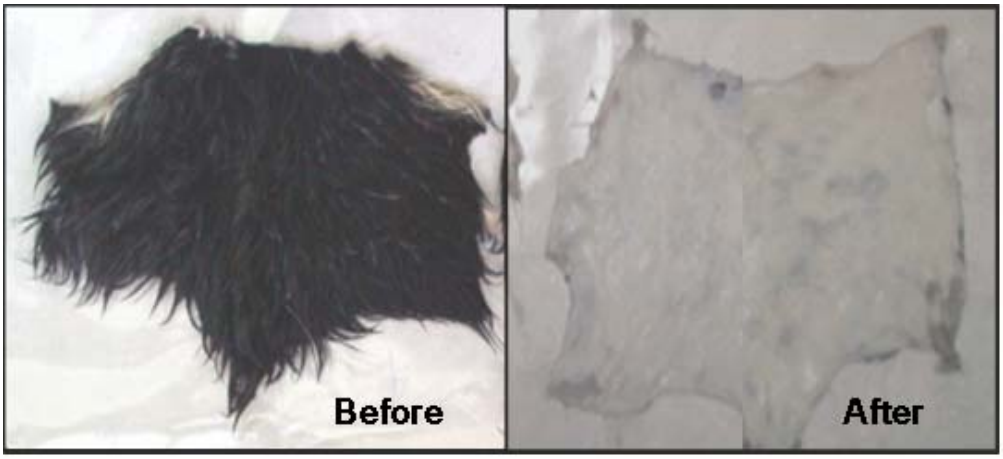

Figure 4.1.7: Dehairing of goat skin with protease

Figure 4.1.8: Hair removal from goat skin along with epidermis and enlarged view

Figure 4.1.9: Dehairing of sheep skin with lime and sulfide

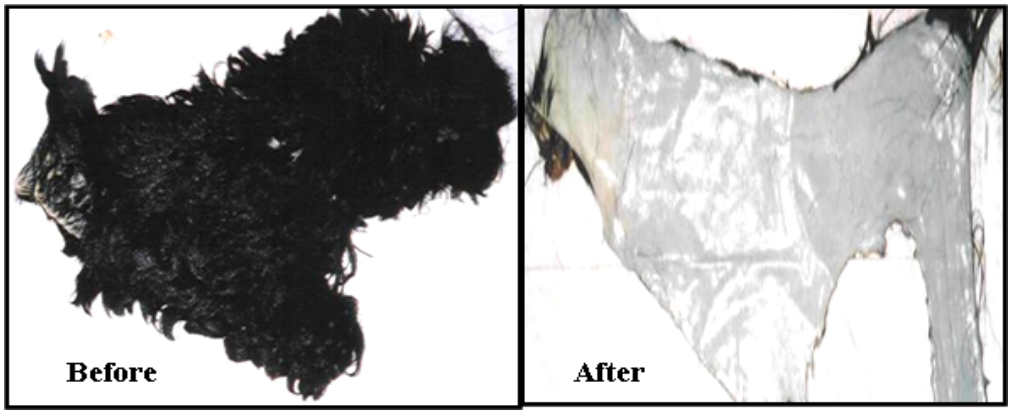

Figure 4.1.10: Dehairing of sheep skin with protease

Majority of the proteases used for dehairing of goat skins were from the strains belonging to the genus *Bacillus* viz. *B. subtilis* (Pillai and Archana, 2008; Sivasubramanian *et al.,* 2008b; Senthilvelan *et al.,* 2012), *B. megaterium* (Rajkumar *et al*., 2011), *B. licheniformis* (Nadeem *et al.,* 2010; Haddar *et al.,* 2011), *B. halodurans* (Prakash *et al.,* 2010), *B. cereus* (Sundararajan *et al.,* 2011; Saleem *et al.,*2012) and *Bacillus* sp. (Raju *et al.,* 1996; Arunachalam and Saritha 2009). Dehairing of goat skins by protease from *Elizabethkingia meningoseptica* resulted in complete removal only after 18 h (Nagal *et al.,* 2010). Some of these studies were conducted as dehairing assays using very small pieces of skins ranging from 2x2 cm to 5x5 cm by dipping the skin pieces in enzyme solutions with or without lime and sulfide held in alkaline pH range and temperatures ranging from 30-40°C which are optimum for the enzyme action (Pillai and Archana, 2008; Nadeem *et al.,* 2010; Prakash *et al.,* 2010; Haddar *et al.,* 2011; Saleem *et al.,* 2012). Haddar *et al.,* (2011)

reported dehairing of goat skin pieces with *B. licheniformis* protease (7000 U/ml) and found no hair removal at 25°C while incomplete dehairing was seen at 30°C and complete hair removal was achieved only at 37°C after incubating for 24 h under shaking conditions. Excessive damage to the skin can occur when dehairing has to be carried out at elevated temperature which is difficult to maintain at commercial scale (Edmonds, 2008). Though one can argue that temperatures vary in different regions of the country and may reach around 40°C in summers in south, skins are normally piled up and left overnight when temperatures are lower than the day temperatures. Therefore, proteases active over a broad temperature range and suitable for application throughout the year and all over the country are advantageous.

Dehairing of goat skins by keratinase from *Streptomyces fradiae* in presence of alkali is reported by Robison *et al.,* (1961). Dehairing of goat skins was studied with proteases from wild and mutant strain of *S. nogalator* and was correlated to the levels of protease secreted by them (Mitra and Chakrabartty, 2005). Verma *et al.,* (2011) reported an alkaline protease active at 80°C and pH 8 from *Thermoactinomyces* sp. RM4 which showed good dehairing of goat skin in 24 h with intact hair and clean pelt.

The survey of literature revealed that the number of reports on dehairing with fungal proteases are very few compared to bacterial proteases and mostly confined to the genera of *Aspergillus* (Malathi and Chakraborty, 1991; Dayanandan *et al.,* 2003; Madhavi *et al.,* 2011), with few reports on dehairing with proteases from *Rhizopus oryzae, C. coronatus, Beauveria* sp., and *Penicillium griseofulvum* (Pal *et al.,* 1996; Laxman *et al.,* 2007; Laxman *et al.,* 2011b; Madhavi *et al.,* 2011).

Although reports on enzymatic dehairing of goat skins are many, reports on dehairing of sheep skin are very few. Pal *et al.,* (1996) reported dehairing of goat and sheep skins with *R. oryzae* protease (5 U/cm^2) after soaking and equilibrating the skins to pH 8 in buffer. Complete hair removal reported after 11-12 h.

Dehairing of cow and buffalo hides

The structural features and thickness of skin and hide varies greatly and hence efficiency of an enzyme in their dehairing may vary accordingly. Therefore, dehairing

of hides is considerably difficult than that of skins (Sivasubramanian *et al.*, 2008b). Dehairing efficiency of the protease towards cow and buffalo hide was investigated by applying 3% protease on the flesh side of hide pieces (100-250 g). After enzyme application, hide pieces were piled and incubated for 16 h and dehairing was performed with blunt knife. A control with conventional lime and sulfide method was also carried out for comparison. The protease was effective for dehairing of both cow and buffalo hides. The dehaired pelts showed smooth and white appearance and were cleaner and whiter compared to lime and sulfide dehaired pelts (Figures 4.1.11 to 4.1.14).

Figure 4.1.11: Dehairing of cow hide with lime and sulfide

Figure 4.1.12: Dehairing of cow hide with protease

4.1.13: Dehairing of buffalo hide with lime and sulfide

Figure 4.1.14: Dehairing of buffalo hide with protease

Proteases from bacterial as well as fungal sources have been used for dehairing of bovine hides. However, reports on dehairing of cow and buffalo hide are limited and mainly restricted to bacterial proteases (Macedo *et al.,* 2005; Zambare *et al.,* 2007; 2011; Pandeeti *et al.,* 2011; Saleem *et al.,* 2012). Reports on dehairing of buffalo hides are mostly from India and fewer compared to that of cow hides. Dehairing of calf skins by proteases from *S. fradiae* in presence of lime is reported by Robison *et al.,* (1961). Anderson, (1998) reported dehairing of bovine hides for 24 h with a commercial protease in presence of lime and protein disulphide redox agent. Laxman *et al.,* (2007) reported dehairing of cow hides using alkaline protease from *C. coronatus* devoid of lime but in presence of small amounts of sodium sulfide. Dehairing of cow hides with protease from *Beauveria* sp. in absence of any added chemicals is also reported recently (Laxman *et al.,* 2011b). Rose *et al.,* (2007) reported lime and sulfide free unhairing of skins/hides using animal and/or plant

enzymes. Saravanbhavan *et al.,* (2005a) reported enzymatic unhairing of cow and buffalo hides with commercial bacterial proteases. Four among the five commercial proteases tested were found promising for dehairing of hide by dip method but resulted in incomplete removal of epidermis with dark coloration on the pelt (Dettmer *et al.,* 2011).

Physical properties of dyed crust

It is essential that there is no damage to the quality of leather in terms of strength, elongation etc. while complete removal of hair is achieved. Over exposure should be avoided to get good quality leather. Therefore dehaired pelts were processed further by conventional methods to obtain dyed crust. Evaluation of physical properties of dyed crust leather obtained from goat skin and cow hide were performed and compared with corresponding control samples dehaired by lime and sulfide method (Table 4.1.3). The values for tensile strength, elongation and tear strength were slightly higher than that of control samples which is desirable. This is in agreement with some of the earlier reports, wherein enzymatic dehairing improved tensile properties and elongation of crust leather (Pal *et al.,* 1996; Dayanandan *et al.,* 2003; Sivasubramanian *et al.,* 2008b).

Table 4.1.3: Physical properties of dyed crust leather

Skin /hide	Dehairing method	Tensile strength (Kg cm^{-2})		Elongation at break (%)		Tear strength (Kg cm^{-1})	
		Parallel	Perpendicular	Parallel	Perpendicular	Parallel	Perpendicular
Goat	Lime-sulfide	233.13	186.85	48.11	71.22	45.10	38.11
	Enzymatic	239.45	192.36	49.04	73.26	46.66	38.87
Cow	Lime-sulfide	290.01	242.71	48.12	68.77	47.01	41.00
	Enzymatic	302.25	250.36	50.23	72.24	47.65	42.04

Histological analysis of dehaired pelts

Reported literature indicates that diffusion of protease to proteolytic sites is important. Once enzyme is penetrated, broad spectrum protease activity can bring about the destruction of various proteins located around the cells of the epidermis and the basal layer (Edmonds, 2008). Complete removal of epidermal layer from skin and hide was observed when dehairing was performed using *C. brefeldianus* protease. The optical micrograph of the stained cross section of goat skin and cow hide confirmed the visible assessment (Figures 4.1.15 & 4.1.16). The microscopic structure of cross section of dehaired pelt clearly distinguishes the enzymatic dehairing from the conventional process of dehairing with respect to the extent of removal of epidermis, hair shaft and follicles. Complete absence of these structures was observed in the sections from enzymatically dehaired pelt of goat skin and cow hide. In case of conventionally dehaired pelts, remnants of epidermal layer and hair shaft were observed. Enzymatically dehaired pelt showed complete and uniform collagen fiber opening in the dermis and corium region, while in conventionally dehaired pelt, the fiber structure appeared to be compact and unevenly opened. Similar observations on differences between conventionally and enzymatically dehaired pelts were also reported by other researchers (Raju *et al.,* 1996; Zambare *et al.,* 2007; Sivasubramanian *et al.,* 2008b; Saleem *et al.,* 2012).

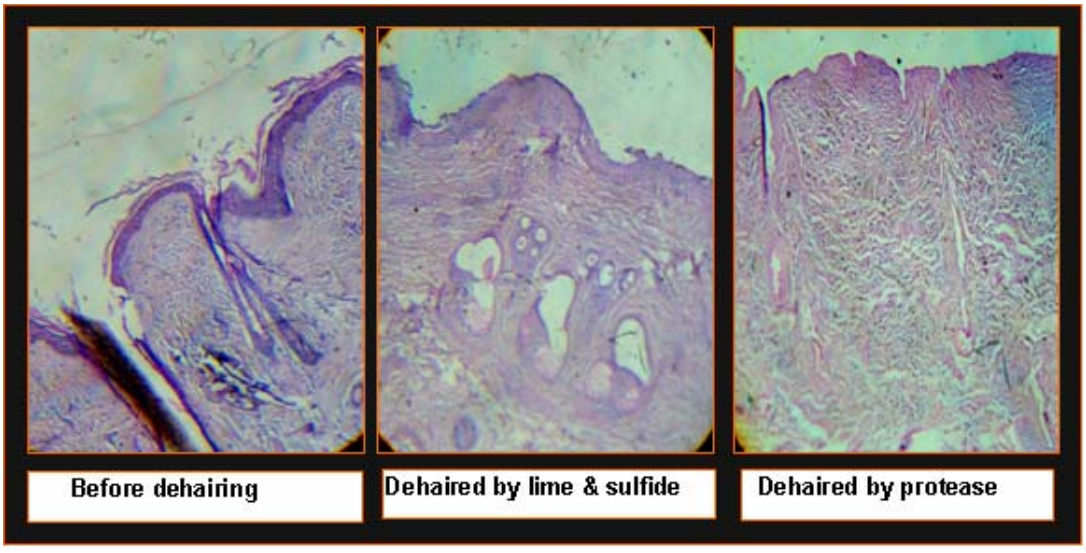

Figure 4.1.15: Histology of goat skin sections before and after dehairing (100x)

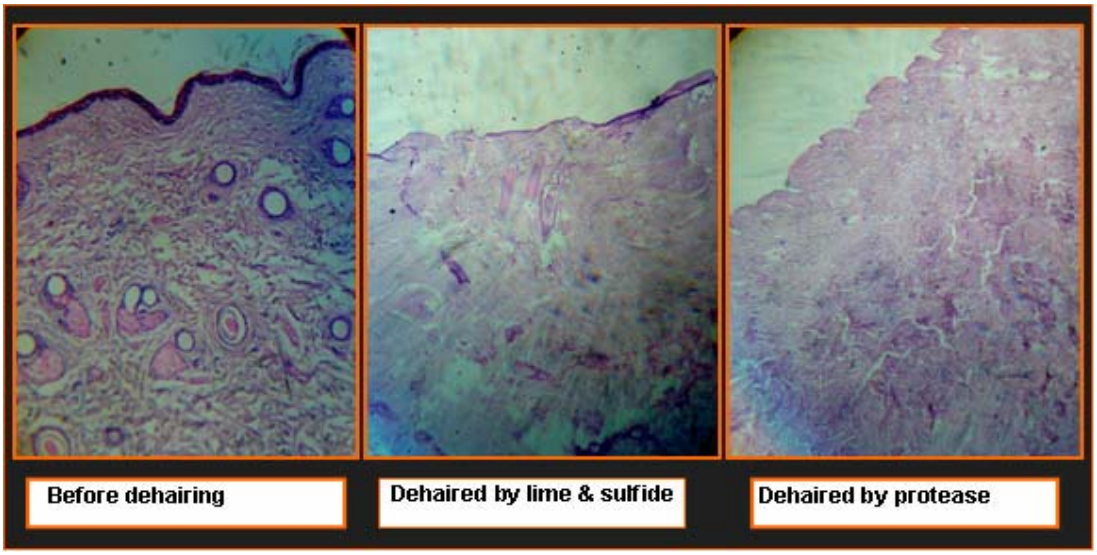

Figure 4.1.16: Histology of cow hide sections before and after dehairing (100x)

SEM analysis of dehaired pelt and dyed crust

Scanning electron microscopic studies were carried out on the grain surface and cross section of dehaired pelt and dyed crust respectively. Grain structure was clean and empty hair follicles were clearly seen in case of enzymatic dehairing. However in conventionally dehaired pelt, hair follicles were not clearly visible due to deposits of lime and sulfide and scuds were seen. Cross section of dyed crust from enzymatic dehairing shows more visible fiber separation compared to those obtained by conventional method (Figures 4.1. 17 & 4.1.18).

