PRODUCTION PURIFICATION AND CHARACTERIZATION OF PROTEASE FROM *BEAUVERIA* SP

Thesis submitted to University of Pune

For the degree of

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

BY

SHIV SHANKAR

UNDER THE GUIDANCE OF

DR. (Mrs.) MALA RAO

&

CO-GUIDANCE OF

DR. (Mrs.) R. SEETA LAXMAN

DIVISION OF BIOCHEMICAL SCIENCES

NATIONAL CHEMICAL LABORATORY,

PUNE - 411 008, INDIA

JUNE 2010

Affectionately Dedicated

To

My Beloved Parents, Wife, Family Members and

Respected Teachers

Research is to see what everybody else has seen and to think what nobody else has thought.

-Albert-Szent-Gyorgi (1893-1986) U. S. Biochemist

TABLE OF CONTENTS

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 and foremost, I would like to extend my sincere gratitude to my research guide Dr. (Mrs.) Mala Rao for introducing me to this exciting field of science and for her dedicated help, advice, inspiration, encouragement and continuous support, throughout my Ph.D. Her enthusiasm, integral view on research and her mission for providing high-quality work, has made a deep impression on me. During our course of interaction during the last six and half years, 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. Every time, I meet her, I can feel "the fire in the belly" which she always talks about. I owe her lots of gratitude for having me shown this way of research. I am really glad to be associated with a person like Dr. Mala Rao in my life.

My special words of thanks should also go to my research co-guide Dr. (Mrs.) R. Seeta Laxman for her continuous support, guidance, cooperation, encouragement and for facilitating all the requirements, going out of her way. She has taught me another aspect of life, that, "goodness can never be defied and good human beings can never be denied". Her constant guidance, cooperation, motivation and support have always kept me going ahead. I owe a lot of gratitude to her for always being there for me and I feel privileged to be associated with a person like her during my life.

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. She and her family members have always been there for me with there supporting hands whenever I needed it the most. The work done with her as a co-worker has taught me how a person can succeed in achieving what seems impossible to begin with…thank you Mam for everything.

I am grateful to Dr. Yogesh Shouche from National Centre for Cell Sciences (NCCS) who gave me permission to work in his laboratory and use the facilities available there for DNA sequencing.

My heartfelt thanks to my seniors Dr. Anish, Dr. Vinod, Dr Arohi, Dr. Ajit and Dr. Sharmili for there 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, Harish, Shalaka, Ashwini, Priya, Dheeraj, Sudarshan, Sarvanan, Dhananjay, Rajkumar, Padmavati, Tanajee, Ishwar, Gyan, Vishnu, Deepali, Rupesh, Arvind, Mehraj and Nilesh for always standing by my side and 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, Ambrish, Sachin, Atul, Aparna, Shiddarth, Umesh, Imran, Ashutosh, Arun, Manje, Sarvesh, Vipul, Akhilesh, JP,

Shashidhar, Anil, Atul Thakur, Sagar, Nagraj, Sandeep and many more for their constant support and cooperation during my stay. Their timely help and friendship shall always be remembered.

My special regards to my teachers because of whose teaching at different stages of education has made it possible for me to see this day. Because of their kindness I feel, was able to reach a stage where I could write this thesis.

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

I owe my deepest gratitude towards my better half for her eternal support and understanding of my goals and aspirations. Her infallible love and support has always been my strength. Her patience and sacrifice will remain my inspiration throughout my life. Without her help, I would not have been able to complete much of what I have done and become who I am. It would be ungrateful on my part if I thank ***Lily*** *in these few words. I am thankful to my son* ***Shivansh*** *for giving me happiness during the last one and half month of my studies.*

A special thanks to my younger brother Rakesh, sister Priyanka, brother in law Rajeev and my sisters childern Nanhy, Mona and Omkar for their love and affection.

My heart felt regard goes to my father in law, mother in law, sister in law Mrs Nirupama and her family, Mrs. Madhumita and her family, Ms Vibha and brother in law Mr. Anand for their love and moral support.

As always it is impossible to mention everybody who had an impact to this work however there are those whose spiritual support is even more important. I feel a deep sense of gratitude for my grand parents, mother, father, who formed part of my vision and taught me good things that really matter in life. Their infallible love and support has always been my strength. Their patience and sacrifice will remain my inspiration throughout my life.I am also very much grateful to all my family members for their constant inspiration and encouragement.

I gratefully acknowledge ***Council of Scientific and Industrial Research****, for providing me financial support and* ***Director, NCL*** *to allow me to carryout my research in this esteemed laboratory.*

Finally, I am thankful to library staff and administrative staff of NCL for cooperation.

SHIV SHANKAR

CERTIFICATE

Certified that the work incorporated in the thesis entitled: "**Production purification and characterization of protease from *Beauveria* sp**", submitted by Mr. Shiv Shankar, 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.) Mala Rao**

Place: Pune (Research Guide)

CERTIFICATE

Certified that the work incorporated in the thesis entitled: "**Production purification and characterization of protease from *Beauveria* sp**", submitted by Mr. Shiv Shankar, 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 Co-guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled " **Production purification and characterization of protease from *Beauveria* sp.**", 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. Mala Rao. 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: **Shiv Shankar**

Place: Pune (Research Scholar)

ABBREVIATIONS

°C	Degree Celsius
Å	Angstrom
Ag	Silver
APS	Ammonium persulphate
ATFB	Alkali treated fungal biomass
Au	Gold
BAP	*Beauveria* alkaline protease
BAPNA	N-α-benzoyl-DL-arginine-p-nitroanilide
BLAST	Basic Local Alignment Search Tool
BME	β- mercaptoethanol
BSA	Bovine serum albumin
CAPS	3-cyclehexylamino 1-propane sulphonic acid
CBB	Commassie brilliant blue
CD	Circular dichroism
CD	Circular dichroism
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
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetra acetic acid
EDTA	Ethylene diamine tetra acetic acid
EDTA	Ethylene diamine tetra acetic acid
FTIR	Fourier transform infra red spectroscopy
g	Gram
GYE	Glucose and yeast extract

h	hour
HPLC	high performance liquid chromatography
kDa	Kilo Dalton
Km	Michaelis Menton Constant
L	liter
M	Molar
MGYP	Malt extract, glucose, yeast extract and peptone
min	Minute
ml	Milliliter
mM	Milli molar
MSC	Mustard seed cake
MTCC	Microbial Type Culture Collection
NaCl	Sodium chloride
NBS	*N*-Bromosuccinimide
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
PEG	Polyethylene glycol
pI	Isoelectric point
PMF	Peptide mass finger printing
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
RNAase	Ribonuclease
rpm	Revolutions per minute
RSM	Response surface methodology
SAED	Selected area electron diffraction
SBTI	Soyabean trypsin inhibitor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	Tris Borate EDTA

TEM	Transmission electron microscope
TEMED	N,N,N'N'- Tetramethyl ethylene diamine
TEMED	N,N,N,N'-Tetramethylethylenediamine
TLCK	*N*-tosyl-L-lysine chloromethyl ketone
TNBS	2,4,6-trinitrobenzenesulphonic acid
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
UV/Vis	Ultraviolet/ visible spectroscopy
Vmax	Maximum velocity
WRK	Woodward's reagent
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
µl	Micro liter

ABSTRACT

Introduction

Proteases constitute a large group of hydrolytic enzymes that cleave peptide bond of protein and degrade them into small peptides and amino acids. Proteases are involved in all the cellular functions of the living organisms and found in all forms of life. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes. Of the industrial enzymes, 75% are hydrolytic. 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 strains with more efficient enzymes with desirable properties required for diverse applications. Most of the commercial proteases are of bacterial origin and have been extensively studied. Comparatively, studies on fungal proteases are limited to species mainly belonging to the genera *Conidiobolus*, *Rhizopus, Aspergillus*, *Penicillium*, *Mucor*, *Tritirachium*, *Fusarium*, *Cephalosporium*, *Paecilomyces.* Enzymes of fungal origin are advantageous due to the ease of cell removal during downstream processing. Proteases have a large variety of applications in various industries and many investigations are focused on the discovery and characterization of novel naturally occurring proteases from sources that have been overlooked.

The present work in the thesis describes molecular characterization of a new protease secreting fungal strain *Beauveria sp* MTCC 5184 isolated from animal dung. Purification and biochemical characterization of the protease will be discussed.

Outline of the thesis

This thesis consists of six chapters.

Chapter 1: Introduction

The ***first chapter*** of the thesis is general introduction which gives a brief review of literature on proteases viz.: history, source of proteases, classification, nomenclature, catalytic behavior, mechanism of action of serine proteases and their industrial applications.

Chapter 2: Purification of alkaline protease from *Beauveria* sp

The ***second chapter*** deals with the identification of fungal strain secreting alkaline protease on the basis of 18S rDNA, ITS sequence and morphology. The 18S rDNA as well as ITS sequence showed 99% nucleotide sequence homology towards *Beauveria felina* but it was morphologically different from *Beauveria felina* NCIM 1314. The nucleotide sequences of 18S rDNA and ITS gene of the *Beauveria* sp are deposited in NCBI database with accession numbers: FJ895305 and FJ895306 respectively. Purification of the alkaline protease to homogeneity has described in this chapter. The protease was produced on 1% glucose and 0.3% yeast extract with 2% mustard seed cake as inducer with a maximum activity of 10-12 U in 3-4 days. The protease was purified to homogeneity using ammonium sulphate saturation (40-70%) followed by DEAE-cellulose ion exchange column chromatography, with a 10.2 fold increase in specific activity and 38.6% recovery. Purity was checked and confirmed on cationic PAGE and HPLC which showed single band/peak.

Chapter 3: Biochemical characterization of protease

The ***third chapter*** deals with the biochemical characterization of the purified protease. The protease was optimally active at pH 9 and 50°C. The enzyme was stable over a wide range of pH (5-11) with maximum stability at pH 7. Protease was stable up to 40°C and half life at 50°C was 2h. The Km and Vmax of the enzyme were found to be 5.1 mg/ml and 29.67 U/ml respectively. The molecular mass of purified protease was estimated to be 29 kDa by SDS-PAGE and 28 kDa by MALDI-TOF. Isoelectric point of the protease was 9.3. The protease was totally inhibited by PMSF, partially inhibited by TPCK and TLCK but no inhibition was observed with EDTA, Iodoacetate and Benzamidine. Protease showed highest activity with casein followed by haemoglobin and least with bovine serum albumin. The protease showed less than 20% inhibition by 10 mM Ca^{2+}, K^{+}, Fe^{3+} whereas less than 40% inhibition was observed by 10 mM Cd^{2+}, Hg^{2+}, and Mn^{2+}. The N-terminal sequence and peptide mass finger printing of purified protease showed partial homology with subtilisin-like proteinase. The 28 kDa protease gave two fragments of 15.6 kDa and 10.2 kDa after autolysis. Chemical modification studies showed serine, tryptophan and aspartic acid to be at the active site of enzyme.

Chapter 4: Stability of protease in organic solvents, detergents and denaturants

The ***fourth chapter*** deals with the effect of organic solvents, detergents and denaturants on protease activity and stability. Various water miscible and water immiscible organic solvents were tested at 28 and 50°C for their influence on stability of protease. More than 60% activity was retained in ethanol, isopropanol, methanol, butanol and DMSO compared to 15% in protease devoid of organic solvent after 48 h at 28°C. At 50°C protease showed highest stability in acetone and DMSO and retained more than 70% activity after 2 h. Protease was stable in β-meracptoethanol, urea, NaCl and Triton-X-100.

Chapter 5: Applications of protease

The ***fifth chapter*** describes the potential applications of the protease. This chapter is divided into two sections.

Section A. Animal tissue culture

This section deals with the application of purified protease from *Beauveria* sp in animal tissue culture. The purified protease can substitute trypsin for separation of cells in animal tissue culture.

Section B. Biosynthesis of gold and silver nanoparticles

This section deals with the synthesis and characterization of gold and silver nanoparticles. The nanoparticles were characterized by UV-Vis spectrophotometer, TEM, SAED, XRD, FTIR and XPS. The gold nanoparticles formed were of different shape and size varied from 1-1.5 μm whereas silver nanoparticles in the range of 200-600 nm. Both gold and silver nanoparticles formed are crystalline in nature.

Chapter 6: Studies on *Conidiobolus coronatus* protease

The ***sixth chapter*** is divided into 2 sections

Section A. Immobilization of *Conidiobolus coronatus* alkaline protease on fungal biomass

This section deals with the identification of the fungal culture and immobilization of protease on fungal biomass. The sequence of 18S rDNA showed maximum homology with *Conidiobolus coronatus.* The protease from *Conidiobolus coronatus* was immobilized on different matrices viz. Ca-alginate, polyacrylamide and alkali treated

fungal biomass (ATFB) with and without crosslinking with glutarldehyde. ATFB with and without glutarldehyde were found to be the best among all the matrices tested. Pre-treating the ATFB with 2% glutaraldehyde showed marginal increase in binding of protease. Temperature optima of free and immobilized protease were similar. The optimum pH of the immobilized enzyme shifted to a more alkaline range (11) as compared with the free enzyme (10). Though there was no change in pH stability, temperature stability slightly increased after immobilization. There were no differences in FTIR of ATFB before and after binding with protease. More than 70% activity of the immobilized protease was retained after three reuses.

Section B. Recovery of Silver from waste X-ray film by alkaline protease from *Conidiobolus coronatus*

This section deals with the recovery of silver from waste X-ray film using alkaline protease from *Conidiobolus coronatus.* The protease hydrolyzed gelatin of waste X-ray films. At the end of the treatment, gelatin layer was completely removed leaving the polyester film clean and silver was recovered in the hydrolysate. Various parameters such as pH, temperature and enzyme concentration on silver removal from the film were studied. Gelatin hydrolysis was monitored by measuring increase in turbidity in the hydrolysate. Gelatin layer was stripped completely with 1.35 U/ml of protease within 6 min at 40˚C, pH 10. Rate of gelatin hydrolysis increased with increase in protease concentration. The enzyme could be effectively reused for four cycles of gelatin hydrolysis. Silver was recovered from the hydrolysate and corresponded to 0.2 % (w/w) weight of X-ray film.

PUBLICATIONS

- **S. Shankar**, S.V. More, R. Seeta Laxman, (2010). Recovery of silver from waste Xray film by alkaline protease from *Conidiobolus coronatus*. *Kathmandu Univ J Sci Engg Technol*, 6: 60-69.

- **Shiv Shankar**, Mala Rao and R. Seeta Laxman. Purification and characterization of an alkaline protease by a new strain of *Beauveria* sp. Under review in Process Biochemistry.

- **S. Shankar** and R. Seeta Laxman. Biochemical characterization of serine alkaline protease from new strain of *Beauveria* sp. Manuscript under preparation.

- **S. Shankar** and R. Seeta Laxman. Biosynthesis of silver nanoparticles from waste Xray film by using protease from new strain of *Beauveria* sp. Manuscript under preparation.

- **S. Shankar** and R. Seeta Laxman. Immobilization of *Conidiobolus coronatus* alkaline protease on fungal biomass. Manuscript under preparation.

PATENTS FILLED

- Laxman, R. S., **Shankar, S.,** Khandelwal, H. B., More**,** S. V., Narasimhan, C. B. K., Palanivel, S. and Balaram, P. Enzymes from *Beauveria* sp. and process for preparation thereof". Indian patent Application No. 249/DEL/2010.

CONFERENCES / ABSTRACTS/ POSTERS

- Snehal V. More, **S. Shankar**, Harish B. Khandelwal & R. Seeta Laxman. Poster entitled, "Production of alkaline protease from C*onidiobolus coronotus* and its properties" presented at International conference "Emerging trends in Biotechnology (ETBT)", December 2009 held in BHU, Varanasi, India.

- Snehal V. more, **S. Shankar** & R. Seeta Laxman. Poster entitled, "Protease from *Conidiobolus coronotus* and its application" presented at 50th annual conference of Association of Microbiologist of India December 2009 held at Pune, India.

- **S. Shankar** & R. S. Laxman. Poster entitled, "Immobilization of protease on fungal biomass from *Conidiobolus coronatus*" presented at International conference in New Horizon in Biotechnology "NHBT" November 2007 held in Trivendrum, Kerala, India.

- Snehal V. More, **S. Shankar** & R. S. Laxman. Poster entitled, "Silver recovery from waste X-ray film by fungal protease" presented at 74$_{th}$ Annual meeting of Society of Biological Chemist, India, November 2005 held in CDRI, Lucknow.

- Attended National Seminar on Crystallography, at NCL, Pune from January 8-11, 2004.

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Enzymes are the catalytic cornerstones of metabolic activities of living being and catalyze most of the reactions in living organisms. Classically enzymes work by providing an alternative path of lower activation energy for reactions and dramatically accelerating its rate. The term enzyme was coined by German physiologist Wilhelm Kuhne and was derived from the Greek word *ενζυμον*. Various workers like Buchner, Sumner, Northrop, Stanley, Phillips and several others showed that enzymes are specialized proteins. Enzyme focused research is of importance not only to biological community but also to process designers/engineers, chemical engineers and researchers working in other scientific fields. Enzymes being proteins possess properties such as specificity towards the reactions they catalyze and the substrates on which they act upon. Apart from basic approach, enzymes are widely studied from an industrial point of view. Enzymes have been used since the dawn of mankind in cheese manufacturing and indirectly via yeasts and bacteria in food manufacturing. 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.

Enzyme Nomenclature

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 the EC number. Accordingly the top level classes based on the mechanism of operation of an enzyme are:

1. Oxidoreductases: catalyze oxidation/reduction reactions
2. Transferases: transfer a functional group
3. Hydrolases: catalyse the hydrolysis of bonds with addition of water
4. Lyases: remove groups from their substrates
5. Isomerases: catalyze isomerisation changes within a single molecule
6. Ligases: join two molecules with covalent bonds

From an industrial standpoint, only a limited number of enzymes are commercially available and few of them have found applications in large quantities. More than 75% of industrial enzymes are hydrolases (Rao et al., 1998). More than fifty commercial industrial enzymes are available and their number is being increased steadily. Today, the enzymes are commonly used in many industrial applications and the demand for more stable, highly active and specific enzymes are growing rapidly. Global market for industrial enzymes is reported to be €1 billion in 1995 (Godfrey and West, 1996) whereas, it was increased to $2.3 billion in 2007 and was expected to increase to over $2.7 billion by 2012, with a compound average annual growth rate (CAGR) of 4% (http://www.encyclopedia.com/doc/1G1-172107942.html) as new enzymes and applications are discovered. Presently the biotechnology industries are dominated by the leather, detergent, starch, textile and fuel alcohol industries accounting for the majority of the total enzymes market, with the feed and food enzymes. Proteases, amylases, pectinases, cellulases, lipases, phytases, chitinases, lactase and xylanases are some of the major hydrolytic enzymes used in industries.

Proteases

Proteases are among the oldest and most diverse families of enzymes known and are involved in every aspect of organism's function. They constitute a very large and complex group of hydrolytic enzymes that degrade proteins into small peptides and amino acids. Proteases catalyze the addition of water across amide (and ester) bonds to cleave using a reaction involving nucleophilic attack on the carbonyl carbon of the scissile bond. They differ widely in their properties such as substrate specificity, active site and catalytic mechanism and possess different profiles for mechanical stress, chemical environment, pH and temperature for stability and activity. Because of their broad substrate specificity, proteases have a wide range of applications such as in leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacture, soysauce production, protein hydrolysate, pharmaceutical industry, waste treatment, silk industry, organic synthesis, recovery of silver from waste photographic film, as well as analytical tools in basic research and have high commercial value (Godfrey and West, 1996). Among the bulk of industrial enzymes, proteases from plant, animal, and microbe constitute around 60 % of the total worldwide enzyme sales (Kunamneni et al., 2003; Merheb-Dini et al., 2009).

Currently, the largest share of the enzyme market has been held by detergent proteases which are active and stable at alkaline pH. They are also important from a physiological point of view, as they are involved in many cellular processes like protein turn over and digestion as well as fungal morphogenesis, spore formation and spore germination. Yet, there is a continued search for proteases having novel properties with known and newer applications

Sources of Proteases

Proteases are ubiquitous and found in all forms of life: plants, animals and microorganism including viruses.

Plant Proteases

Most common plant proteases are bromelain, ficin, papain and zingibain (Lee et al., 1986; Adulyatham and Owusu-Apenten, 2005).

Bromelain: Bromelain (EC 3.4.22.32) is a crude extract from the pineapple (*Ananas comosus*) plant. It is a mixture of sulfur-containing proteases (EC 3.4.22.32 –S, EC 3.4.22.33- F). Bromelain is present in all parts of the pineapple plant but the stem is the most common commercial source. It is active between pH 5 to 9 and stable up to 70°C beyond which it is inactivated. It is used as meat tenderizer, anti-inflammatory agent and in debridement (Secor et al., 2005). The major mechanism of action of bromelain appears to be proteolytic in nature, although evidence suggests an immunomodulatory and hormone like activity acting via intracellular signalling pathways. Bromelain has also been shown to reduce cell surface receptors such as hyaluronan receptor CD44, which is associated with leukocyte migration and induction of proinflammatory mediators (Tochi et al., 2008).

Ficin: It is extracted from latex of ficus and is a sulfhydryl proteinase with cysteine at the active site (EC 3.4.22.3). It preferentially cleaves at tyrosine and phenylalanine residues. Ficin has proven to be a versatile low cost biocatalyst useful in peptide synthesis (Sekizaki et al., 2008).

Papain: It is a cysteine protease (EC 3.4.22.2) extracted from latex of papaya (*Carica papaya*). The crude enzyme has broad specificity due to mixture of several proteases.

The enzyme is active between pH 5 to 9 and is stable up to 80-90°C in presence of substrates. It consists of 212 amino acids stabilized by 3 disulfide bridges. Its catalytic triad is made up of 3 amino acids - cysteine-25 (from which it gets its classification), histidine-159, and asparagine-158. It is extensively used in tenderization of the meat (to break down the tough meat fibers), preparation of highly soluble and flavoured protein hydrolysates, dissociate cells in the first step of cell culture preparations, to make single cell preparation and an ingredient in some toothpastes and mints as teeth-whitener (Kim et al., 2004). The proportion of application of papain in several industries around the world is: 75 % in brew, 10 % in meat, 5 % in fish, 5 % in other food, 2 % in medicine and 3 % in other use (http://en.cnki.com.cn/Articleen/CJFDTOTAL-GDHG200810029.htm).

Animal Proteases

The well-known proteases of animal origin are pepsin, trypsin, chymotrypsin and rennin. Production of these enzymes depends on available stock in slaughterhouse.

Chymotrypsin: It is found in the pancreatic extract of animals (EC 3.4.21.1). The enzyme cleaves peptides at the carboxyl side of tyrosine, tryptophan and phenylalanine although over time it also hydrolyzes other amide bonds, particularly those with leucine-donated carboxyls. It is present in zymogen form and is activated on cleavage by trypsin into two parts that are still connected via an S-S bond. Pure chymotrypsin has main applications in analytical and diagnostic field. It is extensively used in deallergenizing of milk protein hydrolysates.

Pepsin: It is a digestive protease (EC 3.4.23.1) released by the chief cell in the stomach of almost all vertebrates that function to degrade food proteins into peptides. Pepsin is produced in its zymogenic form i.e pepsinogen, whose primary structure has additional 44 amino acids. This zymogen is activated by hydrochloric acid (HCl), which is released from parietal cell in the stomach lining. HCl creates an acidic environment which allows pepsinogen to unfold and cleave itself in an autocatalytic fashion, thereby generating pepsin. Pepsin functions best in acidic environments between pH 1 to 2 and is inactivated above pH 6. Pepsin cleaves preferentially after the N-terminal of aromatic amino acids such as phenyalanine and tyrosine. It is an

aspartyl protease and has resemblance with HIV-1 protease. Pepsin is commonly used in the preparation of $F(ab)^2$ fragments from antibodies.

Rennin: It is an aspartic acid protease (EC 3.4.23.4), produced as an inactive precursor, prorennin in stomachs of all nursing mammals but more specifically in the fourth stomach of cows. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in *k*-casein to generate insoluble para-*k*-casein and C-terminal glycopeptide. 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 together and would form a three dimensional network to trap the aqueous phase of the milk resulting in the formation of calcium phosphocaseinate. This specificity is used to bring about the extensive precipitation and curd formation in cheese making.

Trypsin: It is a serine protease (EC 3.4.21.4) found in the digestive system and is responsible for the breakdown of food proteins. Trypsin has an optimal operating pH and temperature of about 8 and 37°C respectively and predominantly cleaves proteins at the carboxyl side of the lysine and arginine. Trypsin is commonly used in proteomics, since it has a very well defined specificity. Trypsin is also used for the preparation of bacterial media, to dissolve blood clots, treat inflammation and to dissociate dissected cells. Trypsin has limited application in food because the protein hydrolysates generated have bitter taste. Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, trypsin is targeted for biocontrol of insect pests.

Microbial Proteases

Proteases are widely distributed in microbial population viz. bacteria, actinomycetes, viruses and fungi. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes and account for around two-thirds of commercial production worldwide. Alkaline serine proteases (EC 3.4.21) are the most important group of commercial enzymes (Kumar and Takagi, 1999). 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 strains with more efficient enzymes with desirable properties required for their diverse applications. Isolation of new and novel extremophiles organisms such as alkalophiles and thermophiles from diverse habitats secreting proteases that are active and stable under harsh conditions of industry has resulted in renewed interest in microbial proteases with novel properties.

Bacteria: Most of the commercial proteases are of bacterial origin (Prakasham et al., 2006). Though proteases are produced by variety of bacteria such as *Pseudomonas aeruginosa*, *Flavobacterium*, *Clostridium*, *Staphylococcus aureus*, *Achromobacter*, *Thermoactinomyces* and species belonging to *Streptomyce, Bacillus* is the major source which secretes a variety of soluble extracellular enzymes. Alkaline proteases from bacterial source are widely used in detergent formulations due to their activity and stability at high pH (9-11) and temperature (50-60°C). Neutral proteases of bacterial origin are active at pH 5-8 and between 35-40°C. Compared to alkaline proteases, neutral proteases have lower thermo-tolerance. Protein hydrolysate produced using neutral proteases of microbial origin has less bitterness compared to the one using animal trypsin and hence finds application in food industry. Neutral proteases are also used in brewing industry. Alkalophilic bacteria are also known to produce proteases. The first report of alkaline protease by an alkalophilic *Bacillus* sp. strain 221 was published in 1971 by Horikoshi, (1971). Purification and characterization of aqualysin 1 a thermostable alkaline serine protease produced by thermophilic *Thermus aquatiens* YT-1 is reported by Matsuzawa et al. (1988). Subsequently many more proteases from alkalophiles with novel properties are also reported (Horikoshi, 1999).

Virus: Viral proteases are involved in processing of proteins that cause fatal diseases like AIDS and cancer. Mature enzymes encoded within the human immunodeficiency virus type 1 (HIV-1) genome protease (PR); reverse transcriptase (RT) and integrase (IN) are derived from proteolytic processing of a large polyprotein (Gag-Pol). The viral PR catalyzes Gag-Pol processing, which is active as a homodimer (Olivares et al., 2007). The HIV-1 protease is a homodimeric enzyme composed of two identical subunits of 99 amino acids. Each subunit contains the conserved sequence Asp-Thr-Gly that provides the aspartyl group necessary for catalysis. Crystal structures and biochemical studies have shown that major interactions that stabilize the mature dimer

appear in a four-stranded antiparallel β-sheet, where the N-terminal residues of each of the protease monomers form the outer strands of the β -sheet and the C-terminal residues of each monomer form the two inner strands (Weber, 1990; Dunn and Rao, 2004).

Fungi: Filamentous fungi can effectively secrete various hydrolytic enzymes and one of the main groups of secreted enzymes in fungi is protease. Submerged as well as solid state fermentation have been employed for protease production. They usually show better results when cultured in solid-state fermentation as compared to bacteria (Pandey et al., 1999). Fungi are known to produce acid, neutral, alkaline and metallo proteases. A single organism can produce more than one type of protease (Lindberg et al., 1982). Fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity (Rao et al., 1998). One of the first known representatives of proteases was proteinase K, an alkaline enzyme from *Engyodontium album* also known as *Tritirachium album* (Kotlova et al., 2007). Several reports are available on production of proteases by fungi belonging to the genera *Aspergillus* (Coral et al., 2003; Tunga et al., 2003; Wang et al., 2005; Kunert and Kopecjek, 2000; Hajji et al., 2007; Vishwanatha et al., 2010); *Penicillium* (Germano et al., 2003; Agrawal et al., 2004; Oliveira et al., 2006; Zhu et al., 2009; Krishna et al., 2009), *Rhizopus* (Farley and Ikasari, 1992; Kumar et al., 2005), *Humicola* (Aleksieva and Peeva, 2000), *Mucor* (Maheshwari et al., 2000; Alves et al., 2002; Tubesha and Delaimy, 2003; Zheng and Zhao, 2009); *Thermomyces* (Li et al., 1997; Jensen et al., 2002). Other fungi also known to produce extracellular alkaline proteases, for example C*onidiobolus* (Srinivasan et al., 1983; Phadtare et al., 1993; Laxman et al., 2005), *Arthrobotrys olgospora* (Tunlid et al., 1994); *Trichoderma harzianum* (Dunaevsky et al., 2000); *Cephalosporium* sp. KM388 (Tsuchiya et al., 1987); *Metarhizium anisopliae* (Leger et al., 1992). Among fungi, the ability of many species of *Aspergillus* to produce proteases is well known. For example, *Aspergillus oryzae* produces acid, neutral and alkaline proteases.

Alkaline proteases from *Aspergillus* species are used in leather treatment (Godfrey and West 1996). Endo and exoproteases from *A. oryzae* have been used to modify wheat gluten, an insoluble protein, by limited proteolysis thereby facilitating its handling and machining. Genes encoding alkaline proteases from A. *oryzae, A. sojae, A. fumigatus, A. flavus and A. nidulans* have been cloned sequenced and

expressed (Hodgson, 1994). Proteases have been cloned and sequenced in many microorganisms, including *A. niger* (Frederick et al., 1993), *A. nidulans* (Katz et al., 1994), and *A. fumigatus* (Reichard et al., 1995). In some cases these enzymes have been associated with allergenic or inflammatory responses (Hanzi et al., 1993). *A. clavatus* strain was isolated from Brazilian soil as one of the the best protease producer (Tremacoldi and Carmona, 2005).

Other Proteases

Collagenase: Collagen is a fibrous protein and is a constituent of skin, bone, cartilage, tendon and other connective tissue. Its commercial importance in leather and in production of gelatin and glue has long been recognized. Collagen is converted into gelatin by boiling. Collagenases are enzymes capable of degrading collagen and are of two types, (i) low molecular weight serine collagenases (24-36 kDa) which are involved in the production of hormones and pharmacologically active peptides and (ii) high molecular weight metallocollagenases (30-150 kDa) containing zinc, which require calcium for stability and are involved in remodeling the extracellular matrix (Park et al, 2002). Microbial collagenases are reported from bacteria, actinomycetes and fungi. Among fungi, collagenases are reported from *Aspergillus* (Kundu et al., 1974; Sukhosyrova et al., 2003; Mahmoud et al., 2007), *Coccidioides immitis* (Lupan and Nziramasanga, 1986), *Arthrobotrys amerospora* a nematode-trapping fungus (Schenck et al., 1980), antarctic fungus *Arthrobotrys tortor* (Tosi et al., 2001) and *Lagenidium giganteum* a mosquito-parasitizing fungus (Dean and Domnas, 1983). Tosi et al. (2001) have screened fungal species belongs to *Arthrobotrys* for collagenase production and found that collagenase from antarctic strain of *Arthrobotrys tortor* (ATCC 96018) was a constitutive enzyme and produced about three fold higher activities than other species viz. *Arthrobotrys tortor* B114, *Arthrobotrys conoides* CMM 1017 and *Arthrobotrys oligospora* CBS 280.86.

Elastase: Elastin is a fibrous protein and together with collagen determines the mechanical properties of connective tissue. It imparts elasticity and allows the tissues to regain its original shape after stretching or contracting. Elastase is a protease, which breaks down elastin and has applications in food, pharmaceuticals and cosmetics industries (Chen et al., 2007). The specific peptide bonds cleaved by elastase are those on the carboxy side of small hydrophobic amino acids such as

glycine, alanine and valine. Rippon and his colleagues have reported elastase from ringworm fungus for the first time in 1967. Later they screened several fungi including dermatophytes for elastase producers (digestion of elastin incorporated in Czapek-Dox agar) and found crude fungal extracts of *Allescheria boydii* was able to digest elastin (Rippon and Hoo, 1971). Fungal elastases have been reported from *Aspergillus* (Markaryan et al., 1994; Mellon and Cotty., 1995; Alp and Arikan, 2008) and entomopathogenic fungus *Conidiobolus coronatus* (Wieloch and Bogus, 2007). Mellon and Cotty (1996) purified and characterized elastase from *Aspergillus flavus* NRRL 18543.

Keratinase: Keratin is a fibrous protein present in hair, wool, nails, horn, hoofs and the quills of feathers and generally contains large quantities of sulfur-containing amino acids particularly cysteine. Keratinases [EC.3.4.21/24/99.11] are the proteolytic enzymes capable of hydrolyzing highly rigid, strongly cross-linked structural polypeptide, keratin which is recalcitrant to commonly known proteases such as trypsin, pepsin and papain. Keratinases are widely distributed in nature and secreted by variety of organisms belonging to bacteria, actinomycetes and fungi. Dermatophytic fungi long known for their pathogenesis are producers of keratinases (Sohnle and Wagner, 2000). Apart from dermatophytes like *Trichophyton* sp and *Microsporum* sp, keratinases are also produced by *Aspergillus*, *Candida*, *Doratomyces microsporus*, *Chrysosporium, Cladosporium*, *Trichurus*, *Geomyces*, *Fusarium*, *Paecilomyces*, *Myrotheium*, *Penicillium*, *Stachybotrys*, *Scopulariopsis*, *Curvularia* (Wawrzkiewicz et al., 1991; Santos et al., 1996; Gradisar et al., 2000; Gupta and Ramnani, 2006; Vermelho et al., 2010). Keratinases are inducible enzymes and are produced in presence of keratin rich substrates like hair, feather, nails, hoofs etc. Keratinases are active over a wide pH of 7-12 (mostly in alkaline environments) and temperature range 30-80°C with optimal activity at temperatures up to 50°C (Gradisar et al., 2005). The molecular masses of the enzymes range from 20-60 kDa and mainly belong to serine or metalloproteases. A thermostable, alkaline keratinolytic proteinase was produced by C*hrysosporium keratinophilum* (Dozie et al., 1994). Traditionally keratinases have been in use for production of feather meal, fertilizers, glues etc. Further their applications have been extended to other areas such as detergent formulations, cosmetics, leather, medicine and animal feed. Keratinases

are also finding applications in treatment of mad cow disease (degradation of prion) and biodegradable plastic (Gupta and Ramnani, 2006).

Microbial Rennins: Rennin, an aspartic acid protease is important enzyme in cheese manufacture. The enzymes possess high milk-clotting activity and low proteolytic activity, enabling them to be used as substitutes for calf chymosin in the cheese industry. Traditionally rennin is isolated from animal source (calf rennin) but increased demand of religious and ethnic regulations against animal derived enzyme has generated interest in microbial rennnis. Among Mucorales, *M. miehe*i, *M. pusillus* (Arima et al., 1967) and *M. bacilliformis* have been utilized in food industry and fermented beverage. The fungus, *Mucor miehei* are used for the production of rennin in large scale for industrial use (Seker et al., 1999; Tubesha and Delaimy, 2003). Webb et al. (1974) reported that the yield and quality of cheese produced with the protease from *Mucor miehei* were the same as the calf rennet. Compared to calf rennin, microbial rennin ripened cheese more quickly and had no bitter flavours. The milk-clotting activity of the enzyme is due to its selective attack on the *k*-casein fraction, which stabilizes the casein micelle in milk. The split *k*-casein loses its stabilizing activity, and the micelles of casein coagulate in the presence of calcium. The Mucor rennins are produced by solid state fermentation on wheat bran. They are classified as acid proteases (EC 3.4.23.6) since they hydrolyse casein and hemoglobin optimally in acidic range of pH 3.5-4. The *M. pusillus* enzyme is stable in pH range of 3-6 and optimally active at 55°C. They are inhibited by the aspartic protease inhibitors diazoacetyl-DL-norleucine methyl ester and pepstatin. *M. pusillus* and *M. miehei* rennins had similar molecular masses (38.5 and 42 kDa) and isoelectric points (3.9 and 4.1), respectively. They have been purified and crystallized. Both *M. pusillus* and *M. miehei* rennins cleave peptide bonds in synthetic peptides with an aromatic amino acid as the carboxyl donor. Although the Mucor rennins (aspartic proteases) are structurally homologous, they differ from other fungal aspartic proteases and from mammalian proteases. The specificity of Mucor rennins is similar to that of pepsin and calf rennin.

Classification of Proteases

Proteases are classified on the basis of a critical amino acid required for the catalytic function (e.g., serine protease), the pH optimum for their activity (acidic, neutral, or

alkaline protease), their site of cleavage (e.g., aminopeptidases, which act at the free N terminus of the polypeptide chain or carboxypeptidases which act at the C terminus of the polypeptide chain) or their requirement of a free thiol group (e.g., thiol proteinase).

Nomenclature and Terminology

All proteolytic enzymes are designated as peptidases, indicating that they hydrolyze peptide bond. Those enzymes that require the presence of an unsubstituted N or C-terminus in the substrate are designated as exopeptidases while those, which do not require are designated as endopeptidases. The secondary and tertiary structure of protein (substrate) usually prevents attack by exopeptidases. The action of an endopeptidase is generally not favored by the presence of a free N or C- terminus close to the scissile bond. Exopeptidases remove a single amino acid, a dipeptide or a tripeptide from one or other terminus, the basis on which exopeptidases are classified (based on action). Similar consideration of specificity cannot be applied to the endopeptidases. Endopeptidases are divided into four subgroups based on their catalytic mechanism as (i) serine proteases (ii) aspartic proteases (iii) cysteine proteases and (iv) metallo proteases each with a different and special mode of action with different amino acids at their active site. The same catalytic mechanism also operates among the exopeptidases and hence they can be further subdivided in to serine, cycteine, aspartic and metallo- exopeptidases (Kenny, 1999).

Classification of Proteases According To Enzyme Commission

According to the Committee of International Union of Biochemistry and Molecular Biology (1992), proteases are classified in subgroup 4 of group 3 (hydrolases) and assigned with EC.3 (hydroloases), 4 (proteases). The main classes of peptidases are defined by a third numeral (11 to 24) as listed in Table 1.1. The exopeptidases are classified mainly on the basis of their actions. Only peptides with unsubstituted terminus are attacked with the exception of a very small number, grouped as omega peptidases (3.4.19), which can release certain modified terminal residues.

Table 1.1: Classification of Proteases (Peptidases)

E.C. number	Peptidase type	Action
Exopeptidases		
3.4.11-	Aminopeptidase	N-terminal residue released
3.4.13-	Dipeptidase	Acts only on dipeptides
3.4.14-	Dipeptidyl peptidase Tripeptidyl peptidase	N-terminal dipeptide released N-terminal tripeptide released
3.4.15-	Peptidyl dipeptidae	C-terminal dipeptide released
3.4.16	Carboxypeptidase (serine)	C-terminal residue released (serine at active site)
3.4.17-	Carboxypeptidase (metallo)	C-terminal residue released (metal requiring protease)
3.4.18-	Carboxypeptidase (cysteine)	C-terminal residue released (cysteine at active site)
3.4.19-	Omega peptidase	Releases modified residue from N- or C- termini
Endopeptidases		
3.4.21-	Serine endopeptidase	Serine at active site
3.4.22-	Cysteine endopeptidase	Cysteine at active site
3.4.23-	Aspartic endopeptidase	Aspartate at active site
3.4.24-	Metallo endopeptidase	Metal requiring protease
3.4.99-	Endopeptidase of unknown Catalytic mechanism	

Proteases or proteolytic enzymes are enzymes that break peptide bonds between amino acids of proteins. The process is called proteolytic cleavage, a common mechanism of activation or inactivation of enzymes. They use a molecule of water for this and are thus classified as hydrolases. There are currently six classes of proteases:

• Serine proteases

• Threonine proteases

• Cysteine proteases

• Aspartic acid proteases

• Metalloproteases

• Glutamic acid proteases

The threonine and glutamic acid proteases were not described until 1995 and 2004, respectively and are therefore recent additions. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the character of a polarized peptide bond (serine, cysteine and threonine proteases) or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carbonyl group.

Rawlings et al. (2007) have devised a classification scheme based on statistically significant similarities in sequence and structure of all known proteolytic enzymes and term this database MEROPS. The classification system divides peptidases into clans based on catalytic mechanism and families on the basis of common ancestry. At present, over 66,000 peptidase protein sequences have been classified into 50 clans and 184 families (MEROPS release 7.90). Over 26,000 serine peptidases are grouped into 13 clans and 40 families (Page and Di Cera, 2008).

The MEROPS database is a manually curated information resource for peptidases (also known as proteases, proteinases or proteolytic enzymes), their inhibitors and substrates. The database has been in existence since 1996 and can be found at http://merops.sanger.ac.uk. The principle of the database is a hierarchical classification in which homologous sets of peptidases and protein inhibitors are grouped into protein species, which are in turn grouped into families, which are grouped into clans (Rawlings et al., 2010)

Mechanism of Action of Proteases

The mechanism of action of proteases has been a subject of great interest to researchers as it forms a basis for exploring various ways of modifying its activity to make it suitable for its biotechnological application. Studies of the mechanism of proteases have revealed that they exhibit different types of mechanism based on their active-site configuration.

Serine Protease: Serine proteases are characterized by serine at the active site. They are wide spread and reported from bacteria, fungi and viruses. They are found in endopeptidases as well as exopeptidases. Three residues which forms the catalytic triad are essential in the catalytic process i.e His (base), Asp (electrophile) and Ser

(nucleophile). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme (Figure 1.1). The deacylation, which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of Ser residue. The His residue provides a general base and accept the OH group of the reactive Ser residue (Rao et al., 1998).

Aspartic protease: Catalysis by aspartic proteases does not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate (Rao et al., 1998).

Cysteine protease: Catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys and His play the same role as Ser and His respectively as in serine proteases. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps (Rao et al., 1998).

Metalloprotease: The catalytic mechanism by metalloprotease leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Rao et al., 1998).

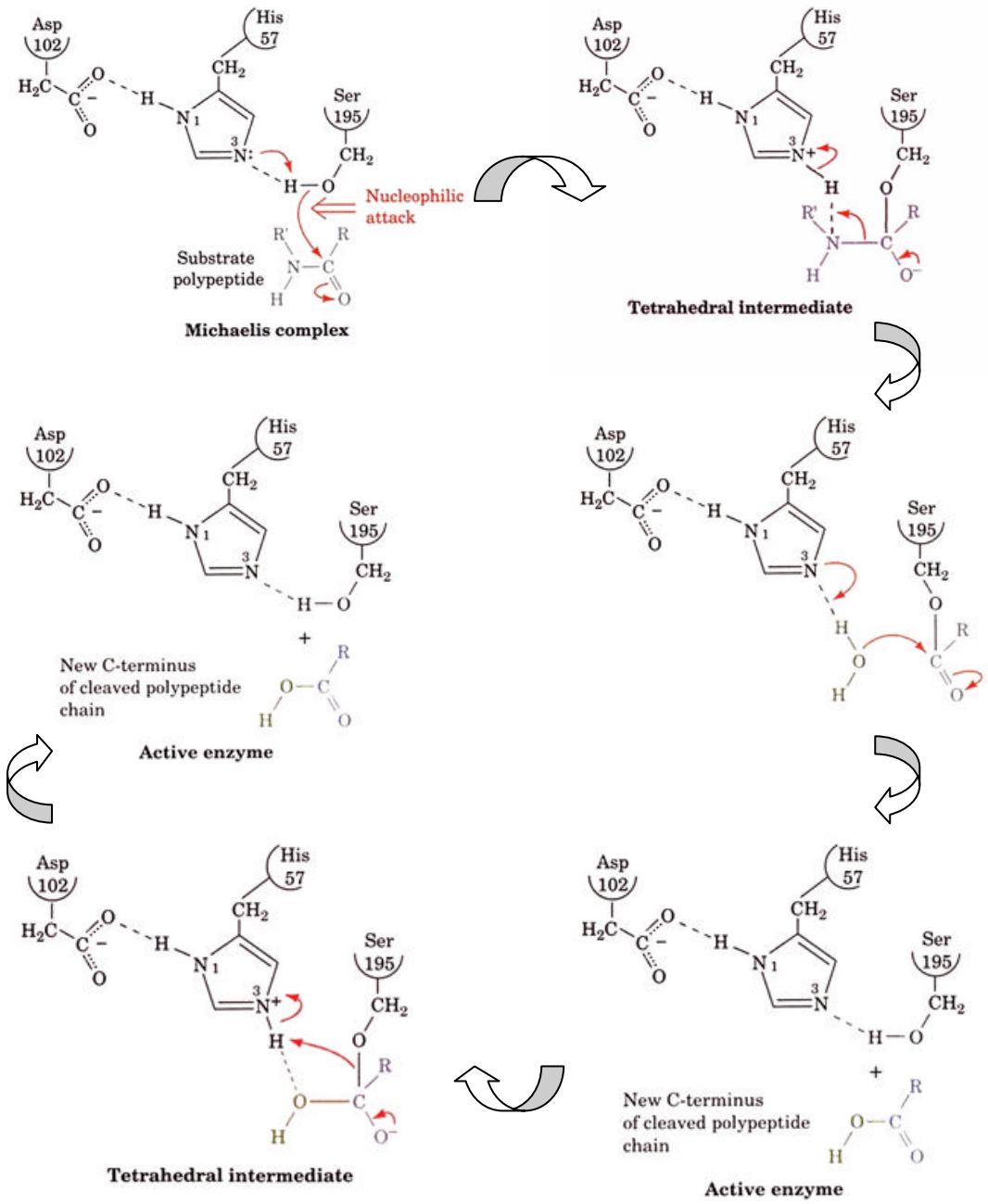


Figure 1.1: Mechanism of action of serine proteases.
(Ref: http://info.bio.cmu.edu/courses/03231/Protease/SerPro.htm)

Types of Serine Proteases

Serine proteases are characterized by a nucleophilic serine residue located in their active site. Apart from this, these proteases are also distinguished by having essential aspartate and histidine residues which, along with the serine, forms the catalytic triad.

They are generally active at neutral and alkaline pH, with optima at pH 7–11, although higher pH optima of 8.5-12 from *Cordyceps militaris* have also been reported (Hattori et al., 2005). They show broad substrate specificity and structural homology to well established proteases and are classified on the basis of their side-chain specificity against the oxidized insulin β-chain. Various types of serine proteases have been described in great detail. A comprehensive account of subclasses of serine proteases is presented below.

Chymotrypsin-like Proteases

Chymotrypsin, a mammalian digestive protease, has structural homology with trypsin, elastase and thrombin. It catalyzes the hydrolytic cleavage of peptide bonds at carboxyl terminal of Phe, Tyr and Trp 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), diisopropylfluorophosphate (DFP) and soyabean trypsin inhibitor (SBTI). The molecular weight is generally around 20 kDa (Gupta et al., 2002).

Subtilisin-like Proteases or Subtilases

Subtilisin-like serine proteases are generally bacterial in origin, although there are reports in favor of other organisms also. They are generally secreted extracellularly for the purpose of scavenging nutrients. This class of proteases cleave aromatic or hydrophobic residues (at position P1), such as tyrosine, phenylalanine and leucine. They are highly sensitive towards 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 (Rao et al., 1998).

Wheat Serine Carboxypeptidase II-like Proteases

These have a different catalytic triad residue in their amino acid sequence and their tertiary folds are also completely different for each structural family. They are most active in the pH range 4–5.5, suggesting an unusual low p*K*a value for its catalytic histidine residue (Liao and Remington, 1990).

Production of Proteases

Fungi elaborate a wider variety of enzymes than bacteria, e.g., *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 can effectively secrete various hydrolytic enzymes in submerged as well as solid state fermentation. Due to the hyphal nature which enable them to penetrate and colonize the substrate, fungi are better suited to solid-state fermentation as compared to bacteria. In solid state fermentation different agricultural residues are used as solid support like wheat bran, rice bran, soyabean cake or combination of two or more substrates (Wang et al., 1974; Sardjono et al., 1998; Kitano et al., 2002; Germano et al., 2003; Haq and Mukhtar, 2004). Reports on production of fungal enzymes including proteases by solid state fermentation are plenty (Chakrabarti et al., 2000; Agrawal et al., 2004; 2005; Chellappan et al., 2006; Anandan et al., 2007). In submerged fermentation, microorganism grows in liquid media supplemented with protein rich sources like soyabean meal, casein, fish flour (Sutar et al., 1992; Boer and Peralta, 2000; Laxman et al., 2005; Hajji et al., 2007). Since cost of enzyme production is mainly due to the growth medium used, hence optimization of fermentation condition is necessary for cost-efficient enzyme production. Most of the commercial proteases are generally produced by submerged fermentation. There is no universal medium for protease production and media composition varies with organism to organism. The major factors influencing the production are nutrients like carbon and nitrogen sources, vitamins and metal ions, environmental factors such as pH and temperature, agitation/aeration etc. Response Surface Methodology (RSM) is a technique used in recent times to optimize the production and increase the yields of enzyme. RSM is a useful model for studying the effect of several factors influencing the response by varying them simultaneously and carrying out a limited number of experiments. The conventional method of optimization involves varying one parameter at a time and keeping the others constant. This often does not bring out the effect of interaction of various parameters as compared to factorial design.

Effect of Carbon and Nitrogen Sources

The production of enzymes is highly influenced by nitrogen and carbon sources. Organic nitrogen sources like soyabean meal, cornstep liquor, soya oil, corn glutan

and inorganic nitrogen sources such as nitrates, ammonium salts, amino acids etc are used for protease production. Complex nitrogen source is preferred as it is slowly degraded in medium 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 (Larchar et al., 1996).

Effect of Aeration and Agitation

Aeration and agitation will have profound influence on production of enzymes especially in fermentors and bioreactors. 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. The variation in agitation speed influences the extents of mixing in the bioreactor or in shake flasks and also affect nutrient availability.

Protease production by *Conidiobolus coronatus,* in 14 L laboratory fermentor on medium containing 1% glucose or sucrose as carbon source and casein and soyabean meal as nitrogen source was reported by Sutar et al. (1992). They obtained 19-20 U/ml in 40 h and productivities of 1600 U/L/h with aeration and agitation rates of 0.5 vvm and 200 rpm respectively. However, enzyme production decreased when aeration was increased above 0.5 vvm with a constant agitation rate at 200 rpm. Agitation beyond 200 rpm resulted in marginal decrease in enzyme activity with a constant aeration rate of 0.5 vvm. Another strain of *Conidiobolus coronatus* produces protease in 14 L laboratory fermentor on medium containing 1% glucose and 0.3% yeast extract as carbon and nitrogen sources and 2-4% soyabean meal as inducer. Maximum activities of 70-90 IU/ml with productivities of 1200-1500 IU/L/h were obtained in 40-48 h with optimal rates of aeration and agitation being 0.5 vvm and 300-400 rpm respectively. When the production was scaled up in 100 L fermentor activities and productivities obtained were 40 -50 IU/ml and 1000-1200 IU/L/h in 40 h (Laxman et al., 2005). The production of 240 U/ml of proteolytic activity was achieved after 72 h in a jar-fermentor under optimal conditions of aeration and agitation being 1 vvm and 600 rpm respectively, using *Aspergillus usami* mut shirousami IFO 6082 (Morimura et al., 1994). In 30 L airlift bioreactor the maximum protease activity of 283.84 U/ml was obtained in 4 days with the controlled aeration

rate of 0.9 vvm operated in a bubble column mode, which was close to that obtained from the shak-flask cultivation in 7 days. Aeration and agitation had a great influence on the production of acid protease from *A.niger*. Maximum activity of 200 U/ml was achieved in 8-days in a fermentor under the optimal conditions of 1 vvm and 500 rpm (Yang and Lin, 1998). In 5 L stirred-tank bioreactor, a maximum protease activity of 238.77 U/ml was obtained in 6 days of cultivation when aeration rate and agitation speed were controlled at 0.6 vvm and 150 rpm, respectively (Rao et al., 2006).

Fungal Alkaline Proteases

Studies on protease production by different species of *Entomophthora* by Jonsson, (1968) revealed that *E. coronata* and *E. virulenta* produce maximum activities in 40 h when grown in liver based medium in the temperature range of 24 – 32°C and pH range of 7.2- 7.9. The liver base in the medium could be successfully replaced by meat scrap, rapeseed oil meal, cottonseed nutrients, milk powder, and meat hydrolysate etc with similar yields. *Acremonium chrysogenum*s ATCC11550 is known to produce approximately 1 g/L extracellular alkaline protease (Yagi et al., 1972). Production of Proteinase K from *Tritirachium album* Limber by submerged fermentation in 1% glucose, 1% casein peptone and salts containing medium is reported by Ebeling et al. (1974). Protease production by *Cephalosporium* sp. strain KM388 in organic medium containing polypeptone, meat extract, and glucose as well as semi-synthetic medium containing $NaNO_3$, glucose, and yeast extract is reported by Kimura and Tsuchiya (1982). Production of extracellular protease (activity equivalent to 4.4 g of trypsin) from *Conidiobolus* sp. in submerged fermentation using a medium containing glycerol, casein, peptone and soluble starch was reported by Srinivasan et al. (1983). Phadatare et al. (1993) have reported the production of alkaline protease by *Conidiobolus coronatus* (NCL 86.8.20). The fungus produced 30 U/ml in 48 h in medium containing 3% sucrose, 0.38% ammonium nitrate, 2% tryptone, 0.2% potassium chloride, 0.1% potassium di-hydrogen phosphate and 2% casein in submerged fermentation using 24 h old preinduced inoculum grown in the same medium. Laxman et al. (2005) have reported optimization and scale up of production of alkaline protease from *Conidiobolus coronatus*. The protease production was complete in short fermentation cycles of 2–3 days comparable to bacterial fermentations. The organism utilized carbon sources such as starch, sucrose, lactose, glucose and fructose, nitrogen sources such as di-ammonium hydrogen

phosphate, casamino acids, peptone and yeast extract yeast extract for production. Soyabean meal at 2–3% was found to be the best inducer. The production was scaled up to 100 L in instrumented fermentor with yields comparable to those in shake flasks (Laxman et al., 2005). Extracellular protease production by *Beauveria bassiana* GK2016 grown on a semi-liquid medium containing gelatin as the sole carbon and nitrogen is reported by Bidochka and Khachatourians (1987). Production of extracellular protease by fungus *Scedosporium apiospermum* was highest in modified Czapek-Dox medium with 0.1% peptone and 1% glucose as nitrogen and carbon sources (Larcher et al., 1996). The production of protease by *Aspergillus tamarii* was investigated in submerged fermentation using different substrates by Boer and Peralta (2000). The proteolytic activity was detected even in minimal medium (containing 0.2% NH_4NO_3 as nitrogen source) supplemented with glucose, sucrose or starch. Low basal proteolytic activity was detected with casein or gelatin as the only substrate (16–20 U/ml). The enrichment of protein medium with glucose increased the protease production up to 6 times (110 U/ml). The highest proteolytic levels were obtained in cultures supplemented with 1% wheat bran (120 U/ml) and 1% soybean meal (161 U/ml). Increasing the soybean meal to 2% or more caused a significant decrease in the production of extracellular protease probably due to catabolite repression. Poza et al. (2001) have reported high yields of extracellular protease from *Candida caseinolytica* on medium containing 2.4% skim milk powder as inducer. Ingale et al. (2002) have reported alkaline protease production by *Basidiobolus* (N.C.L. 97.1.1) and effect of 'darmform' morphogenesis and cultural conditions on enzyme production. Growth under stationary conditions in liquid medium containing salts such as ammonium chloride resulted in 'darmform' morphogenesis. High levels of protease activity were reported in cultures initiated with darmform morphogenesis, whereas little or no protease secretion was observed with mycelial inocula. *Fusarium culmorum* produced alkaline serine protease on wheat gluten-containing medium (Pekkarinen et al., 2002). A novel extracellular protease EPg222 was produced by *Penicillium chrysogenum* (Pg222), an isolate from dry-cured hams in nutrient broth medium containing NaCl and myofibrillar protein extract (Benito et al., 2002). Optimization of an extracellular protease production by *Chrysosporium keratinophilum* IMI 338142, isolated from site polluted with organic pollutants was reported by Singh (2002). Activities were higher in glucose-gelatin medium compared to glucose–aspargine medium and reached maximum after 15 days of incubation at pH 8, 40°C. Effect of sugars viz. arabinose,

maltose, mannose and fructose on protease production showed that they suppressed enzyme production with fructose being most repressive. Tremacoldi and Carmona (2005) have reported production of extracellular alkaline protease by *Aspergillus clavatus* which was optimally active at 40°C and pH 9.5. The highest protease activity (38 U/ml) was obtained in liquid Vogel medium containing 1% glucose and 1% casein as carbon and nitrogen sources after 6 days of incubation at 25°C. *Aspergillus fumigatus* Fresenius TKU003 produced extracellular proteases and chitinases when grown on shrimp and crab shell powder (SCSP) medium (Wang et al., 2005). Rao et al. (2006) have optimized protease production by *Beauveria bassiana* using response surface methodology and reported 280.72 U/ml in 7 days. Production of extracellular serine protease by the nematode trapping fungus *Dactylellina varietas* on PL-4 liquid medium was reported by Yang et al. (2007). *Paecilomyces lilacinus* produced alkaline protease on medium containing glucose and tryptone as carbon and nitrogen sources (Kotlova et al., 2007). *Pleurotus ostreatus* produces a subtilisin like extracellular protease (PoSl) in submerged culture on potato broth supplemented with 0.5% yeast extract (Palmieri et al., 2001). Chi et al. (2007) have reported production of alkaline protease by marine yeast strain *Aureobasidium pullulans* and optimization of fermentation conditions to improve the yields. Highest activities of 7.2 U/ml were reported in the optimized sea water medium containing 2.5% soluble starch and 2% $NaNO_3$ at pH 6, 24.5°C for 30 h. The protease had an optimum pH and temperature of 9 and 45°C respectively. Basu et al. (2008) have reported production and characterization of extracellular protease from mutant *Aspergillus* niger AB100 grown on fish scale. They obtained 55.470% and 73.34% of protease production in comparison to optimum when grown on fish scale and soyabean meal respectively as sole carbon and nitrogen source.

Proteases from Thermophilic Fungi

Thermostable proteases active at high temperatures are required for the degradation of proteins into amino acids and peptides especially in baking, brewing and detergent industries (Haki and Rakshit, 2003). A source of one of the most thermostable fungal acid protease is a thermophilic strain of *Penicillium duponti* isolated from compost. The enzyme was produced in submerged culture on rice bran medium at 50°C with vigorous aeration and agitation (Hashimoto et al., 1972). Production, purification and characterization of thermomycolase, the extracellular serine protease of the

thermophilic fungus *Malbranchea pulchella* var. *sulfurea* is reported by Ong and Gaucher (1976). The protease was produced on casein containing medium and was repressed by glucose, peptides, amino acids, or yeast extract. *Scytalidium thermophilum,* a thermophilic fungus produced extracellular neutral and alkaline proteases along with endoglucase activities on yeast extract-peptone medium containing 2% microcrystalline cellulose (Ifrij and Ogel, 2002). Production reached maximum after 3 days when endoglucanase activity was low. Mehreb-Dini et al. (2009) have reported production of metalloprotease from the thermophilic fungus *Thermoascus aurantiacus* in solid state fermentation. Protease showed optimum pH at 5.5 and 10.5 when using casein and 5.5 when using azocasein as a substrate.

Genetic and Protein Engineering of Fungal Proteases

In the biotechnological application of enzymes, the cost of enzyme is usually a major factor. To make the process economical it is necessary to decrease the enzyme cost by increasing the enzyme yields and productivities. The following methods are often employed to increase the yields: a) optimization of fermentation parameters including media formulations b) strain improvement. Strain improvement can be done by conventional methods like mutagenesis or asexual/parasexual recombination or protoplast fusion. Advanced methods of strain improvement include recombinant DNA technology and protein engineering. Recombinant DNA technology improves the understanding on structure function relationships of protease. It provides an excellent tool for the manipulation and control of gene expression. The objective of gene cloning is mainly for the over production of commercially important enzymes and in recent years most of the enzymes are produced by genetically engineered microorganisms. Protein engineering is used to determine the three-dimensional structure of protein and introduce changes in amino acid sequence by site-directed mutagenesis to improve stability towards temperature and pH of the enzyme (Rao et al., 1998).

Rao et al. (1998) have extensively reviewed cloning of proteases. Relation between secretion of extracellular protease and fungal morphology was studied in recombinant *Aspergillus niger* prepared by making a fusion protein gene of protease with glucoamylase-GFP, green fluorescence protein (Xu et al., 2000). In *Yarrowia lipolytica,* the amino acid sequence of protease AXP was deduced from its nucleotide sequence and it showed sequence homology to several proteases. In *Yarrowia*

lipolytica, pH of the medium, determine the secretion of either acid or alkaline protease (Gonzalez-Lopez et al., 2002). Expressed sequence tags (EST) of cDNA clones from *Conidiobolus coronatus* were sequenced to analyze gene expression during growth on insect cuticle. Sixty percent of homology among fungal sequences included chitinases, subtilisins, trypsin, metalloprotease and aspartyl proteases having potential to degrade host tissue. *Conidiobolus coronatus* produced fewer types of hydrolases, antimicrobial agents, toxic secondary metabolites and no ESTs with putative roles in the generation of antibiotics compared to *Metarhizium anisopliae*. Instead, *Conidiobolus coronatus* produced higher proportion of ESTs encoding ribosomal proteins and enzymes of intermediate metabolism that facilitate its rapid growth (Freimoser et al., 2003). The *spr1* gene, a putative serine protease gene from *Monacrosporium megalosporum* is homologous to the putative cuticle-degrading serine proteases *PII* and *Azo1* gene from the nematode-trapping fungus, *Arthrobotrys oligospora*. The *spr1* gene expression depends on the pH and was not regulated by either carbon or nitrogen source, or exogenous protein (Kanda et al., 2008). *EPg222* gene encoding protease from *Penicillium chrysogenum* (Pg222) was cloned in *Pichia pastoris* (Benito et al., 2006). The gene for extracellular protease (Dv1) and its promoter were cloned using degenerate primer and chromosome walking technique. The *Dv1* gene showed low homology with other nematode trapping fungus (Yang et al., 2007).

Sequence Homology

Comparative genomics is the analysis and comparison of genomes from different species. Nucleotide sequences common to the genomes of several diverged species are indicative of shared biology whereas differences in genomic sequence and structure may shed light on what makes species distinct. Although alignment is based on the identification of similar sequences, similarity is not equivalent to homology. In general, alignments may be used to specify relationships other than common ancestry such as structural or functional similarities (**Dewey and Pachter**, 2006). Homology among proteins and DNA is based on the sequence similarity. Some sequences are similar but not homologous. Multiple sequence alignment (MSA) is a pair-wise alignment to incorporate more than two sequences of protein and nucleotide at a time (Higginis and Sharp, 1988). Multiple alignments are often used to identifying conserved sequence to locate the active site of enzyme. Alignments are also used to in

establishing evolutionary relationships by constructing phylogenetic trees. Multiple sequence alignment of three or more sequences can be done by using bioinformatic tool, CLUSTAL W. *XPR2* gene encoding an alkaline extracellular protease (AEP) from *Yarrowia lipolytica* shows 42.6% homology to the fungal proteinase K (Davidow et al., 1987). Seven genes for serine protease of the subtilisin family (*SUB*) were isolated from *Trichophyton rubrum.* Based on the sequence data, SUB2 of *Trichophyton rubrum* shows ancestral lineage with *Aspergillus SUBs* (Jousson et al., 2004). The 15 N-terminal amino acid sequence of the ES1 protease from *Aspergillus clavatus* revealed 93 and 86% homology with serine proteases of *Aspergillus fumigatus* and *Aspergillus viridunitans* respectively (Hajji et al., 2007).

Industrial Application of Fungal Proteases

Proteases find wide range of applications in food, detergent, leather and pharmaceutical industries. Stringent pollution control norms have resulted in development of environmental friendly technologies based on enzymes, especially proteases. Proteases are one of the three important commercial enzymes, amyloglucosidases and glucose isomerases being the other two. Both crude as well as pure preparations are used in the industries. For example, crude proteases are used in bulk quanties in leather and detergent industries while pharmaceutical industries require small but pure protease preparations. Some of the applications are described below.

Detergents: 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 present in detergents (Rao et al., 1998; Kumar and Takagi, 1999). The protease is most suitable for detergent application if its isolelectric pH (pI) coincides with the pH of the detergent solution. More recently 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., 2002). Some of the fungal proteases are also reported to be suitable for detergent application (Phadatare et al., 1993; Tanksale et al., 2001; Hajji et al., 2007).