Effect of enzyme concentration on dehairing of goat skin

Though goat skins have a thickness of 1- 2 mm which is similar to sheep skins (1-3 mm), the structure of goat skins is very much firmer. Sheep skins due to the immense amount of hair roots and fat cells within the skin the structure is relatively weak. Hence, if the dehairing of goat skin is achieved by an enzyme, it is most likely to work for dehairing of sheep skins. Since *C. brefeldianus* protease was effective in dehairing all types of skins/hides tested, optimization of dehairing conditions such as effect of enzyme concentration and time was carried out before large scale trials were conducted with goat skin as a representative of skin type.

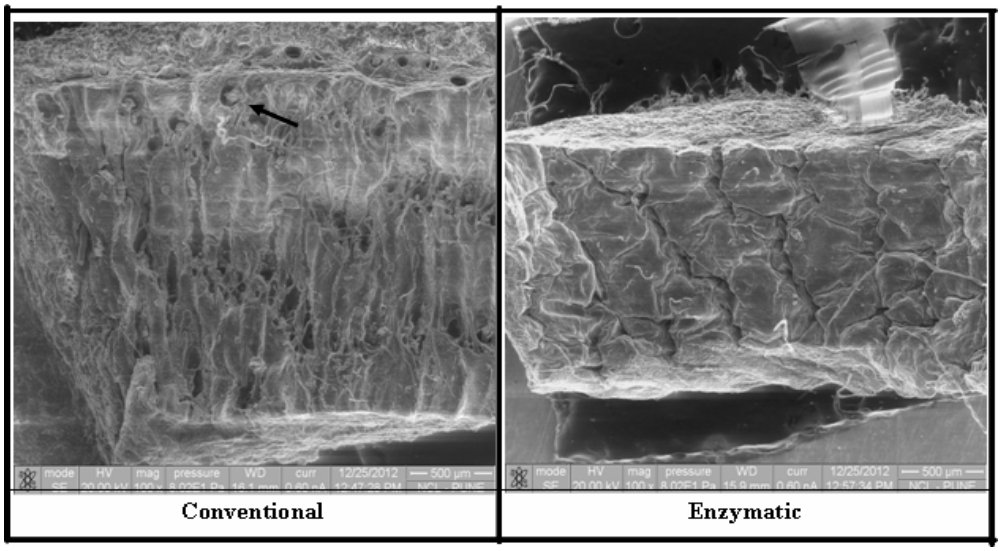

Figure 4.1.17: SEM showing of grain surface of dehaired goat pelts

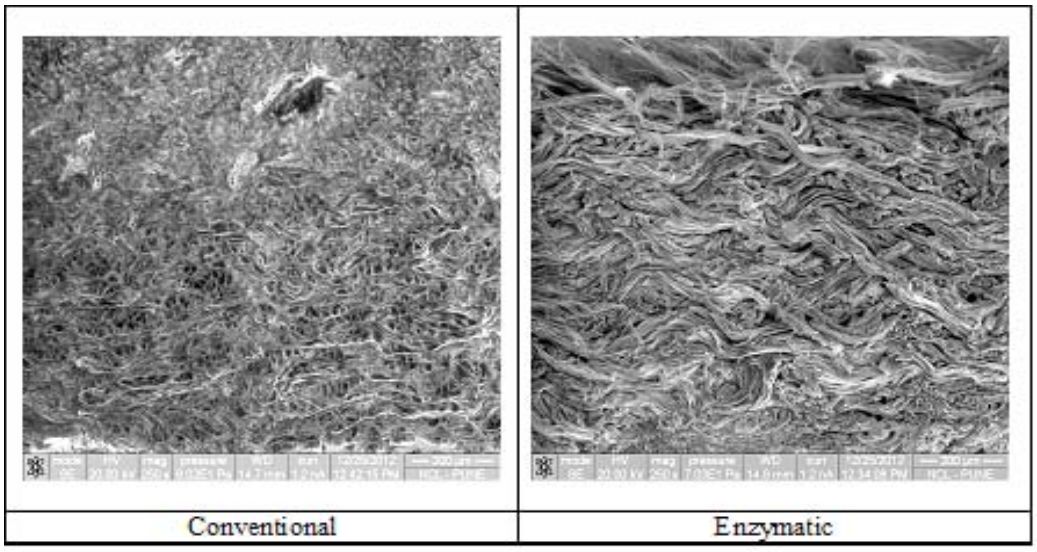

Figure 4.1.18: SEM showing cross section of dyed crust of goat skins

Dehairing was performed on goat skin pieces (300 to 800g) with protease concentrations ranging from 0.2 to 2.0% for 16 h. The enzyme was effective for dehairing of goat skin even at lower enzyme concentrations of 0.2 to 0.5% with 65-90% dehairing and increasing the concentration to 1% resulted in 98% dehairing (Figure 4.1.19).

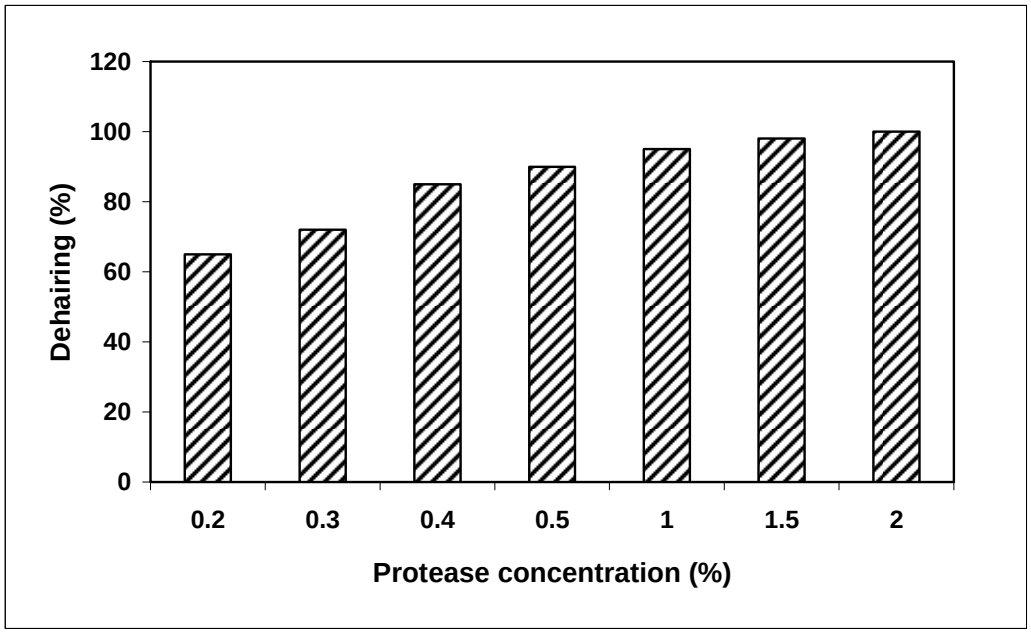

Figure 4.1.19: Effect of protease concentration on dehairing of goat skin

Time course of dehairing of goat skins

Time span required for dehairing was investigated on goat skin pieces using 1.5% protease. Extent of hair removal was examined at regular time intervals. No dehairing was observed in 2 h and was initiated only after 6 h and increased with incubation time. Around 70-75%, dehairing was seen after 12 h and was completed within 16 h (Figure 4.1.20). Pillai and Archana, (2008) reported that keratinolytic protease from *B. subtilis* required 18 h to dehair goat skin piece by dip method when used in presence of 0.1% lime. Rajkumar *et al.,* (2011) carried out dehairing of goat skin using *B. megaterium* RRM2 protease and required 12 h of treatment time for visible dehairing activity. Dayanandan *et al.,* (2003) studied dehairing efficiency of *A. tamarii* alkaline protease (applied in combination with 10% kaolin) on goat skin and complete dehairing was observed after 18 and 12 h with 1 and 2% enzyme respectively. They observed that decreasing the protease concentration to 0.5% gave moderate dehairing even after 24 h while addition of 1% sulfide to 0.5% protease resulted in complete dehairing in 24 h. Dehairing of goat skins by protease from *Elizabethkingia meningoseptica* resulted in loosening of hair after 12 h and complete removal was observed only after 18 h (Nagal *et al.,* 2010). Saravanbhavan *et al.,*

(2005a) reported enzymatic unhairing of cow and buffalo hides for 18 h with commercial bacterial proteases.

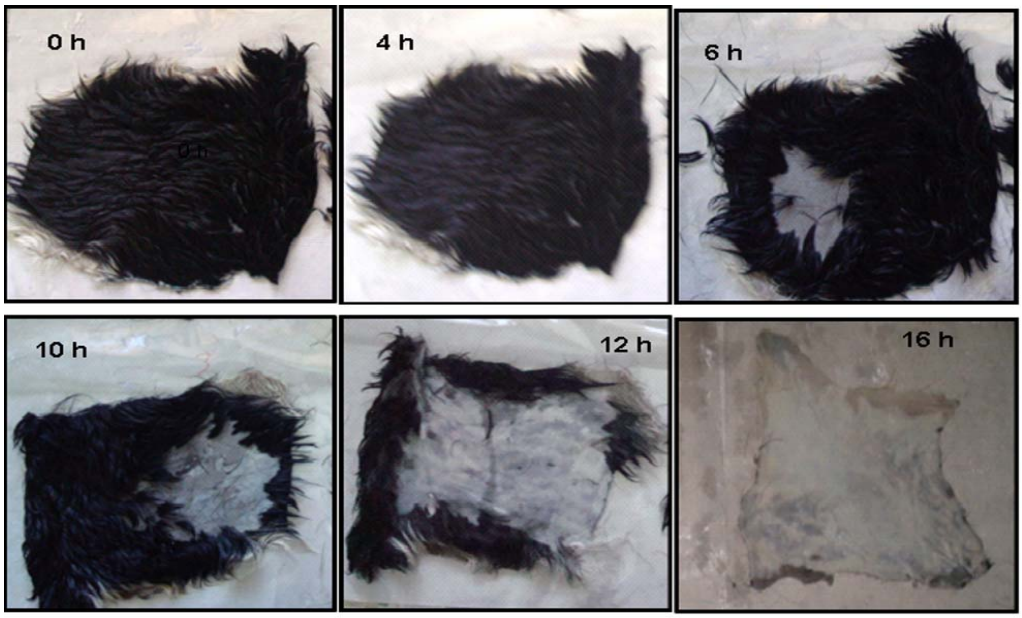

Figure 4.1.20: Time course of dehairing for goat skin

Fiber opening of goat skin

The processing of hides and skins to leather involves several stages. Pre tanning and tanning processes are known to contribute major pollution load in leather processing due to involvement of different chemical operations. These different operations and pH variations result in the emission of large pollution loads such as BOD, COD, chlorides, sulphates, chromium and sludge. Saravanabhavan *et al.*, (2005b) described an integrated three step bio-based process, which limits the pH range of the process to 4 - 8 and substantially decrease the chemical consumption from 380 to 50 kg for tanning one ton of raw skins. This involved application of protease and amylase for dehairing and fiber opening respectively instead of conventional lime-sulfide dehairing and liming operation. This was followed by pickle–basification free chrome tanning. Amylases and lipases are generally used solely or in combination with protease for fiber opening and degreasing respectively (Thanikaivelan *et al.,* 2002; Choudhary *et al.,* 2004). In present study, we examined the fiber opening of goat skin dehaired by *C. brefeldianus* protease using amylase alone and in combination with lipase. Four halves of goat skins were dehaired with 2% protease. Wet weight of dehaired skin was noted for each half. One-half goat skin was used for each trial. One

percent amylase was used alone or in combination with 0.5% lipase with 100% water (based on wet weight of dehaired skin). Treatment was given in drum with run at 10 rpm. Drum was run for 15 min followed by 45 min rest for 3 h. The increase in wet weight of dehaired skin after swelling was noted and expressed as percentage increase in weight (Table 4.1.4). It is evident that amylase at 1% concentration resulted in fiber opening slightly less than that of control (10% lime) where as 1% amylase in combination with 0.5% lipase resulted in swelling better than the control. Sarvanabhavan *et al.,* (2005b) optimized amylase concentration for opening up of fiber bundles in three step tanning process. The fiber opening was better with 0.5 to 1.0 % amylase.

Table 4.1.4: Fiber opening of enzymatically dehaired pelts (goat)

Swelling with			Weight of dehaired pelt before swelling (g)	Weight of dehaired pelt after swelling (g)	Increase in weight (%)
Lime	Amylase	Lipase			
10%	-	-	600	860	43.33
-	1%	--	580	820	41.37
-	1%	0.5%	600	880	46.66

Dehairing of goat skins at large scale

The extent of hair integrate varies in different areas of the same skin. Hair in the backbone and neck region is tougher to remove due to presence of excessive proteoglycans. Hence, it is necessary to examine the dehairing potential of protease with whole skins. After optimizing the conditions of dehairing on skin pieces, dehairing of goat skins was validated at large scale using 10 goat skins. Skins were soaked, washed and cut into two halves. The right halves of the skins were applied with the enzyme preparations and treated as experimental skins. The left halves were the controls and were dehaired by following a conventional lime sulfide method using 3.0% sodium sulfide and 10% lime. Two enzyme concentrations (1.5 and 2%) were used for dehairing. Paste of enzyme and 10% water was applied on the flesh side and the skins were piled and covered with gunny cloth and left for overnight. Next day

(after about 16 h), dehairing was performed with blunt knife on wooden beam in similar manner used in leather industry. Hair could be easily removed from the central area of skin, whereas it required slight force to unhair the peripheral and backbone part. While complete dehairing was observed with 2% protease, few hair patches were observed with 1.5% protease (Figure 4.1.21**)**. The dehaired pelts obtained by enzymatic method showed smooth and white appearance due to removal of epidermis and no grain damage was seen. Nadeem *et al.,* (2010) reported complete dehairing of goat skin pieces with *B. licheniformis* protease in 12 h but on further incubation up to 15 h, considerable grain damage was observed. Pal *et al.,* (1996) reported dehairing of goat and sheep skin by *R. oryzae* protease at pH 8 and 12 h.