Leather: Leather manufacture is one of the highly polluting industries and generates solid wastes as well as liquid effluents. The major source of pollution is from dehairing step in the pretanning operations due to the use of hazardous chemicals like lime and sulphide. Leather processing involves number of steps such as soaking, dehairing, bating, degreasing and tanning etc. Though proteases have been used for bating for more than a century, their use for soaking and dehairing is more recent. The purpose of soaking is to swell the hide and addition of a small amount of protease to soaking liquor was found to facilitate water uptake and reduce the time required for swelling. Since skin and hair consist of proteins, their selective removal with proteases is environmentally friendly. Conventional dehairing involves treatment with lime and sulphide to solubulize the proteins at the hair root and loosen the hair for removal. However, the hair is destroyed and removed in the form of pulp leading to high BOD and COD in the effluents. Crude proteases are being used increasingly to remove non-collagenous and globular proteins since they act under milder conditions and hair can be recovered as a valuable by-product (Rao et al., 1998; Kumar and Takagi, 1999; Gupta et al., 2002; Foroughi et al., 2006). However, care should be taken that the protease preparation is collagenase free and should not act on the skin protein, namely collagen. Non-collageneous proteins remaining after dehairing are removed during bating operation to make the leather soft and supple. Earlier bating methods used animal feces as source of proteases, which were replaced by pancreatic trypsin. At present mostly bacterial and to some extent fungal proteases are being used for alkaline and acid bating respectively. Few fungal proteases have been reported to be suitable and find application in leather industry (Malathi and Chakraborty, 1991; Laxman et al., 2007).

Food: Proteases can hydrolyze protein from plants, fish or animal to produce hydrolysates of well-defined peptide profile. These protein hydrolysates are extensively used as food for children and invalids. Papain, ficin and bromelain are mostly used for this purpose. The proteases are also used for meat tenderization and solubilisation of fish protein concentrate having high nutritional value (Gupta et al., 2002). Alcalase has been used to produce less bitter and debittered hydrolysate from whey. Alkaline protease from *B. amyloliquefaceins* is 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 also used for decolourisation of blood. During the preparation of bread, proteases are added 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). 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).

Tabe 1.2 represents the commercially available fungal proteases which find application in food industries.

Pharmaceutical: Pharmaceutical and clinical applications of proteases include their use in digestive aids and treatment of burns. Collagenases with alkaline protease activity are increasingly used for therapeutic application in the preparation of slow-release dosage forms. A new semi-alkaline protease with high collagenolytic activity was produced by *Aspergillus niger* LCF9 which hydrolyzed various collagen types and liberated low molecular weight peptides of potential therapeutic use without release of amino acids (Barthomeuf et al., 1992). Elastase from *B. subtilis* 316M immobilized on bandages is used in treatment of wounds and burns. The 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).

Silver recovery: Silver is one of the precious and noble metals used in large quantities in the photographic industry. Around 25% of the world's silver requirement is met through recycled silver of which 75% is obtained from photographic waste (Nakiboglu et al., 2003). The waste X-ray photographic films containing 1.5-2% black metallic silver spread in gelatin is good source for silver recovery. 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 bound to gelatin (protein) it is possible to release the bound silver by hydrolyzing the gelatin by proteases (Nakiboglu et al., 2001). Alkaline proteases from *Bacillus sp* (Fujiwara and Yamamoto, 1987) and fungi (Ingale et al., 2002; Shankar et al., 2010) have been used successfully to recover silver from spent photographic films.

Management of industrial and household waste: The proteases solubilize proteinaceous waste and thus help in lowering the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities opened up a new era

in the use of proteases in waste management. Proteases can be effectively used for degradation of protein containing waste and help in clearing pipes and to remove clogs in blocked drainage pipes. They have immense application in converting wastes generated from poultry, fish, meat industries and slaughter house to value added products like fish meal, feather meal and feed. Large quantities of waste are generated in the form of feather, hair, left over protein rich solids from meat industry. For example, feathers constitute around 5% of the body weight of the animal and proteases with keratinolytic activity are being successfully used for hydrolysis of keratin rich products like hair and feather to prepare animal feed and food, amino acids and peptides (Kumar and Takagi, 1999; Gupta et al., 2002).

Silk degumming: One of the least explored areas for the use of proteases is the silk industry and only a few patents have been filed describing the use of proteases for the degumming of silk. Sericin, which is about 25% of the total weight of raw silk, covers the periphery of the raw silk fibers, thus providing the rough texture of the silk fibers. This sericin is conventionally removed from the inner core of fibroin by conducting shrink-proofing and twist-setting for the silk yarns, using starch. The process is generally expensive and therefore an alternative method suggested is the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing (Gupta et al., 2002). Alkaline proteases remove serecin covering the silk fibroin and increase the luster without damaging the properties of the fiber (Gulrajani et al., 2000).

Other Applications: Some of the other applications of proteases include their use in basic research, peptide synthesis, desizing of fabrics, optical resolution of amino acids, for dissociation of cells from monolayer animal cell culture etc. Their selective peptide bond cleavage is used in the elucidation of structure function relationship, in the synthesis of peptides, and in the sequencing of proteins. Protease catalyzed peptide synthesis have several advantages over the chemical methods since it catalyzes reactions with unparalleled chiral (enantio-) and positional (regio-) selectivities. Therefore, proteases can be used in both simple and complex transformations without the need for the tedious blocking and deblocking steps that are common in enantio- and regioselective organic synthesis. Such high selectivity also affords efficient reactions with few by-products thereby making enzymes an environmentally friendly alternative to conventional chemical catalysts (Schmid et al.,

2001). Alkaline protease from *Conidiobolus coronatus* was able to replace trypsin in animal cell cultures (Chiplonkar et al., 1985). Alkaline protease from *Conidiobolus coronatus* was capable of resolving the racemic mixtures of DL-phenylalanine and DL-phenylglycine conventionally done by Subtilisin Carlsberg (Sutar et al., 1992). Alcalase acted as catalyst for resolution of N-protected amino acid esters (Kumar and Takagi, 1999).

Table 1.2: Commercial fungal proteases and their applications

Name	Source	pH	Temp (^{o}C)	Application
Amano Pharmaceutical Co.				
Prozyme P 'Amano' 6	*Aspergillus* sp.	8	45	Food
Acid protease 'Amano' II	*Rhizopu*s 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

REFERENCES

- Adulyatham, P. and Owusu-Apenten, R. (2005). *J Food Sci,* 70: C231-C234.
- Agrawal, D., Patidar, P., Banerjee, T. and Patil, S. (2004). *Proc Biochem*, 39: 977-981.
- Agrawal, D., Patidar, P., Banerjee, T. and Patil, S. (2005). *Proc Biochem,* 40: 1131–1136.
- Aleksieva, P. and Peeva, L. (2000). *Enz Microb Technol*, 26: 402-405.
- Alp, S. and Arikan, S. (2008). *J Basic Microbiol*, 48: 1-7.
- Alves, M. H., Campos-Takaki, G. A., Porto, A. L. F. and Milanez, A. I. (2002). *Braz J Microbiol,* 33.
- Anandan, D., Marmer, W. N. and Dudley, R. L. (2007). *J Ind Microbiol Biotechnol*, 34: 339–347.
- Arima. K., Iwasaki, S. and Tamura, G. (1967). *Agric Biol Chem*, 31: 540-545.
- Barthomeuf, C., Pourrat, H. and Pourrat, A. (1992). *J Ferment Bioeng*, 73: 233-36.
- Basu, B. R., Banik, A. K. and Das, M. (2008). *World J Microbiol Biotechnol*, 24: 449-455.
- Benito, M. J., Connerton, I. F. and Cordoba, J. J. (2006). *Appl Microbiol Biotechnol,* 73: 356-365.
- Benito, M. J., Rodriguez, M., Nunez, F., Asensio, M. A., Bermudez, M. E. and Cordoba, J. J. (2002). *Appl Environ Microbiol,* 68: 3532-3536.
- Bidochka, M. J. and Khachatourians, G. G. (1987). *Appl Environ Microbiol,* 53: 1679-1684.
- Boer, C. G. and Peralta, R. M. (2000). *J Basic Microbiol,* 40: 75-81.
- Chakrabarti, S. K., Matsumura, N. and Ranu, R. S. (2000). *Curr Microbiol*, 40: 239–244.
- Chellappan, S., Jasmin, C., Basheer, S. M., Elyas, K.K. Bhat, S. G. and Chandrasekaran, M. (2006). *Proc Biochem*, 41: 956–961.
- Chen, Q., Guoqing, H. and Jinling, W. (2007). *J Food Eng,* 80: 490-496.
- Chi, Z., Ma, C., Wang, P. and Li, H. F. (2007). *Biores Technol,* 98: 534-538.
- Chiplonkar, J. M., Gangodkar, S. V., Wagh, U. V., Ghadge, G. D., Rele, M. V. and Srinivasan, M. C. (1985). *Biotechnol Lett,* 7: 665-668.

- Coral, G., Arikan, B., Unaldi, M. N. and Guvenmez, H. (2003). *Annals of Microbiol,* 53: 491-498.
- Davidow, L. S., O'Donnell, M. M., Kaczmarek, F. S., Pereira, D. A., Dezeeuw, J. R. and Franke, A. E. (1987). *J Bacteriol*, 169: 4621-4629.
- Dean, D. D. and Domnas, A. J. (1983). *Exp Mycol*, 7: 31-39.
- Dewey, C. N. and Pachter, L. (2006). *Human Molecular Genetics,* 15: 51-56.
- Dozie, I. N. S., Okeke, C. N. and Unaeze, N. C. (1994). *World J Microbiol Biotechnol,* 10: 563-567.
- Dunaevsky, T. E., Gruban, T. N., Beliakova, G. A. and Belozersky, M. A. (2000). *Biochem* (Moscow), 65: 723-727.
- Dunn, B. M. and Rao, M. (2004). Human immunodeficiency virus 1 retropepsin. In *Handbookof Proteolytic Enzymes,* edited by Barrett, A. J., Rawlings, N. D. and Woessner, J. F., 2nd ed Elsevier, London , pp 144-154.
- Ebeling, W., Hennrich, N., Kloclow, M., Metz, H., Orth, H. D. and Lang, H. (1974). *Eur J Biochem,* 47: 91-97.
- Farley, P. C. and Ikasari, L. (1992). *J Gen Microbiol,* 138: 2539-2544.
- Foroughi, F., Keshavarz, T. and Evans, C. S. (2006). *J Chemical Technol Biotechnol,* 81: 257-261.
- Frederick, G. D., Rombouts, P. and Buxton, F. P. (1993). *Gene,* 15: 57-64.
- Freimoser, F. M., Screen, S., Hu, G. and Leger, R. S. (2003). *Microbiol*, 149: 1893-1900.
- Fujiwara, N. and Yamamoto, K. (1987). *Hakkokogaku,* 65: 531-534.
- Germano, S., Pandey, A., Osaku, C. A., Rocha, S. N. and Soccol, C. R. (2003). *Enz Microb Technol*, 32: 246-251.
- Godfrey, T. and West, S. (1996). Industrial Enzymology, 2nd edn. NY: Macmillan Publishers, Inc. ISBN 0-33359464-9. 3.
- Gonzalez-Lopez, C. I., Szabo, R., Blanchin-Roland, S. and Gaillardin, C. (2002). *Genetics,* 160: 417-427.
- Gradisar, H., Friedrich, J., Krizjaj, I. and Jerala, R. (2005). *Appl Environ Microbiol,* 71: 3420-3426.
- Gradisar, H., Kern, S. and Friedrich, J. (2000). *Appl Microbiol Biotechnol*, 53: 196-200.

- Gulrajani, M. L., Agarwal, R. and Chand, S. (2000). *Ind J Fibre textile Research*, 25: 138-142.
- Gupta, R. and Ramnani, P. (2006). *Appl Microbiol Biotechnol*, 70: 21-33.
- Gupta, R., Beg, Q. K. and Lorenz, P. (2002). *Appl Microbiol Biotechnol,* 59: 15-32.
- Hajji, M., Kanoun, S. Nasri, M. and Gharshallah, N. (2007). *Proc Biochem,* 42: 791-797.
- Haki, G. D. and Rakshit, S. K. (2003). *Biores Technol,* 89: 17-34.
- Hanzi, M., Shimizu, M., Hearn, V. M. and Monod, M. (1993). *Mycoses,* 36: 351-356.
- Haq, I. and Mukhtar, H. (2004). *J Basic Microbiol,* 44: 280-287.
- Hashimoto, H., Iwaasa, T. and Yokotsuka, T. (1972). *Appl Microbiol,* 24: 986-992.
- Hattori, M., Isomura, S., Yokoyama, E., Ujita, M. and Hara, A. (2005). *J Biosci Bioeng,* 100: 631-636.
- Higgins, D. G. and Sharp, P. M. (1988). *Gene,* 73: 237-244.
- Hodgson, J. (1994). *Nat Biotechnol*, 12: 789-790.
- Horikoshi, K. (1971). *Agric Biol Chem*, 35: 1407-1414.
- Horikoshi, K. (1999). *Microbiol Molecul Biol Reviews*, 63: 735-750.
- http://en.cnki.com.cn/Article_en/CJFDTOTAL-GDHG200810029.htm
- http://info.bio.cmu.edu/courses/03231/Protease/SerPro.htm
- http://merops.sanger.ac.uk
- http://www.encyclopedia.com/doc/1G1-172107942.html
- Ifrij, I. H. and Ogel, Z. B. (2002). *Biotechnol Lett,* 24: 1107-1110.
- Ingale, S., Rele, M. and Srinivasan, M. C. (2002). *W J Microbiol Biotechnol,* 5: 403-408.
- Jensen, B., Nebelong, P., Olsen, J. and Reeslev, M. (2002). *Biotechnol Lett,* 24: 41-45.
- Jonsson, A. G. (1968). *Appl Environ Microbiol,* 16: 450-457.
- Jousson, O., Lechenne, B., Bontems, O., Mignon, B., Reichard, U., Barblan, J., Quadroni, M. and Monod, M. (2004). *Gene,* 339: 79-88.
- Kanda, S., Aimi, T., Kano, S., Ishihara, S., Kitamoto, Y. and Morinaga, T. (2008). *Mycological Res,* 163: 63-72.

- Katz, M. E., Ricea, R. N. and Cheetham, B. F. (1994). *Gene*, 150: 287-292.
- Kenny, A. J. (1999). Introduction: Nomenclature and classes of Peptides, in Proteolytic Enzymes Tools and Targets (springer-Verlag Berlin Heidelberg, NY), 1-8.
- Kim, J., Kim, J., Chang, S., Yun, S. (2004). EP Patent 1,401,385, Multiple layered patches for teeth whitening.
- Kimura, T. and Tsuchiya, K. (1982). *App Environ Micro,* 43: 654-658.
- Kitano, H., Kataoka, K., Furukawa, K. and Hara, S. (2002). *J Biosci Bioeng,* 93: 563-567.
- Kotlova, E. K., Ivanova, N. M., Yusupova, M. P., Voyushina, T. L., Ivanushkina, N. E., and Chestukhina, G. G. (2007). *Biochemistry (Moscow*), 72: 117-123.
- Krishna, K. V., Gupta, M., Gupta, N., Gaudani, H., Trivedi, S., Patil, P., Gupta, G., Khairnar, Y., Borasate, A. and Mishra, D. (2009). *International J Microbiol Res*, 1: 14-18.
- Kumar, C. G. and Takagi, H. (1999). *Biotechnol Adv*. 17: 561–594.
- Kumar, S., Sharma, N. S., Saharan, M. R. and Singh, R. (2005). *Proc Biochem,* 40: 1701-1705.
- Kunamneni, A., Ellaiah, P. and Prasad, S. D. (2003). AAPS *Pharmatech*, 4 Article 56 (http://www.aapspharmscitech.org).
- Kundu, A. K., Manna, S. and Pal, N. (1974). *Ind J Exp Biol,* 12: 441-443.
- Kunert, J. and Kopecek, P. (2000). *Mycoses*, 43: 339–347.
- Larcher, G., Cimon, B., Symoensa, F., Tronchin, G., Chabasse, D. and Bouchara, J. P. (1996). *Biochem J,* 315: 119-126.
- Laxman, R. S., More, S. V., Rele, M. V., Rao, B. S., Jogdand, V. V., Rao, M. B., Deshpande, V. V., Naidu, R. B., Manikandan, P., Kumar, D. A. and Kanagaraj (2007). Process for the preparation of alkaline protease. USP 7186546.
- Laxman, R. S., Sonawane, A. P., More, S. V., Rao, B. S., Rele, M. V., Jogdand, V. V., Deshpande, V. V. and Rao, M. B. (2005). *Proc Biochem,* 40: 3152-3158.
- Lee, Y. B., Sehnert, D. J. and Ashmore, C. R. (1986). *J Food Sci*, 51: 1558-1559.

- Leger, R. J. S., Frank, D. C., Roberts, D. W. and Staples, R. C. (1992). *Eur J Biochem,* 204: 991-1001.
- Liao, D. I. and Remington, S. J. (1990). *J Biol Chem*, 265: 6528-6531.
- Li, C. D., Yang, Y. J. and Shen, C. Y. (1997). *Mycol Res*, 101: 18-22.
- Lindberg, R. A., Rhodes, W. G., Eirich, L. D. and Drucker, H. (1982). *J Bact,* 150: 1103-1108.
- Lupan, D. M. and Nziramasanga, P. (1986). *Infect Immun,* 51: 360-361.
- Maheshwari, R., Bharadwaj, G. and Bhat, M. K. (2000). *Microbiol Mol Biol Rev*, 64: 461-488.
- Mahmoud, Y. A. G., El-Souod, S. M. A., El-Shourbagy, S. M. and El-Badry, A. S. M. (2007). *Annals Microbiol*, 57: 109-113.
- Malathi, S. and Chakraborty, R. (1991). *App Environ Micro*, 57: 712-716.
- Markaryan, A., Morozova, I., Yu, H. and Kolattukudy, P. E. (1994). *Infect Immun,* 62: 2149-2157.
- Matsuzawa, H., Tokugawa, K., Hamnoki, M., Mizoguchi, M., Taguchi, H., Terada, T., Knon, S. and Ohta, T. (1988). *Eur J Biochem*, 171: 441-447.
- Mehreb-Dini, C., Cabral, H., Leite, R. S. R., Zanphorlin, L. M., Okamoto, D. N., Rodriguez, G. O. B., Juliano, L., Arantes, E. C., Domes, E. and Da Silva, R. (2009). *J Agric Food Chem,* 57: 9210-9217.
- Mellon, J. E. and Cotty, P. J. (1995). *Mycopathol,* 131: 115-120.
- Mellon, J. E. and Cotty, P. J. (1996). *Appl Microbiol Biotechnol,* 46: 138-142.
- Morimura, S., Kida, K. and Sonoda, Y. (1994). *J Ferment Bioeng,* 77: 183-187.
- Nakiboglu, N., Toscali, D. and Nisli, G. (2003). *Turk J Chem.* 27: 127-133.
- Nakiboglu, N., Toscali, D. and Yasa, I. (2001). *Turk J Chem,* 25: 349-353.
- Olivares, I., Mulky, A., Boross, P. I., Tozser, J., Kappes, J. C., Lopez, G. C. and Menendez, A. L. (2007). *J Molecular Biol,* 372: 369-381.
- Oliveira, L. A., Porto, A. L. F. and Tambourgi, E. B. (2006). *Biores Technol,* 97: 862-867.
- Ong, P. S. and Gaucher, G. M. (1976). *Can J Microbiol*, 22: 165-176.
- Page, M. J. and Di Cera, E. (2008). *Cell Mol Life Sci,* 65: 1220-1236.
- Palmieri, G., Bianco, C., Cennamo, G., Giardina, P., Marino, G., Monti, M. and Sannia, G. (2001). *Appl Environ Microbiol*, 67: 2754-2759.

- Pandey, A., Selvakumar, P., Soccol, C. R. and Nigam, P. (1999). *J Microbiol*, 77: 149-162.
- Park, P. J., Lee, S. H., Byun, H. G., Kim, S. H. and Kim, S. K. (2002). *J Biochem Mol Biol*, 35: 576-582.
- Pekkarinen, A. I., Jones, B. L. and Niku-Paavola, M. L. (2002). *Eur J Biochem,* 269: 798-807.
- Phadatare, S. U., Srinivasan, M. C. and Deshpande, V. V. (1993). *Enzyme Microb Technol,* 15: 72–76.
- Poza, M., de Miguel, T., Sieiro, C. and Villa, T. G. (2001). *J Appl Microbiol*, 91: 916-921.
- Prakasham, R. S., Rao, C. S. and Sharma, P. N. (2006). *Biores Technol*, 97: 1449-1454.
- Rao, Y. K., Lu, S. C., Liu, B. L. and Tzeng, Y. M. (2006). *Biochemical Eng J*, 28: 57–66.
- Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). *Microbiol Mol Biol Rev,* 62: 597-635.
- Rawlings, N. D., Morton, F. R., Kok, C. Y., Kong, J. and Barrett, A. J. (2007). *Nucleic Acids Res*. 36: D320–325.
- Rawlings, N. D., Barrett, A. J. and Bateman, A. (2010). *Nucleic Acids Res*, 38: D227–D233.
- Reichard, U., Monod, M. and Ruchel, R. (1995). *FEMS Microbiol Lett,* 130: 69-74.
- Rippon, J. W. and Hoo, M. S. (1971). *Appl Microbiol,* 22: 471-472.
- Santos, R. M. D. B., Firmino, A. A. P., de Sa, C. M. and Felix, C. R. (1996). *Curr Microbiol*, 33: 364-370.
- Sardjono, Zhu Y, and Knol W (1998). *J Agric Food Chem*, 46: 3376-3380.
- Schenck, S., Chase, Jr. T., Rosenzweig, W. D. and Pramer, D. (1980). *Appl Environ Microbiol,* 40: 567-570.
- Schmid, A., Dordick, J. S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. (2001). *Nature*, 409: 258-268.
- Secor, Jr. E. R., Carson, W. F., Cloutier, M. M., Guernsey, L. A., Schramm, C. M., Wu, C. A., Thrall, R. S. (2005). *Cellular Immunol,* 237: 68-75.
- Seker, S., Beyenal, V. and Tanyolac, A. (1999). *J Food Sci,* 64: 525-529.

- Sekizaki, H., Toyota, E., Fuchise, T., Zhou, S., Noguchi, Y. and Horita, K. (2008). *Amino Acids,* 34: 149-153.
- Shankar, S., More, S. V. and Laxman, R. S. (2010). *KUSET,* 6: 60-69.
- Singh, C. J. (2002). *Mycopathol,* 156: 151-156.
- Sohnle, P. G. and Wagner, D. K. (2000). Fungal infections, cutaneous, In: Lederberg J (Ed) Encyclopaedia of Microbiology, 2nd Ed. Academic, San Diago, 451-459.
- Srinivasan, M. C., Vartak, H. G., Powa,r V. K. and Sutar, I. I. (1983). *Biotechnol Lett*, 5: 285-288.
- Sukhosyrova, E. A., Nikitina, Z. K., Yakovleva, M. B., Veshchikova, E. V. and Bykov, V. A. (2003). *Bull Exper Biol Med,* 135: 447-451.
- Sutar, I. I., Srinivasan, M. C. and Vartak, H. G. (1992). *W J Microbiol Biotechnol*, 8: 254-258.
- Tanksale, A., Chandra, P. M., Rao, M. and Deshpande, V. (2001). *Biotechnol Lett,* 23: 51-54.
- Tochi, B. N., Wang, Z., Xu, S. Y. and Zhang, W. (2008). *Pakistan J Nutrition*, 7: 513-520.
- Tosi, S., Annovazzi, L., Tosi, I., Iadarola, P. and Caretta, G. (2001). *Mycopathol,* 153: 157-162.
- Tremacoldi, C. R. and Carmona, E. C. (2005). *World J Microbiol Biotechnol,* 21: 169-172.
- Tsuchiya, K., Arai, T., Seki, K. and Kimura, T. (1987). *Agric BioChem,* 51: 2959-2965.
- Tubesha, Z. A. and Al-delaimy, K. S. (2003). *International J Dairy Technology,* 56: 237-241.
- Tunga, R., Shrivastava, B., Banerjee, R. (2003). *Proc Biochem,* 38: 1553-1558.
- Tunlid, A., Rosen, S., Ek, B. and Rask, L. (1994). *Microbiol,* 140: 1687-1695.
- Vermelho, A. B., Mazotto, A. M., de Melo, A. C. N., Vieira, F. H. C., Duarte, T. R., Macrae, A., Nishikawa, M. M. and da Silva Bon, E. P. (2010). *Mycopathologia,* 169: 57-65.
- Vishwanatha, K. S., Appu Rao, A. G. and Singh, S. A. (2010). *Appl Microbiol Biotechnol*, 85: 1849-1859.

- Wang, H. L., Vespa, J. B. and Hesseltine, C. W. (1974). *Appl Microbiol,* 27: 906-911.
- Wang, S. L., Chen, Y. H., Wang, C. L., Yen, Y. H. and Chern, M. K. (2005). *Enzyme Microb technol*, 36: 660-665.
- Wawrzkiewicz, K., Wolski, T. and Lobarzewski, J. (1991). *Mycopathol,* 114: 1-8.
- Webb, B. H., Johnson, A. H. and Ford, J. A. A. (1974). Fundamentals of Dairy Chemistry, (2nd edn. Westport, CT, USA: AVI).
- Weber, I. T. (1990). *J Biol Chem*, 265: 10492-10496.
- Wieloch, W. and Bogus, M. I. (2007). Proteo-, chitino- and lipolytic enzymes production by entomopathogenic fungus *Conidiobolus coronatus*. Sympozjum F Poster at Zjazd Polskiego Towarzystwa Biochemicznego,
- Xu, J., Wang, L., Ridgway, D., Gu, T. and Young, M. M. (2000). *Biotechnol Prog,* 16: 222-227.
- Yagi, J., Yano, T., Kubochi, Y., Hattori, S., Ohashi, M., Sakai, H., Jomon, K. and Ajisaka, M. (1972). *J Ferment Technol,* 50: 592-599.
- Yang, F. C. and Lin, I. H. (1998). *Enzyme Microb Technol*, 23: 397-402,
- Yang, J., Liang, L., Zhang, Y., Li, J., Zhang, L., Ye, F., Gan, Z. and Zhang, K. Q. (2007). *Appl Microbiol Biotechnol,* 75: 557-565.
- Zheng, X. T. and Zhao, X. H. (2009). *Microbiol,* 36: 193-197.
- Zhu, H., Tian, Y., Hou, Y. and Wang, T. (2009). *Mol Biol Rep,* 36: 2169-2174.

CHAPTER 2

PURIFICATION OF PROTEASE FROM *BEAUVERIA* SP

ABSTRACT

The isolate secreting protease was identified as a new strain of *Beauveria* sp on the basis of morphology, 18S rDNA and ITS sequence homologies. The 18S rDNA as well as ITS sequence of the new strain showed 99% homology with *Beauveria felina* but it was morphologically very different. *Beauveria felina* (NCIM 1314) showed cottony growth whereas the new isolate showed creamish colony growth with white erect stalks. The new isolate was deposited in Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigardh with accession number MTCC 5184. The nucleotide sequences of 18S rDNA and ITS gene from the new isolate of *Beauveria* sp have been deposited in the NCBI GenBank database and assigned accession numbers: FJ895305 and FJ895306 respectively. An extracellular alkaline serine protease (BAP) was produced by *Beauveria* sp MTCC 5184 on simple and inexpensive medium consisting of 1% glucose, 0.3% yeast extract and 2% mustard seed cake as inducer under submerged fermentation conditions. The *Beauveria* alkaline protease (BAP) was purified to homogeneity using ammonium sulphate precipitation at 40-70% saturation followed by DEAE-cellulose ion exchange column chromatography, with 10.02 fold increased in specific activity and 38.6% recovery. A single peak observed on high performance liquid chromatography (HPLC) gel filtration column confirmed the homogeneity of the preparation.

INTRODUCTION

The correct identification of fungi is of great practical importance not only in the industry but also in clinical, pathology, biotechnology and environmental studies. The earliest system for fungal classification up to species level relied on morphological characters, mainly those of reproductive structures. However, this method of classification presents critical limitations such as sterility of fungal cultures that have not developed reproductive structures or morphological similarity among the members of different species (Santos et al., 2010). Other features beside morphology, like susceptibilities to toxins, chemicals and antifungal drugs, molecular techniques, biochemical tests, secondary metabolites, fatty acid composition, cell wall composition and spectrometry spectrum have been used in classification and also in identification.

Morphology

Classification systems of organisms are historically based on observable phenotypic characteristics. Most of the fungi are microscopic and the study of their morphology requires the use of the light microscope. During vegetative phase, fungi can be recognized by the cultural morphology such as colony color, hyphal branching, hyphal constriction and formation of septa, a short distance from the point of origin of hyphal branches (Nontachaiyapoom et al., 2010). The pigmentation and shape of these hyphae and the presence or absence of septa is required for accurate identification. The fungus can also be classified on the basis of spore structure and type of spores.

Molecular Techniques

For poorly differentiated filamentous fungi, morphological identification is time consuming and provides insufficient taxonomic resolution. In contrast, molecular methods are universally applicable. The aim of molecular studies in biodiversity is phylogenetic and taxonomic. 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). 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 analyse the inter- and intra-specific variation in fungi (Sanon et al., 2009). The main reasons for the popularity of rDNA are that it is a multiple-copy, non-protein coding gene, whose repeated copies in tandem are homogenized by concerted evolution, and it is therefore 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**).** The ITS regions are much more variable, but sequences can be aligned with confidence only between closely related taxa. The evolutionary distance is generally displayed in the form of trees and wide diversity of algorithm is available to construct them. Two basic methods are available: distance matrix methods, resulting in phenograms and maximum-parsimony methods, resulting in cladograms. Among the classical DNA-based methods, G+C content of the nuclear DNA (nDNA) is determined. The G+C content of nDNA has been established for many fungi, primarily yeasts. A difference of 2% in the G+C content has been considered to indicate that two strains should be assigned to different species (Guarro et al*.,* 1999). The G+C content can be determined by using the T_m from the S-shaped melting curve of the DNA.

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) is particularly significant for taxonomy. This technique involves digesting DNA samples with a panel of restriction enzymes. The patterns 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.

Spectrometry Spectrum

Recent genotyoic approaches though useful for rapid identification of microorganisms are not routinely used due to technical limitations such as protocol complexities, reagents cost, choices of specific primers for each species, sensitivity to mutations etc.

The spectroscopic or spectrometry spectrum of any compound is known to give a unique fingerprint. Fourier transform infrared (FTIR) is an old and powerful technique for identifying types of chemical bonds in a molecule. This technique is used for characterizing the chemical composition of very complex probes and has been successfully applied in various fields of quality control and for the identification of filamentous fungi and yeasts. The current state of identification and characterization of filamentous fungi and yeasts by FTIR is reviewed recently by Santos et al. (2010).

Proteases

For most commercial purposes, crude protease preparations are generally employed while pure preparations are needed for pharmaceutical and other medical applications. The purified enzyme is also required for property studies and better understanding of structure function relationship. Recovery of crude cell free preparation at the end of fermentation is the first step in down stream processing. If the production of protease is by submerged fermentation, crude cell free protease can be obtained from the fermented broth either by filtration (Bidochka and Khachatourians 1987) or by centrifugation (Sharma et al., 2006). Protease can be extracted from fermented koji after solid state fermentation with buffers/surfactants/salts at suitable pH by vortexing/shaking (Agrawal et al., 2005). Several methods are employed for concentration of the culture filtrate viz. salt or solvent precipitation (Tunga et al., 2003; Hajji et al., 2007, Fernandez-Lahore et al., 1998); ultrafiltration through membrane (Bohdziewicz 1994); dialysis against polyethyleneglycol (Chaia et al., 2000) and lypholization (Bernal et al., 2006).

Protease can be purified by a combination of chromatographic procedures such as affinity chromatography (Kumar and Takagi 1999), ion-exchange chromatography (Sharma et al., 2006), hydrophobic interactions (Batish et al., 2003) and gel filtration (Sharma et al., 2006; Hajji et al., 2007). In addition, other procedures such as Fast performance liquid chromatography (FPLC) (Abbas et al., 1989), preparative poly acrylamide gel electrophoresis (PAGE) (Phadatare et al., 1992) and converging-diverging foam fractionation (Banerjee et al., 1993) have also been used for the purification of the proteases. The homogeneity of the enzyme preparation is checked and confirmed by more than one of the following methods: sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or by Matrix assisted laser

desorption ionization- time of flight (MALDI-TOF) or by gel-filtration or by isoelectric focusing and the pure enzyme is then used for further characterization. In recent years, the potential use of microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Alva et al., 2007). Many investigations are focused on the discovery and characterization of novel naturally occurring proteases from sources that have been overlooked (Goud et al., 2009). Search for new and more active enzymes has renewed the interest in proteases from fungal strains isolated from diverse and hitherto unexplored habitats.

A fungus, *Beauveria* sp MTCC 5184 secreting alkaline protease was isolated from animal dung. The present chapter describes the identification of the fungus using molecular techniques like ITS and 18S rDNA sequence homologies as well as purification of its alkaline protease to homogeneity.

MATERIALS AND METHODS

Malt extract, yeast extract and peptone were obtained from M/s Hi-Media Chemicals, India. Hammerstein casein was obtained from M/s Sisco Research Laboratories, India. Enzymes and chemicals for PCR and sequencing were obtained from Bangalore Genie (India). All other chemicals were of analytical grade. Mustard seed cake was obtained from local market.

Microorganism

Beauveria sp MTCC 5184 was isolated from animal dung and was deposited in the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, (IMTECH), Chandigarh, Pune, India. It was maintained on MGYP agar (malt extract, 0.3%; glucose, 1%; yeast extract, 0.3%; peptone, 0.5% and agar, 2%) slants and sub-cultured once in a month and preserved at 4°C after growth and sporulation.

Identification of the Microorganism

PCR amplification and sequencing of 18S rDNA and ITS 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 7 days old MGYP slant. The flasks were incubated on a rotary shaker (200 rpm) for 48 h at 28°C. The contents were centrifuged at 8000 rpm for 15 min, washed repeatedly to remove the media constituents. 3-5 g of wet mycelium was freeze-dried in liquid nitrogen and ground 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, 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 and ITS gene: PCR amplification of 18S ribosomal DNA was performed using commercially available primers NS1-F (GTA GTC ATA TGC TTG TCT C), NS8-R (TCC GCA GGT TCA CCT ACG GA) and the internal transcribed spacer (ITS) region using primers ITS1-F (TCC GTA GGT GAA CCT GCG G) and ITS4-R (TCC TCC GCT TAT TGA TAT GC) (White et al., 1990). The polymerase chain reaction (25 µl) was set to amplify the 18S rDNA and ITS 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-1.25 µl each, distilled water-16.60 µl, and 1 unit of *Taq* DNA polymerse-0.20 µl. 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 and ITS gene amplification were: initial denaturation-95°C for 3 min; followed by 35 cycles of 94°C for 1 min, 57°C for 30 sec, 72°C for 2 min and final extension at 72°C for 10 min. 5 µl 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), ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) for sequencing (White et al., 1990). The sequencing reaction mixes were 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).

Protease Production

Spores from 2-3 weeks old MGYP slant was used for inoculum development. 48 h old vegetative inoculum (10% v/v) grown in MGYP was used to inoculate the experimental flasks. Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 50 ml GYE (glucose, 1%; yeast extract, 0.3%) medium with 2% mustard seed cake (w/v) as inducer. Flasks were incubated at 28°C, 200 rpm for 3-5 days. Samples were removed periodically, centrifuged at 10,000 rpm for 10 min and the clear supernatant was used as the source of crude enzyme.

Determination of Alkaline Protease Activity

Protease as caseinolytic activity was estimated at 50°C, pH 9 according to Laxman et al. (2005). The reaction mixture contained an aliquot of suitably diluted enzyme and 10 mg Hammerstein casein 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. Amount of tyrosine produced is calculated from a pre-calibrated graph of absorbance at 280 nm against tyrosine concentration. One unit of activity is defined as the amount of enzyme required to release 1 μM of Tyr/min under the assay conditions.

Protein Estimation

Protein was estimated according to Lowry et al. (1951) and Bradford (1976) with bovine serum albumin (BSA) as the standard. During the course of purification, protein was determined by measuring the absorbance at 280 nm as described by (Jagannathan et al, 1956).

Purification of Protease

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

Ammonium sulphate precipitation: Protein precipitation by salting out technique was carried out using ammonium sulphate with constant and gentle stirring. The crude culture filtrate was subjected to fractional ammonium sulphate precipitation with 10%

increments. The pellet from each fraction (10,000 rpm, 20 min) was suspended in 50 mM phosphate buffer pH 7 and the supernatant was used for the next fractionation step. Dialysis was carried out in dialysis tubing of 10 kDa MW cut off against 100 volumes of 50 mM phosphate buffer pH 7 for 16 h with one change after 4 h. Increase in volume after dialysis was noted.

Ion exchange chromatography: Dialyzed ammonium sulphate fraction was loaded on a DEAE-cellulose column (2.5 cm x 25 cm) previously equilibrated with 50 mM phosphate buffer pH 7. The column was eluted with same buffer at a flow rate of 18-20 ml/h and 2 ml fractions were collected. Protein and protease activity in the fractions were estimated.

Polyacrylamide gel electrophoresis: The purity of the enzyme preparation was judged by visualizing protein and activity bands. Cationic PAGE was run with 7.5 % 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, a vertical strip of the gel was cut and incubated in the assay buffer (0.1 M carbonate-bicarbonate, pH 9) for 10 min. Kept the gel on glass plate and was then overlaid by an equal sized exposed X-ray film for detection of the enzyme activity in the gel by contact print technique for 10 min at 37°C. The X-ray film was removed and washed with hot water (60-70°C) until the protease bands were visible. The vertical strip of gel was cut into 9 equal pieces horizontally and homogenized in 0.1 M potassium phosphate buffer, pH 7 followed by centrifugation at 10,000 rpm for 10 min. The supernatant obtained from each fraction was checked for protease activity. Silver staining was used to visualize protein bands on the gel Morrissey (1981).

High performance liquid chromatography (HPLC): 100 μg of purified protein was loaded on HPLC gel filtration TSK G 2000 SW pre-packed column (7.5 mm x 600 mm) and eluted with 50 mM phosphate buffer, pH 7 at a flow rate of 0.7 ml/min.

RESULTS AND DISCUSSION

Morphological Characteristics of Fungus

The fungal strain was isolated from rabbit dung. The fungus was grown aerobically as white cottony mycelium. Small stalks start forming after 3-4 days which mature into white hairy erect stalks after 7-10 days of growth. The stalk (synnemata) was creamish white coloured and erected.

The wet mount of well grown fungus in submerged fermentation was prepared on glass slide. Microscopic observation (100X) showed that the organism formed thick network of thin mycelia and older cultures showed numerous spores. Conidia were hyaline, 1-celled, ovoid and not produced in gelatinous material.

The fungus was grown over a pH range of 5 to 9 with an optimum at 6.5-7.5 and the temperature range of 15 to 35°C with an optimum at 28°C.

Isolation of Genomic DNA

The genomic DNA when run in 0.8% agarose gel was found to be intact and of high molecular weight (Figure 2.1). The spectrophotometric analysis of the DNA showed that the ratio of absorbance at 260_{nm} vs 280_{nm} was 2 indicating that the preparation was pure and free from protein contamination.

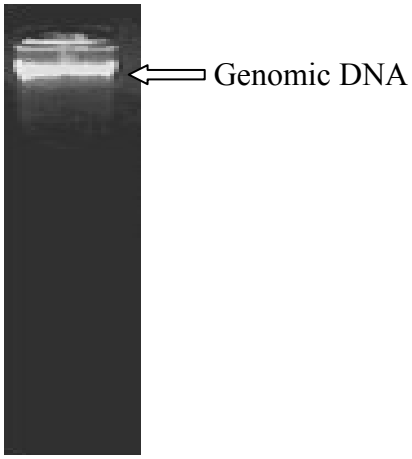


Figure 2.1: Agarose gel electrophoresis of genomic DNA

PCR Amplification and Sequencing of 18 S rDNA and ITS Gene

Both 18S rDNA and ITS gene were amplified as a single band. The PCR amplified products of 18S rDNA and ITS gene were about 1.8 kb and 0.6 kb respectively (Figure 2.2).

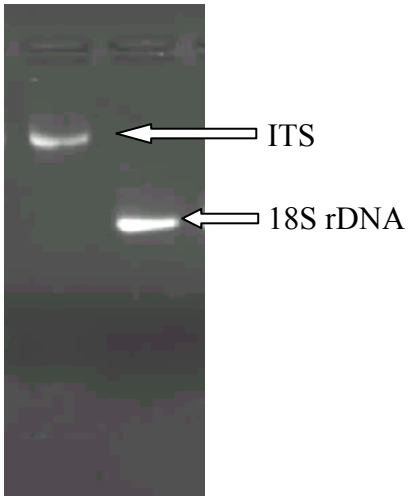


Figure 2.2: Agarose gel electrophoresis of PCR amplified product of 18S rDNA and ITS gene.

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 sequences of 18S rDNA and ITS genes from new isolate of *Beauveria* sp MTCC 5184 have been deposited in NCBI GenBank database and assigned accession numbers FJ895305 and FJ895306 respectively.

Sequence of 18S rDNA Gene

The 18S rDNA sequence (1726 bp) of *Beauveria* 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 1726 bp 18S rDNA sequence (Table 2.1).

Table 2.1: Sequence of 18S rDNA gene

5′AAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTATACAGCGAAACTGCGAATGGCT
CATTATATAAGTTATCGTTTATTTGATAGCACCTTACTACTTGGATAACCGTGGTAATTCT
AGAGCTAATACATGCTAAAAATCCCGACTTCGGAAGGGATGTATTTATTAGATACAAAACC
AATGCCCTTCGGGGCTCACTGGTGATTCATGATAACTTCGCGAATCGCACAGCCTTGCGCT
GGCGATGGTTCATTCAAATTTCTTCCCTATCAACTTTCGATGTTTGGATATGGGCCAAACA
TGGTTGCAACGGGTAACGGAGGGTTAGGGCTCGACCCCGGAGAAGGAGCCTGAGAAACGGC
TACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGT
GACAATAAATACTGATACAGGGCTCTATAGGGTCTTGTAATCGGAATGAGTACAATTTAAA
TCTCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCT
CCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCGTAGTTGAACCTTGGGCCTGGC
TGGCCGGTCCGCCTCACCGCGTGCACTGGTCCGGCCGGGCCTTTCCCTCTGTGGAACCCCA
TGCCCTTCACTGGGTGTGGCGGGGAAACAGGACTTTTACTTTGAAAAAATTAGAGTGCTCC
AGGCAGGCCTATGCTCGAATACATTAGCATGGAATAATAAAATAGGACGTGCGGTTCTATT
TTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGGCATCAGTATTCAG
TTGTCAGAGGTGAAATTCTTAGATCTACTGAAGACTAACTACTGCGAAAGCATTTGCCAAG
GATGTTTTCATTAATCAGGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAG
TCTTAACCATAAACTATGCCGACTAGGGATCGGACGATGTTATTACTTGACTCGTTCGGCA
CCTTACGAGAAATCAAAGTGCTTGGGCTCCAGGGGGAGTATGGTCGCAAGGCTGAAACTTA
AAGAAATTGACGGAAGGGCACCACCAGGGGTGGAGCCTGCGGCTTAATTTGACTCAACACG
GGGAAACTCACCAGGTCCAGACACAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATTT
TGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGAT
AACGAACGAGACCTTAACCTGCTAACTAGCCCGTATTGCTCCGGCAGTACGCTGGCTTCTT
AGAGGGACTATCGGCTCAAGCCGATGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTT
AGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAGCCAGCGAGTACTCCCTTGGCCGAA
AGGCCTGGGTAATCTTGTTAAACTCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTC
TTCAACGAGGAATCCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCT
TTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCAGTGAGGCGTCCGGACTGGCC
CAGAGAGGTGGGCAACTACCACTCAGGGCCGGAAAGTTCTCCAAACTCGGTCATTAGAGGA
AGTAAATACAACGAGAAT 3′

Sequence of ITS Gene

The ITS sequence (506 bp) of *Beauveria* sp was obtained by sequencing with the primers ITS1 to ITS4. The sequences so obtained by each set of primer were then overlapped to get 506 bp ITS sequence (Table 2.2).

Table 2.2: Sequence of ITS gene

5′TGCGGAGGGATCATTACCGAGTTTCTAACTCCATACCTTTGTGAACATACCTATCGTTG
CTTCGGCGGGTCCGTCCCGGAGCTGGCAGTGCACGGCCAGCCCCGGAGCCAGACGCCCGCC
GAGGACCCCAAACTCTTGTTTTTATAGTGGATCTTCTGAGTCTTATACAAAATAAATTAAA
ACTTTCAGCAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG
TACTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCAGGGCCCGTCCGCGGGACC
TGGCGTTGGGGATCGGCTGCCCCTGGCGGCTGCCGGCCCTGAAATACAGTGGCGGTCTCTT
CGCGACCTCCCCTGCGTAGTAGTGATACCTCGCAGCCGGATAGCGGAGCGGCCACGCCGTA
AAACCCCCTACTTCTCAAGG 3′

BLAST Search and Phylogenetic Tree

The 18S rDNA and ITS nucleotide sequence were analyzed with the GenBank database using BLAST program. The 18S rDNA sequence showed 99% homology with 18S rDNA of *Beauveria felina*, 98% with *Nectria cinnabarina* and *Stilbocrea macrostoma;* 97% with *Bionectria pityrodes*, *Cordyceps sinensis*, *Paecilomyces lilacinus*, *Nectria cinnabarina* and *Nectria curta* (Table 2.3). The ITS sequence showed homology with ITS region of *Beauveria felina* and *Marine ascomycete* (Table 2.4).

Multiple sequence alignment was performed with Clustal W programme. The phylogenetic tree was constructed by using neighbour-joining method which indicated that the new isolate to be a strain of *Beauveria* sp (Figure 2.3 and 2.4). The morphology of the new isolate was compared with a strain of *Beauveria felina* (NCIM 1314) from National Collection of Industrial Microorganisms (NCIM), India. Although 18S rDNA and ITS sequences of MTCC 5184 showed 99% homology with *Beauveria felina,* there were marked differences in their morphology. The isolate MTCC 5184 formed white erect stalks within 7-10 days while *B. felina* NCIM 1314 grown as a flat colony with cottony growth and no stalks were formed even after prolonged incubation (Figure 2.5). On the basis of morphology, 18S rDNA and ITS sequence homology, the isolate MTCC 5184 was designated as a new strain of *Beauveria* sp.

Table 2.3: First 10 BLAST hit of 18S rDNA

Sequences showing significant alignment		Score (bits)	Homology	References
AY261369.1	*Beauveria felina* strain CBS 250.34 18S	3157	99%	Luna-Conde, (2003)
AY261367.1	*Beauveria felina* strain HL-51-ALSP16-I003 18S	3153	99%	Luna-Conde, (2003)
AY261368.1	*Beauveria felina* strain CBS 173.71 18S	3145	99%	Luna-Conde, (2003)
AY489693.1	*Stilbocrea macrostoma* strain GJS73-26 18S	2996	98%	Castlebury et al., 2004
AB023945.1	*Paecilomyces lilacinus* gene for 18S	2968	97%	Endo and Sugiyama (Unpublished)
AB237663.1	*Nectria cinnabarina* genes for 18S	2964	98%	Watanabe et al., (2006)
AB067701.1	*Cordyceps sinensis* gene for 18S	2964	97%	Kinjo,(Unpublished)
AY357275.1	*Nectria curta strain* UMB 39.01 18S	2963	97%	Belliveau and Barlocher, (2005)
AB003949.1	*Nectria cinnabarina* gene for 18S	2959	97%	Ogawa and Sugiyama, (2000)
AY249900.1	*Bionectria pityrodes* strain CBS 246.78 18S	2946	97%	Luna-Conde, (2003)

Table 2.4: First 10 BLAST hit of ITS sequence

Sequences showing significant alignment		Score (bits)	Homology	References
AY261369.1	*Beauveria felina* strain CBS 250.34 18S	928	99%	Luna-Conde, (2003)
EF495156.1	*Marine ascomycete* sp. HF01016 18S	918	99%	Bao et al., (2007)
AY261367.1	*Beauveria felina* strain HL-51-ALSP16-I003 18S	911	99%	Luna-Conde (2003)
U18956.1	*Beauveria felina* 5.8S rRNA gene and ITS 1 and 2	874	100%	Shih and Tzean, (Unpublished)
U35286.1	*Beauveria felina* 5.8S rRNA gene & ITS I and II	857	97%	Shih et al., (Unpublished)
Z54106.1	*Beauveria felina* 5.8S rRNA gene & ITS 1 and 2	857	97%	Shih et al., (Unpublished)
AY261368.1	*Beauveria felina* CBS 173.71 18S rRNA gene, ITS1	776	94%	Luna-Conde, (2003)
AM410612.1	*Ascomycete* sp. VTT D-041035	614	88%	Suihko et al., (2007)
AY952467.1	*Stilbella fimetaria* D99026	553	86%	Lehr et al., (2006)
EU045572.1	*Emericellopsis pallida* strain XJURML-3	547	86%	Su et al., (Unpublished)

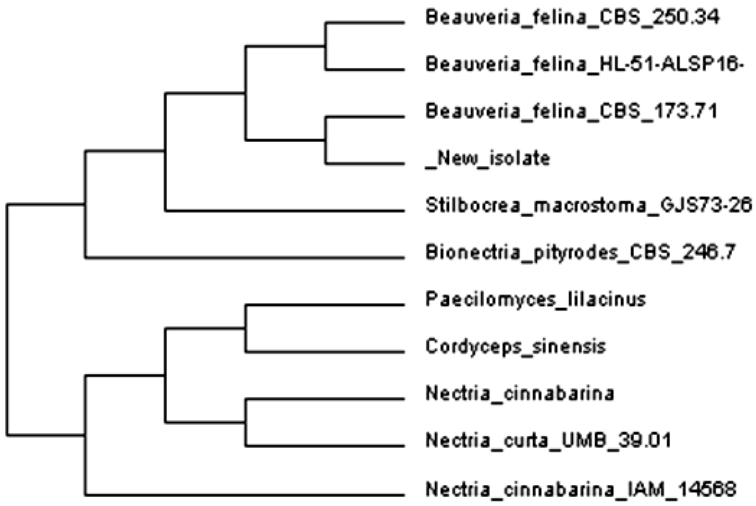


Figure 2.3: Dendrogram showing phylogenetic relationship between the 18S rDNA sequences of *Beauveria sp*. MTCC 5184 and other fungus.

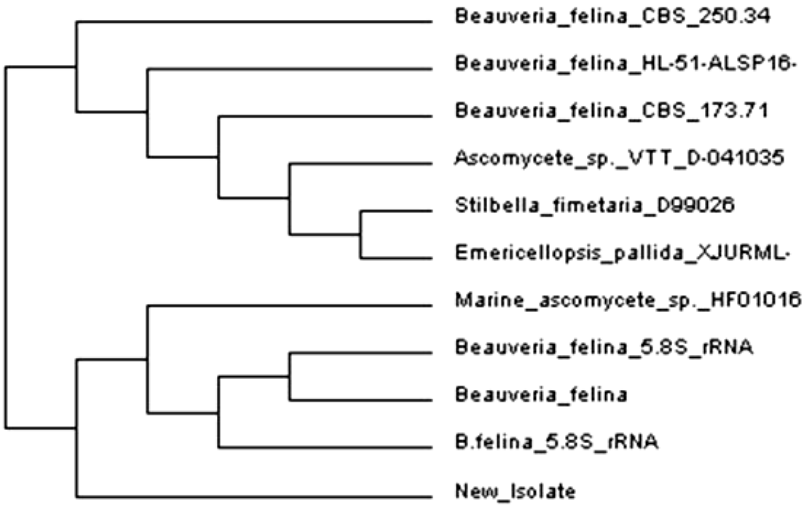


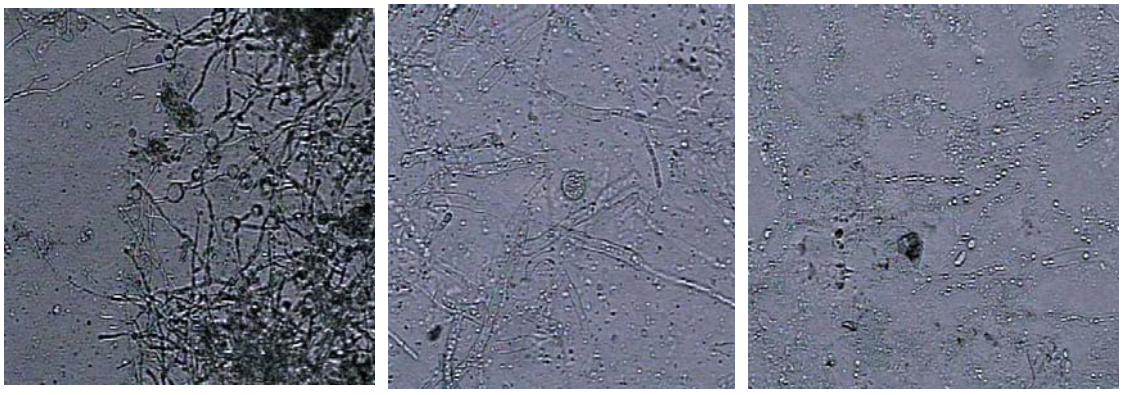
Figure 2.4: Dendrogram showing phylogenetic relationship between the ITS sequences of *Beauveria sp*. MTCC 5184 and other fungus.

Beauveria felina NCIM 1314 *Beauveria* sp MTCC 5184

Figure 2.5: Comparison of morphological characterstics

Protease Production Profile

Protease was produced by growing *Beauveria* sp MTCC 5184 in GYE medium supplemented with 2% mustard seed cake at 28°C, 180 rpm. Growth was very thick in the initial stages of the fermentation (48 h) and as slowly liquefied after 72-96 h. Microscopic observation showed thick and crowded mycelial network in the initial stages of the fermentation and secretion of protease was accompanied by cell lysis (Figure 2.6).



48 h of growth 72 h of growth 96 h of growth

Figure 2.6: Mycelia growth pattern of fungus during protease production

Very low activities were recorded in first 48 h and increased slowly to reach maximum after 3-4 days. The pH increased from 7 to 8.5 with the progress of fermentation time (Figure 2.7). At the end of the fermentation, the contents were filtered on muslin cloth to remove undigested mustard seed cake. The filtrate was centrifuged at 10, 000 rpm for 10 min and the supernatant was used as the crude enzyme broth.

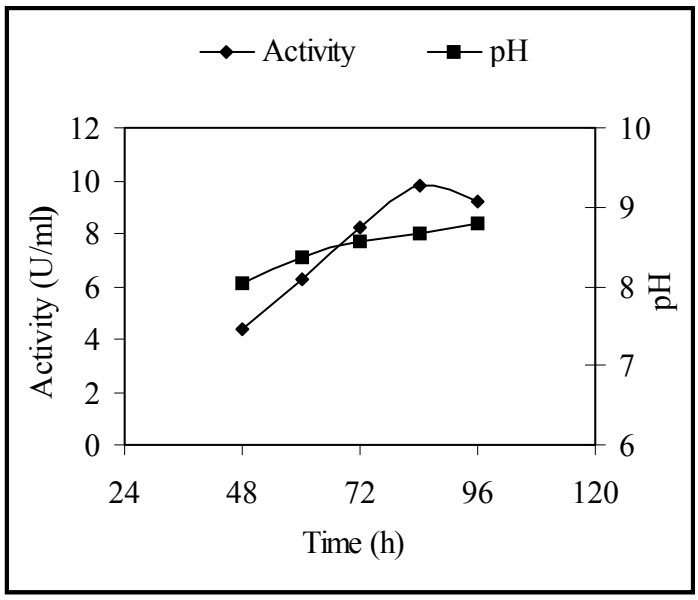


Figure 2.7: Fermentation profile for the production of protease

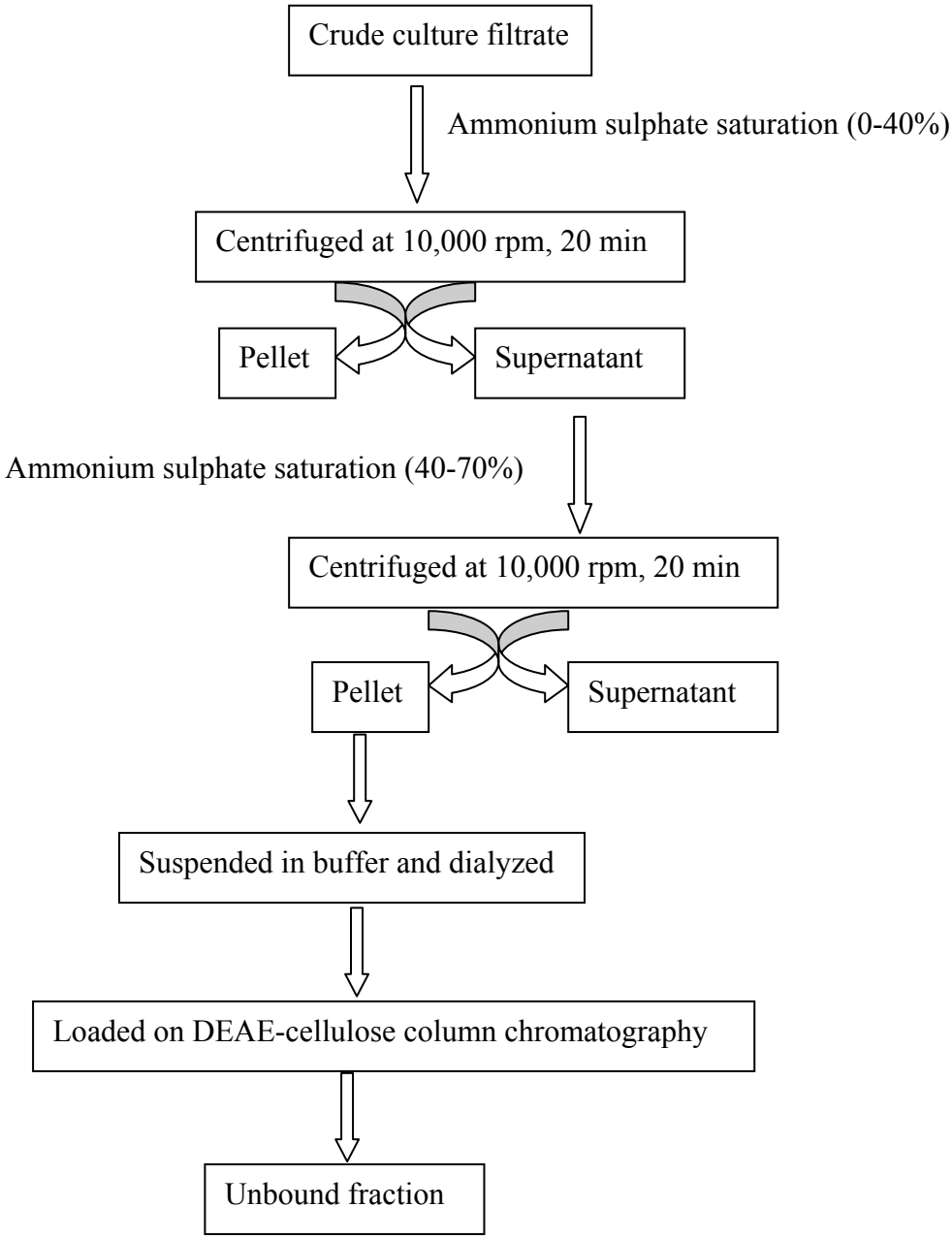
Protease production by fungi like *Aspergillus* (Coral et al., 2003; Wang et al., 2005; Hajji et al., 2007); *Thermomyces (*Jensen et al., 2002), C*onidiobolus* (Phadtare et al., 1993; Laxman et al., 2005), *Arthrobotrys oligospora* (Tunlid et al., 1994); *Fusarium* (Ueda et al., 2007); *Paecilomyces lilacinus* (Kotlova et al., 2007) in submerged fermentation is normally carried out in mineral salts medium supplemented with inducers like casein, soyabean and its products, agricultural byproducts like oil seed cakes, fish meal etc. Maximum activities are reported in 3-5 days.

Purification of *Beauveria* sp MTCC 5184 Protease

Initially, ammonium sulphate precipitation was carried out in narrow range with 10% increments and the precipitate obtained from each step was dialysed and protease activity and protein concentration were determined. Specific activity was found to be highest in 40-50, 50-60, and 60-70% fractions. Therefore, for large scale purification

crude broth was precipitated at 0-40% to remove unwanted proteins and the supernatant was further precipitated at 40-70% to obtain partially purified protease.

Purification Steps



Dialyzed 40–70% ammonium sulphate fraction when subjected to DEAE-cellulose column chromatography showed that around 56% of the protease was eluted in un-adsorbed fractions while all the impurities were bound to the matrix. There was only a single protein peak in unbound fraction which coincided with the protease activity peak (Figure 2.8).

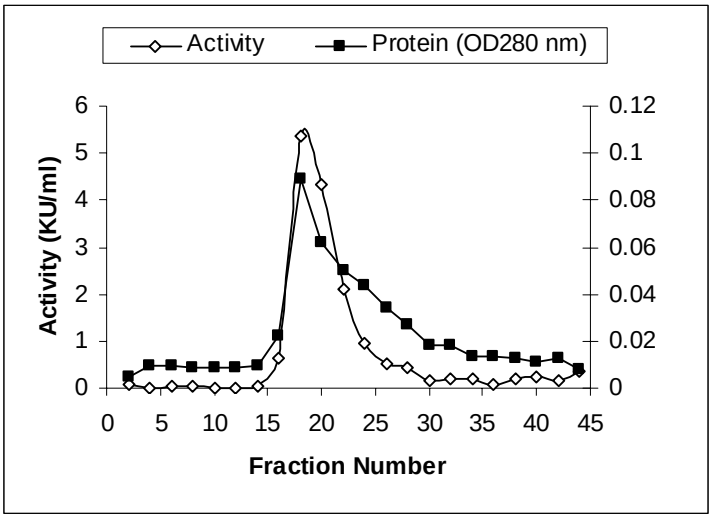


Figure 2.8: Elution profile of protease on DEAE cellulose column chromatography

Silver staining

Activity staining

Fraction No	Protease Activity (U/ml)
1	0.0364
2	0.0381
3	0.0990
4	0.4934
5	**0.9432**
6	0.5985
7	0.1111
8	0.0376
9	0.0493
Protease activity in fractions	

Figure 2.9: Cationic PAGE of pooled fractions from DEAE cellulose column

Fractions with protease activity were pooled, concentrated on speed vac and loaded on cathodic PAGE which showed a single band both by protein as well as activity staining (Figure 2.9). When the cationic PAGE gel was cut into 9 pieces and protease activity was determined, the 5th fraction showed maximum protease activity which corresponds to the protein band of gel and digested gelatin area of X-ray film.

The results of the purification of *Beauveria* protease are summarized in Table 2.3. Specific activity of the protease increased from 6.025 U/mg for the crude protease to 60.39 U/mg after the final purification step with a 10.02 fold purification. The overall yield of 38.6% for purified protease is one of the highest values reported.

This is similar to the yields obtained by Sharma et al. (2006) who reported 29 fold purification of protease from *Aspergillus oryzae* AWT 20 using CM Sephadex C-50 column and Sephadex G-100 columns with 35.2% yield of purified protease. Basu et al. (2008) have reported 26 fold purification of a protease from *Aspergillus niger* AB100 with 21.50% yield. Protease from *Beauveria bassiana* was purified by 5 fold with recovery of 22% and specific activity of 3.76 U/mg protein (Zibaee and Bandani, 2009).

Table 2.3. Summarization of purification of protease

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude culture filtrate	9783	1624	6.025	100	1
Ammonium sulphate (40-70%)	6799	739.6	9.198	69.5	1.5
DEAE-cellulose (Unbound fraction)	3780	62.6	60.39	38.6	10.02

The purified protease showed single peak at the retention time of 40 min on HPLC which confirms the purity of protease (Figure 2.10).