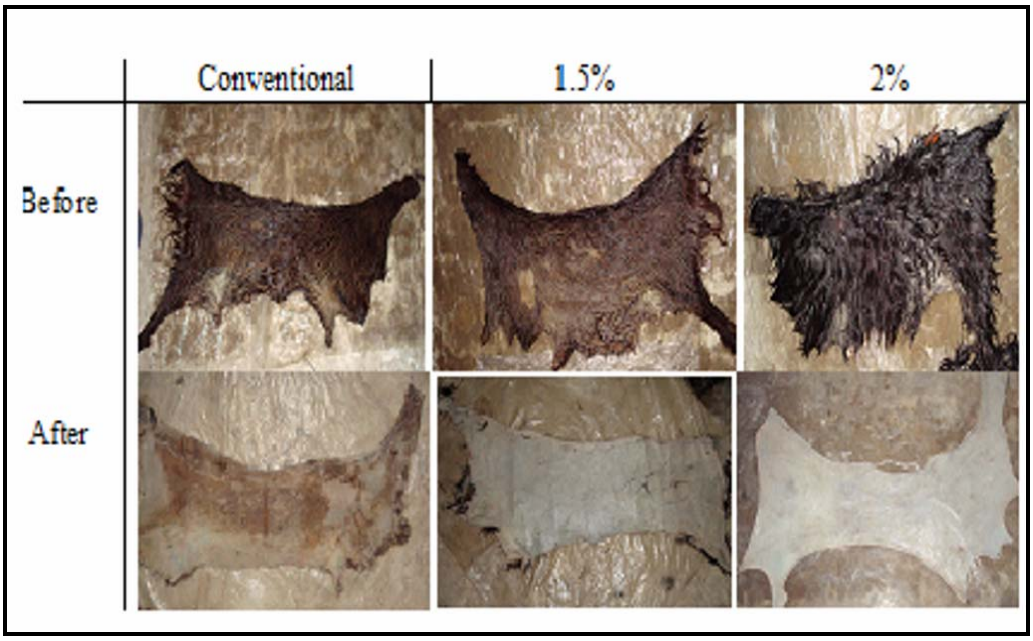

Figure 4.1.21: Large scale trials for dehairing of goat skins

Dehairing of cow sides

Wet salted cow hides were soaked, washed and cut into two halves. The left halves were dehaired by conventional method of lime (10%) sulfide (3%). The right halves of the skins were applied with the enzyme preparations and treated as experimental skins. Three enzyme concentrations (1.5, 2, and 2.5%) were used dehairing. Paste of enzyme and 10% water was applied on the grain side and the skins were piled grain to grain, covered with gunny cloth and left for overnight. Next day, dehairing was performed with blunt knife on wooden beam. Dehairing increased with increase in

enzyme concentration and complete dehairing (100%) was observed with 2.5% protease after 18 h (Table 4.1.5**)**. The resultant dehaired pelt showed white appearance due to removal of epidermis. In this experiment, enzyme was applied on grain side instead of flesh side (as applied in the earlier experiment) to facilitate better penetration of enzyme to reach up to hair follicles taking into account that the thickness of hide is much more than skin. These results suggest that the extent of hair and epidermis removal was not affected by the mode of enzyme application (enzyme application either on the flesh side or grain side).

Table 4.1.5: Effect of protease concentration on dehairing of cow hide

Weight of cow hide (kg)	Protease (%)	Dehairing (%)
7	1.5	70
6	2.0	90
9	2.5	100

Conclusions

The crude protease was effective in lime and sulfide free dehairing of goat/sheep skins and cow/buffalo hides at 1.5 and 2.5% concentration respectively. Dehairing was completed in 16-18 h. The resultant dehaired pelts showed white appearance with smooth feel due to removal of epidermis and undamaged grain structure. Hair obtained by enzymatic method was intact and there was absence of short hair in dehaired pelts. Histological examination of enzymatically dehaired pelt also substantiated above findings, wherein complete removal of epidermis and absence of short hair could be seen. Enzymatically dehaired skin/hide showed well-opened fiber structure. These results were also supported by SEM studies. The crude alkaline protease preparation from *C. brefeldianus* exhibits other associated activities like elastase, keratinase, azocollagenase and chondroitinase, which are useful in improving dehairing efficacy by removal of proteoglycans of the skin in the back bone and neck region. The enzyme preparation is free from true collagenase activity and no damage to the grain is seen which is critical for good quality leather. The present enzyme possesses excellent dehairing efficacy towards all the skin and hide type tested, which are major source of raw material for leather industry.

Advantages of *C. brefeldianus* protease preparation are:

i) the crude preparation has elastase, keratinase as well as chodroitinase activities
ii) does not have collagenase activity
iii) active at ambient temperature and neutral pH thus exclusive control may not be required.
iv) Does not require any added chemicals like lime and/or sulphide
v) The hair obtained was intact and of good quality.
vi) Cleaner and whiter pelts

In view of this, the present protease may prove itself as an excellent dehairing agent for eco-friendly leather processing. Large scale trials of dehairing of goat skins and cow hides were successful and its commercialization is being explored.

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SECTION 2

APPLICATION OF PROTEASE IN DEGUMMING OF SILK

Abstract

Traditionally, degumming of silk is carried out with soap and alkali at higher temperature or by boiling at elevated temperature and/ or pressure for 1-2 h. It is a high resource consuming process and generates effluents with high impact on environment. In contrast, enzymatic degumming process is advantageous as it would save energy, water, chemicals and effluent treatment costs. Degumming of Chinese bivoltine silk with six indigenous microbial alkaline proteases was investigated and compared with commercial enzymes as well as with conventional degumming process. The enzymatic degumming was carried out between of 40 to 65°C while conventional alkali soap degumming was performed at 95°C. Among the six proteases tested, two fungal and two actinomycete proteases were promising which showed weight loss similar to conventional method (19.58 to 21.78%). *C. brefeldianus* and BOA-2 proteases were found to be best which showed complete degumming within 15-30 min and at low enzyme concentrations. Degummed fibers showed no significant difference in tensile strength or elongation at break indicating no damage to the fiber which was confirmed by scanning electron microscopic studies. Recovery of sericin from degumming liquid after enzymatic degumming and analysis by SDS–PAGE revealed the presence of three distinct proteins with molecular weights ranging from 19.6 kDa to 26.6 kDa or slightly higher. Enzymatic hydrolysis of pure sericin obtained from silk cocoons with *C. brefeldianus* protease was investigated. Analysis of peptides obtained after hydrolysis for 30 min with low protease concentrations showed a major peak corresponding to 1092 Da while with higher concentration, major peptide formed was of lower molecular weight (516 Da). Effect of time on hydrolysis showed that 15 to 30 min was sufficient to hydrolyse the sericin to peptides with molecular weights in the range of 516-1092 Da.

Introduction

According to a legend, silk was discovered in China at around 2700 B.C. Silkworms were damaging mulberry trees of one Chinese emperor. To protect mulberry trees from the silkworm attack, his wife detached silkworm cocoons from trees but unknowingly dropped it into hot tea. She was surprised to see that the cocoon could be unwound to produce a lustrous fabric which we now know as silk. At the time, the secret of silk was closely guarded and reserved only to China's emperors and other important people. As time passed, the secret to the creation of silk spread to India and Europe. Now the whole world knows about silk (http://frogglemedia.files.wordpress.com/2008/03/silkworms).

Sericulture

Sericulture refers to the controlled large-scale cultivation of silk producing organisms to obtain silk cocoons and its reeling into fiber. Silk is further weaved to convert the yarn to fabric. The word 'sericulture' is derived from the Greek word 'Sericos' meaning 'Silk' and the English 'Culture' meaning 'Rearing'. Silk is one of the most beautiful and precious gift given to us by nature and known as ***'****Queen of fibers'*. Silk has fascinated man since antiquate due to its splendor, sensuous and marvelous feeling. Cloths made up of silk have always surrounded by aura of magnificence. Other natural fibers and more predominantly synthetic fibers overshadowed it over the past few years. However, recently its importance to textile industry has again increased due to the current preference for natural products and the consequential boost in demand for fabrics made up of natural fibers. The radiant look for which silk is appreciated comes from the triangular prism-like structure of fibers, which allows silk cloth to refract incoming light at different angles. Although the production of silk could not reach up to the level of other natural and synthetic fibers, it will continue to occupy its special position as a fiber for exceptionally high quality garments. The silk is gaining increasing importance day by day because of its exclusive qualities, which are rarely found in any other fibers. It is recognized for its characters such as luster, water absorption, heat retention, smooth feel, comfort and could survive man made fibers such as nylon and polyesters etc. Silk is the strongest of all the natural fibers and is a poor conductor of electricity making it comfortable to wear in cool weather (http://www.fibre2fashion.com/industry-article/23/2262/chemical-processing-of-silk2.asp; Laxman, 2012).

Structure and composition of silk fiber

Silkworm after maturation builds a cocoon around itself by extruding a viscous protenious secretion from two large glands present in the lateral part of body. The fluid is extruded through two ducts in the head of the silkworm into a common spinneret. Another protenious secretion, extruded from distinct gland, covers the viscous part. These two components are cemented together by emerging into the air, coagulating and producing a firm continuous filament (Sonthisombat and Speakman, 2004). The fiber produced by spinning process has two main parts, the fibrous structure is known as fibroin and the cementing glue part is known as sericin (Figure 4.2.1).

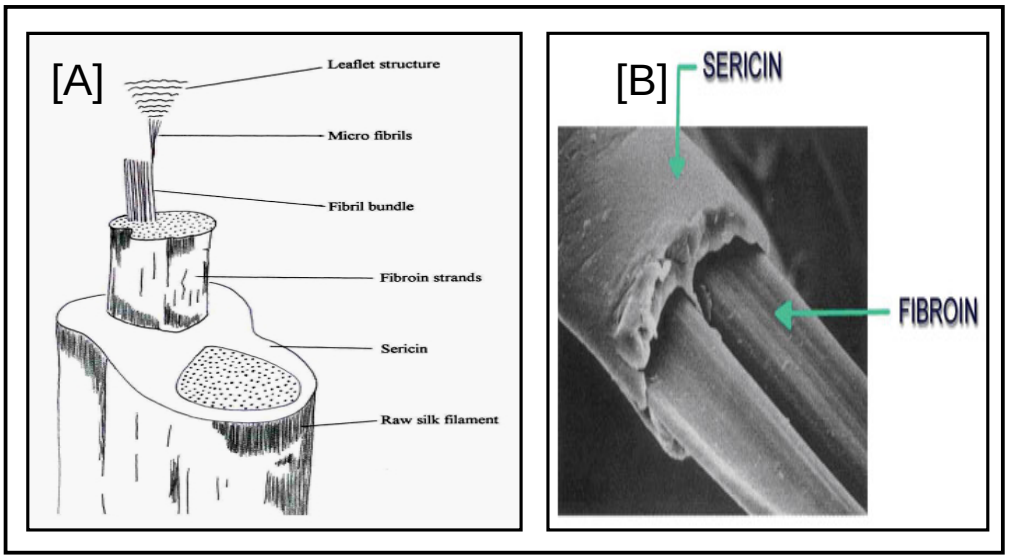


Figure 4.2.1: Structure of silk fiber: A-Schematic representation (Sonthisombat and Speakman, 2004); B-Scanning electron micrograph (Shao and Vollrath, 2002)

Fibroin accounts for about 70 to 80 % of the total silk whereas sericin that gives harsh and stiff feeling to the silk fiber, contributes 20 to 30% in the silk (Wu *et al*., 2008). Apart from fibroin and sericin, waxes and inorganic matter are also present in trace amounts. The overall composition of silk is given in Table 4.2.1.

The amino acid composition of *Bombax mori* silk fibroin and sericin has been listed in Table 4.2.2.

Table 4.2.1: Overall composition of silk (Rajasekhar, 2011)

Components	Availability (%)
Fibroin	70-80
Sericin	20-30
Wax	0.4-0.8
Carbohydrate	1.2-1.6
Inorganic Matter	0.7

Table 4.2.2: Amino acid composition of fibroin and sericin (residues/1000residues)

Amino acids	Fibroin	Sericin
Glycine	446.0	127.0
Alanine	294.0	55.1
Valine	22.0	26.6
Leucine	5.3	7.2
Isoleucine	6.6	5.5
Serine	121.0	319.7
Threonine	9.1	82.5
Aspartic acid	13.0	138.4
Glutamic acid	10.2	58.0
Lysine	3.2	32.6
Arginine	4.7	28.6
Histidine	1.4	13.0
Tyrosine	51.7	34.0
Phenylalanine	6.3	4.3
Proline	3.6	5.7
Tryptophan	1.1	-
Methionine	1.0	0.5
Cystine	2.0	1.4

(Sonthisombat and Speakman, 2004)

Fibroin is made up of eighteen amino acids and majority of them contain non-polar side chains glycine, alanine and serine, which together constitute about 82%, of which about 10% is serine (Sen and Babu, 2004). It is a glycoprotein composed of two

protein subunits of 370 and 25 kDa linked by disulfide bond. Fibroin filament is composed of crystalline and amorphous domain (Padamwar and Pawar, 2004). The characteristics orientation and crystalline structure tends to make it insoluble in water. Sericin is a group of gelatinous proteins made up of 18 amino acids most of which are rich in polar amino acids. Serine and aspartic acid constitute approximately 33.4 and 16.7% respectively of total amino acids **(**Myung *et al.,* 2008). Sericin is easily dissolved in a boiling aqueous solution in the presence of soap, alkali, organic acid or synthetic detergents (Freddi *et al*., 2003; Laxman, 2012). Sericin occurs mainly in amorphous forms and to a certain extent in beta sheet structure. The cocoon of *Bombax mori* silkworm mainly consists of three sericin polypeptides having molecular masses of the 400, 250 and 150 kDa as determined by SDS-PAGE (Takasu *et al.*, 2002).

Silk processing

The processing of silk from cocoon to finished fabric involves series of operations like reeling, degumming, weaving and dying. In reeling process, silk yarn unwinds from the cocoons by treating them in hot water. The removal of sericin from the raw silk is a key step in silk processing to obtain an ideal fiber for textile industry and known as 'degumming'.

Silk degumming

Due to the rigid, rough and drab nature of raw silk due to presence of sericin envelop, it is necessary to remove the sericin and other impurities associated with it to make the silk yarn shiny and soft. In addition, degumming is also important for handling the yarn in knitting and dying process.

Degumming process may be primarily considered as process of cleavage of peptide bonds of sericin by the hydrolytic method and its subsequent removal from fibroin by solubilization or dispersion in water (Gulrajani *et al*., 2000a). General methods suggested for degumming of silk are those, which involved extraction of sericin with water at elevated temperature in presence of soap or alkali, high pressure and high temperature, degumming in acidic solutions and enzymatic degumming (Chopra and Gulrajani, 1994; Gulrajani *et al*., 2009). Mahamoodi *et al.* (2010) studied degumming of Persian silk with microwave irradiation alone and in combination with soap and

alkali (sodium carbonate). The results showed that microwave irradiation alone had lower degumming efficiency compared with the degumming with microwave irradiation along with soap or alkali. However, the recommended standard method of degumming is based on Marseilles soap which is obtained from olive oil. Due to the high cost of this soap, degumming is generally carried out using non standard soaps based on sodium stearate. However, this soap requires in very high quantity and treatment time for degumming is 2 h at boiling temperature. Therefore, a combination of soap and alkali has been recommended to overcome these demerits (Chopra and Gulrajani, 1994). Degumming with soap and alkali solution has become most popular method and widely exploited in silk processing. The mechanism of sericin removal by this process involves swelling of sericin due to soap at boiling temperature and high pH and its subsequent emulsification in the degumming solution and elimination from silk fiber. Although chemical and physical degumming processes are effective for degumming, there are also some disadvantages associated with these methods, such as uneven degumming, strength loss of fibers, and high resources consumption with respect to water and energy as well as high output of effluents with polluting substances (Laxman, 2012). Processing of raw silk produces about $5x10^4$ tones of sericin worldwide annually which is mostly discarded in wastewater (Wu *et al.*, 2008).