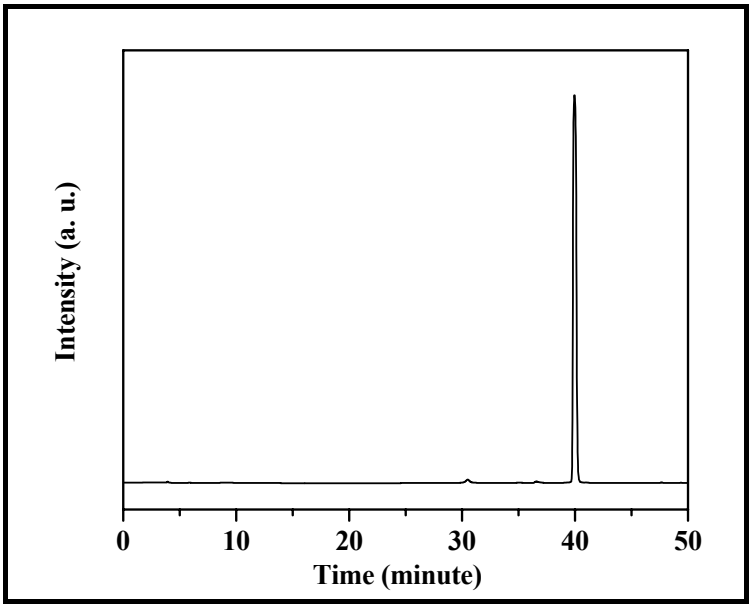


Figure 2.10: HPLC profile of purified protease showing a single peak

CONCLUSION

The organism used in the present study belongs to new strain of *Beauveria sp.* The 18S rDNA and ITS sequences showed maximum homology with *Beauveria felina* whereas morphologically they were different. The fungus was grown over a pH range of 5 to 9 with an optimum at 6.5-7.5 and the temperature range of 15 to 35°C with an optimum at 28°C. The highest protease activity of 10-12 U secreted in 3-4 days on GYE + 2% MSC. The protease was purified in two steps of ammonium sulphate precipitation followed by ion-exchange chromatography with 10.02 fold purification and 38.6% recovery. Specific activity increased from 6.02 to 60.3. On cathodic PAGE protease showed a single band by protein staining as well as by activity staining. The homogeneity of purified protease was also confirmed by HPLC which showed single peak.

REFRENCES

- Abbas, C. A., Groves, S. and Gander, J. E. (1989). *J Bacteriol,* 171: 5630-5637.
- Agrawal, D., Patidar, P., Banerjee, T. and Patil, S. (2005). *Proc Biochem,* 40: 1131–1136.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). *Nucleic Acids Res,* 25: 3389–3402.
- Alva, S., Anupama, J., Savla, J., Chiu, Y. Y., Vyshali, P., Shruti, M., Yogeetha, B. S., Bhavya, D., Purvi, J., Ruchi, K., Kumudini, B.S. and Varalakshmi, K.N. (2007). *Afr J Biotechnol*, 6: 576-581.
- Banerjee, R., Agnihotri, R. and Bhattacharyya, B. C. (1993). *Bioprocess Engg*, 9: 245-248.
- Bao, S., Huang, H., Fang, Z., Cai, H. and Sun, Q. (Unpublished). The ITS sequence of marine fungus HF01016.
- Basu, B. R., Banik, A. K. and Das, M. (2008). *World J Microbiol Biotechnol*, 24: 449–455.
- Batish, S., Hunter, A., Ashby, A. M. and Johnstone, K. (2003). *Physiological Mol Plant Pathol,* 62: 13–20.
- Belliveau, M. J. and Barlocher, F. (2005). *Mycol Res,* 109: 1407-1417.
- Bernal, C., Cairo, J. and Coello, N. (2006). *Enz Microbial Technol,* 38: 49-54.
- Bidochka, M. J. and Khachatourians, G. G. (1987). *Appl Environ Microbiol,* 53: 1679-1684.
- Bohdziewicz, J. (1994). *Proc Biochem.* 29: 109-118.
- Bradford, M. M. (1976). *Analytical Biochem,* 72: 248-254.
- Bunyard, B. A., Nicholson, M. S. and Royse, D. J. (1996). *Fungal Gene Biol,* 20: 243–253.
- Castlebury, L. A., Rossman, A. Y., Sung, G. H., Hyten, A. S. and Spatafora, J. W. (2004). *Mycos Res*, 108: 864-872.
- Chaia, A. A., De-Simone, S. G., Petinate, S. D. G., Lima, A. P. C. A., Branquinha, M. H. and Vermeiho, A. B. (2000). *Braz J Microbiol,* 31: 25-29.
- Coral, G., Arikan, B., Unaldi, M. N. and Guvenmez, H. (2003). *Annals Microbiol,* 53: 491-498.

- Edel, V., Steinberg, C., Gautheron, N. and Alabouvette, C. (1996). *Mycol Res,* 101: 179–187.
- Endo, M. and Sugiyama, J. (Unpublished). Phylogenetic analysis of Paecilomyces and related genera.
- Fernandez-Lahore, H. M., Fraile, E. R. and Cascone, O. (1998). *J Biotechnol,* 62: 83-93.
- Goud, M. J. P., Suryam, A., Lakshmipathi, V. and Singara Charya, M. A. (2009). *Afr J Biotechnol*, 8: 354-360.
- Guarro, J., Gene, J. and Stchigel, A. M. (1999). *Clin Microbiol Rev,* 12: 454-500.
- Hajji, M., Kanoun, S., Nasri, M. and Gharshallah, N. (2007). *Proc Biochem,* 42: 791-797.
- Jagannathan, V., Singh, K. and Damodaran, M. (1956). *Biochem J*, 63: 94-105.
- Jensen, B., Nebelong, P., Olsen, J. and Reeslev, M. (2002). *Biotechnol Lett,* 24: 41-45.
- Kinjo, N. (Unpublished). Phylogenetic study on *Cordyceps sinensis*.
- Kotlova, E. K., Ivanova, N. M., Yusupova, M. P., Voyushina, T. L., Ivanushkina, N. E., and Chestukhina, G. G. (2007). *Biochemistry (Moscow*), 72: 117-123.
- Kumar, C. G. and Takagi, H. (1999). *Biotechnol Adv*. 17: 561–94.
- Laemmli, U. K. (1970). *Nature,* 227: 680–685.
- Laxman, R. S., Sonawane, A. P., More, S. V., Rao, B. S., Rele, M. V., Jogdand, V. V., Deshpande, V. V. and Rao, M. B. (2005). *Proc Biochem,* 40: 3152–3158.
- Lehr, N. A., Meffert, A., Antelo, L., Sterner, O., Anke, H. and Weber, R. W. (2006). *FEMS Microbiol Ecol*, 55: 105-112.
- Lodhi, M. A., Ye, G. N., Weeden, N. F. and Reisch, B. I. (1994). *Plant Mol Biol Reporter*, 12: 6-13.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). *J Biol Chem,* 193: 265–275.

- Lunda-Conde, M. J. (2003). Marine fungi as producer of new cyclic peptides with cytotoxic activity. Thesis, Universidad de Leon, Departamento de Biologia Celulary Anatomia, Campus de Vegazana s/n, Leon, Spain, In press.
- Morrissey, J. H. (1981). *Analytical Biochem,* 17: 307-331.
- Nontachaiyapoom, S., Sasirat, S. and Manoch, L. (2010). Isolation and identification of *Rhizoctonia*-like fungi from roots of three orchid genera, *Paphiopedilum*, *Dendrobium*, and *Cymbidium*, collected in Chiang Rai and Chiang Mai provinces of Thailand. Mycorrhiza DOI 10.1007/s00572-010-0297-3.
- Ogawa, H. and Sugiyama, J. (1997). Evolutionary relationships of the cleistothecial genera with *Penicillium*, *Geosmithia*, *Merimbla* and *Sarophorum* anamorphs as inferred from 18S rDNA sequence divergence. Inn. Samson, R. A. and Pitt, J. I. (Eds.); Integration of modern taxonomic methods for *Penicillium* and *Aspergillus* classification: 149-161; Harwood Academic Publishers, Amsterdam, Netherlands (2000).
- Phadatare, S. U. Srinivasan, M. C. and Deshpande, V. V. (1992). *Eur J Biochem*, 205: 679-686.
- Phadatare, S. U., Srinivasan, M. C. and Deshpande, V. V. (1993). *Enz Microb Technol,* 15: 72-76.
- Sanger, F., Niklen, S. and Coulson, A. R. (1977). *Proc Natl Acad Sci* USA, 74: 5463–5467.
- Sanon, K. B., Ba, A. M., Delaruelle, C., Duponnois, R. and Martin, F. (2009). *Mycorrhiza*, 19: 571–584.
- Santos, C., Fraga, M. E., Kozakiewicz, Z. and Lima, N. (2010). *Res Microbiol*, 161: 168-175.
- Sharma, J., Singh, A., Kumar, R. and Mittal, A. (2006). *The Internet J Microbiol,* 2.
- Shih, H. and Tzean, S. (Unpublished). Comparison of nucleotide sequence of 5.8S rDNA and flanking regions of *Beauveria* sp.
- Shih, H., Yuan, G. and Tzean, S. (Unpublished). The phylogeny of *Beauveria* spp. based on 5.8S rDNA and flanking internal transcribed spacers.
- Su, J., Kuang, H. and Rahman, E. (Unpublished). A new record species of *Emericellopsis pallida* from Xinjiang, China.

- Suihko, M. L., Alakomi, H. L., Gorbushina, A., Fortune, I., Marquardt, J. and Saarela, M. (2007). *Systematic App Microbiol*, 30: 494-508.
- Tunga, R., Shrivastava, B., Banerjee, R. (2003). *Proc Biochem,* 38: 1553-1558.
- Tunlid, A., Rosen, S., Ek, B. and Rask, L. (1994). *Microbiol,* 140: 1687-1695.
- Ueda, M., Kubo, T., Miyatake, K. and Nakamura, T. (2007). *Appl Microbiol Biotechnol,* 74: 331–338.
- Vaneechoutte, M., Rossau, R., DeVos, P., Gillis, M., Janssens, D., Paepe, N., DeRouck, A., Fiers, T., Claeys, G. and Kersters, K. **(**1992). *FEMS Microbiol Lett,* 93: 227–234.
- Wang, S. L., Chen, Y. H., Wang, C. L., Yen, Y. H. and Chern, M. K. (2005). *Enz Microbial Technol*, 36: 660–665.
- Watanabe, N., Hattori, M., Yokoyama, E., Isomura, S., Ujita, M. and Hara, A. (2006). *J Biosci Bioeng*, 102: 365-368.
- White, T. J., Bruns, T., Lee, S. and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., editors. PCR protocols: a guide to methods and applications. New York: Academic press, Inc., p. 315–22.
- Zibaee, A. and Bandani, A. R. (2009). *Biocont Sci Technol*, 19: 797-808.

CHAPTER 3

BIOCHEMICAL CHARACTERIZATION OF PROTEASE

ABSTRACT

The molecular mass of purified protease was estimated to be 29 kDa and 28 kDa on SDS-PAGE and MALDI-TOF respectively. The protease exhibited maximum activity at 50°C, pH 9 and had an isoelectric point of 9.3. The enzyme was stable in pH range of 5 to 11 and temperature up to 50°C. Half life of the alkaline protease at 50°C and pH 7 was 2 h. The K_m and V_{max} of the protease were found to be 5.1 mg/ml and 29.67 U/ml respectively. The protease was totally inhibited by Phenylmethylsulphonyl fluoride (PMSF) while partial inhibition was observed with L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) and *N*-tosyl-L-lysine chloromethyl ketone (TLCK). Ethylene diamine tetra acetic acid (EDTA), Iodoacetate and Benzamidine did not inhibit the enzyme. Metal ions such as Cd^{2+}, Hg^{2+} and Mn^{2+} at 10 mM concentration inhibited the enzyme partially (<40%). The N-terminal sequence of purified protease was Ala Met Ala Thr Pro His Val Ala Pro Leu Val Leu Tyr Gly Val Ala which showed partial homology with subtilisin-like proteinase. Peptide mass finger printing (PMF) of purified protease showed partial homology with subtilisin-like proteinase. The autocatalytic products of the purified protease yielded two peptides with molecular masses of 10.2 and 15.6 kDa. The PMSF, N-bromosuccinimide (NBS) and Woodward's reagent (WRK) inhibited the protease indicating the presence of serine, tryptophan and aspartic acid at the active site of enzyme. Circular dichroism (CD) studies showed that the protease has 87.76% α-helix, 0.47% β-sheet and 11.77% aperiodic structure.

INTRODUCTION

The requirements of many industrial applications of proteases are very different from their physiological properties. Very few proteases from naturally occurring organisms without any modification are used in industrial processes. Therefore exploitation of biodiversity to provide microorganisms that produce proteases well suited for their diverse applications coupled with molecular techniques to improve yields/ properties are considered to be one of the promising alternatives in future. Despite the extensive research carried out on proteases, there is a need for proteases with novel properties that may have new biotechnological applications. Hence in recent years, the search has focused for newer proteases with novel and unusual properties. Looking into the depth of microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties and suitable for commercial exploitation. The multitude of physicochemically diverse habitats has challenged the nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products and processes.

The proteases are characterized on the basis of biochemical properties like optimum pH and stability, optimum temperature and stability, molecular weight, N-terminal sequence, ioselectric point, critical amino acid required for the catalytic function, substrate specificity. The biochemical and physicochemical properties of some of the fungal alkaline proteases have been summarized (Table 3.1)

In general alkaline proteases require metal ions for their maximum activity. The most commonly used metal ions are Ca^{+2}, Mg^{+2} and Mn^{+2}. The Ca^{2+} ion is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline protease at higher temperatures (Lee et al. 1996; Kumar 2002). Other metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} are also used for stabilizing proteases (Rattray et al. 1995; Johnvesly and Naik 2001). These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at higher temperatures. The presence of Ca^{+2} is known to activate the protease and increase the thermostability as mentioned by Kotlova et al. (2007). However, Li et al. (2007) have reported slight inhibition of protease by Ca^{+2} in addition to inhibition by Hg^{+2}, Co^{+2}, Cd^{+2}, Ni^{+2}, Mg^{+2}, and Mn^{+2}. Metal ions like Hg^{+2}; Cu^{+2}, Ag^{+2}, Fe^{+2} and Zn^{+2} are inhibitory to majority of proteases (Moallaei et al, 2006; Pena-Montes et al, 2008). Selective inhibition of elastase by Ca^{+2} during enzymatic dehairing of hide or skin with the

calcium insensitive proteases help in avoid the detrimental effect of elastase on leather quality. The metal ion dependency or sensitivity of a particular protease may be advantageous to its applicability in some cases.

The important features of alkaline proteases are their ability to discriminate among competing substrates 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. In enzymatic dehairing process of leather manufacture it is essential to retain the grain structure intact to obtain the high quality leather. Therefore the protease preparation has to be totally collagenase free. Besides these substrates, alkaline proteases also hydrolyze many synthetic and chromogenic substrates. Synthetic substrates have been routinely used for the elucidation of site of cleavage of the substrate by the enzyme. Synthetic substrates for elastase specific enzymes are succinyl-Ala-Ala-Ala-p-nitroanilide, succinyl-Ala-Pro-Ala-p-nitroanilide, succinyl-Ala-Ala-Pro-Leu-p-nitroanilide and glutaryl-Ala-Ala-Pro-Leu-p-nitroanilide, for chymotrypsin or subtilisin like proteases AAPF (N- Suc-Ala-Ala-Pro-Phe-p-nitroanilide), for trypsin-like proteases BAPNA and N-benzoyl- Phe-Val-Arg-p-nitroanilide and for esterase specific enzymes are benzoyl-L-Arg- ethyl ester and BTEE whereas N-Suc-Ala-Ala-Pro-Leu-pNA can be cleaved by both elastase and chymotrypsin.

The active site directed chemical modification is an important tool for studying structure–function relationship of enzymes and other biologically active proteins. Site-directed mutagenesis is widely used for the same purpose, which needs not only the cloned gene but also the three-dimensional structure of the protein. In contrast, active-site-directed chemical modification can be done without knowing the protein structure (Gote et al., 2007). Chemical modification is an alternative (and complementary) approach to genetic modification for studying the structure-function-stability relationship of enzymes and for modifying their activity-stability properties (Siddiqui et al 2009).

This wealth of structural information together with that from spectroscopic, kinetic and other biochemical investigations have led to a general consensus concerning the overall reaction mechanism. The central catalytic machinery of the

serine proteases consists of an Asp-His-Ser triad was identified by Blow et al. (1969), where the Asp-His pair is believed to increase the nucleophilicity of the serine so as to facilitate its attack on the carbonyl carbon of the scissile bond. It now seems well established that the only charge transfer internal to the members of the catalytic triad is the abstraction of a proton from the serine by the histidine during the nucleophilic attack. This proposal has become known as the one-proton transfer mechanism in order to distinguish it from the two proton transfer or charge-relay system (Barbosa et al., 1996).

This chapter describes the physiochemical and biochemical characterization of purified protease from *Beauveria* sp MTCC 5184. The chemical modifications studies are also described.

Table 3.1 Physiochemical properties of fungal alkaline proteases

Organism	MW	pI	N-Terminal	Opt. pH	Opt Temp	pH Stability	Temp. Stability	Inhibitors	Reference
Aspergillus clavatus	35 kDa		APTHQEGA PWGLAAI	9.5	40		Half life 18 min at 50°C	PMSF, Chymostatin	Tremacoldi et al. (2007)
Aspergillus clavatus ES1	32 kDa		A-L-T-T-Q-SG-A-P-W-G-L-G-S-I	8.5	50°C	7-9	Half life 30 min at 50°C	PMSF	Hajji et al. (2007)
Aspergillus fumigatus TKU003	124 k Da	8.3		8	40	6-10 for 30 min	Stable upto 30 min at 50°C. half life 30 min at 60°C	PMSF	Wang et al. (2005)
Aspergillus nidulans HA-10	42 kDa			8	35	6-10 1h		PMSF and slightly by EDTA, BME and PCMB	Charles et al. (2008)
Aspergillus nidulans PW1	37	4.5	ALTSQSGA PW	8.5	40	24h at pH 8-11 at 37°C	Half life 15 min at 80°C		Pena-Montes et al. (2008)
Aspergillus niger AB100				7	50	80% activity after 24h at pH7	12 and 78% loss in activity after 1h at 40 and 60°C respectively	EDTA	Basu et al. (2008)
Aureobasidium pullulans 10	32 kDa			9	45	4-12	90% activity remain after 1h at 30°C. Half life 1 hr at 45°C	PMSF	Ma et al. (2007)
Beauveria brongniartii	27 kDa	8				half life 2h at 50°C			Erlacher et al. (2006)

Beauveria bassiana		7.5		7.5-9.5					Urtz and Rice, (2000)
Beauveria bassiana	35 kDa +- 2 kDa			8.5	37-42		40% activity after 30 min at 40°C & 5 min at 50°C	PMSF	Bidochka and Khachatouri ans, (1987)
Beauveria felina				10	45			PMSF	Agrawal et al. (2005)
Clonostachys rosea	33 kDa	10.5	ATQSNAQ	11	60	5-12		PMSF , streptomyces subtilisin inhibitor,	Zhao et al. (2005)
Cordyceps militaris	23.4 kDa		IVGGVSVK ICVFPYQV CLCVNNC ANNGGTVI	8.5-12				leupeptin, DFP TLCK , PMSF chymostatin.	Hattori et al. (2005)
Cordyceps sinensis	31		Ala-Leu-Ala-Thr-Gln-His-Gly-Ala-Pro-Trp	7	40		60%activity after 30min at 50°C	PMSF	Li et al. (2007)
Dactylella shizishanna	35 k Da		AEQTDST WGL	10	55	83% activity retain after 2h at pH 8-11	70% activity retain after 30 min at 35°C	PMSF	Wang et al. (2006)
Engyodontium album BTMFS10	38 kDa			11	60				Chellappan et al. (2006)

Fusarium sp BLB	27 kDa		IVVGTTAASGGDFPIIVSIYYQGRAR	9.5	50	3 to 9 for 1 h at 37°C		DIPF and PMSF	Ueda et al. (2007)
Hirsutella rhossiliensis OWVT-1	32 kDa		SVTDQQGADCGLARISHRE	7	40	5-6 for 2hr	Half life 10 min at 45°c	PMSF, SS1 and CI-2	Wang et al. (2007)
Kocuria rosea	240 kDa			10	40	90% activity after 1hr at 9-11		SBTI, AEBSF, chymostatin, & antipain;	Bernal et al. (2006)
Monascus purpureus CCRC31499	40 kDa	7.9		8	40	5-9			Liang et al. (2006)
Paecilomyces lilacinus	33 kDa			11			40% after 3h at 60°C	PMSF, pCMB, Hg	Kotlova et al. (2007)
Scopulariopsis brevicaulis	39 kDa			8.0	40			PMSF	Anbu et al. (2005)
Trichoderma reesei strain QM9414	25	7.3		6					Dienes et al. (2007)
Trichophyton vanbreuseghemii	37 kDa			8				PMSF, TPCK and chymostatin. TLCK and SBTI had significant effect	Moallaei et al. (2006)

MATERIALS AND METHODS

Malt extract, yeast extract, peptone and agar (all from M/s Hi Media chemicals, India), glycerol and D-glucose, Coomassie blue G-250 and R-250, DEAE-cellulose, acrylamide, and ammonium persulphate (Qualigens ExcelaR, India), ampholines and molecular mass markers (BioRad, India), Hammerstein casein (M/s Sisco Research Laboratories), sodium dodecyl sulphate (SDS), 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) were purchased from Sigma Chemical Company (USA). All other chemicals used were of analytical grade.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE): The molecular weight of protease was determined by SDS-PAGE. SDS-PAGE was carried out as described by Laemmli, (1970) using 6% (w/v) stacking gel and 13% (w/v) separating gel. Commassie brilliant blue staining was used to visualize protein bands on the gel. The molecular mass of the enzyme was estimated using a molecular mass markers kit (BioRad, India).

Matrix assisted laser desorption ionization –Time of flight (MALDI-TOF): The molecular mass of purified protease was also determined by MALDI–TOF mass spectrometry using a Voyager DE-STR (Applied Biosystems) equipped with a 337 nm nitrogen laser. 5 μl of the purified enzyme was mixed with 30 μl of sinapinic acid and spotted on MALDI target plate and dried in oven at 50°C and analyzed.

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 (acetate, pH 5; citrate, pH 6; phosphate, pH 7; Tris-HCl, pH 8; carbonate, pH 9 and 10; sodium phosphate–NaOH, pH 11 and KCl-NaOH, pH 12). Stability of protease was examined by incubating the enzyme at 28°C in buffers at pH values ranging from 3 to 11 for 1 h. Residual activity was estimated as described earlier and expressed as percentage of the initial activity taken as 100%.

Effect of Temperature on Protease Activity and Stability

Optimum temperature was determined by estimating the protease activity at pH 9 and temperatures ranging from 30 to 70ºC for 10 min. Thermal stability was examined by incubating the enzyme at temperatures ranging from 4 to 70°C for 1 h. Thermal stability was also examined by incubating the enzyme at 50°C, pH 9 up to 2 h. Residual activity was measured at 50°C, pH 9 and expressed as percentage of initial activity taken as 100%.

Isoelectric focusing

Isoelectric point of the protease was determined by the modified straight tube method using ampholines in the narrow pH range of 8-10. Gels were prepared in tube by mixing 10 % polyacrylamide gel according to Laemmli (1970) using ampholines instead of TEMED. 100 µg purified enzyme was mixed for each tube before addition of ampholine. One tube without enzyme was taken as control. Electrophoresis was carried out at 25 mA and 100 V at 4°C for 16 h. Electrophoresis was terminated when current flow in the gel was stopped. Control was used for measuring pH. Gels were cut into 13 fractions of 1 cm each for activity determination and pH determination. One gel was stained by silver staining

Determination of Michaelis -Menten constant

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 the double-reciprocal Lineweaver-Burk plot equation.

N-terminal Sequencing

The 60 µg of purified protease were loaded on 10% SDS PAGE. Electrophoresis was carried out at 20 mA for 3 hour and the semidry electro blotting was done to transfer the protein on polyvinylidene fluoride (PVDF) membrane. The protein was transferred in 3-cyclehexylamino 1-propane sulphonic acid (CAPS) buffer pH-11 by passing 20 mA current for 3 hour. To visualize band, the PVDF membrane was washed with milli Q water for 3-4 times then stained with Ponsue S. The pink band appeared on the PVDF membrane was cut and subjected to N-terminal sequencing on

a Perkin Elmer sequencer type Procise (Applied Biosystems, Foster City, CA, USA) by Edman degradation method.

Tryptic Digestion

Commassie brilliant blue (CBB) stained SDS PAGE band was excised and washed with 400 µl of 50% acetonitrile (3 times for 15 min) solution to remove the CBB stain. The spots were dehydrated by using 100% acetonitrile solution for 10–15 min until the spots turn opaque white and then dried using speed vaccum. Protein was then reduced and alkaylated by soaking in 100 µl of dithiothreitol (10 mM) solution and then in 100 µl of iodoacetamide (25 mM) solution. The band was then washed with 25 mM ammonium bicarbonate solution and digested with sequencing grade bovine trypsin (Sigma) solution. Molar ratio of protein to trypsin was maintained between 1:10 and 1:20, approximately. Peptides from the gel after overnight trypsin digestion were extracted using 50% acetonitrile and 5% trifluoroacetic acid (TFA), dried and stored at -20°C till further analysis.

Mass Spectrometry

Peptides were reconstituted in 5 µl of 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), then spotted and air dried on 96×2 Teflon coated plate. CHCA (α-cyano hydroxy cinnamic acid) was added as a matrix onto the pre-spotted peptide spots. The Peptide mass fingerprinting (PMF) was obtained using a Voyager-DE-STR MALDI-TOF mass spectrometer (Applied Biosystems). MALDI-TOF mass spectra were recorded in the mass range of 800–4000 Da. The protein was identified by using MASCOT.

Autocatalytic Activity of Purified Protease

The 300 µg of purified protease (300 µg /ml) was incubated at 50°C at pH 9 and 50 µl samples were removed at 1 h interval and analyzed in MALDI-TOF.

Effect of Protease Inhibitors and Chelators

For the determination of protease type, purified protease was pre-incubated at 28°C with following inhibitors: PMSF, EDTA, iodoacetic acid, TPCK, TLCK,

dimethylsulphoxide (DMSO) and benzamidine hydrochloride in 100 mM Tris-HCl buffer (pH 8) for 1 h. Control without inhibitor was taken as 100%.

Effect of Metal Ions

Purified protease was incubated at 28°C with 1 mM and 10 mM metal ions added as chlorides of Ca, Cd, Co, Cu, Fe, Hg, K, and Na for 30 min and the residual protease activity was measured as described earlier and expressed as percentage of activity without metal taken as 100%.

Hydrolysis of Natural Substrates

The purified protease was incubated with different natural substrates like 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 trichloroacetic acid soluble fraction was measured at 280 nm. One unit of enzyme activity is defined as the amount of enzyme required to cause an increase in absorbance by one unit at 280 nm per minute.

Protease Activity towards Azocoll

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 is defined as the amount of enzyme required to cause an increase in absorbance by one unit at 580 nm per minute.

Protease Activity towards Elastin-orcin

The reaction mixture contained an aliquot of suitably diluted protease enzyme and 20 mg elastin-orcin in 0.1 M sodium carbonate 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 enzyme activity is defined as the amount of enzyme required to cause an increase in absorbance by one unit at 578 nm in one minute.

Protease Activity towards Azocasein

The reaction mixture contained an aliquot of suitably diluted protease and 1 mg azocasein in 0.05 M sodium carbonate 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, the reaction was terminated by addition of 500 µl of 10% TCA. After cooling on ice for 15 min, contents were centrifuged at 8000 rpm for 10 min. To 800 µl of supernatant, 200 µl of 1.8 M NaOH was added and absorbance was measured at 420 nm. One unit of enzyme activity is defined as the amount of enzyme required to cause an increase in absorbance by one unit at 420 nm per minute.

Protease Activity towards N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA)

The reaction mixture contained an aliquot of suitably diluted protease enzyme in 0.1 M sodium carbonate buffer pH 9.0 in a total volume of 500 µl. Added 500 µl of 1.5 mM BAPNA prepared in 0.1 M sodium carbonate buffer pH 9 and incubated at 50°C for 60 min, the reaction was terminated by addition of 250 µl of 10% acetic acid. Centrifuged and absorbance of supernatant was measured at 410 nm. One unit of enzyme activity is defined as the amount of enzyme required to cause an increase in absorbance by one unit at 410 nm in one minute.

CD Measurement

CD spectra were recorded in a Jasco-J715 spectropolarimeter at ambient temperature using a cell of 1 mm path length. Replicate scans were obtained at 0.05 nm resolutions, 1 nm band width and at a scan speed of 100 nm/min. Spectra were averages of three scans with the base line subtracted spanning from 260 to 190 nm in 0.05 nm increments. The CD spectrum of purified protease (10 µM) was recorded in 50 mM phosphate buffer pH 7. Secondary structure content of purified protease was calculated using the algorithm of the K_2D_2 program.

Chemical Modification Studies

During chemical modification studies, the residual activity of the modified protease was determined by caseinolytic activity. Protease incubated in the absence of modifying reagents served as control. 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.

The protease (10 μM) in 10 mM phosphate buffer, pH 7 was incubated with (0.005-0.05 mM) PMSF prepared in DMSO, (1-10 mM) WRK prepared in same buffer, (1-10 mM) phenylglyoxal prepared in methanol, (1-10 mM) DEPC freshly prepared in absolute ethanol, (0.01 -0.1 mM) NBS prepared in the same buffer and (0.1-1.0 mM) DTNB prepared in the same buffer, in a final volume of 1 ml at 28°C, for 30 min and residual activity was determined. The methanol and ethanol concentration in the reaction mixture did not exceed 2 % (v/v) and should have no effect on the activity and stability of the protease during the incubation period.

Relationship of Concentration of PMSF and Time of Incubation with Inhibition

The protease (10 μM) in 10 mM phosphate buffer, pH 7 was incubated with PMSF (0.002-0.008 mM) prepared in , at 28°C, for 20 min. Aliquots were removed at 5 min intervals and residual activity determined. Protease sample incubated in the absence of PMSF served as the control.

Ligand Protection or Substrate Protection

This was carried out by pre-incubating the protease (10 μM) with varying concentration of casein (1 – 10 mg) at 4°C followed by addition of 10 μM PMSF, 5 mM WRK and 10 μM NBS and incubated at 4°C for 30 min and residual protease activity was determined.

RESULTS AND DISCUSSION

Molecular Weight of Protease

SDS-PAGE: The molecular mass of purified protease was 29 kDa on SDS-PAGE (Figure 3.1) which is similar to the molecular weight of 27 kDa protease from *Beauveria brongniartii* (Erlacher et al., 2006). However, it is slightly lower compared to the molecular weights of proteases from two different strains of *Beauveria bassiana*. The molecular weight of protease from *Beauveria bassiana* GK2016 was reported to be 35 kDa (Bidochka and Khachatourians, 1987) while the cuticle-degrading protease produced by *B. bassiana* isolate B1 was found to be 47 kDa (Zibaee and Bandani, 2009). The molecular masses of alkaline serine proteases generally fall in the range of 18 to 30 kDa (Gupta et al., 2002) with a few exceptions. The highest molecular weight of 124 kDa for fungal protease from *Aspergillus fumigates* TKU003 is reported by Wang et al. (2005). Small molecular weight proteases from *Conidiobolus* sp (6.8 kDa) and *Kurthia spiroforme* (8 kDa) are reported by Sutar et al. (1991) and Steele et al. (1992) respectively.

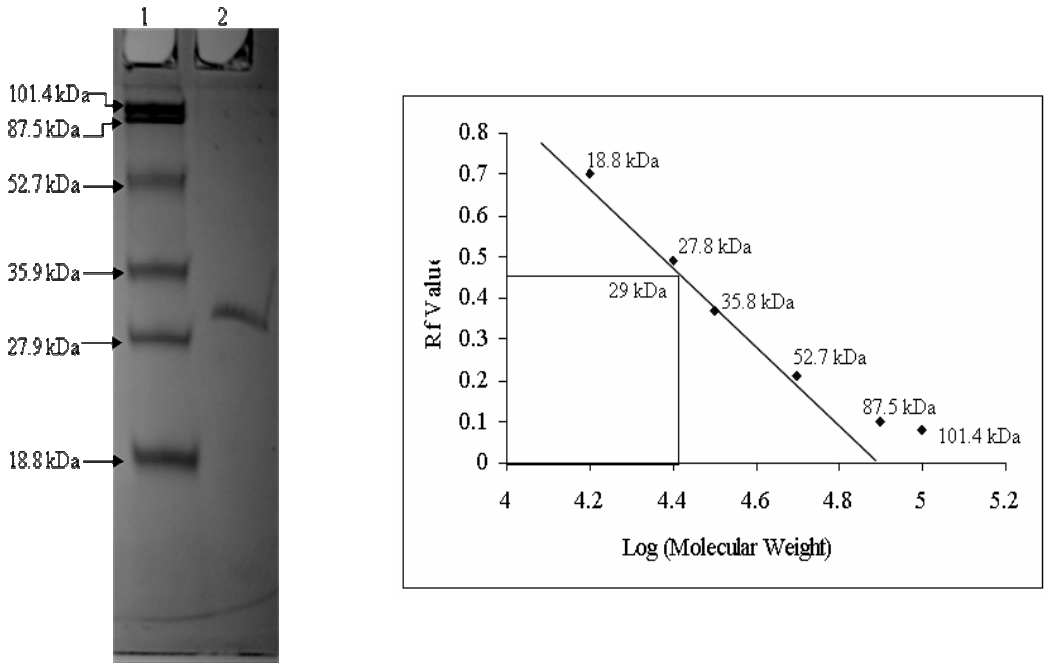


Figure 3.1: SDS-PAGE of purified protease and the molecular weight markers. Lane 1, molecular weight markers: lysozyme (18.8 kDa); soyabean trypsin inhibitor (27.9 kDa); carbonic anhydrase (35.9 kDa); ovalbumin (52.7 kDa); bovine serum albumin (87.5 kDa) and phosphorylase b (101.4 kDa). Lane 2, purified protease.