In contrast, exploitation of enzymes (alkaline or neutral proteases) in degumming process is advantageous. Enzymatic degumming involves hydrolysis of sericin at milder conditions of temperature and pH. It also requires low input of energy and chemicals (Laxman, 2012; Rajasekhar *et al.*, 2011). The effluent treatment is easier for the waste generated by enzymatic degumming. In addition, recovery of sericin which is a valuable byproduct of silk degumming is easier with enzymatic degumming process.

The use of enzymes in the silk industry is relatively unexplored. Early attempt of enzymatic degumming was made by KANEGFUACHI spinning Co. by using extract and secretion of silkworm enzyme (Kanegufuachi, 1917). However exploitation of enzymes in the silk industry is relatively new and has generated a lot of interest in the last twenty years (Gulrajani, 1992; Chopra and Gulrajani, 1994; Gulrajani *et al.*,

2000a, 2000b; Freddi *et al.,* 2003; Arami *et al.,* 2007). Animal, plant and microbial proteases are reported for degumming of silk. Microbial proteases used for degumming are mainly from *Bacillus* species though few fungal proteases are also used (Freddi *et al.,* 2003; Gulrajani *et al.,* 2000a; Arami *et al.,* 2007; Anghileri *et al*., 2007). However, most of these studies are carried out with commercial enzymes such as alcalase, degummase, savinase and reports of degumming with fungal proteases are very few while there are no reports on degumming with actinomycete proteases.

Applications of sericin

Sericin is a natural protein exhibiting unique moisturizer absorption property and shows several biological activities like antioxidation, antimicrobial, tyrosinase inhibition and anticancer activity. Sericin and its diverse applications have been reviewed by various researchers (Zhang, 2002; Padmawar and Pawar, 2004; Sehnal, 2008). Laxman (2012) recently reviewed the applications of proteases in silk industry including applications of sericin and sericin hydrolysates in diverse areas. Sericin can be cross-linked, copolymerized, and blended with other macromolecular materials to produce artificial polymers with improved properties, used as medical and degradable biomaterial and preparation of hydrogel etc. (Zhang, 2002; Teramoto *et al*., 2005). Zhang *et al.* (2006) prepared bio conjugates of silk sericin with insulin (SS–Ins). In vitro studies indicated that the conjugate had 2.3 and 2.7 times more half-life than that of bovine serum albumin–insulin (BSA–Ins) conjugate and intact insulin, respectively and had 4 times longer pharmacological activity than native insulin. Lower molecular weight sericin peptides (≤ 20 kDa) are used in cosmetics like skin care and hair care products, health products and medications (Zhang, 2002; Myung *et al.,* 2008). Tyrosinase is responsible for biosynthesis of skin melanin, sericin has inhibitory action against tyrosinase and thus it became valuable ingredient of skin care products. Feeding of sericin containing food accelerates mineral absorption, helps to relives constipation and suppresses colon tumorigenesis development in rats (Sasaki *et al.,* 2000a; 2000b; 2000c). Sericin proteins having higher molecular masses are useful in production of biomaterial, compound polymer, and hydrogels. Film made up of sericin and fibroin was found to be supporting growth and attachment of cells in animal cell culture (Minoura *et al.,* 1995). A thin membrane prepared by cross-linking

water-soluble urethane resin and sericin was used as separating membrane for water and ethanol (Mizoguchi *et al.*, 1991). Air filters coated with sericin enhanced its function due to antioxidant and antimicrobial properties of sericin (Sarovart *et al*., 2003).

As mentioned earlier, sericin has lot of biological activities such as antioxidant, tyrosinase inhibition activity and pharmacological functions such as anticoagulation, anti-cancer activities, cryoprotection and promotion of digestion. However, as sericin as such is an intestinal indigestible protein but the absorbability of small sericin peptides in the intestinal mucous membrane is better than proteins. Therefore, to improve the functional and nutritional properties of native sericin, recent efforts are directed towards production of bioactive peptides by enzymatic hydrolysis which could be optimally applied for functional food manufacture or nurtures for human nutrition and health (Wu *et al*., 2008). In recent years, selected sericin peptides are gaining importance in anti-aging skincare formulations. These formulations provide cellular nutrition and stimulation for up-regulating dermal matrix renewal and restore attractive youthful skin appearance and support general skin health.

This section deals with another application of alkaline protease. Enzymatic degumming of silk with microbial proteases and the properties of the degummed fiber are presented. Recovery of sericin from degumming waste liquor as well as hydrolysis of pure sericin and the molecular size distribution of the liberated oligopeptides by SDS PAGE and MALDI-TOF were investigated.

Materials and Methods

Materials

Beef extract, malt extract, peptone, yeast extract were procured from M/s HiMedia Chemicals, India. Chinese bivoltine silk twists (two-ply) and silk cocoons were obtained from Central Silk Board, Bangalore, India. Commercial proteases viz. papain, palkobate and trypsin were procured from M/s Sisco Research Laboratories, India, Maps India Ltd and M/s Loba-Chemi Ltd, India respectively. Sodium dodecyl sulphate (SDS), trifluoroacetic acid (TFA) and sinapinic acid were procured from M/s Sigma Chemical Co, USA. Molecular weight markers for SDS PAGE were purchased from M/s BioRad, India. All other chemicals used were of analytical grade.

Methods

Microorganism and protease production

C. coronatus (PTA-4132), *C. brefeldianus* (MTCC 5185), *Beauveria* sp. (MTCC 5184), and an alkali-tolerant fungal strain (BOA-10) and two alkalophilic actinomycetes isolated from Sambhar lake brine (BOA-2 and BOA-3) were used for protease production. Protease production was carried out in shake flasks at 28°C on a rotary shaker. Inoculum was developed from three to seven days old stock culture in MGYP (*C. coronatus, C. brefeldianus, Beauveria* sp) and alkaline Mikami (BOA-2, BOA-3, and BOA-10) media. After 24–48 h, 10% (v/v) was transferred to production medium. Production medium (MGYP or alkaline Mikami) contained soybean meal (*C. coronatus*, *C. brefeldianus*, BOA-2, BOA-3 and BOA-10) or mustard seed cake (*Beauveria* sp) as inducers. Protease production by *C. brefeldianus* was carried out as described earlier. Flasks were incubated for 48–96 h depending on the microorganism used (Laxman *et al.*, 2002, Laxman *et al.*, 2005; Shankar *et al.*, 2011). At the end of fermentation, cell-free extracts were collected by centrifugation and used for the degumming experiments.

Protease assay

Protease assay was performed as described earlier at respective optimum pH and temperature for individual proteases.

Degumming procedure

Chinese bivoltine silk twists were dried in hot air oven at 95-100°C for 3-4 h to remove traces of moisture. Enzymatic degumming was performed in a glass beaker with solid to liquid ratio of 1:30; protease concentrations varying from 20 to 200 IU/ g of silk yarn and in 0.1 M buffer of required pH. Phosphate buffer for pH 7, Tris HCl buffer for pH 8 and carbonate bicarbonate for pH 9 & 10 were used. Degumming was carried out in Julabo water bath set to temperatures ranging from 40-65°C with intermittent rotation of the silk fiber. Degumming of silk by conventional alkali-soap method was carried out with Marseilles soap and sodium bicarbonate at 95°C for 1 h with solid to liquid ratio of 1:30. All the degumming experiments were carried out in duplicate. Weight loss comparable to conventional method is taken as complete degumming as it is considered to remove sericin completely.

Determination of weight loss

After degumming, silk fibers were washed with hot water at 65°C for 20 min followed by washing with cold water (cold wash) with intermittent shaking. Finally, silk fibers were kept in hot air oven at 95-100°C for drying to reach a constant weight (5-6 h). Weight loss of treated silk denotes the quantitative evaluation of the degumming process. It was measured by calculating the difference in weight of silk fiber before and after degumming and expressed in terms of percentage weight loss.

Scanning electron microscopy

Morphological characterization of silk fiber was performed by means of scanning electron microscope. The filaments of untreated, conventionally degummed and enzymatically degummed silk samples were scanned on scanning electron microscope Model Stereoscan-440 from LEICA–Cambridge, UK.

Testing of physical properties

An Instron 6255 was used to determine the strength and elongation of silk fiber. Ten measurements were made on each sample and mean of the results are reported.

Extraction of sericin proteins from degumming liquid

As sericin was visible as insoluble sediment at the bottom of the tube, its separation from degumming liquor after degumming process was attempted. It could be easily

separated by centrifugation at 1000 rpm for 5 minutes. Sericin pellet (insoluble sericin) was washed with deionized water to remove soluble sericin as well as impurities. The washed pellet was suspended in 0.05 M carbonate bicarbonate buffer, pH 9. The uniform suspension was filtered through 0.2 micron filter to remove the suspended particles and obtain a clear solution which was used as source of sericin proteins for further studies. The schematic diagram of the procedure is illustrated in Figure 4.2.2.

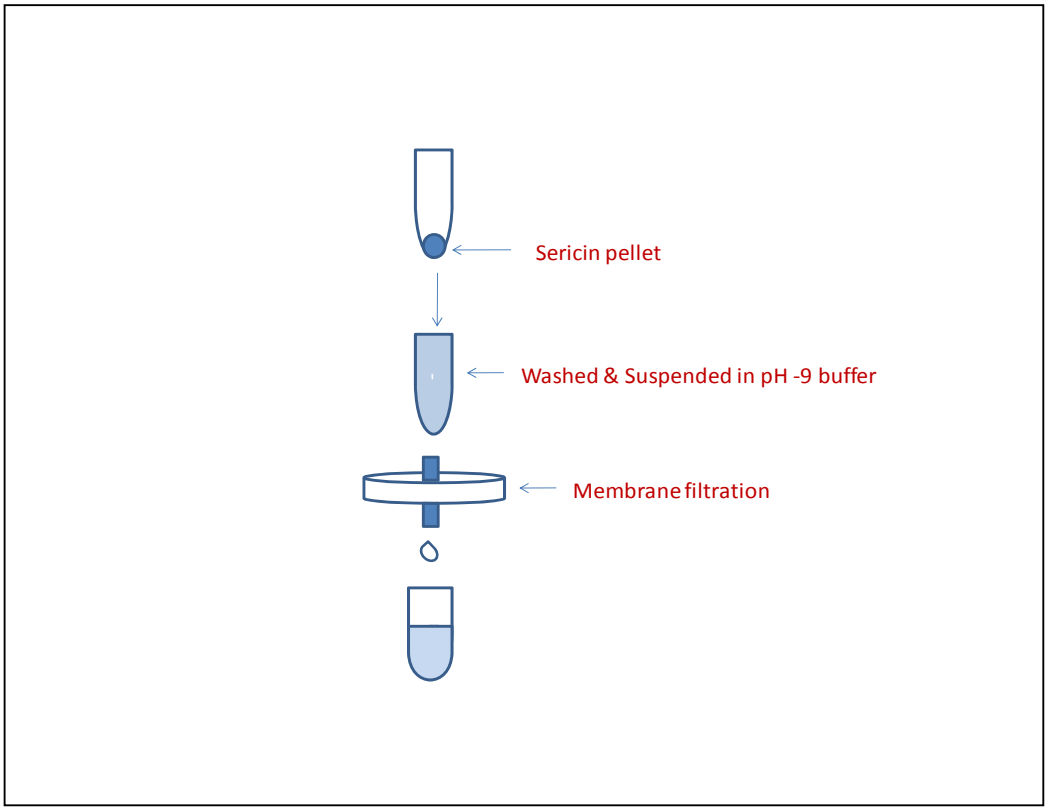


Figure 4.2.2: Schematic diagram of extraction sericin proteins

SDS –PAGE of sericin proteins from degumming liquor

Molecular weight distribution of extracted sericin proteins from degumming liquid was determined by SDS PAGE as described by Laemmli (1970). The protein bands were visualized by silver staining method. Low molecular weight markers for SDS PAGE were used as reference proteins.

Extraction of sericin proteins from coccons

Cocoons were cut into small pieces, washed thoroughly with deionized water and autoclaved at 121°C for 45 min. The solid to liquid ratio was 1:100. After autoclaving, solution containing soluble sericin was filtered to remove fibroin. The filtrate was subjected to lyophilization to obtain sericin powder and stored at 4°C till use.

Hydrolysis of sericin extracted from cocoons by C. brefeldianus protease

For enzymatic hydrolysis, sericin solution was prepared in 0.05 M carbonate bicarbonate buffer, pH 9. Hydrolysis was carried out with 2% sericin concentration in a total volume of 200 µl at 50°C, pH 9. Two sets of hydrolysis conditions were studied. In one set, hydrolysis was carried out for 30 min with protease concentration varying from 1 to 4 IU. In the second set, hydrolysis was carried out with 4 IU of protease with hydrolysis time varying from 15 to 60 min. At the end of hydrolysis, enzyme was inactivated by boiling for 5 min. The mixture was centrifuged at 10000 rpm for 10 min. Supernatant was subjected to gradient gel SDS–PAGE (5–20%) and protein bands were visualized by silver staining method as well as MADLI-TOF analysis.

MALDI-TOF of sericin hydrolysates

The lower weight molecular masses of sericin peptides were analyzed by MALDI–TOF mass spectrometry using Absciex (Applied Biosystems) unit. Four micro liters of hydrolysates was mixed with 16 µl of sinapinic acid and spotted on MALDI target plate, allowed to air dry and analyzed.

Results and discussion

Silk fiber consists of two elements, i.e. inner layer called silk fibroin and the outer layer called sericin. The different solubility of the two silk proteins is the base of the degumming process. The enzymatic action is very specific and the treatment can be performed under mild conditions of pH and temperature that can be finely controlled without triggering the hydrolytic degradation of the fibroin to avoid strength loss as well as uniformly degum the silk. Sericin is a globular and water soluble protein and is accessible to protease action before fibroin can be attacked. Secondly, the characteristic fibrous structure and hydrophobic nature of fibroin tends to make it difficult for enzymatic attack. Alkaline proteases are reported to be more effective than neutral and acidic proteases (Freddi *et al*., 2003). Degumming of silk (weight loss) can be influenced by various factors such as source and type of enzyme, time, temperature, enzyme concentration etc., and need to be optimized. It has to be ascertained that no damage is done to the fiber while sericin is completely removed. Degumming is mainly monitored as percent weight loss and by visual assessment to check the luster and feel of the fiber. In addition, mechanical properties like tensile strength and elongation at break have to be tested to ascertain that no damage is done to the strength of the fiber.

Degumming of silk with alkaline proteases from different sources

Degumming of silk was carried out at a fixed enzyme concentration of 200 IU/g with six alkaline proteases produced in our laboratory. Weight loss was compared with commercial proteases viz. papain (plant), palkobate (bacterial) and trypsin (animal) as well as with conventional method of soap and alkali. The weight loss after degumming with the proteases produced in the laboratory ranged between 15.74 to 25.74%, while conventional method resulted in 21.4% weight loss (Table 4.2.3). Among the commercial proteases tested, papain and Palkobate gave similar weight loss of 22.74 and 22.54% respectively, while trypsin gave lower weight loss of 16.30%. Among the six indigenous proteases tested, BOA-2, BOA-3, *C. brefeldianus* and *Beauveria* sp. proteases showed weights loss of 25.74, 23.97, 25.03 and 23.63%, respectively, which were slightly higher, compared to conventional method indicating that lower enzyme concentrations may be enough for complete degumming.

Table 4.2.3: Comparison of weight loss of silk with different proteases

Enzyme Source	Degumming condition	Weight loss (%)
Conidiobolus coronatus PTA-4132	40°C, pH 10.0	15.74
Conidiobolus brefeldianus MTCC 5185	50°C, pH 9.0	25.03
Alkalitolerant fungus (BOA-10)	40°C, pH 10.0	16.77
Beauveria sp MTCC 5184	50°C, pH 9.0	23.63
Actinomycete-1 (BOA-2)	65°C, pH 10.0	25.74
Actinomycete-2 (BOA-3)	65°C, pH 10.0	23.97
Papain	50°C, pH 9.0	22.74
Palkobate	65°C, pH 7.0	22.54
Trypsin	40°C, pH 8.0	16.30

The remaining two fungal proteases viz. *C. coronatus* and BOA-10 showed a weight loss of 15.74 and 16.77% which was similar to that of trypsin (16.29%). There was increase in luster, smooth feel and shine after enzymatic degumming with microbial proteases. After degumming, fibers were more voluminous compared to untreated ones due to falling apart of fibers after sericin removal (Figure 4.2.3). Alkaline proteases are reported to be more effective than neutral and acidic proteases (Freddi *et al.,* 2003). Puri (2001) studied the silk-degumming efficiency of an alkaline protease from *Bacillus* sp. RGR-14 which showed a weight loss of 7.5% after 5 h incubation. Krishnaveni and Rajkumar (2010) studied effect of enzymatic degumming with biodegummase, papain, trypsin and pepsin on dyeing of silk and reported higher weight loss (21%) compared with soap (16%). Degumming of raw silk yarn for 1 h with three alkaline proteases from M/s Genecor showed weight loss of around 23% (Anghileri *et al.,* 2007). Gulrajani *et al.* (2000a) reported weight loss of 19.8% by degumming of raw mulberry silk with a fungal protease for 3 h. Arami *et al.* (2007) investigated the feasibility of degumming Persian silk with alcalase and savinase individually and in different combinations and compared with soap degumming. They reported weight loss of 22.10, 21.4 and 22.02% after degumming for 1 h with alcalase, savinase and soap respectively.

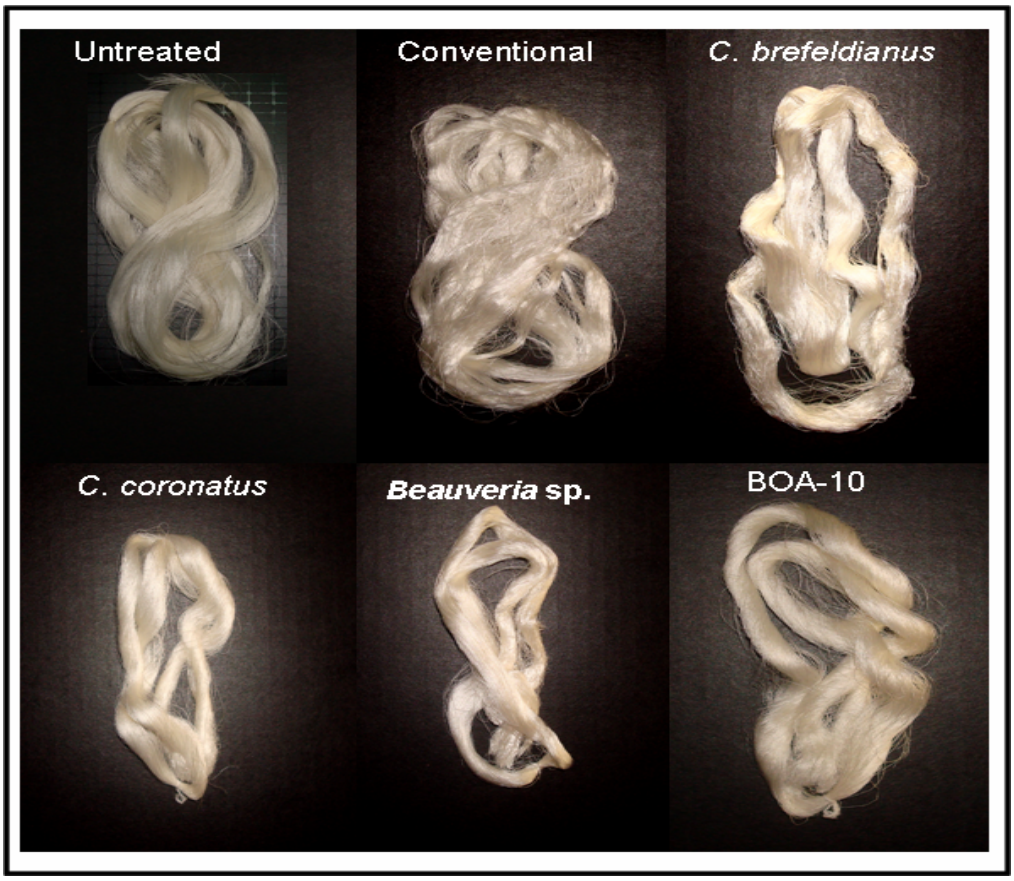


Figure 4.4.3: **Visible appearance of the treated and untreated Chinese bivoltine silk fibers showing difference in shine**

Effect of enzyme concentration

Since most of the proteases showed higher weight loss compared to conventional method, effect of enzyme concentration on degumming was studied in the concentration range of 20-200 IU/g to optimize enzyme concentration required for complete degumming. Figure 4.2.4 shows the effect of fungal protease concentration on weight loss of silk after degumming by fungal proteases. Among the three proteases tested, *C. brefeldianus* was found best followed by *Beauveria* sp. and *C. coronatus*. In general, increase in enzyme concentration resulted in increase in weight loss for all the used proteases. However, the increase was not exponential. It is observed that 50 IU of *C. brefeldianus* protease was sufficient for complete degumming while 150 IU were required for similar weight loss with *Beauveria* sp. protease. However, complete degumming was not achieved even with 200 IU of *C. coronatus* protease. Both the actinomycete proteases showed complete

sericin removal with 50 IU (Figure 4.2.5) indicating high degumming potential these proteases.

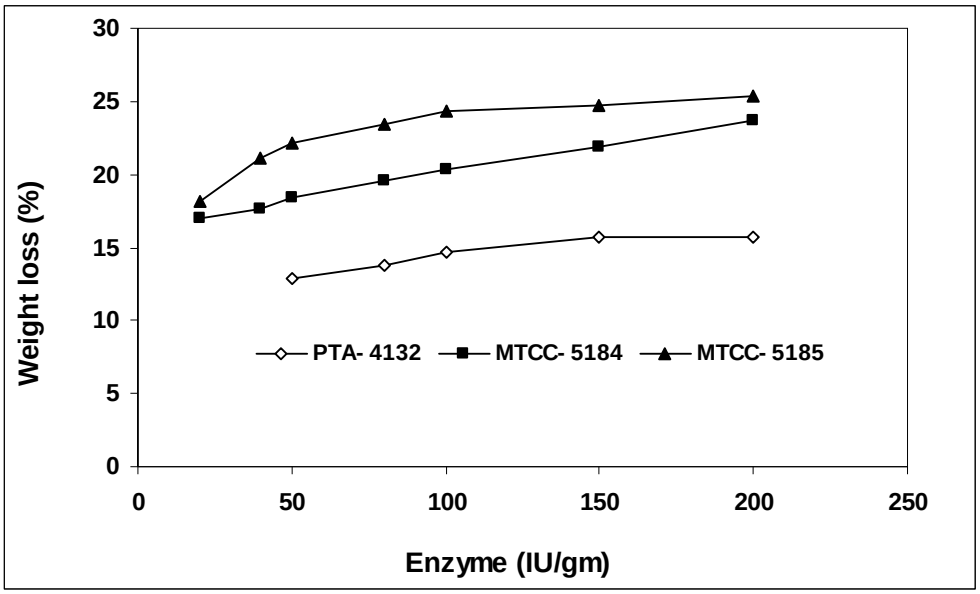


Figure 4.2.4: Effect of enzyme concentration on weight loss after degumming with fungal proteases

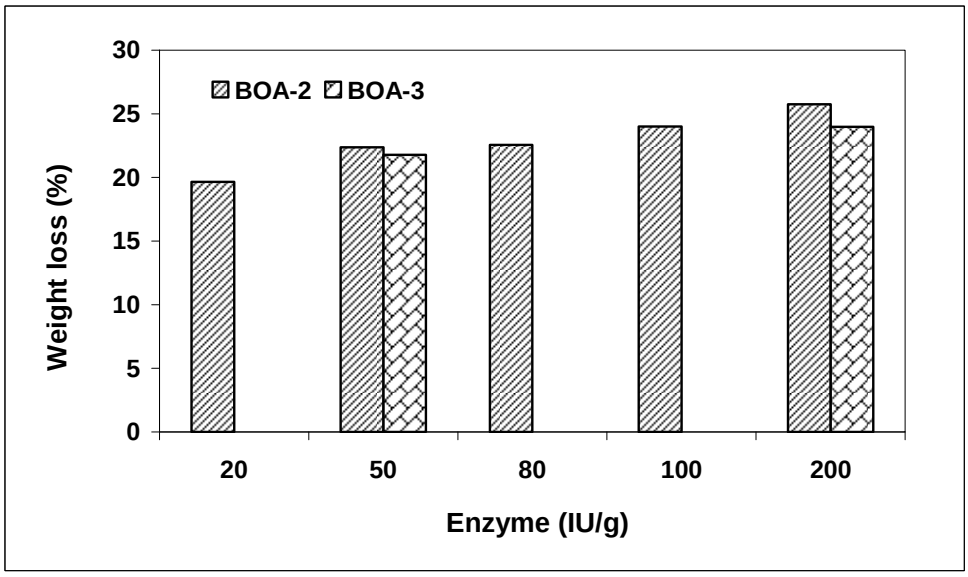


Figure 4.2.5: Effect of enzyme concentration on weight loss after degumming with actinomycete proteases

Comparison of the performance of all the used proteases at a fixed enzyme concentration of 50 IU showed that proteases from *C. brefeldianus*, and both the

actinomycetes were able to degum the silk fibers completely since the weight loss was comparable to the weight loss obtained by conventional method (Figure 4.2.6). *Beauveria* sp, alkalitolerant fungus BOA-10 and *C. coronatus* resulted in 18.43, 12.89 and 12.50% weight loss respectively.

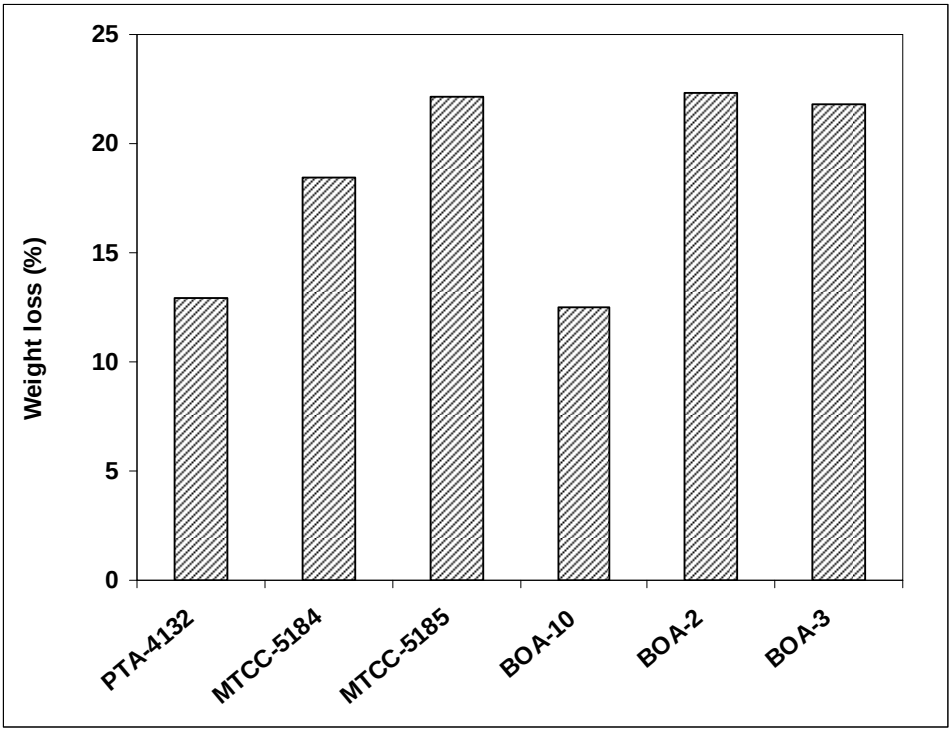


Figure 4.2.6: Comparison of weight loss after degumming with different proteases at fixed enzyme concentration of 50 (IU/g)

Degumming of Bivoltine silk yarn with 8 different commercially available enzymes viz. degummase 1000 L, Protosol, Trypsin, Alcalase, Protease A, Protease N, Pepsin and Protease M was reported by Gulrajani *et al.* (1996). Most of the enzymes at 15% concentration showed a weight loss of around 24% while degummase showed similar weight loss at 25% concentration. Degumming of plain and crepe silks with 15% alcalase for 3 h gave weight loss of 18.5 and 16.3% respectively (Gulrajani *et al.,* 2000b). Gulrajani *et al.* (1999) reported biopolishing of Tasar silk with degummase with 7.69% weight loss. Gulrajani *et al.* (1998) reported degumming of Indian and Chinese dupion silk with commercial proteases. Degumming of Indian dupion silk for 1 h with 25% Protease M Amano, papain, Protease A Amano 2, Protease N Amano, degummase and alcalase showed weight loss of 15.7, 9.6, 8.3, 19.5, 19.7 and 8.6% respectively. The weight loss for Chinese

dupion silk was between 14.2 to 19% after degumming with above enzymes under identical conditions. Arami *et al.* (2007) reported increase in weight loss with increase in enzyme concentration from 0.25 to 1g/L beyond which there was no further increase. Similar observation of increase in weight loss with increase in enzyme concentration was made by Gulrajani e*t al.* (2000a). Since *C. brefeldianus* and BOA-2 proteases were most effective in degumming, further optimization of degumming conditions was performed at an enzyme to substrate ratio of 50 IU/g.

Effect of temperature

In general, enzymatic degumming with proteases is reported in the temperature range from 37 to 60°C (Chopra and Gulrajani, 1994; Gulrajani *et al*., 2000a; Freddi *et al*. 2003; Arami *et al*., 2007). Enzymes are active in a temperature range, which varies with enzyme to enzyme. In general, activity increases with increase in temperature up to a certain value beyond which they are inactivated and lose activity due to denaturation. Some times the presence of substrate protects the enzyme from inactivation. Therefore, it is important to optimize the temperature for degumming. Effect of temperature on degumming with *C. brefeldianus* and BOA-2 proteases showed that the weight loss increased with increase in temperature up to 50°C and 65°C for *C. brefeldianus* and BOA-2 proteases, respectively (Figure 4.2.7).