MALDI-TOF: MALDI-TOF analysis of purified protease showed a major peak corresponding to the molecular weight of 28.2 kDa (Figure 3.2). This value is nearly similar to that obtained by SDS-PAGE. The minor peak corresponding to that of 15.7 kDa seems to be the product of autoproteolysis. Very few reports are available on the molecular weight determination by MALDI-TOF. No differences in the molecular mass of proteases in SDS PAGE and MALDI-MS was observed by Hattori et al., (2005) and Palmeiri et al. (2001) whereas Pekkarinen et al., (2002) reported differences in molecular weight of protease by SDS PAGE (26.8 kDa) and MALDI-TOF (28.663 kDa). A low molecular weight serine protease from spider venom gland extract by MALDI-TOF was reported by Devaraja et al. (2008).

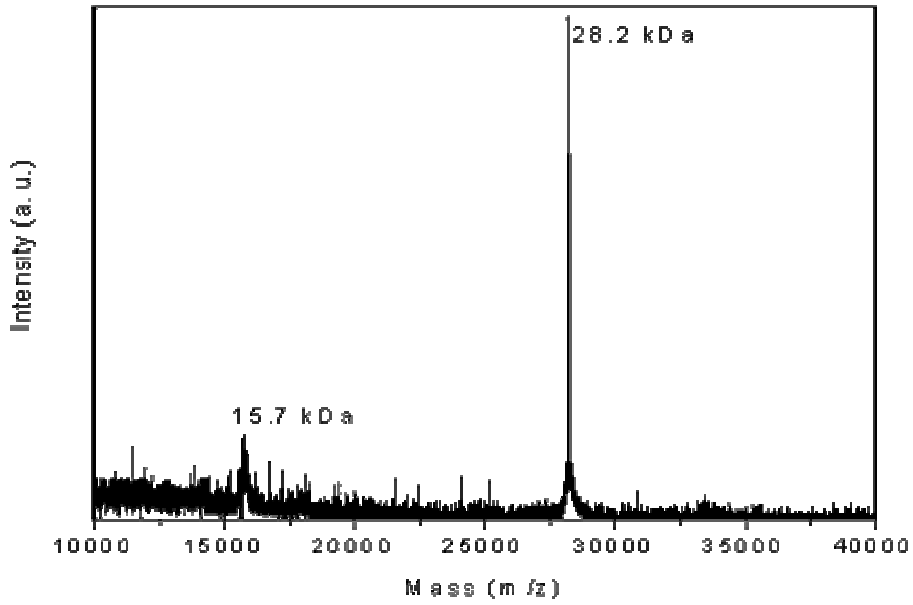


Figure 3.2: MALDI–TOF of purified protease showing a major peak with molecular weight 28.2 kDa.

Isoelectric Focusing

Alkaline proteases mostly have their isoelectric points near to their pH optimum in the range of 8–11. The pH of the fractions and activity and the silver stained gel are shown in (Figure 3.3a and b). The pI of purified protease was found to be 9.31 which were slightly higher compared to majority of fungal alkaline proteases which have pI in the range of 7 to 8.5. This could be due to the presence of higher percentage of basic amino acids in the protein as compared to acidic amino acids. However, the pI of *Beauveria brongniartii* protease is reported to be 8.0 (Erlacher et al., 2006) which is 1 unit lower than the protease from *Beauveria* sp MTCC 5184. The higher pI of

10.5 was reported for serine-like protease from nematophagous fungus *Clonostachys rosea* (Zhao et al., 2005) whereas, the lower pI of 4.5 was reported for alkaline protease from *Aspergillus nidulans* (Pena-Montes et al., 2008). The proteases are most suitable for detergent application if their pI coincides with the pH of the detergent solution. For example, two commercial alkaline proteases, Esperase and Savinase marketed by Novo have very high isoelectric points (pI 11) and can withstand higher pH ranges and are used for detergent applications.

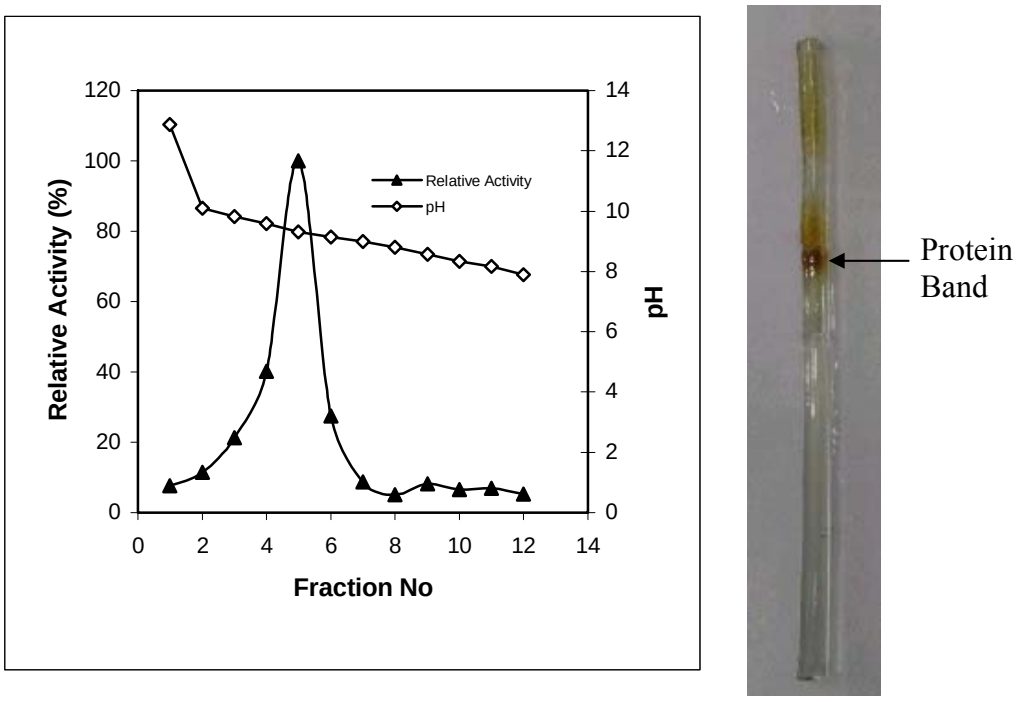


Figure 3.3: Isoelectric focussing of purified protease

Effect of pH on Enzyme Activity and Stability

The pH activity profile of the purified protease is shown in (Figure 3.4). The protease was active in the pH range of 5–12, with an optimum at pH 9 and showed more than 60 and 85% activity at pH 8 and 10 respectively. The optimum pH of majority of fungal proteases falls in the range of 8 to 12 while optimum pH of proteases from different strains of *Beauveria* varied from 7.5-9.5 (Table 3.1). The optimum pH for the cuticle-degrading proteases produced by *Beauveria bassiana* and *Beauveria brongniartii* is 8 (Erlacher et al., 2006; Zibaee and Bandani, 2009) while the protease from another strain *Beauveria bassiana* GK2016 is reported to have an optimum pH of 8.5 (Bidochaka and Khachatourians, 1987). The extracellular protease BBP from a

B. bassiana isolate had an alkaline pH optimum in the range 7.5–9.5 (Urtz and Rice, 2000).

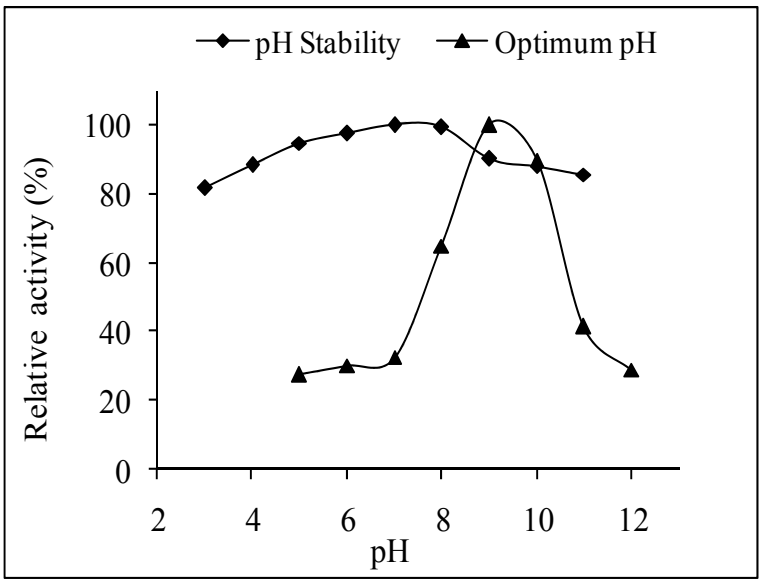


Figure 3.4: Optimum pH and stability of purified protease

The protease from *Beauveria* sp MTCC 5184 was highly stable in pH range 3-11 with maximum stability at pH 7 which is important for its industrial importance (Figure 3.4). Most of the fungal proteases are stable in the pH range of 5 to 10, especially at neutral pH. *B. brongniartii* protease was most stable at pH 8 and 20°C and the half life of the enzyme in presence of 20 mM $CaCl_2$ was 33 h (Erlacher et al., 2006). Hajji et al. (2007) have reported that the serine protease from *Aspergillus clavatus* ES1 to be stable at 4°C and in pH 8-9 which retained about 68 and 54% of its initial activity at pH 7 and 10 respectively. *Aspergillus nidulans* HA-10 protease was stable at 37°C and in pH range of 6 to 10 for 1 h (Charles et al., 2008) while protease from *Fusarium* sp BLB was stable in pH range of 3 to 9 for 1 h at 37°C (Ueda et al., 2007).

Effect of Temperature on Enzyme Activity and Stability

The protease was active in the temperature range of 30 and 60°C with an optimum at 50°C (Figure 3.5). The protease showed 92 and 94% activity at 40 and 60°C respectively whereas activity sharply decline at 70°C which may be due to the denaturation of protein at higher temperature. The optimum temperature for fungal

alkaline proteases ranges from 35 to 60^{o}C (Table 3.1). In contrast, bacterial proteases have higher temperature optima as high as 85°C (Rhaman et al., 1994) and 115°C (Nakanishi and Yamamoto 1974). Bidochka and Khachatourians, (1987) and Zibaee and Bandani, (2009) have reported optimum temperatures of 37-45^{o}C and 45°C respectively for proteases from *Beauveria bassiana*. However, the protease from *Beauveria brongniartii* is reported to have a lower optimum temperature of 20°C (Erlacher et al., 2006).

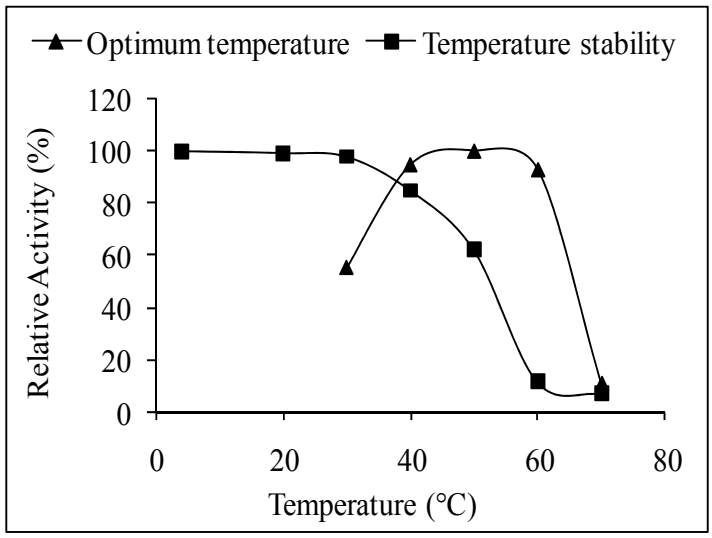


Figure 3.5: Optimum temperature and stability

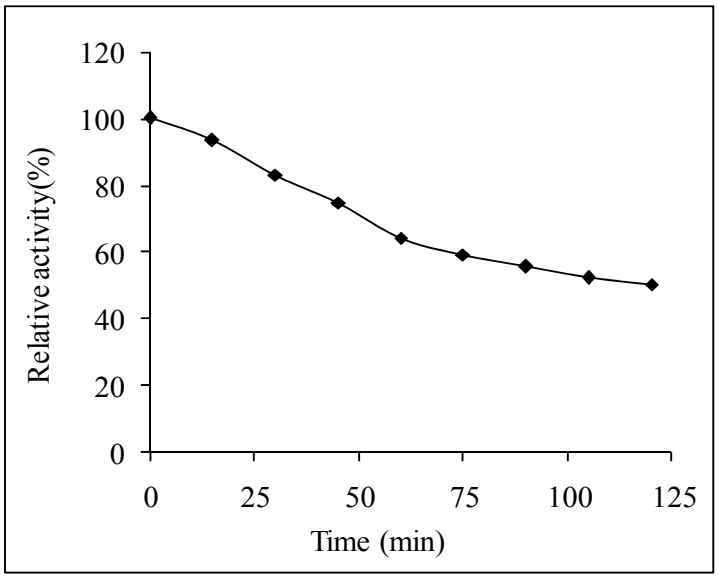


Figure 3.6: Stability of protease at 50°C

The thermal stability profile of the protease showed that the enzyme retained full activity at room temperature (30°C) while there was marginal loss of 15% at 40°C after 1 h (Figure 3.5). Fungal alkaline proteases are generally stable up to 50-60°C whereas the bacterial proteases exhibit higher thermal stabilities. Bacterial proteases are more preferred compared to fungal proteases as detergent additives due to their higher thermal stabilities. However, the high pH stability of the fungal alkaline proteases and their ease of isolation compared to the bacterial enzymes are attracting more attention for their use as additives in detergents. Protease from *Beauveria bassiana* retained 40% activity after 30 min at 40°C or 5 min at 50°C (Bidochka and Khachatourians, 1987). Protease from *Aspergillus niger* AB100 was stable at 30°C for 1 h while 88 and 22% activity was retained at 40 and 60°C respectively (Basu et al., 2008). At its optimal temperature (50°C), the *Beauveria* sp MTCC 5184 protease had a half life of 2 h (Figure 3.6) which is similar to the half life of 2 h at 50°C for protease from *Aspergillus clavatus* ES1 (Hajji et al., 2007).

Effect of Substrate Concentration and Determination of K_m and V_{max}

Effect of casein concentration on protease activity was determined by incubating the enzyme with 0.5 mg to 10 mg of casein at 50°C, pH 9. The protease activity was increased with increase in substrate concentration and maximum protease activity was observed at 0.5% casein concentration (5mg) beyond which there was slight inhibition in activity (Figure 3.7).

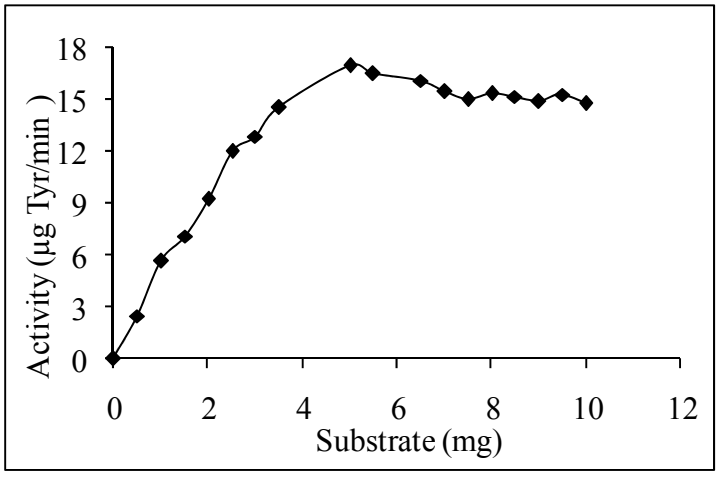


Figure 3.7: Effect of substrate concentration on protease activity

The K_m and V_{max} of purified protease were calculated from double reciprocal Lineweaver-Burk plot and found to be 5.1 mg and 29.67 U/ml respectively (Figure 3.8). *B. brongniartii* protease showed the K_m of 0.228 and 0.202 mM with N-Suc-Ala-Ala-Pro-Phe-pNA and N-Suc-Ala-Ala-Pro-Leu-pNA. Kinetic parameters of these proteases in crude extract showed that the V_{max} values were 1.85 and 1.08 U/mg protein in addition to K_m values that measured 0.74 and 1.06 mM for Pr1 (subtilisin-like protease) and Pr2 (cuticle degrading protease), respectively from *B. bassiana* B1 (Zibaee and Bandani, 2009).

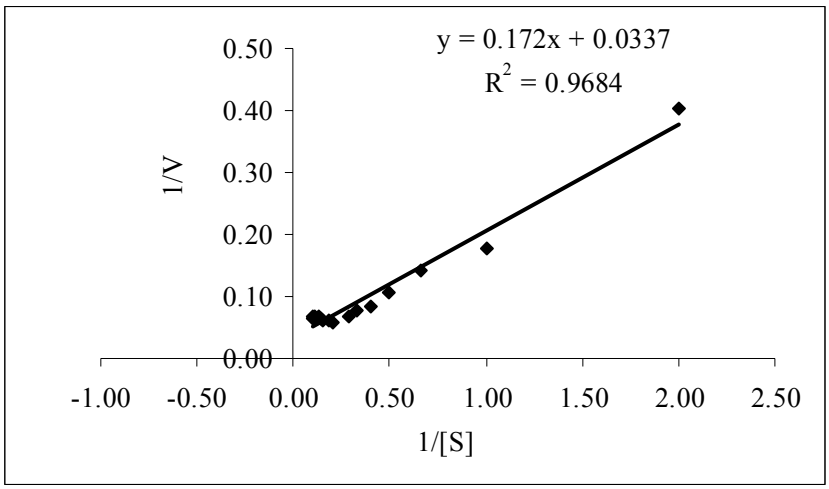


Figure 3.8: Double reciprocal Lineweaver Burk Plot

N-terminal Sequence of Purified Protease

The purified protease band observed on SDS-PAGE was transferred to PVDF membrane by semidry electroblotting. The transferred protein was of good quality and showed single pink band with Ponsue S stain. The band was cut and dried between whatman filter paper and stored in ependorf tube for N-terminal sequence determination. The N terminal sequence of protein was determined by Edmans degradation principle by the instrument PROCISE cLC. The N-terminal sequence of the first 16 amino acid residues of the purified alkaline protease from *Beauveria* sp MTCC 5184 was found to be Ala-Met-Ala-Thr-Pro-His-Val-Ala-Pro-Leu-Val-Leu-Tyr-Gly-Val-Ala. Table 3.2 showed the first 10 BLAST hit of N terminal sequence. The N-terminal amino acid sequence of *Beauveria* sp MTCC 5184 showed similarity to those of subtilisin like protease Mp1 from *Magnaporthe poae* (Sreedhar et al., 1999), peptidase S8 and S53 subtilisin from *Spirosoma linguale* DSM 74 (Lucas et al., Unpublished), subtilisin-like serine protease pepD from *Aspergillus niger* (Pel et

al., 2007), protease from *Aspergillus niger* (Jarai et al., 1994), S8A subfamily serine protease from *Listeria grayi* and hypothetical protein from *Gibberella zeae*. The highest homology was observed with the hypothetical protein from *Gibberella zeae.* Among protease highest homology was observed with subtilisin-like serine protease from *Magnaporthe poae* followed by subtilisin like protease from *Spirosoma linguale* and *A. niger*. The *Beauveria* sp MTCC 5184 protease differs from that of *Magnaporthe poae* with only three residues in the first 13 amino acids. The amino acid Ser-354 and Gly-355 in *Magnaporthe poae* alkaline protease was replaced by Ala-8 and Pro-9 in purified protease (Figure 3.9). In case of protease from *A. niger* amino acids Thr-356, Gly-357 and Iso-359 in place of Ala-8, Pro-9 and Val-11.

Table 3.2: First 10 BLAST hit of N terminal protein sequence

Accession No	Description	Max score	Query coverage	References
XP380982.1	hypothetical protein FG00806.1 [*Gibberella zeae* PH-1]	38.0	87%	Unpublished
ZP02152454.1	response regulator [*Oceanibulbus indolifex* HEL-45]	34.1	75%	Unpublished
Q9Y778.1	subtilisin-like proteinase Mp1 [*Magnaporthe poae*]	34.1	75%	Sreedhar et al. (1999)
XP391581.1	hypothetical protein FG11405.1 [*Gibberella zeae* PH-1]	34.1	93%	Unpublished
XP001905571.1	hypothetical protein [*Podospora anserina* S mat+]	33.7	75%	Espagne et al. (2008)
EEU47305.1	hypothetical protein [*Nectria haematococca* mpVI 77-13-4]	33.3	75%	Coleman et al. (2009)
YP003389455.1	peptidase S8 and S53 subtilisin kexin sedolisin [*Spirosoma linguale*]	32.0	81%	Lucas et al. (Unpublish)
XP001393694.1	subtilisin-like serine protease pepD-*Aspergillus niger*	31.6	75%	Pel et al. (2007)
AAA32703.1	protease [*Aspergillus niger*]	31.6	75%	Jarai et al. (1994)
ZP04443150.1	S8A subfamily serine protease [*Listeria grayi* DSM 20601]	31.2	62%	Unpublished

Beauveria sp MTCC 5184			AMATPHVAPLVLYGVA	
XP 380982.1	hypothetical protein (*Gibberella zeae* PH-1)	357	MATPHIAGLVLYGI	370
ZP 02152454.1	response regulator (*Oceanibulbus indolifex* HEL-45)	11	AMATPAVAPLVL	22
Q9Y778.1	Subtilisin-like proteinase (*Magnaporthe poae*)	348	MATPHVSGLVLY	359
XP 391581.1	hypothetical protein (*Gibberella zeae* PH-1)	358	MAAPHVAGLVLY	369
XP 001905571.1	hypothetical protein (*Podospora anserina*)	367	MASPHVAGLVLY	378
EEU47305.1	hypothetical protein (*Nectria haematococca* mpVI)	387	MAAPHVAGLVLY	398
YP 003389455.1	peptidase S8 and S53 subtilisin (*Spirosoma linguale*)	368	MATPHVAGILLYG	380
XP 001393694.1	subtilisin-like serine protease pepD (*A. niger*)	350	MATPHVTGLILY	361
AAA32703.1	protease (*Aspergillus niger*)	350	MATPHVTGLILY	361
ZP 04443150.1	S8A subfamily serine protease (*Listeria grayi*)	632	MATPHVAALV	641

Figure 3.9: Comparison of N-terminal amino acid sequence of the purified alkaline protease from *Beauveria sp.* with other proteins.

Peptide Mass Fingerprinting

Large-format 2D gel electrophoresis systems, Edman microsequence analysis and proteins staining with specific antibodies have been used to systematically identify proteins and establish cellular databases. There are, however, significant problems associated with these approaches. Most proteins are only present in the low quantity, which is significantly below the level at which automated sequencers can reliably operate. The relatively slow speed of the Edman process also means that the number of proteins is too great to permit large-scale characterization within any useful period of time. The use of monoclonal antibodies, while both rapid and sensitive, requires the ready availability of a large pool of specific antibody probes. New methods have been developed using a combination of protease digestion, matrix-assisted laser-desorption ionization (MALDI) mass spectrometry (MS) and screening of peptide-mass databases that offer significant increase in the speed at which proteins can be identified (Pappin, 1997). Peptide-mass fingerprints can prove as discriminating as linear peptide sequences, but can be obtained in a fraction of the time using less protein. In many cases, this allows for a rapid identification of a sample protein before committing it to protein sequence analysis (Pappin et al., 1993).

The purified protease band visualized by Coomassie staining on SDS-PAGE was removed from the gel and the protein was digested with trypsin. The Peptide mass fingerprinting (PMF) was obtained using a Voyager-DE-STR MALDI-TOF mass spectrometer (Applied Biosystems). MALDI mass spectra were recorded in the mass range of 800–4000 Da (Figure 3.10). Peptide masses obtained were searched using the Mascot program for protein identification. PMF of Tryptic digest of purified 28 kDa protease showed partial homology with only subtilisin-like protease 3 precursors from *Microsporum canis* in the PMF database. Out of 67 mass values

searched only 11 mass values matched with *Microsporum canis* protease. For search parameter mass values were monoisotopic and peptide mass tolerence were +/- 1 Da. The PMF result indicates that protease from new isolate of *Beauveria* sp MTCC 5184 is subtilisin like protease. Erlacher et al. (2006) reported peptide mass mapping of subtilisin like protease from *B. brongniartii* based on tryptic digestion and analysis of the peptide fragments with nano-HPLC-ESI-MS and found no homology in PMF database. Anandan et al. (2007) have reported alkaline protease from *Aspergillus tamarii* showed homologous with the alkaline protease fragment (MW 35,154 Da) isolated from *Aspergillus fumigatus.* Yadav et al. (2010) have reported plant subtilase with unique peptide mass fingerprints. Dienes et al. (2007) have identified the PMF of the unknown component from the SDS-PAGE and isoelectric focusing were almost identical, confirming that the prominent bands on SDS-PAGE and on isoelectric focusing gels were caused by the same protein.

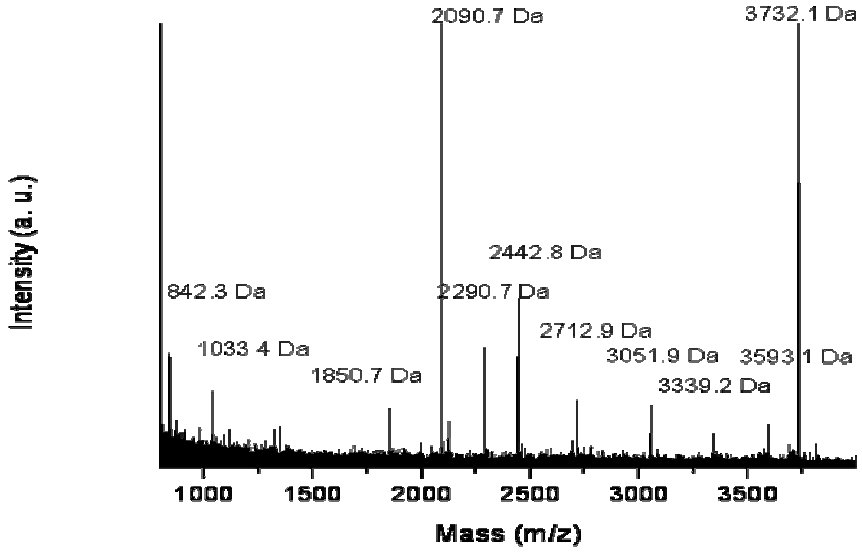


Figure 3.10: PMF of purified protease

Autocatalytic Activity of Purified Protease

Initially there was only one peak of 28.2 kDa. Intensity of 28.2 kDa peak decreased whereas peak of 15.6 kDa and 10.2 kDa peak increased with time (Figure 3.11). After 5 h 28.2 kDa peak vanished where as only 15.6 kDa and 10.2 kDa peaks remain indicated that the 15.6 and 10.2 kDa peaks are the autocatalytic product of 28.2 kDa peak. After 5 h of incubation there was no protease activity remained in the sample

indicated that the 15.6 and 10.2 kDa did not have protease activity. Protease activity was only in 28.2 kDa protein.

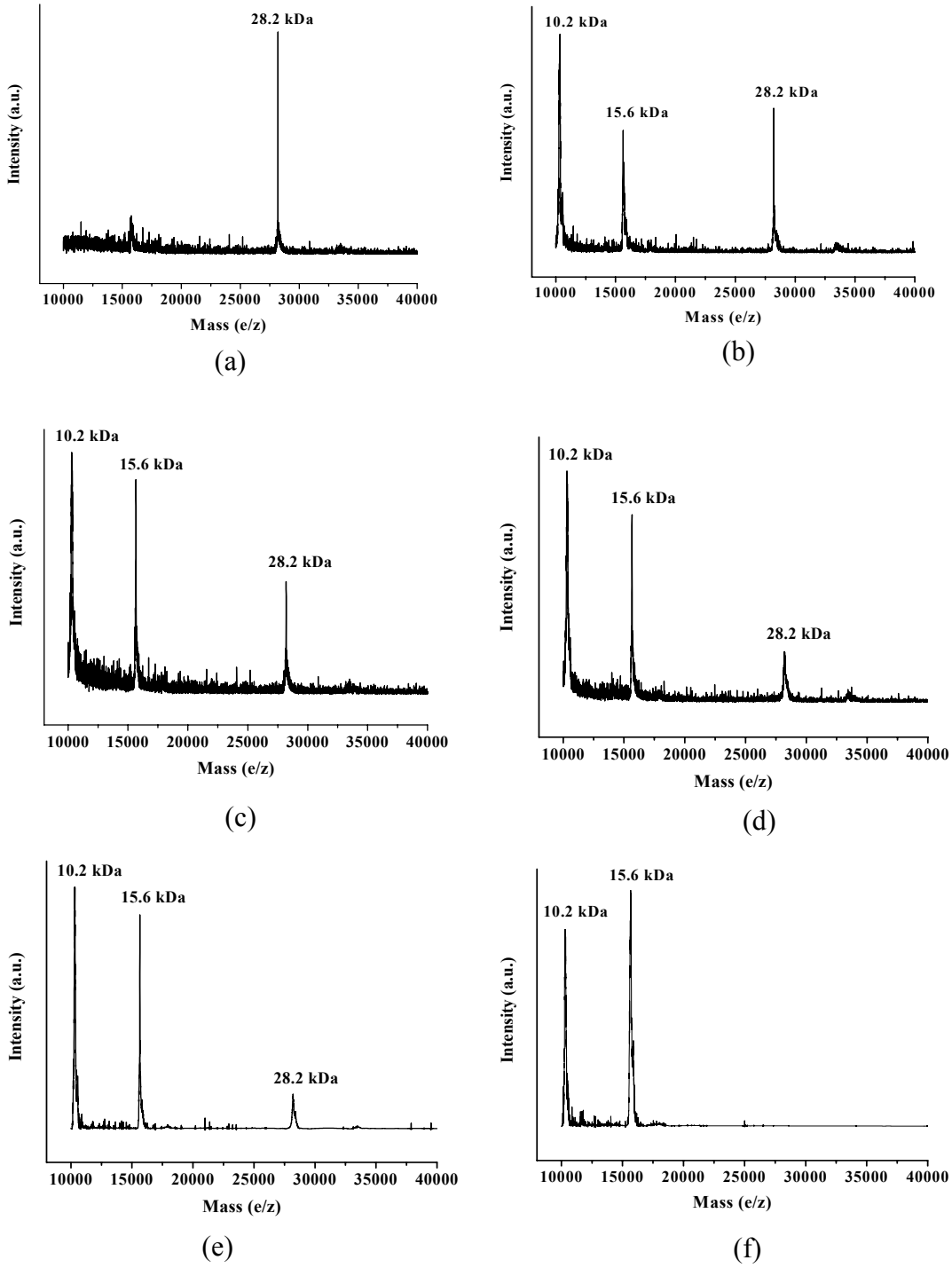


Figure 3.11: Autocatalytic activity of purified protease at 50°C. Samples were withdrawn after (a) 0 h (b) 1 h (c) 2 h (d) 3 h (e) 4 h (f) 5 h.