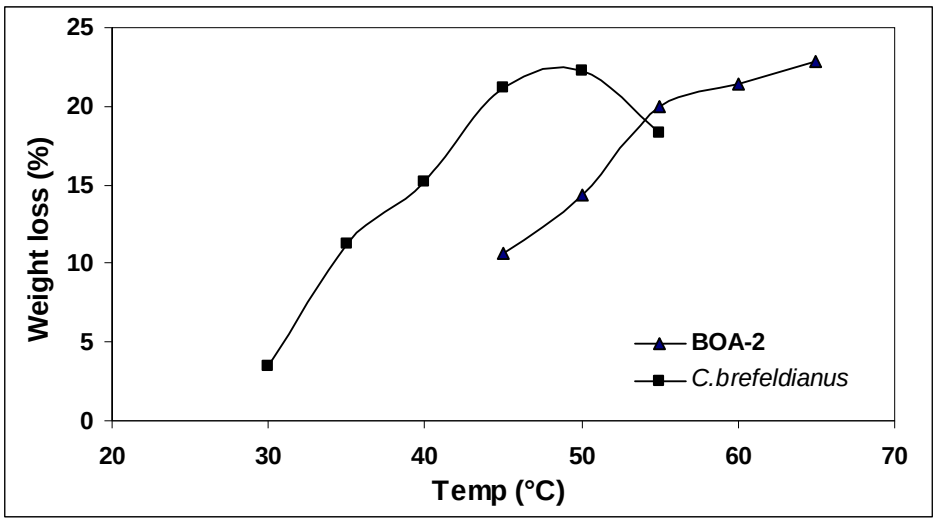


Figure 4.2.7: Effect of temperature on weight loss after degumming

Turbidity also increased with increase in temperature indicating the release of insoluble sericin from the silk fibers due to degumming (data not shown). Higher weight loss with *C. brefeldianus* and BOA-2 proteases was observed at 50°C and between 60°C and 65°C, since the proteases were optimally active at these temperatures.

Effect of time

Effect of time on degumming was studied with *C. brefeldianus* and alkalophilic actinomycete BOA-2 proteases. It can be seen from the Figure 4.2.8 that weight loss after degumming increased with incubation time but 70-75% of the degumming was complete within first 15 min and thereafter the increase was slow. Weight loss after 1 h with *C. brefeldianus* and BOA-2 proteases was 22.49 and 23.87%, respectively, while the weight loss after 15 min was 16.73 and 20.37% respectively. These values seem to suggest that treatment time to be shorter than the reported values in literature.

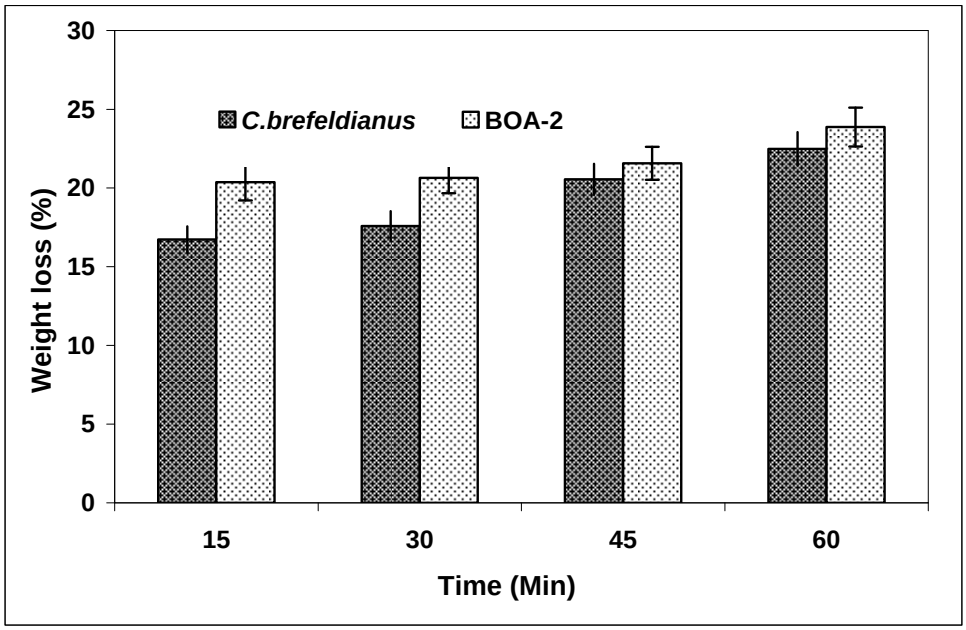


Figure: 4.2.8: Effect of time on weight loss after enzymatic degumming

Chopra and Gulrajani (1994) reported sericin removal to be linear in first 25 min for Chinese bivoltine silk. Gulrajani *et al.* (1996) studied degumming with several proteases and found that most of the degumming was complete in first one hour and further increase in treatment time from 1h to 3h did not bring about significant

weight loss. Gulrajani *et al.* (1998) reported that increase in enzyme concentration showed increase in weight loss and less time was required to achieve the same weight loss. Degumming with fungal protease (3 ml/g of fabric) resulted in 19.8% weight loss in 3 h (Gulrajani *et al.,* 2000a). Arami *et al.* (2007) studied degumming of Persian silk and reported sericin removal of 21.52% with alcalase in 30 min, 20.08% with savinase in 60 min while it was 22.58% with soap in 120 min. Puri *et al.* (2001) studied the silk degumming efficiency of an alkaline protease from *Bacillus sp.* RGR-14 which showed a weight loss of 7.5% after 5 h incubation. Rinsey Johnny and Karpagam Chinnammal (2012) performed degumming of Mulberry silk yarn with *Bacillus* protease for 6 h and resultant weight loss was found to be 25.68%.

Structural properties

Physical properties through light on the structural integrity of the fiber and adverse effects of degumming treatment. These studies help to control quality, because it allows choosing less invasive degumming process thereby reducing the extent of fiber degradation (Anghileri *et al.,* 2007) .

Enzymatically degummed silk yarns showing weight loss similar or slightly lower to conventional method were tested for tensile strength and elongation at break (Table 4.2.4). It was observed that there were no significant differences in tensile strength or elongation by enzymatic degumming indicating no strength loss.

Table 4.2.4: Physical properties of the degummed fiber

Treatment	Enzyme concentration (IU/g silk)	Weight loss (%)	Tensile strength (g/den)	Elongation at break (%)
Untreated	-	-	3.5±0.23	19±1.59
Conventional	-	21.40	3.8±0.25	15±2.16
C. brefeldianus	40	21.10	3.6±0.16	17±1.37
C. coronatus	200	15.74	3.5±0.22	16±1.69
BOA-10	200	16.77	3.6±0.18	18±0.86
Beauveria sp.	40	19.58	3.8±0.25	17±1.54
BOA-2	20	19.63	4.0±0.25	17±1.27
BOA-3	50	21.78	4.1±0.25	17±1.17

The above results were also corroborated by scanning electron microscopic studies. This observation is in agreement with Gulrajani *et al.* (1996; 1998) and Arami *et al.* (2007). Arami *et al.* (2007) reported that the enzymatic treatment improved the strength and elongation of the silk yarn with better mechanical properties (strength and elongation at break) compared to the degummed silk obtained by the soap degumming method. The above results of no loss in strength of the degummed fiber were also corroborated by scanning electron microscopic studies.

SEM studies

Scanning electron micrographs of untreated and degummed silk fibers with conventional method and *C. brefeldianus* protease are showed in (Figure 4.2.9). It is observed that the fibers are clubbed together in a bundle by means of sericin in case of untreated silk yarn whereas fibers have fallen apart after degumming with protease. Untreated yarn contains sericin, which is seen as deposits on the surface of filaments holding them together. Degumming with optimum conditions resulted in the removal of the sericin deposits and separation of fibers (Figure 4.2.9). Similar observations of nonuniform sericin deposits in untreated and undegummed silk and their removal after enzymatic degumming, as observed under scanning electron microscopic studies, are reported by other others (Arami *et al.*, 2007; Gulrajani *et al.*, 2000a). The cleaning of the surface by the proteases is responsible for the improvement in the luster of the treated samples.

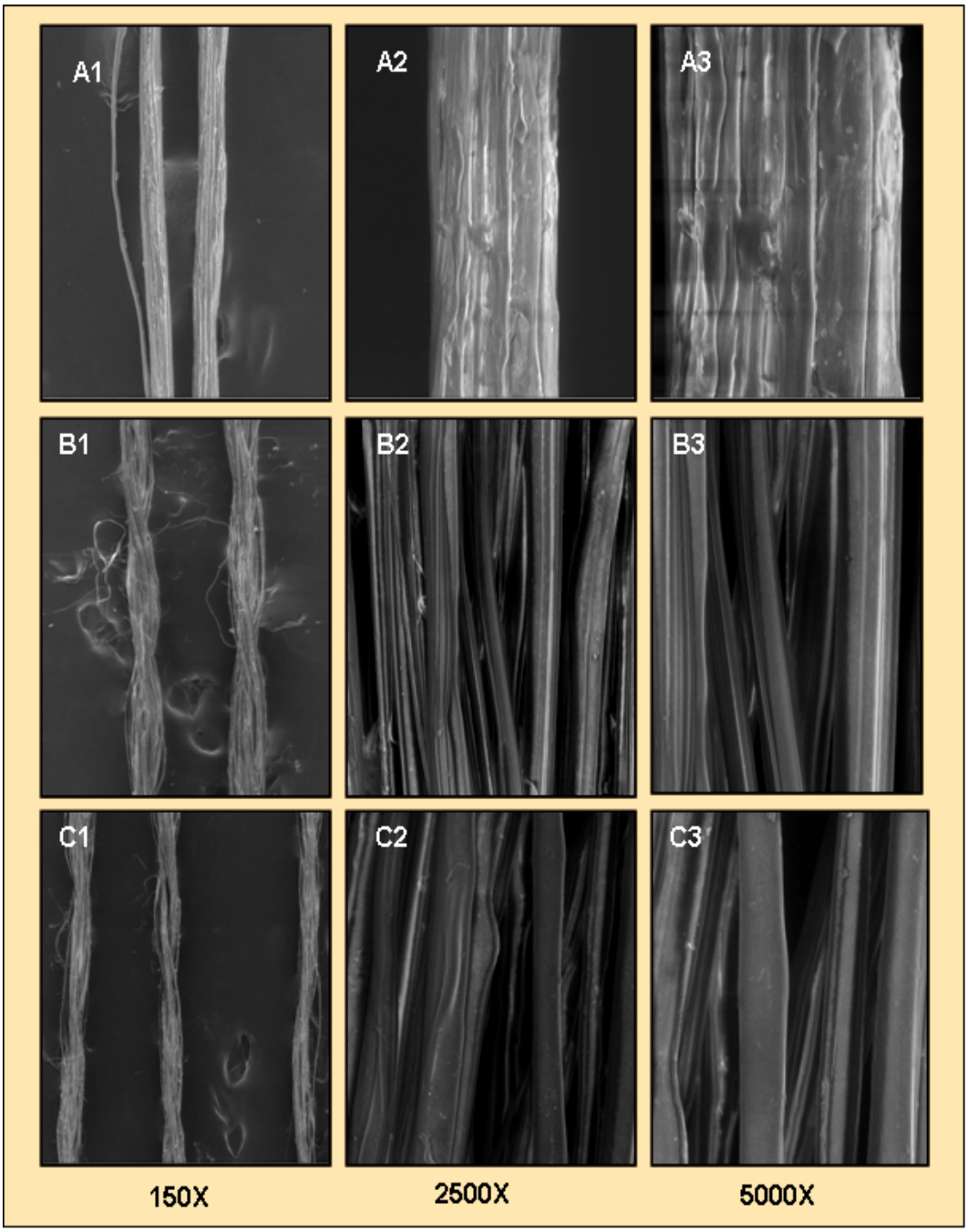


Figure 4.2.9: Scanning electron micrographs of silk fibers. A1 to A3- Untreated; B1 to B3- Conventionally treated; C1 to C3- *C. brefeldianus* protease treated.

Extraction of sericin proteins from degumming liquid

Sericin is a water soluble macromolecular protein which helps to hold the fibers of silk together to form bundles. In conventional degumming process, most of the sericin

is lost as it is drained out as effluent which causes adverse environmental impact. In addition, there is huge loss of valuable sericin which has versatile applications (Zhang, 2002). The structural and functional properties of sericin obtained depend on the degumming method applied for its extraction (Chopra and Gulrajani, 1994; Padamwar and Pawar, 2004; Gulrajani *et al.*, 2009). Soap makes sericin swell, then emulsifies it in the degumming bath, and removes it from the filaments (Mahmoodi *et al.*, 2010). Gulrajani *et al.* (2009) developed a membrane filtration technique for recovery of sericin proteins obtained from three different methods of degumming viz. high temperature high pressure (HTHP), alkaline, and soap plus alkali (SPA) which included series of operations. Contrarily, recovery of sericin from degumming liquid of enzymatically degummed silk is comparatively simple. In the present study also, three methods of degumming viz. HTHP, soap and alkali and enzymatice degumming with *C. brefeldianus* protease were investigated. Enzymatic degumming resulted in formation of solution with suspended sericin particles and forms sedimentation in static condition (Figure 4.2.10).

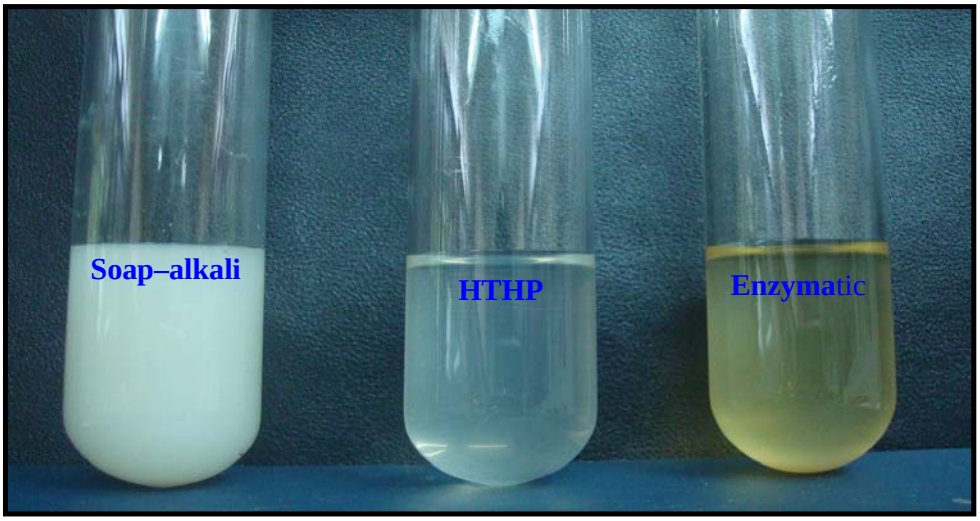


Figure 4.2.10: Degumming solutions

Degumming by soap and alkali resulted in emulsified sericin solution, where as degumming at high temperature and high pressure (HTTP) forms sericin solution with gelling properties due to the characteristic random coil structure of sericin, which tends to get converted to beta sheet structure at lower temperature, which result in gel formation (Padamwar and Pawar, 2004).

SDS –PAGE of sericin proteins from degumming liquor

The clear sericin sample (washed insoluble sericin after filtration through 0.2 micron filter) from degumming liquid was subjected to SDS PAGE to analyze the molecular weight range of peptides. Three samples obtained after degumming with three enzyme concentrations were analyzed. Three distinct proteins as evidenced by SDS–PAGE. The molecular weight of larger protein was slightly more than 26.6 kDa, while rest of the proteins has their molecular weights in the range of 26.6 kDa to 19.6 kDa (Figure 4.2.11).

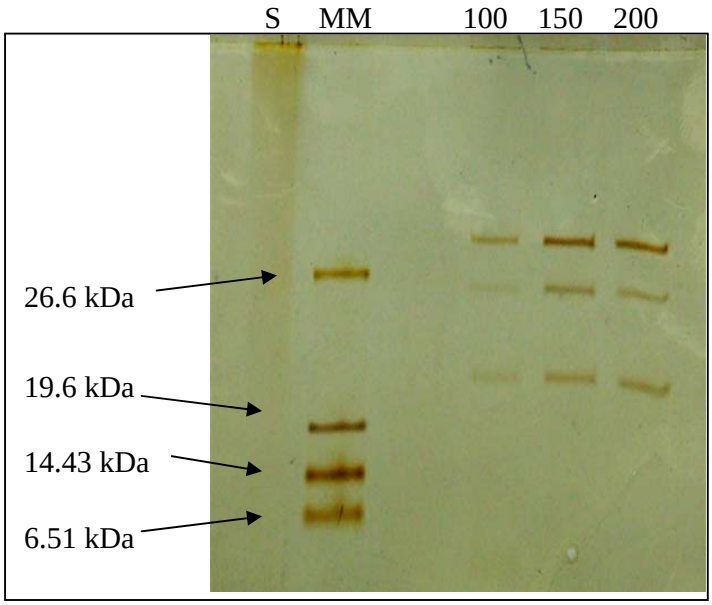


Figure 4.2.11: SDS-PAGE of sericin proteins

S: Sericin by HTHP; MM: molecular weight markers;
Hydrolsates from 100 IU; 150 IU and 200 IU protease

Terada *et al.* (2005) obtained sericin having molecular weight <70 kDa (less than 70kDa) by alkaline hydrolysis. This fraction was further fractionated by gel exclusion chromatography to get three fractions of varying molecular weight range viz. sericin fraction L (20-70 kDa), sericin fraction M (10-40 kDa) and sericin fraction S (less than 30 kDa). Mitogenic activity of these fractions when tested on hybridoma cells showed that though all the fractions supported better proliferation compared to control without sericin, better results in terms of viable cell density were obtained with sericin having molecular weight in the range of 20 to 70 kDa. The molecular weight range of

sericin proteins separated from degumming liquid obtained from *C. brefeldianus* fall in the molecular weight range of 19-30 kDa and hence may find application in cell culture medium.

Hydrolysis of sericin extracted from silk cocoon with C. brefeldianus protease

Encouraged by the above results, enzymatic hydrolysis of pure sericin extracted from cocoons with *C. brefeldianus* protease and the molecular weight distribution of the peptides was investigated. Two controls (without enzyme and without sericin) were also included. Sericin hydrolysates obtained were then subjected to electrophoresis on SDS gradient to visualize the sericin peptides. As can be seen, there are two major protein bands corresponding to molecular weight of 26.6 kDa and a lower molecular weight protein of about 6.5 kDa (Figure 4.2.12).

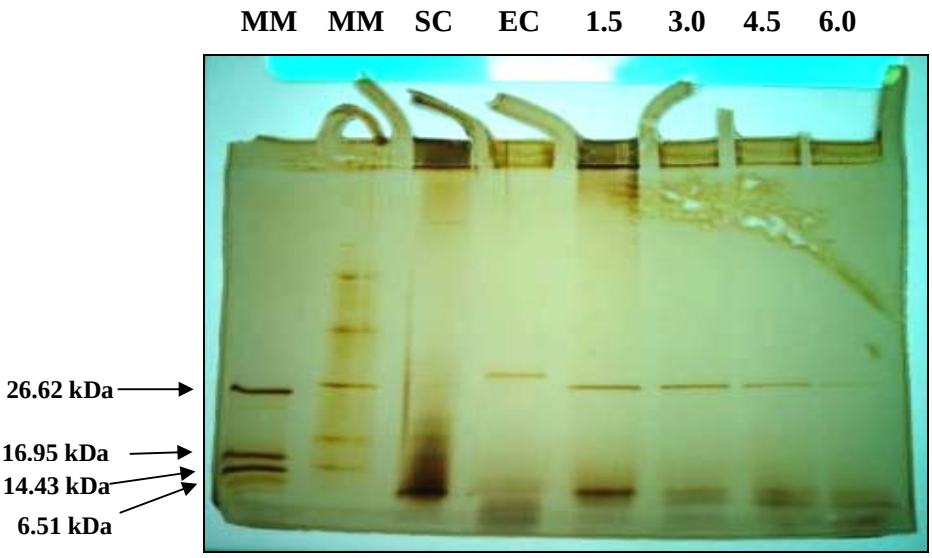


Figure 4.2.12: SDS PAGE of enzymatic hydrolysates of cocoon sericin

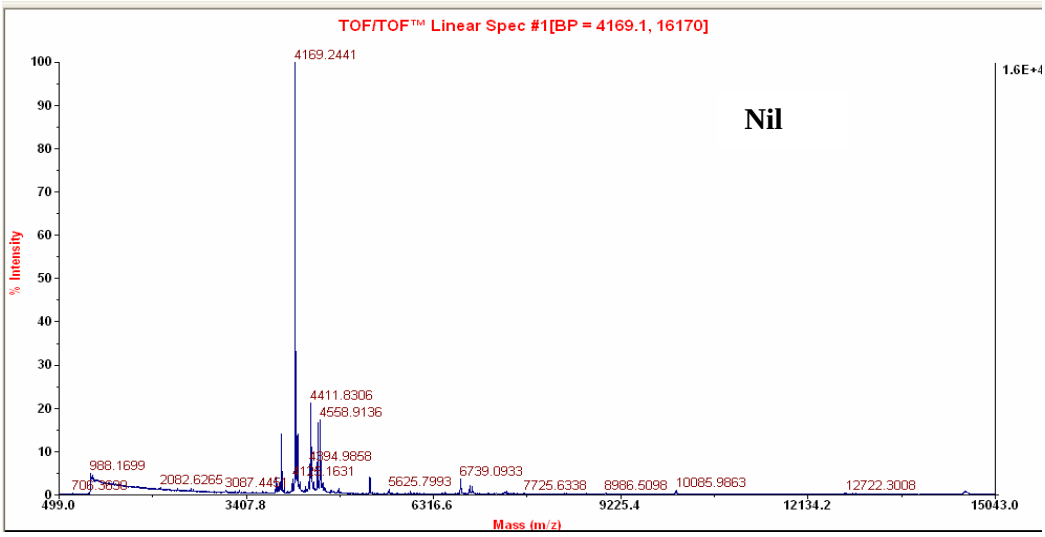
MM: Molecular weight markers; SC: Sericin control; EC: Enzyme control; Lanes 5 to 8: Enzymatic hyolysates with 1.5 IU; 3.0 IU; 4.5 IU and 6.0 IU of protease

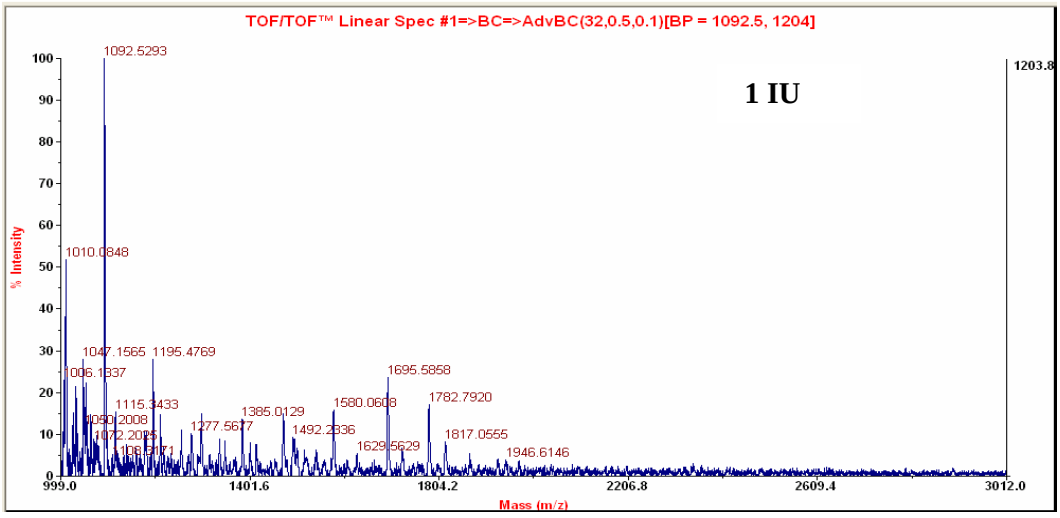
Intensities of band corresponding to 26.6 kDa decreased with corresponding increase of the faster moving band when the protease concentration increased. However their exact molecular masses could not be calculated as they were faster than the lowest molecular weight marker. This indicates that the protease brought about hydrolysis of larger sericin protein into smaller peptides having molecular masses below 6.5 kDa.

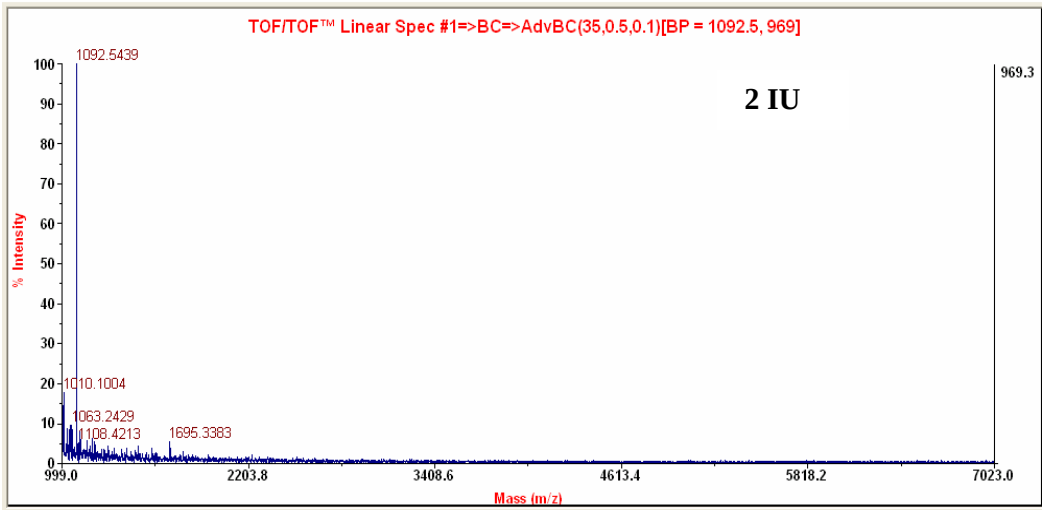
Watanabe *et al.* (2007) reported hydrolysis of three sericin fraction s-A, s-M and s-P with silk gland fibroinase indicating its action on sericin in addition to fibroin. Analysis of the peptides on 7% SDS-PAGE revealed that intensity of sericin band decreased with time and after 3 h only faint band was seen. They further separated sericin peptides of less than as well as larger than 10 kDa by RP-HPLC and the sequences of peptides smaller than 10kDa were determined**.** Wu *et al.* (2008) studied the production of bioactive peptides from sericin obtained by high temperature and high pressure (HTHP) with five commercial proteases. Hydrolysis with Protease P (Amno 6, from *Aspergillus melleus*) under its optimal conditions produced hydrolysate with main molecular weight distribution of 250–4000 Da having the major amino acid constituents (Ser and Thr) up to 30%. Freeze-dried sericin hydrolysate showed the markedly high antioxidant activities and the tyrosinase-inhibitory effects. Therefore, it will be interesting to investigate various biological activities of sericin peptides produced by *C. brefeldianus* protease.

MALDI analysis of sericin hydrolysates

The molecular weights of small peptides derived by enzymatic hydrolysis were too low and could not be identified by SDS-PAGE. Therefore, the molecular weights of the oligopeptides were analyzed by MALDI-TOF. Initially, effect of protease concentration on the molecular weight distribution was investigated. The MALDI-TOF profiles of the hydrolysate obtained with 0, 1, 2, 3 and 4 IU of protease are presented in Figure 4.2.13. Control without enzyme (Nil) showed a sharp peak corresponding to the molecular mass of 4169 Da. With minimum protease concentration of 1 IU, the oligopeptides were distributed in the molecular range of 1010 to 1817 Da with a major peak at 1092 Da while with 2 IU protease, intensities of other peaks decreased with a major peak at 1092 Da. However further increase in enzyme concentrations (3-4 IU) for hydrolysis of sericin resulted in further degradation of these peptides with molecular weight distribution in the range 516 to 954 Da with major peak at 516 Da.


TOF/TOF™ Linear Spec #1[BP = 4169.1, 16170]
Nil
% Intensity
Mass (m/z)
4169.2441
4411.8306
4558.9136
988.1699
2082.6265
5625.7993
6739.0933
7725.6338
8986.5098
10085.9863
12722.3008


TOF/TOF™ Linear Spec #1=>BC=>AdvBC(32,0.5,0.1)[BP = 1092.5, 1204]
1 IU
% Intensity
Mass (m/z)
1092.5293
1010.0848
1047.1565
1195.4769
1006.1337
1695.5858
1782.7920
1580.0608
1385.0129
1115.3433
1277.5677
1492.2836
1817.0555
1946.6146


TOF/TOF™ Linear Spec #1=>BC=>AdvBC(35,0.5,0.1)[BP = 1092.5, 969]
2 IU
% Intensity
Mass (m/z)
1092.5439
1010.1004
1063.2429
1108.4213
1695.3383