Effect of Protease Inhibitors and Chelators

Among the various types of serine proteases known, chymotrypsin/trypsin and subtilisin like proteases are most important. Different inhibitors are used to establish the identity and type of the protease. Chymotrypsin, a mammalian digestive protease which has structural homology with trypsin, elastase and thrombin is inhibited by TPCK. These proteases are mainly of animal origin and those belonging to various species of *Streptomyces*, like *S. erythreus*, *S. fradiae* and *S. griseus*. Subtilisin-like serine proteases are generally bacterial in origin, although there are reports from other sources (Dhar and Kaur, 2010; Donatti et al., 2008; Kudryavtseva et al., 2010). This class of proteases is specific for aromatic or hydrophobic residues (at position P1), such as tyrosine, phenylalanine and leucine. They are highly sensitive towards PMSF, diisopropyl-fluorophosphate (DFP) and potato inhibitor. This class of protease is well represented by various species of *Bacillus*, like *B. amyloliquifaciens*, *B. licheniformis* and *B. subtilis* (Rao et al., 1998).

Table 3.4: Effect of inhibitors and chelators

Inhibitor	Concentration of inhibitors (mM)	Inhibition (%)
Control	-	0
TPCK	5	7.33
TLCK	5	12.12
PMSF	1	97.75
EDTA	20	1.51
Iodoacetate	10	0.30
DMSO	10%	0.04
Benzamidine	5	3.62

Effect of different inhibitors on protease activity is presented in Table 3.4. The protease was strongly inhibited by 1 mM PMSF indicating the enzyme to be a serine protease. No inhibition was observed in presence of 20 mM EDTA or 10 mM iodoacetate indicating that it is neither a metlloprotease nor a cysteine protease. The stability of protease in the presence of EDTA is very useful for application as detergent additive in view of the fact that chelating agents are components of most

detergents. Trypsin and chymotrypsin inhibitors TPCK and TLCK at 5 mM concentration did not significantly inhibit indicating that the protease has neither trypsin nor chymotrypsin like activity. Benzamidine which is a trypsin inhibitor also had no inhibitory effect. This is in agreement with the extracellular protease from *B. bassiana* which was not inhibited by TPCK or TLCK at 10 mM concentration (Bidocka and Khachatourians, 1987). Proteinases from *Cephalosporium* sp. KM388 were not inhibited by TPCK or TLCK (Tsuchiya et al., 1987). At 100 µM concentration, TLCK inhibited the trypsin-like protease, P-1-1from *Cordyceps militaris* protease by 21% while at 1 mM concentration; the inhibition was 56% (Hattori et al., 2005).

Effect of Metal Ions on Protease Activity and Stability

Effect of various metal ions on purified protease is presented in Table 3.5. At 1 mM concentration, there was no significant inhibition by any of the metals tested except Fe^{3+} which showed around 20% loss in activity. Maximum inhibition of around 40% was observed with 10 mM Hg^{2+} and Cd^{2+} while Na^{+}, Co^{2+} and Cu^{2+} did not inhibit the protease at this concentration. Hg^{2+} was found to be inhibitory for proteases from *Aspergillus niger* AB100 (Basu et al., 2008) and *Cordyceps sinensis* (Li et al., 2007). Cuticle-degrading protease from *Beauveria bassiana* was activated by 5 mM Ca^{2+} (149%) and Mg^{2+} (131%) while Mn^{2+}, Na^{+}, K^{+} decreased the activity by 74, 25 and 63% respectively (Zibaee and Bandani, 2009). Li et al. (2007) have reported the protease from *Cordyceps sinensis* was inhibited drastically with Hg^{2+}, Cu^{2+} and Mn^{2+} whereas Cd^{2+}, Co^{2+} and Ni^{2+} moderately reduced the activity. The addition of Cu^{2+} and Zn^{2+} inhibited the protease from *Aspergillus clavatus* ES1 by 38 and 84%, respectively while the protease was completely inhibited by Co^{2+} (Hajji et al., 2007). Basu et al. (2008) have reported activation of protease from *Aspergillus niger* AB100 by metal ions such as Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+}. The protease being stable in presence of metals such as Cd, Cu and Hg at 100 mM concentration is useful for its applications. At 1 mM concentration Cu, Hg and Zn strongly inhibited the protease from *B. felina* (Agarwal et al., 2005).

Table 3.5: Effect of chloride salt of metal ions on protease activity

Metals added	Relative Activity (%)	
	1mM	**10 mM**
None (control)	100	100.00
$CaCl_2$	92.99	81.75
$CdCl_2$	96.99	63.50
$CoCl_2$	103.86	112.356
$CuCl_2$	109.42	100.02
$FeCl_3$	81.67	82.66
$HgCl_2$	90.38	63.16
KCl	81.71	87.63
$MnCl_2$	101.51	73.98
NaCl	100.94	99.01

Activity towards Natural and Synthetic Substrates

The important feature of alkaline protease is their ability to discriminate among competing substrates and utility of these enzymes often depends on their substrate specificity. 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, bovine serum albumin and heamoglobin was determined. The protease was able to degrade the above substrates to varying degrees. It was more active against casein compared to haemoglogin and bovine serum albumin (Table 3.6). These results are in agreement with other reports where the proteases are more active towards casein compared to bovine serum albumin. The proteases from *Conidiobolus* sp (Sutar et al., 1991); *Cephalosporium* sp. KM388 (Tsuchiya et al., 1987) and *Beauveria bassiana* (Bidocka and Khachatourians, 1987) have been reported to be more active towards casein compared to bovine serum albumin. Protease Ds1 from the nematode-trapping fungus *Dactylella shizishanna* was particularly effective at degrading casein and skimmed milk, while being less effective at hydrolysing BSA (Wang et al., 2006). Since haemoglobin and albumins are present in the body secretions, such proteases will find application in detergent formulation.

Table 3.6: Activity towards substrates

Substrate	Activity (KU/ml)
Casein	11.5
Hemoglobin	5.45
BSA	0.58
Azocoll	1.84*
Elastin-orcin	0.011*
Azocasein	16.05*
BAPNA	0.018*

* - Activity in U/ml

CD Spectra Analysis

Circular dichroism is a common tool for the analysis of the proteins secondary structure. The CD spectrum in the far UV region (260–190 nm) is related to the alpha helix and the beta sheet content of a protein (Spelzini et al., 2005). To evaluate the secondary structure of purified protease from *Beauveria* sp MTCC 5184, the CD spectra of protease was analyzed. The estimated secondary structure contents from the CD analysis were 87.76% α- helix, 0.47% β-sheet and 11.77% aperiodic structure (Figure 3.12). Suzuki et al. (2005) have reported a protease grifolisin from fruiting bodies of the fungus *Grifola frondosa*, a maitake mushroom. The α -helix, β structure and random coil contents of the purified protease (grifolisin) were calculated to be 43.2%, 28.2% and 28.4%, respectively. Yadav et al. (2009) have reported a plant subtilisin like protease has 29% of α-helix and 38% of β-sheet content as determined by CD spectra.

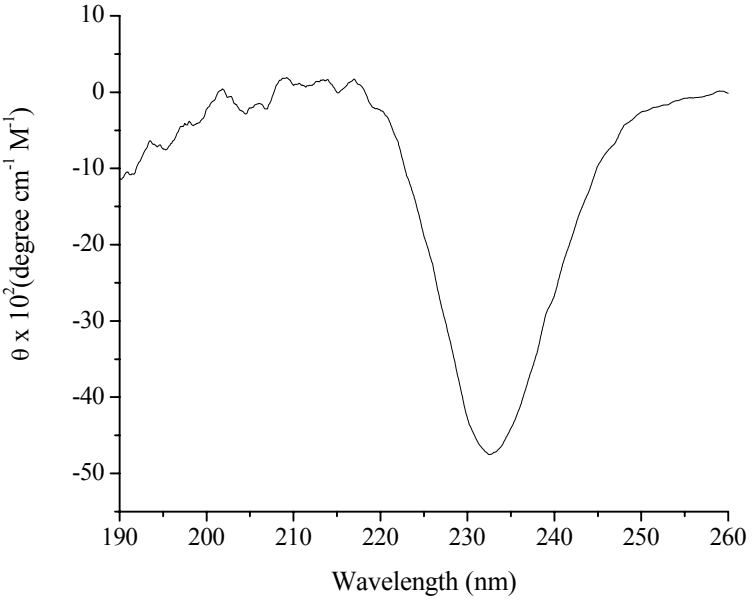


Figure 3.12: Far-UV CD spectra analysis of purified protease to analyze the secondary structure. Purified protease dissolved in 0.05 M phosphate buffer pH 7.0 and the CD spectra were recorded from 260 to 190 nm at 25°C.

Chemical Modification

Active site directed chemical modification is an important tool for studying structure-function relationship of enzymes and other biologically active proteins. Site-directed mutagenesis is widely used for the same purpose, which needs not only the cloned gene but also the three-dimensional structure of the protein. In contrast, active-site-directed chemical modification can be done without knowing the protein structure (Gote et al., 2007).

The importance of amino acid functional groups for the activity of protease was determined by chemical modification studies using amino acid side chain-specific chemical reagents. The results of the inactivation studies of protease are presented in Table 3.7. Lack of inhibition by 1 mM DTNB and 10 mM Phenylglyoxal suggested the non-involvement of cysteine and arginine in the protease catalysis. The protease was completely inhibited by 9 mM WRK, 60 µM NBS and 20 µM PMSF whereas 40% inhibition was with 10 mM DEPC. Besides histidine, DEPC also reacts with tyrosine, cysteine and lysine residues (Gote et al., 2007). The complete inhibition of protease activity with PMSF, WRK and NBS indicated the presence of serine, aspartic acid and tryptophan at or near the active site of protease, since these amino acids are

essential for the activity of protease. It is remarkable that serine, histidine, and aspartic acid come together in the folded structure to form a triad that is an essential element of the active site in all the serine proteases (Phadtare et al., 1997).

Nucleophilicity of the catalytic Ser is typically dependent on a catalytic triad of Asp, His and Ser residues, commonly referred to as the charge relay system. Many serine peptidases employ a simpler dyad mechanism where Lys or His is paired with the catalytic Ser. Other serine peptidases mediate catalysis via novel triads of residues, such as a pair of His residues combined with the nucleophilic Ser (Page et al., 2008). In present work we observed the novel triads of residues such as Ser, Asp and Trp which mediate the catalysis of serine like protease. Sheng et al. (2006) reported protease from *Beauveria brongniartii* has three active sites (D140, H170 and S326), which are characteristic of the subtilisin-like serine proteases. Besides the characteristic residues of the catalytic triad (Asp, His, Ser), reported to be present in the subtilisins, an essential Trp and thiol residue were detected in the active site of *Conidiobolus* alkaline protease. Presence of cys residue at or near the active site of serine protease has reported from *Cordyceps sinensis* (Li et al 2007, Ma et al., 2007). Page et al. (2008) have summarized the catalytic units in all serine peptidase families, primary specificity and the fold that harbors them.

Table: 3.7: Effect of modification agent on protease activity

Modifying Agent	**Concentration**	**Possible amino acid modification**	**Residual activity (%)**
DEPC	10 mM	Histidine	60
DTNB	1 mM	Cysteine	100
NBS	50 µM	Tryptophan	7
Phenylglyoxal	10 mM	Arginine	93.6
PMSF	20 µM	Serine	0
WRK	10 mM	Aspartic acid	0

The inactivation of protease was dependent on the concentration of the PMSF added. The plots of residual activity versus time of inactivation for various PMSF concentrations were linear (Figure 3.13).

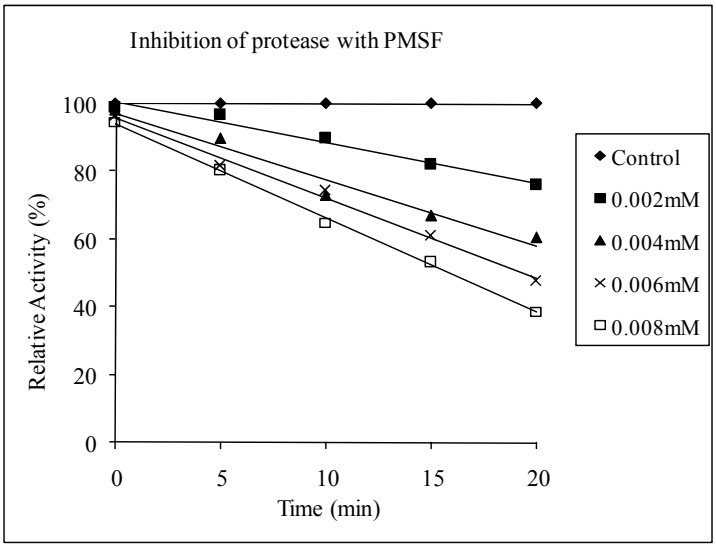


Figure 3.13: Inhibition kinetics of protease with respect to concentration of PMSF and incubation time

Ligand Protection

To ascertain whether the amino acids, serine, aspartic acid and tryptophan were present at the active site of protease, substrate protection assay was carried out. The protease showed decreasing trend of inhibition in presence of inhibitors when protease was pre incubated with increasing concentration of substrate (Table 3.8). Protease without inhibitors showed no inhibition where as with 0.01 mM inhibitor concentration showed complete inhibition. When protease was preincubated with 10 mg of casein followed by addition of 5 mM WRK, 0.01 M PMSF and 0.01 NBS showed inhibition of approximately 9, 26 and 16 % respectively. This indicated that the substrate competes with the inhibitors for the active site and presence of aspartic acid, serine and tryptophan at the active site of protease.

Table 3.8: Ligand protection assay

Sample	Inhibition (%)		
	0.01 mM PMSF	5 mM WRK	0.01 mM NBS
Enzyme without inhibitors	0	0	0
Enzyme	95.02	75.26	87.06
Enzyme + 1mg casein	74.91	66.03	84.85
Enzyme + 2mg casein	70.78	59.32	69.95
Enzyme + 3mg casein	64.06	53.22	64.09
Enzyme + 4mg casein	60.71	45.06	48.93
Enzyme + 5mg casein	52.65	40.25	43.25
Enzyme + 6mg casein	45.22	31.10	38.03
Enzyme + 7mg casein	41.73	26.27	34.06
Enzyme + 8mg casein	34.80	19.32	24.25
Enzyme + 9mg casein	31.56	12.87	16.27
Enzyme + 10mg casein	26.26	8.72	16.28

CONCLUSION

The protease isolated from *Beauveria* sp MTCC 5184 has molecular weight of 29 kDa by SDS-PAGE and 28.2 kDa by MALDI-TOF. Th PI of purified protease was 9.3. Optimum pH and temperature were 9 and 50°C respectively with stability in the pH range of 3-11 and half-life at 50°C was 2 h. K_m and V_{max} was found to be 5.1 mg and 29.67 U/ml respectively. N terminal sequence was Ala-Met-Ala-Thr-Pro-His-Val-Ala-Pro-Leu-Val-Leu-Tyr-Gly-Val-Ala which showed partial homology with subtilisin like protease. The PMF of tryptic digest of purified protease also showed partial homology with subtilisin like protease. The autocatalytic activity of purified protease (28.2 kDa) showed the 15.6 and 10.2 kDa proteins as product. The protease was a serine type protease with neither trypsin nor chymotrypsin like activities. Protease was stable in all metal ions except Fe^{3+}, Hg^{2+} and Cd^{2+} in which it showed 30-40% inhibition. It showed maximum activity against casein followed by haemoglobin and BSA. The purified protease at pH 7 has 87.76% α- helix, 0.47% β-sheet and 11.77% aperiodic structure as determined by CD spectra. Chemical modification studies showed the presence of critical amino acids serine, aspartic acid and tryptophan present at or near the active site of protease.

REFERENCES

- Agrawal, D., Patidar, P., Banerjee, T. and Patil, S. (2005). *Proc Biochem,* 40: 1131–1136.
- Anandan, D., Marmer, W. N. and Dudley, R. L. (2007). *J Ind Microbiol Biotechnol*, 34: 339–347.
- Anbu, P., Gopinath, S. C. B., Hilda, A., Lakshmipriya, T. and Annadurai, G. (2005). *Enz Microbial Technol,* 36: 639–647.
- Barbosa, J. A. R. G., Saldanha, J. W. and Garratt, R. C. (1996). *Protein Engg*, 9: 591-601.
- Basu, B. R., Banik, A. K. and Das, M. (2008). *World J Microbiol Biotechnol*, 24: 449–455.
- Bernal, C., Cairo, J. and Coello, N. (2006). *Enz Microb Technol,* 38: 49-54.
- Bidochka, M. J. and Khachatourians, G. G. (1987). *App Environ Biol,* 53: 1679-1684.
- Blow, D. M., Birktoft, J. J. and Hartley, B. S. (1969). *Nature,* 221: 337-340.
- Charles, P., Devanathan, V., Anbu, P., Ponnuswamy, M. N., Kalaichelvan, P. T., and Hur B. K. (2008). *J Basic Microbiol*, 48: 347–352.
- Chellappan, S., Jasmin, C., Basheer, S. M., Elyas, K. K., Bhat, S. G. and Chandrasekaran, M. (2006). *Proc Biochem,* 41: 956-961.
- Coleman, J. J., Rounsley, S. D., Rodriguez-Carres, M., Kuo, A., Wasmann, C. C., Grimwood, J., Schmutz, J., Taga, M., White, G. J., Zhou, S., Schwartz, D. C., Freitag, M., Ma, L. J., Danchin, E. G., Henrissat, B., Coutinho, P. M., Nelson, D. R., Straney, D., Napoli, C. A., Barker, B. M., Gribskov, M., Rep, M., Kroken, S., Molnár, I., Rensing, C., Kennell, J. C., Zamora, J., Farman, M. L., Selker, E. U., Salamov, A., Shapiro, H., Pangilinan, J., Lindquist, E., Lamers, C., Grigoriev, I. V., Geiser, D. M., Covert, S. F., Temporini, E., Vanetten, H. D. (2009). The genome of *Nectria haematococca*: contribution of supernumerary chromosomes to gene expansion. *PLoS Genet*. 5: e1000618.
- Devaraja, S., Nagaraju, S., Mahadeswaraswamy, Y. H., Girish, K. S. and Kemparaju, K. (2008). *Toxicon,* 52: 130-138.
- Dhar, P. and Kaur, G. (2010). *African J Biochem Res,* 4: 65-72.
- Dienes, D., Borjesson, J., Hagglund, P., Tjerneld, F., Liden, G., Reczey, K. and Stalbrand, H. (2007). *Enz Microb Technol,* 40: 1087–1094.

- Donatti, A. C., Furlaneto-Maia, L., Fungaro, M. H. P. and Furlaneto, M. C. (2008). *Curr Microbiol,* 56: 256–260.
- Erlacher, A., Sousa, F., Schroeder, M., Jus, S., Kokol, V., Cavaco-Paulo, A. and Guebitz, G. M. (2006). *Biotechnol Lett,* 28:703–710.
- Espagne et al. (2008). *Genome Biol,* 9: R77.
- Gote, M. M., Khan, M. I. and Khire, J. M. (2007). *Enz Microb Technol*, 40: 1312–1320.
- Gupta, R., Beg, Q. K., Khan, S. and Chauhan, B. (2002). *App Microbiol Biotechnol*, 59: 15-32.
- Hajji, M., Kanoun, S., Nasri, M. and Gharsallah, N. J. (2007). *Proc Biochem*, 42: 791-797.
- Hattori, M., Isomura, S., Yokoyama, E., Ujita, M., and Hara, A. (2005). *J Biosci Bioengg,* 100: 631-636.
- Jarai, G., Kirchherr, D. and Buxton, F. P. (1994). *Gene*, 139: 51-57.
- Johnvesly, B. and Naik, G. R. (2001). *Proc Biochem*, 37: 139-44.
- Kotlova, E. K., Ivanova, N. M., Yusupova, M. P., Voyushina, T. L., Ivanushkina, N. E. and Chestukhina, G. G. (2007). *Biochem (Moscow*), 72: 117-123.
- Kudryavtseva, N. N., Gvozdeva, E. L., Sofin, A. V. and Valueva, T. A. (2010). *App Biochem Microbiol,* 46: 324–330.
- Kumar, C. G. (2002). *Lett Appl Microbiol*, 34: 13-17.
- Laemmli, U. K. (1970). *Nature* 227: 680-685.
- Lee, J. K., Kim, Y. O., Kim, H. K., Park, Y. S. and Oh, T. K. (1996). *Biosci Biotechnol Biochem*, 60: 840-46.
- Li, H. P., Hu, Z., Yuan, J. I., Fan, H. D., Chen, W., Wang, S. J., Zheng, S. S, Zheng, Z. L. and Zou, G. L. (2007). *Phytother Res*, 21: 1234–1241.
- Liang, T. W., Lin, J. J., Yen, Y. H., Wang, C. L. and Wang, S. L. (2006). *Enz Microbial Technol*, 38: 74–80.
- Lucas et al., Unpublished
- Ma, C., Ni, X., Chi, Z., Ma, L. and Gao, L. (2007). *Marine Biotechnol*, 9: 343–351.
- Moallaei, H., Zaini, F., Larcher, G., Beucher, B. and Jean-Philippe Bouchara, J. P. (2006). *Mycopathologia,* 161: 369–375.

- Nakanishi, T. and Yamamoto, T. (1974). *Agric. Biol. Chem.* 38: 2391.
- Page, M. J. and Di Cera, E. (2008). *Cell Mol Life Sci*, 65: 1220 – 1236.
- Palmieri, G., Bianco, C., Cennamo, G., Giardina, P., Marino, G., Monti, M. and Sannia, G. (2001). *Appl Environ Microbiol*, 67: 2754-2759.
- Pappin, D. J. C. (1997). Peptide mass fingerprinting using MALDI-TOF mass spectrometry. Methods in Molecular Biology, vol. 211: Protein seqeuencing protocols, 2nd ed. Edited by: Smith, B. J. Humana Press Inc., Totowa, NJ pp. 211-219.
- Pappin, D. J. C., Hojrup, P. and Bleasby, A. J. (1993). *Current Biol*, 3: 327-332.
- Pekkarinen, A. I., Jones, B. L. and Niku-Paavola, M. L. (2002). *Eur J Biochem,* 269: 798-807.
- Pel et al. (2007). *Nature Biotechnol*, 25: 221-231.
- Pena-Montes, C., González, A., Castro-Ochoa, D. and Farres, A. (2008). *Appl Microbiol Biotechnol,* 78: 603–612.
- Phadtare, S., Rao, M. and Deshpande, V. (1997). *Arch Microbiol,* 166: 414-417.
- Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). *Microbiol Mol Biol Rev,* 62: 597-635.
- Rattray, F. P., Bockelmann, W., Fox, P. F. (1995). *Appl Environ Microbiol,* 61: 3454-3456.
- Rhaman, R. N. Z., Razak, C. N., Ampon, K., Basri, M., Yunus, W. and Saleh, A. B. (1994). *Appl. Microbiol. Biotechnol.* 40: 822-827.
- Sheng, J., An, K., Deng, C., Li, W., Bao, X . and Qiu, D. (2006). *Current Micobiol*, 53: 124–128.
- Siddiqui, K. S., Parkin, D. M., Curmi, P. M. G., De Francisci, D., Poljak, A., Barrow, K., Noble, M. H., Trewhella, J. and Cavicchioli, R. (2009). *Biotechnol Bioengg*, 103: 676-686.
- Spelzini, D., Farruggia, B. and Pico, G. (2005). *J Chromatography B*, 821: 60–66.
- Sreedhar, L., Kobayashi, D. Y., Bunting, T. Y., Hillman, B. I. and Belanger, F. C. (1999). *Gene,* 235: 121–129.

- Steele, D. B., Fiske, M. J., Steele, B. P. and Kelley, V. C. (1992). *Enz Microb Technol*, 14: 358-360.
- Sutar, I. I., Srinivasan, M. C., and Vartak, H. G. (1991). *Lett* 13: 119-124.
- Suzuki, N., Nishibori, K., Oodaira, Y., Kitamura, S., Michigami, K., Nagata, K., Tatara, Y., Lee, Y. R. and Ichishima, E. (2005). *Phytochem*, 66: 983–990.
- Tremacoldi, C. R., Monti, R. and Selistre-de-Arurjo, H. S. (2007). *World J Microbiol Biotechnol,* 23:295-299.
- Tsuchiya, K., Arai, T., Seki, K. and Kimura, T. (1987). *Agric Bio Chem,* 51: 2959-2965.
- Ueda, M., Kubo, T., Miyatake, K. and Nakamura, T. (2007). *Appl Microbiol Biotechnol,* 74: 331–338.
- Urtz, B.E. and Rice, W.C. (2000). *Mycological Res,* 104: 180-186.
- Wang, B., Wu, W. and Liu, Z. (2007). *Mycopathologia,* 163: 169–176.
- Wang, R. B., Yang, J. K., Lin, C., Zhang, Y. and Zhang, K. Q. (2006). *Lett Appl Microbiol*, 42: 589–594.
- Wang, S. L., Chen, Y. H., Wang, C. L., Yen, Y. H. and Chern, M. K. (2005). *Enz Microbial Technol*, 36: 660–665.
- Yadav, S. C., Jagannadham, M. V., Kundu, S., Jagannadham, M. V. (2010). *Biophysical Chem,* 139: 13–23.
- Zibaee, A. and Bandani, A.R., (2009). *Biocont Sci Technol,* 19: 797-808.
- Zhao, M. L., Huang, J. S., Mo, M. H. and Zhang, K. Q. (2005). *Fungal Diversity,* 19: 217-234.

CHAPTER 4

STABILITY OF PROTEASE IN ORGANIC SOLVENTS, DETERGENTS AND DENATURANTS

ABSTRACT

The protease from *Beauveria* sp MTCC 5184 was stable in presence of organic solvents, denaturants and commercial detergents. The stability of protease in organic solvents indicates its suitability for application in the peptide synthesis and in organic solvent based reactions. The protease was highly stable in the presence of 10% BME, 6 M urea and 3 M NaCl. The residual activity of 80% was found in case of 2% Triton-X-100. Besides organic solvents and denaturants protease was also stable in presence of commercial detergents and SDS. It retained residual activity of more that 90% at 30°C for 30 min in the presence of commercial detergents which indicates the suitability of enzyme for its use as detergent additives.

.

INTRODUCTION

Proteases catalyze the hydrolysis of protein in aqueous solution whereas it catalyzes peptide synthesis in reverse direction in organic solvents or in solvents with low water content. The use of proteases in peptide synthesis has numerous advantages over chemical synthesis, e.g. regio- and stereo-selectivity, absence of racemization, lack of requirement of side-chain protection and milder non-hazardous reaction conditions. In recent years proteases have received great attention due to its application in production of certain oligo-peptide as a viable alternative to chemical synthesis (Wang et al., 2008). The use of proteases in peptide synthesis is limited because reactions occurred in organic media and proteases are less specific and less stable in organic solvents (Ghorbel et al., 2003). The rate of peptide synthesis is low in the presence of organic solvents because of denaturation or inactivation of the enzymes. Therefore, it is necessary to search for proteases, which are naturally stable in the presence of organic solvents used in the synthetic reaction. Fagain, (2003) has reviewed the different aspects for the stability of protein. 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). The proteases stable in organic solvent have been reported from microorganisms, plants and animals. The effect of organic solvent on the stability of proteases have been reported for α-chymotrypsin, thermolysin, subtilisin CLECs (Fernandes et al., 2005), subtilisin BPN (Sears et al., 1999), aspartic proteinase, Cardosin A (Sarmento et al., 2003), phytoprotease such as araujiain, funastrain and papain (Barberis et al., 2006). The protease from *Pseudomonas aeruginosa* PST-01 stable in organic solvent and has been extensively studied (Ogino et al., 1999; 2000; 2001; 2007). Gupta and Khare, (2009) have reviewed the organic solvent stable proteases from bacterial origin whereas few reports are available on proteases from fungi, such as *Beauveria brongniartii* (Erlacher et al., 2006), *Aspergillus nidulans* (Pena-Montes et al., 2008).

The present chapter deals with the fungal alkaline protease stable in organic solvents as well as in detergents (commercial) and denaturants such as β-mercaptoethanol (BME), Urea, NaCl and Triton-X.

MATERIALS AND METHODS

Acetone, 1-butanol, benzene, chloroform, dimethylsulphoxide (DMSO), ethanol, hexane, isopropanol, methanol and β-mercapto ethanol (BME) were obtained from Qualigens, India. NaCl and Urea from s.d fine chemical pvt Ltd. SDS was purchased from Sigma Chemical Company (USA). Triton X-100 from Loba cheime. Commercial detergents were obtained from local market.

Effect of Organic Solvents on Protease Stability

The purified protease was incubated with water missible and water immiscible organic solvent at 28°C and 50°C. Following organic solvents were used: acetone, 1-butanol, benzene, chloroform, dimethylsulphoxide, ethanol, hexane, isopropanol and methanol. Purified protease was incubated with 25% (v/v) organic solvents (effective concentration) at 28°C, pH 7. Samples were removed at different time intervals and residual activity was estimated. Sample without organic solvent served as control. Initial activity with respective solvents was taken as 100%.

Effect of BME and Triton X-100 on Protease Activity

To study the effect of BME and Triton X-100, 10 μM of purified protease in 50 mM phosphate buffer pH 7 was pre-incubated with 1 to 10% (v/v) BME and 0.2 to 2% Triton X-100 at 28°C for 30 min. Residual activity was measured at 50°C, pH 9 and expressed as percentage of activity without any denaturants (control) taken as 100%.

Effect of Urea and NaCl on Protease Activity

Stability was checked under two sets of conditions, a) 10 μM of purified protease in 50 mM phosphate buffer pH 7 was pre-incubated with 1 to 6 M urea and 1 to 3 M NaCl at 28°C for 30 min, b) 10 μM of purified protease in 50 mM phosphate buffer pH 7 was pre-incubated with 6 M urea and 3 M NaCl at 28°C up to 2 h. Samples were removed at regular intervals and residual activity was measured at 50°C, pH 9 and expressed as percentage of activity without urea and NaCl taken as 100%.

Effect of Commercial Detergents on Protease Stability

For detergent stability purified protease was incubated with SDS and commercial detergents with a final concentration 0.7 mg/ml at 30°C for 30 min. Detergents were heated at 100°C for 10 min before use. Samples were removed after 30 min and

residual activity was estimated at pH 9 and 50° C and expressed as percentage of initial activity with respective detergents taken as 100%.

RESULTS AND DISCUSSION

The physiological parameters of organic solvents are presented in Table 4.1. The effects of organic solvents (25%, v/v) on the stability of the purified proteases were studied at 28°C and 50°C. The purified protease was incubated with 25% (v/v) organic solvents and the residual activity was determined under normal assay conditions.

Table 4.1: Physicochemical parameter of the organic solvents

Solvent	Formula	BP (°C)	MP (°C)	Density (g/cm)	Dielectric constant	Relative polarity
Water	H_2O	100	0.00	1.00	78.54	1.000
Acetone	C_3H_6O	56.2	-46	0.786	20.7	0.355
Ethanol	C_2H_6O	78.5	-114.1	0.789	24.6	0.654
Isopropanol	C_3H_8O	82.4	-88.5	0.785	18.3	0.546
Methanol	CH_4O	64.6	-98	0.791	32.6	0.762
Benzene	C_6H_6	80.1	5.5	0.879	2.28	0.111
1-Butanol	$C_4H_{10}O$	117.6	-89.5	0.81	17.8	0.602
Chloroform	$CHCl_3$	61.7	-63.7	1.498	4.81	0.259
DMSO	C_2H_6OS	189	18.4	1.092	47	0.444
Hexane	C_6H_{14}	69	-95	0.659	1.89	0.009

(http://www.erowid.org/psychoactives/chemistry/chemistry_info5.shtml)
(http://www2.onu.edu/~b-myers/organic_solvents.html)

Effect of Organic Solvent on Purified Protease

At 28°C the protease from *Beauveria* sp MTCC 5184 was highly stable in presence of organic solvents tested. More than 80% activity was retained in ethanol, isopropanol, methanol, butanol and DMSO whereas less than 30% residual activity was retained in presence of acetone, benzene, chloroform and hexane after 24 h. Control without solvent showed around 26.66% residual activity under identical conditions (Table 4.2).

At 50°C the protease was highly stable up to 1 h in presence of all the organic solvents tested with residual activities ranging from 60 to 90%. After 2 h, the stability

was highest in acetone and DMSO with residual activities of around 70% while in all other solvents, residual activity was around 30-40% (Table 4.3).