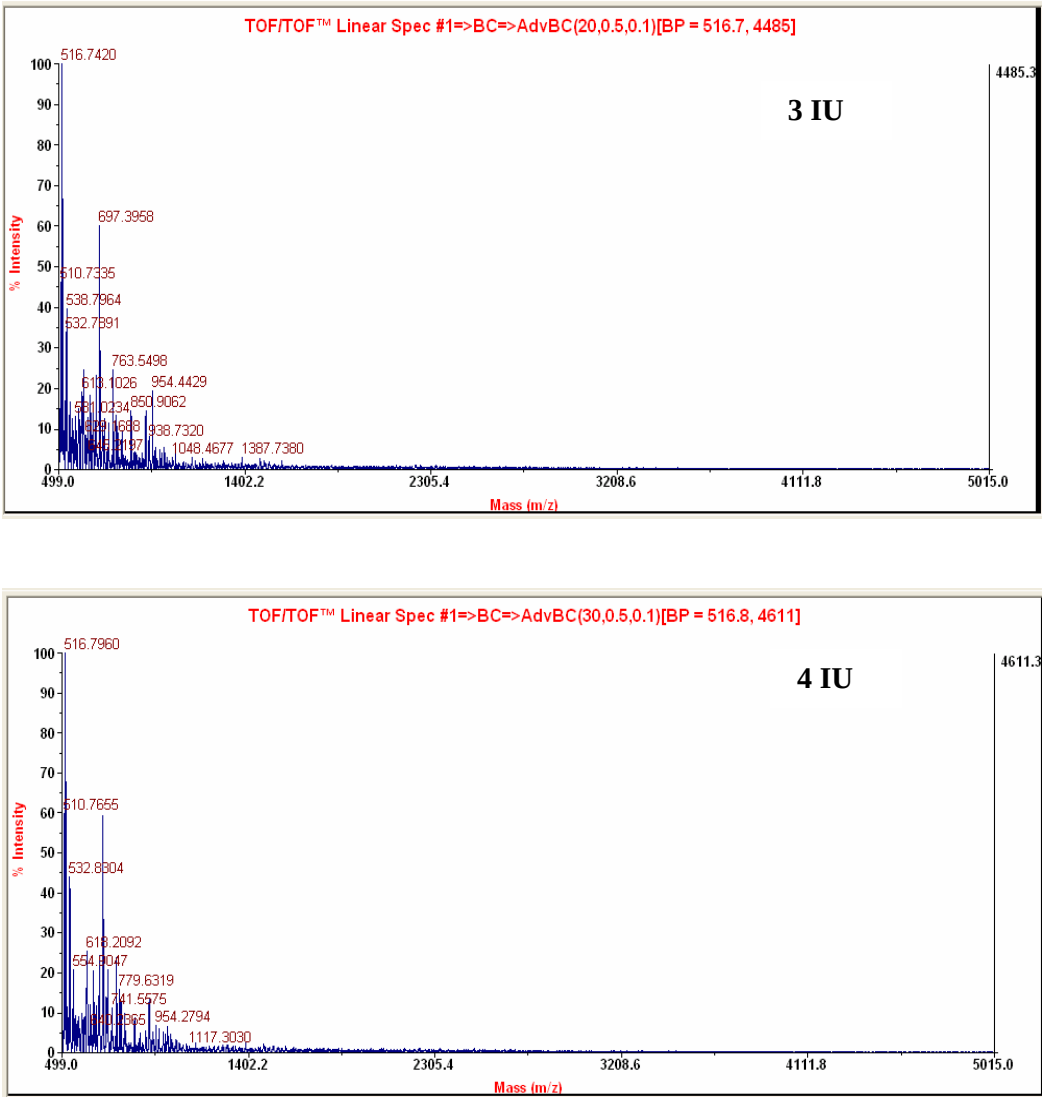
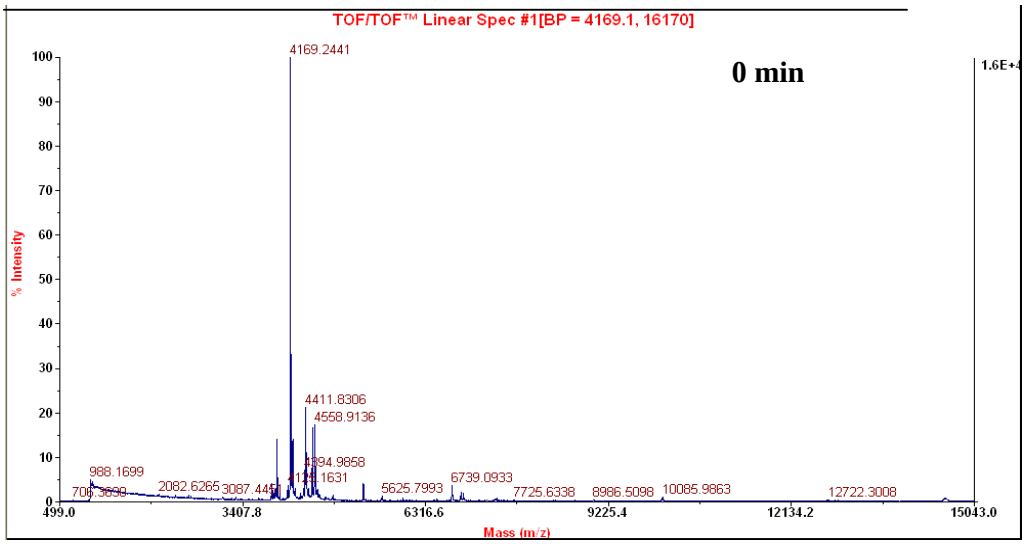
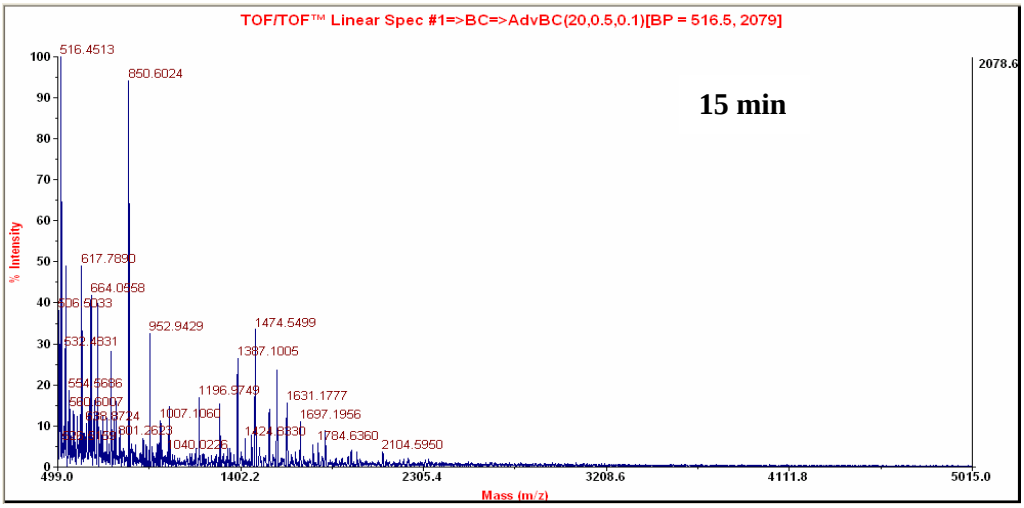
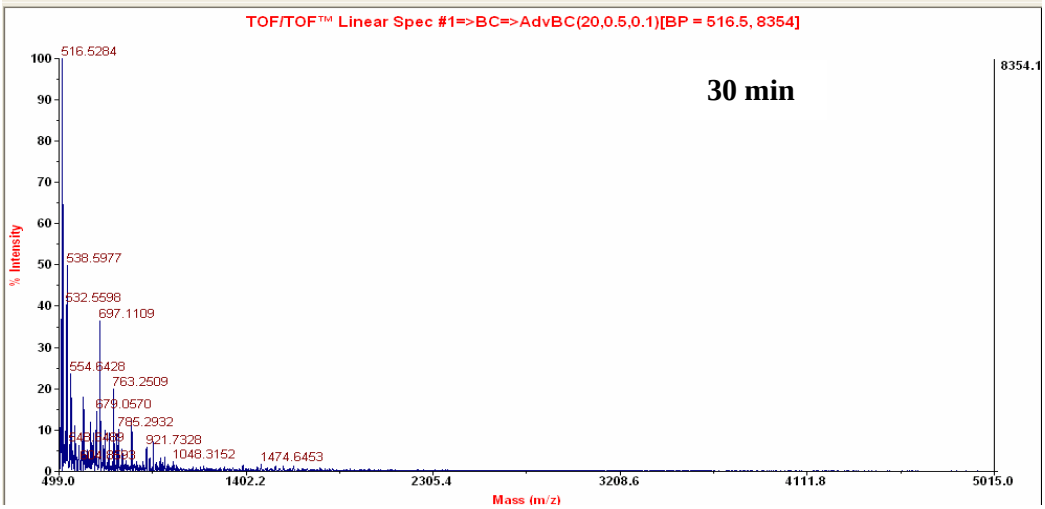


Figure 4.2.13: Effect of enzyme concentration

Similarly the time course of enzymatic hydrolysis of sericin was performed with fixed enzyme concentration of 4 IU. In this case also, initially (0 min), a major peak corresponding to 4169 Da along with few minor peaks was visible. After 15 min incubation, that peak completely disappeared with simultaneous appearance of several low molecular peaks having their molecular weights in the range of 516 to 1697 Da. Two major peaks corresponding to 516 and 850 Da were visible. On further incubation up to 30 min, even the 850 peak disappeared and only 516 Da peak appeared as a major one. There was no significant difference in the pattern on further incubation indicating that the hydrolysis is nearly complete (Figure 4.2.14).


TOF/TOF™ Linear Spec #1[BP = 4169.1, 16170]
0 min
% Intensity
Mass (m/z)
4169.2441
4411.8306
4558.9136
988.1699
2082.6265
5625.7993
6739.0933
7725.6338
8986.5098
10085.9863
12722.3008


TOF/TOF™ Linear Spec #1=>BC=>AdvBC(20,0.5,0.1)[BP = 516.5, 2079]
15 min
% Intensity
Mass (m/z)
516.4513
850.6024
617.7890
664.0958
952.9429
1474.5499
1387.1005
1196.9749
1631.1777
1697.1956
1784.6360
2104.5950


TOF/TOF™ Linear Spec #1=>BC=>AdvBC(20,0.5,0.1)[BP = 516.5, 8354]
30 min
% Intensity
Mass (m/z)
516.5284
538.5977
532.5598
697.1109
554.6428
763.2509
679.0570
785.2932
921.7328
1048.3152
1474.6453

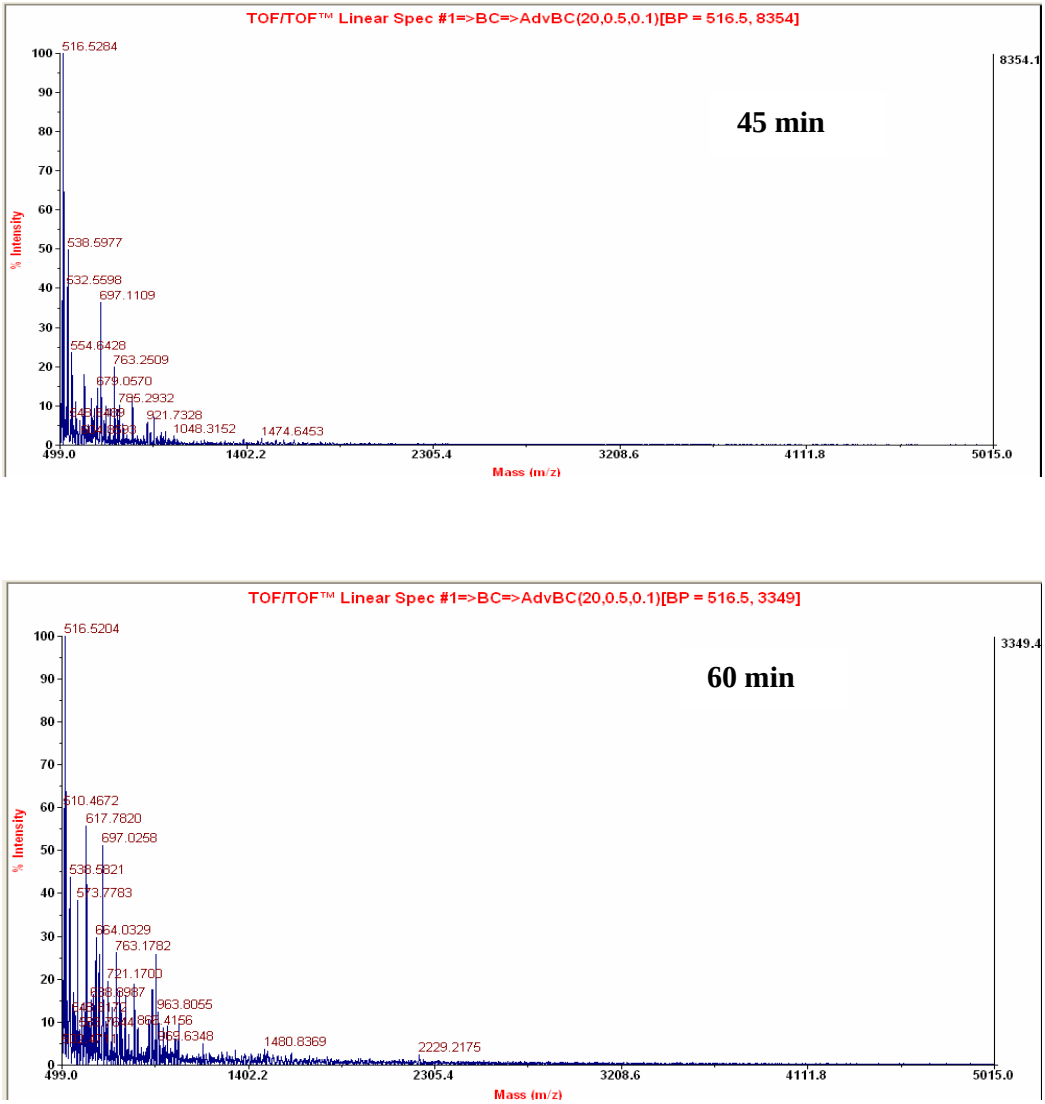


Figure 4.2.14: Effect of treatment time

This suggests that two major low molecular weight pepetides corresponding to 516 and 1092 Da can be obtained by the enzymatic hydrolysis of sericin in 15- 30 min with 1-2 IU of protease. Myung *et al.* (2008) performed hydrolysis of sericin (obtained from cocoon by hot water treatment) by immobilized trypsin in bioreactor. The low molecular weight oligopeptides were separated by an integrated with ultra filtration membrane system. Oligopeptides with a molecular weight of 1500–1900 Da were permeated through the membrane. MALDI- TOF analysis indicated the molecular weight of the oligopeptides to be in the range of few thousand with major peaks corresponding to 921 Da, 1519 and 1814 Da. Fan *et al.* (2010) prepared silk

sericin hydrolysates (SSH) using six different proteases originated from plant, animal and microorganism. Hydrolysis was performed for 11 h between 37 to 65°C and pH 7 to 8.5 and the molecular size distribution profile of the hydrolysates was determined by high-performance liquid chromatography (HPLC) on a gel permeation chromatography column. They evaluated the antioxidative and free radical-scavenging activities of the peptides and found that different protease hydrolysates possessed different antioxidative activity and free radical-scavenging activities. Their results revealed that highest antioxidant activity was exerted by alcalase hydrolysates. Further they stated that peptide fraction with molecular weight ranging from 200 to 3000 Da was probably associated with higher antioxidant activity and the SSH can be used as a food supplement or used in the pharmaceutical and medical industries. Therefore, the hydrolysates of sericin may have some biological activities which need to be further investigated in detail.

Conclusions

The six indigenous alkaline proteases tested for degumming showed varying degrees of weight losses indicating partial to complete degumming. Among them, *C. brefeldianus* and BOA-2 proteases were found to be best which were effective in complete degumming within 15 to 30 min at low enzyme concentrations as well as unlike the conventional method requiring 60 min for the same. Recovery of sericin from waste degumming liquor after enzymatic degumming with *C. brefeldianus* and its molecular weight analysis by SDS PAGE showed presence of three proteins in the molecular weight range of 19.6 to 26.6 kDa. Hydrolysis of sericin extracted from cocoons by the protease revealed the presence of small molecular peptides as analyzed by SDS PAGE and MALDI-TOF, the size of which depended on the hydrolysis conditions such as enzyme concentration and time. Sericin oligopetides have several applications in diverse fields. Preparation and separation of these peptides and testing their biological activities is essential for their biotechnological applications.

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