Table 4.2: Stability of protease in presence of organic solvents at 28°C

Type of the solvent	Solvent	Residual Activity (%)		
		0h	24 h	48 h
Control	None	100	26.66	14.26
Water miscible	Acetone	100	23.06	18.19
	Ethanol	100	81.60	67.88
	Isopropanol	100	84.18	61.15
	Methanol	100	91.10	68.14
Water immiscible	Benzene	100	30.47	13.32
	Butanol	100	82.73	78.50
	Chloroform	100	24.66	13.83
	DMSO	100	93.11	75.28
	Hexane	100	25.54	11.02

Table 4.3: Stability of protease in presence of organic solvents at 50°C

Type of the solvent	Solvent	Residual Activity (%)		
		0h	1h	2h
Control	Nil	100.00	69.03	35.87
Water miscible	Acetone	100.00	99.07	70.45
	Ethanol	100.00	98.50	45.21
	Isopropanol	100.00	71.26	46.16
	Methanol	100.00	93.49	46.09
Water immiscible	Benzene	100.00	61.85	40.66
	Butanol	100.00	82.07	46.38
	Chloroform	100.00	77.14	38.55
	DMSO	100.00	87.52	72.14
	Hexane	100.00	76.18	29.43

Among the organic solvents used in the study DMSO and methanol were found to be best for the stability of protease. Enzymes, in general, get denatured or

give very low rates of reaction in solvent media because of the unfolding, structural disfunctioning and stripping of the essential water layer from the enzyme molecule. The presence of organic solvents alters the catalytic process of enzyme by disruption of hydrogen bonds and hydrophobic interactions as well as changes in the dynamics and conformation of the protein (Barberis et al 2006). The performance of enzyme in organic solvents can be attributed to many factors. The protein-solvent interactions favor exclusion of the solvent molecules from the enzyme hydration layer resulting in compaction of the enzyme because of that unfavorable surface energy rise which stabilizes the enzyme (Sellek & Chaudhuri, 1999). Gupta and Khare (2007) have found protease from *Pseudomonas aeruginosa* PseA was more stable in organic solvents benzene, cyclohexane, isooctane and *n*-dodecane and was comparatively less stable in the presence of hydrophilic solvent whereas Ogino et al (1999) have reported protease from *Pseudomonas aeruginosa* PST-01 was more stable in water-soluble organic solvent. Barberis et al (2002) have reported cysteine protease morrenain b II from *Morrenia brachystephana* (*Asclepiadaceae*) was stable in organic solvent which had application in peptide synthesis by using PheOMe and Asp as substrate. Castillo et al (2005) have studied the effect of organic solvent on the stability of α-chymotrypsin and pig-liver esterase and found that these enzymes were inactivated in pure organic solvents. Many workers have correlated the stability of protease in organic solvent with reference to their properties such as dielectric constant, water solubility, Hildebrand solubility, three dimensional solubility parameter spaces and log *P* (Barberis et al 2002). Goek et al (2003) have reported that protease was inhibited and less stable in the organic solvent with log Po/w value between 1.5-3.5 and more stable in organic solvent with log Po/w value between 4-8.8 as compare to control (without any organic solvent), whereas in some cases the relation between stability of protease in organic solvent and solvent polarity (log *P* value) was not found (Ogino et al 1999; Gupta et al 2005; Rahman et al., 2006). It can be concluded that organic solvent stability of the enzyme depends on the nature of organic solvents. This means that replacement of some water molecules in an enzyme with organic molecules sometimes stabilizes the structure of the enzyme. Ogino et al (2007) have substituted the amino acid residues by side directed mutagenesis and found that the amino acid residues located at the surface of the protein molecule played an important role for the stability of enzyme in the organic solvents.

Effect of Denaturants and Salts on Protease Activity

The protease from *Beauveria* sp MTCC 5184 retained full activity in 10% BME at 28°C (Figure 4.1) indicating the absence of disulphide bonds in the protease. The protease retained more than 80% residual activity in presence of 2% Triton X-100 (Figure 4.2). It was stable in presence of urea and NaCl and showed slight increase in stability with increase in concentration of urea and NaCl (Figure 4.). In presence of 6 M urea and 3 M NaCl, protease was stable up to 2 h and retained more than 85% and 75% of initial activity respectively whereas in control without denaturants the residual activity was less than 70% (Figure 4.4).

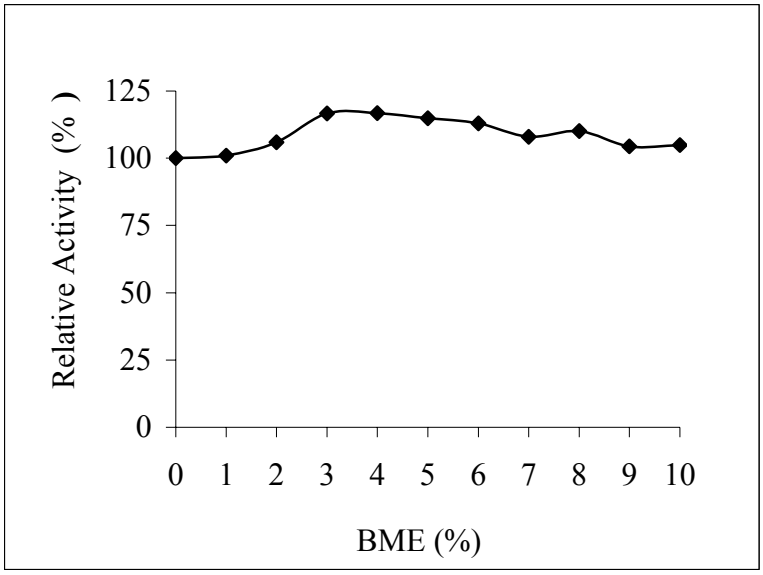


Figure 4.1: Stability in β-mercaptoethanol (0-10 % v/v)

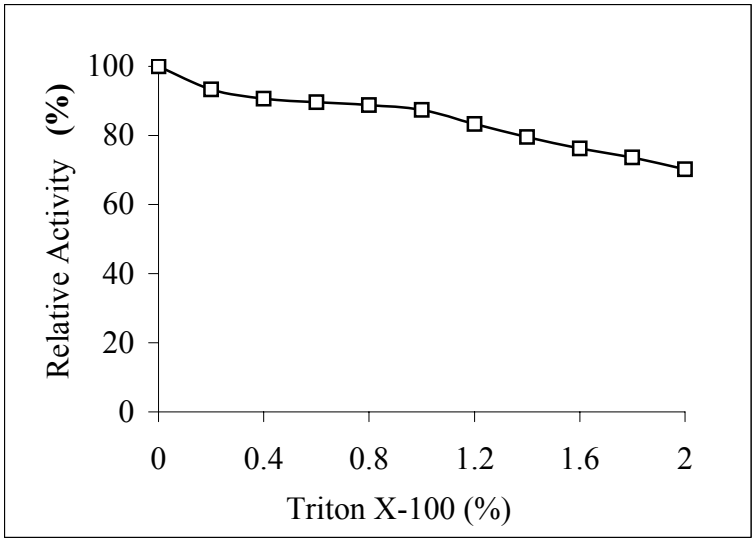


Figure 4.2: Effect of detergents (0.2- 2%)

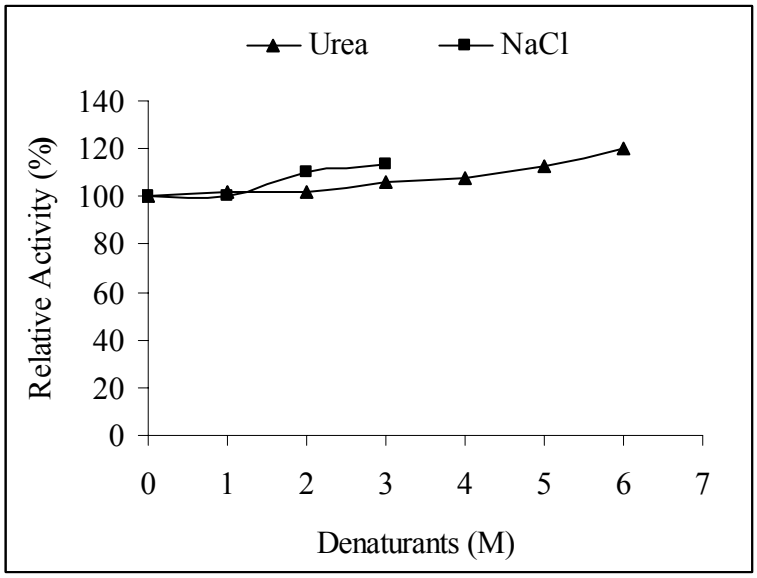


Figure 4.3: Stability of protease in presence of urea and NaCl

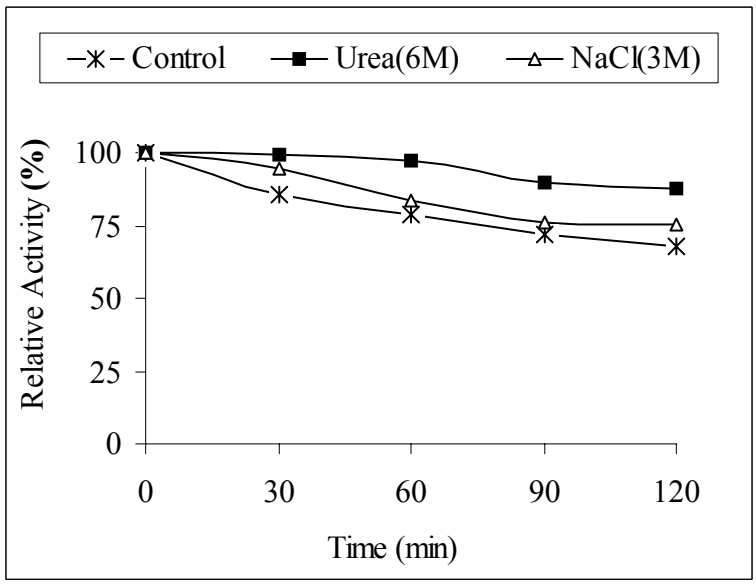


Figure 4.4: Stability in 6 M urea and 3 M NaCl.

Gupta et al. (2005) have reported an organic solvent stable protease from *Pseudomonas aeruginosa* PseA that was also stable in urea (1-5 mM), Triton X (0.1-0.5%) and BME (1-5 mM). Ruiz and Castro (2006) have studied the stability of protease in organic solvent containing different concentration of NaCl and found that high salt concentration (1.5 M NaCl) in different organic solvent increased the stability of protease. Hajji et al. (2007) have reported protease from *Aspergillus*

clavatus ES1 stable in the presence of the non-ionic surfactants like Tween 80 and Triton X-100 for 1 h at 4°C.

Stability of Protease in Commercial Detergents

The application of protease in detergent formulation requires that the protease/ enzyme to be stable in high pH values in the presence of detergents. The protease from *Beauveria* sp MTCC 5184 was found to be stable and compatible in presence of the locally available detergents (Ariel, Rin, Surf Excel and Tide) and SDS. The protease showed more than 90% residual activity as compared to initial activity in respective detergents after 30 min of incubation at 30°C. As the protease produced by the isolate *Beauveria* sp was stable over a wide range of pH values, temperatures, broader substrate specificity and also showed compatibility with various commercial detergents tested, it can be used as an additive component for the detergent, in order to improve the washing performance of heavy-duty laundry detergents.

Table 4.4: Stability in presence of commercial detergents

Detergents (0.7 mg/ml)	Residual activity (%) after 30 min
None (control)	91.20
Ariel	96.10
Rin	98.57
SDS	92.65
Surf excel	94.26
Tide	93.34

Phadtare et al. (1993) have reported protease from *Conidiobolus coronatus* stable and compatable in presence of commercial detergents. Since very few reports are available on the use of fungal proteases in detergent industry, therefore there is a growing need to exploit fungal proteases for commercial exploitation in detergent industry. Hajji et al. (2007) have reported protease from *Aspergillus clavatus* ES1 stable in the presence of 0.1% SDS and retained more than 90% activity compared to control (without SDS) when incubated at 4°C for 1 h. Gupta et al. (2008) have reported protease from *Virgibacillus pantothenticus* (MTCC 6729) stable in presence of the locally available detergents. The protease showed more than 50% activity after

3 h of incubation at 40°C. Sindhu et al. (2009) have reported alkaline protease from *Penicillium godlewskii* and found to be suitable for application in detergent industry. The addition of proteases to detergents considerably increases the cleaning effect by removing protein stains and the consumption of surface active substances, thereby decreasing the pollution load (Esposito et al., 2010).

CONCLUSIONS

Till date few reports are available on the purification of solvent stable proteases from fungal sources. In the present study we have reported the stability of purified protease in organic solvent as well as in salt, denaturants and detergents. Due to the stability of the purified alkaline protease from *Beauveria* sp MTCC 5184 in organic solvent it can be used to catalyze peptide synthesis reactions. The Protease has high optimal pH and thermal stability, which are characteristics of the protease used in detergent formulations.

REFERENCES

- Barberis, S., Quiroga, E., Arribére, M. C. and Priolo, N. (2002). *J Mol Catal B: Enz,* 17: 39–47.
- Barberis, S., Quiroga, E., Morcelle, S., Priolo, N. and Luco, J. M. (2006). *J Mol Catal B: Enz,* 38: 95–103.
- Castillo, B., Pacheco, Y., Al-Azzama, W., Griebenowa, K., Devi, M., Ferrer, A. and Barletta, G. (2005). *J Mol Catal B: Enz,* 35: 147–153.
- Erlacher, A., Sousa, F., Schroeder, M., Jus, S., Kokol, V., Cavaco-Paulo, A. and Guebitz, G. M. (2006). *Biotechnol Lett,* 28: 703–710.
- Esposito, T. S., Marcuschi, M., Amatal, I. P. G., Carvalho, L. B. and Bezerra, R. S. (2010). *J Agric Food Chem,* 58: 6433–6439.
- Fagain, C. O. (2003). *Enz Microb Technol,* 33: 137-149.
- Fernandes, J. F. A., McAlpine, M. and Halling, P. J. (2005). *Biochemic Eng J,* 24: 11–15.
- Geok, L. P., Razak, C. N. A., Rahman, R. N. Z. A., Basri, M. and Salleh, A. B. (2003). *Biochemic Eng J,* 13: 73–77.
- Ghorbel, B., Kamoun, A. S. and Nasri, M. (2003). *Enz Microb Technol,* 32: 513–518.
- Gupta, A. and Khare, S. K. (2007). *Enz Microb Technol,* 42: 11–16.
- Gupta, A. and Khare, S. K. (2009). *Critical Rev Biotechnol*, 29: 44–54.
- Gupta, A., Roy, I., Khare, S. K. and Gupta, M. N. (2005). *J Chromatogr A,* 1069: 155–161.
- Gupta, A., Joseph, B., Mani, A. and Thomas, G. (2008). *World J Microbiol Biotechnol*, 24: 237–243.
- Hajji, M., Kanoun, S. Nasri, M. and Gharshallah, N. (2007). *Proc Biochem,* 42: 791-797.
- http://www.erowid.org/psychoactives/chemistry/chemistry_info5.shtml.
- http://www2.onu.edu/~b-myers/organic_solvents.html.
- Ogino, H., Uchiho, T., Doukyu, N., Yasuda, M., Ishimi, K. and Ishikawa, H. (2007). *Biochemic Biophy Res Comm,* 358: 1028–1033.
- Ogino, H., Uchiho, T., Yokoo, J., Kobayashi, R., Ichise, R. and Ishikawa, H. (2001). *Appl Environ Microbiol,* 67: 942–947.

- Ogino, H., Yokoo, J., Watanabe, F. and Ishikawa, H. (2000). *Biochem Eng J,* 5: 191-200.
- Ogino, H., Watanabe, F., Yamada, M., Nakagawa, S., Hirose, T., Noguchi, A., Yasuda, M. and Ishikawa, H. (1999). *J Biosci Bioeng,* 87: 61–68.
- Pena-Montes, C., González, A., Castro-Ochoa, D. and Farres, A. (2008). *Appl Microbiol Biotechnol,* 78: 603–612.
- Phadatare, S. U., Srinivasan, M. C. and Deshpande, V. V. (1993). *Enzy Microb Technol,* 15: 72–76.
- Rahman, R. N. Z. A., Geok, L. P., Basri, M. and Salleh, A. B. (2006). *Enzy Microb Technol,* 39: 1484–1491.
- Ruiz, D. M. and De Castro, R. E. (2006). Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon *Natrialba magadii. J Indust Micro Biotechnol*, 10.1007/s10295-006-0174-4.
- Sarmento, A. C., Oliveira, C., Pires, E., Halling, P. and Barros, M. (2003). *J Mol Catal B: Enz,* 21: 19-23.
- Sears, P., Witte, K. and Wong, C. H. (1999*). J Mol Catal B: Enz,* 6: 297-304.
- Sellek, G. A. and Chaudhuri, J. B. (1999). *Enz Microb Technol,* 25: 471-482.
- Sindhu, R., Suprabha, G. N. and Shashidhar, S. (2009). *Afr J Microbiol Res,* 3: 515-522.
- Wang, S. L., Yang, C. H., Liang, T. W. and Yen, Y. H. (2008). *Bioresour Technol*, 99: 3700-3707.

CHAPTER 5

APPLICATIONS OF PROTEASE

SECTION A

APPLICATION OF PURIFIED PROTEASE IN ANIMAL TISSUE CULTURE

ABSTRACT

Alkaline protease from *Beauveria* sp MTCC 5184 has been tested as a substitute for trypsin in animal cell culture. The protease separated cells within 2 min at 37°C and pH 7. The separated cells were floating in the medium whereas the cells which were not separated adhered to the bottom of the plate. The separated cells were viable.

INTRODUCTION

Cells are microscopic structure filled with an aqueous medium and enclosed in a semi-permeable plasma membrane. They combine in millions in orderly fashion to form whole organism. The building processes of animals and plants are different, and each type of organism is formed of many types of tissues, in which the cells are assembled and bound together in different ways. In both animals and plants, however, an essential part is played in most tissues by the extracellular matrix. This complex network of secreted extracellular macromolecules has many functions, the first and foremost being, it forms a supporting framework. It helps in holding the cells and tissues together, and in animals, it provides an organized environment within which migratory cells can move and interact with one another in orderly ways. In connective tissue, the extracellular matrix is plentiful and cells are sparsely distributed within it. Two main classes of extracellular macromolecules make up the matrix: (1) polysaccharide chains of the class called glycosaminoglycans (GAGs), which are covalently linked to protein in the form of proteoglycans, and (2) fibrous proteins, including collagen, elastin, fibronectin, and laminin, which have both structural and adhesive functions. The proteoglycan molecules in connective tissue form a highly hydrated, gel-like "ground substance" in which the fibrous proteins are embedded. The polysaccharide gel resists compressive forces on the matrix while permitting the rapid diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells. The collagen fibers help in both strengthening and organizing the matrix. The rubber like elastin fibers give it resilience and many matrix proteins help the cells to attach in appropriate locations (Albert et al., 2002).

Trypsin, a protease of animal origin, is widely employed in animal cell culture. This section of the chapter deals with the possibility of replacing trypsin by the alkaline protease from *Beauveria* sp MTCC 5184 in the animal tissue culture for the separation of cells.

MATERIALS AND METHODS

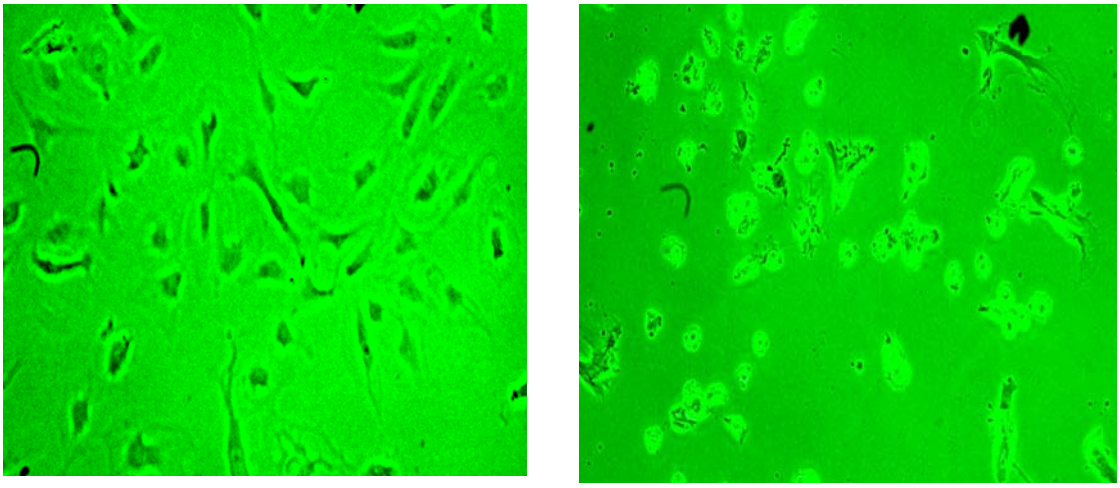
Cells and tissues: Mammalian Endothelial cell lines were maintained on minimum endothelial cell basal medium -2 (EBM-2).

Protease enzyme: Purified alkaline protease was prepared from *Beauveria sp* MTCC 5184 as described in chapter 2.

Dissociation of cells and tissues: The mammalian endothelial cells were grown on endothelial cells basal medium-2 (EBM-2) medium. After 48 h of growth, media was removed from plate in a falcan tube. The adherent cells at the bottom of the plate were washed with 2 ml of 1 mM EDTA solution to remove the floating cells and dead cells. 200 μg of protease in 4 ml of 1 mM EDTA solution was added in the plate and incubated at 37 °C for 2 min and observed under microscope. Cells were flushed out, collected in fresh falcan tube and spinned at 2000 rpm for 2 min. The supernatant was removed and cell pellet was re-suspended in 1 ml fresh EBM-2 medium and transferred to new petri plate.

RESULTS AND DISCUSSION

The proteolytic activity of protease from *Beauveria* sp was optimum at pH 9 and 50°C, to which animal cells cannot be exposed. Hence the experiment was carried out at neutral pH and 37°C. Microscopic observation of the endothelial cells treated with purified protease showed that the cells were separated. The separated cells were found to be floating in the media whereas the cells which were not separated were attached at the bottom of the plate (Figure 5.1a & b). The separated endothelial cells were plated in fresh EBM-2 medium and found viable. Thus the purified protease can substitute trypsin in animal cell culture for separation of cells. The cells get separated because of the action of protease that destrot proteinaceous connection between cells and the surface of the flask/plate in which they were grown. Harvesting of cells using protease results in the release of single cells, which is ideal for subculturing as each cells will then divide and grow. Protease is commonly used in combination of EDTA, which enhance the action of protease because EDTA chelates the Ca^{2+} required by some adhesion molecules that facilitate cell-cell or cell-matrix interaction.



(a) (b)

Figure 5.1: Mammalian endothelial cells (a) before protease treatment (b) after protease treatment.

Some of the protease other than trypsin found application in animal tissue culture experiments. A neutral protease from *Bacillus polymyxa* ATCC 21993 found application in animal tissue culture (Iryie, 1976). The neutral protease (Dispase) isolated from *Bacillus polymyxa* effectively seperated intact epidermis from the

dermis and intact epithelial sheets in culture from the substratum (Stenn et al., 1989). Alkaline proteases from *Conidiobolus* sp. were also able to act as a substitute for trypsin used in the preparation of animal cell cultures (Chiplonkar et al., 1985).

In trypsinization of animal tissues, as the name indicates trypsin is widely used in this process. However trypsin is extracted from the pancreas (mainly collected from slaughter houses), and which is subjected to variations especially in proportions of chymotrypsin and trypsin. A uniform enzyme can be produced from *Beauveria* sp in unlimited quantities under controlled conditions to perform the same application.

CONCLUSION

An alkaline protease from *Beauveria* sp can efficiently separate mammalian endothelial cells without affecting the cell viability by degrading the inter-cellular matrix, which adhere the cells together. Therefore alkaline protease from *Beauveria* sp can be exploited in animal tissue culture for separation of cells instead of trypsin.

REFERENCES

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (2003). Cell junctions, cell adhesion, and the extracellular matrix. Inn: Molecular Biology of the Cell, pp 1752- 1859.
- Chiplonkar, J. M., Gangodkar, S. V., Wagh, U. V., Ghadge, G. D., Rele, M. V. and Srinivasan, M. C. (1985). *Biotechnol Lett*, 7: 665–68.
- Iryie, Y. (1976). Neutral protease useful for animal tissue and cell culture. US Patent 3930954.
- Stenn, K. S., Link, R., Moellmann, G., Madri, J. and Kuklinska, E. (1989). *J Investigative Dermatol*, 93: 287–290.

SECTION B

BIOSYNTHESIS OF GOLD AND SILVER NANOPARTICLES

ABSTRACT

The preparation and characterization of gold and silver nanoparticles are discussed in a great number of publications. The use of microorganisms in the synthesis of nanoparticles emerges as an eco-friendly and exciting approach. Gold nanoparticles were synthesized by incubating crude culture filtrate with gold salt (AuCl) at 28°C on rotatory shaker. Silver nanoparticle was synthesized by incubating purified protease from *Beauveria* sp with waste X-ray film at 28°C on rotatory shaker. The nanoparticles synthesized were characterized by UV-vis spectroscopy, X-ray diffraction (XRD), Transmission electron microscopy (TEM), selected area electron diffraction (SAED), Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectrometer (XPS). The nanoparticles are synthesized as single particle or in aggregates. The nanoparticles formed were of different size and shape. The size of gold and silver nanoparticles was 1-1.5μm and 200-600 nm respectively. XRD spectra showed that nanoparticles formed were crystalline in nature. One type of plane was formed in the case of gold nanoparticle whereas four types of plane were formed in silver nanoparticle. XPS data revealed that the nanoparticles are in metallic form

INTRODUCTION

Nanotechnology can be defined as the science and engineering involved in the design, synthesis, characterization, and application of materials and devices whose smallest functional organization in atleast one dimension is on the nanometer scale or one billionth of a meter (Silva, 2004). The term "nano" is taken from a Greek word "nanos", which means "little old man" or "dwarf". At the nanoscale dimensions, the material properties change significantly differing completely from their bulk counterparts. As the size of material decreases, the percentage of surface atoms increases, thus increasing the reactivity and making them highly reactive catalysts. Among the surface atoms, atoms sitting on the edges and corners are more reactive than those in planes. Also, the percentage of atoms at the edges and corners increases with decrease in the particle size and therefore, smaller metal particles are preferred for catalysis (Klabunde, 2000). Methods for the synthesis of nanoparticles are mainly classified into: "top down" and "bottom up" approaches and combinations thereof (Silva, 2004).

Top down: Top down techniques begin with a macroscopic material or group of materials and incorporate smaller-scale details into them. The best known example of a "top down" approach is the photolithography technique used by the semiconductor industry to create integrated circuits by etching patterns in silicon wafers.

Bottom up: Bottom up approaches, on the other hand, begin by designing and synthesizing custom-made molecules that have the ability to self-assemble or self-organize into higher order mesoscale and macroscale structures.

In the bottom up approach, the synthesis protocols can be further divided into physical methods, chemical methods and biological methods. Various physical methods have been successfully employed for nanomaterial synthesis such as solvated metal atom dispersion (Stoeva et al., 2002), radiolysis (Doudna et al., 2003), thermal decomposition (Hou et al., 2004), spray pyrolysis (Suh and Suslick, 2005), ultrasonication (Pol et al., 2005), vapor deposition (Perekrestov, 2005), laser ablation (Zhu et al., 2006) and photoirradiation (Sakamoto et al., 2006). However, chemical methods have several advantages over physical methods; therefore chemical methods are widely accepted for nanomaterials synthesis. Nanomaterials such as metals, metal oxides and semiconductor nanoparticles can be synthesized by chemical methods by

reduction or oxidation of metal ions. Although, above-mentioned synthetic procedures result in good control over shape, size and crystallinity, they very often involve the use of hazardous reagents, volatile solvents and intense physico-chemical conditions. Therefore, currently researchers are more interested towards development of environmentally benign procedures for nanoparticle synthesis that do not use toxic chemicals. As a result, researchers have been focusing their attention towards the biological processes for nanomaterial synthesis as it operates at environmentally benign conditions. Synthesis of nanoparticles using biological entities has great interest due to their unusual optical, chemical, photoelectrochemical and electronic properties (Chandrasekharan and Kamat, 2000; Peto et al., 2002; Krolikowska et al., 2003; Kumar et al., 2003). During the process of nanoparticle synthesis, the biomolecules secreted by microorganisms restrict the growth of particles over nanometer dimensions and thus act as capping/stabilizing agent. Biological methods for nanomaterials synthesis involve either microorganisms or biomolecules. Among the microorganisms bacteria and yeasts are well known for biosynthesis of nanoparticles. Some well-known examples of bacteria synthesizing nanoparticle include magnetotactic bacteria (magnetite nanoparticles), diatoms (siliceous materials) and Slayer bacteria (gypsum and calcium carbonate layers) (Bharde et al., 2006). The fungi also used for nanoparticle synthesis are *Fusarium* oxysporum and *Verticillium (*Mukherjee et al., 2002; Ahmad et al., 2003, Senapati et al., 2004). In addition to the microbial synthesis of nanomaterial, extracts from various parts of plants have also been employed for gold and silver nanoparticle synthesis (Shankar et al., 2003; 2004; 2005).

Nanotechnology has created a kind of revolution as this new area encompasses physics, chemistry, materials science and engineering and also biology and medicine. Several applications of nanoparticles are in the field of non-linear optics devices (Maier et al., 2001), sensors (Caski et al., 2002), catalysis (Roucoux et al., 2002), drug/gene delivery vehicles (Otsuka et al., 2003), surface enhanced raman spectroscopy (Li et al., 2004), plasmonics devices (Maier et al., 2005), therapeutic agents (Liu et al., 2005), fuel cells (Fichtner, 2005), cosmetics (Cengiz et al., 2006), coating materials (Baglioni and Giorgi, 2006), diagnostic tools (Lawrie et al., 2006) and solar cells (Yae et al., 2007). Gold and silver nanoparticles show interesting electronic and structural properties and can be used in biomedical applications.

This section of the chapter describes the synthesis of gold nanoparticles by using crude culture filtrate and silver nanoparticle by using purified protease of *Beauveria* sp MTCC 5184. Characterization of nanoparticles synthesized are also described.

MATERIAL AND METHODS

Protease Production and Protease Assay

Protease was produced and purified as described in chapter 2. Protease assay was done according to Laxman et al. (2005).

Biosynthesis of Gold Nanoparticle

For synthesis of gold nanoparticles, 1 ml of 10^{-2} M AuCl was added to 100 ml of crude culture filtrate having protease activity of 0.9 U/ml in a 500 ml Erlenmeyer flask and agitated at 28°C, 200 rpm. Periodically, aliquots of the reaction were removed and analyzed in UV-Vis spectrophotometer. Sample collected after 24 h of reaction was characterized by XRD, TEM, SAED, FTIR and XPS.

In a control experiment, the harvested culture filtrate was incubated in the absence of AuCl, thereafter was characterized for the presence of gold nanoparticles. As expected this reaction did not result in the formation of gold nanoparticles. In another experiment, the reduction of AuCl in sterile deionized water in the absence of culture filtrate was studied by TEM. This control experiment was also negative as no gold nanoparticles could be detected.

Biosynthesis of Silver Nanoparticle

For synthesis of silver nanoparticles, 5 g of duly washed waste X-ray film was cut into small pieces of 2 cm x 2 cm and added to 50 ml of purified protease (0.9 U/ml) in a 250 ml Erlenmeyer flask and agitated at 28°C, 200 rpm. Periodically, aliquots of the reaction were removed and subjected to UV-Vis spectroscopy. On completion of the reaction the stripped X- ray film was separated from the black solution and silver nanoparticles were collected by centrifugation at 15000 rpm for 30 min. The precipitate was washed twice with Milli Q water and the unbound proteins were removed by treating with 80% (v/v) ethanol. Reactions were also performed in the presence of denatured protease (obtained after boiling for 10 min) separately.

UV-Vis Spectroscopy

The UV-Vis spectroscopy measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm.

Transmission Electron Microscopy (TEM)

The morphology of the gold and silver nanoparticles was determined by TEM. The gold and silver nanoparticles were prepared by drop-coating the particles suspended in aqueous medium on the carbon coated copper TEM grids. TEM measurements were performed on a JEOL Model 1200 EX transmission electron microscope operated at an accelerating voltage of 80 kV.

X-ray Diffraction (XRD)

The films of gold and silver nanoparticles were prepared on glass slide by drop-coating with nanoparticle solution. The films on glass slide was then subjected to X-ray diffraction, which were performed on a transmission mode on a Philips PW 1830 instrument operated at 40 kV and a current of 30 mA with Cu Kα radiation.

Fourier Transform Infrared Spectroscopy (FTIR)

The solutions containing gold and silver nanoparticles were dropped on silicon wafer and air-dried. The films on Si wafers were subjected to Fourier transform infrared spectroscopic (FTIR) studies, which were carried out in a Shimadzu FTIR-8201 PC instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1}. In order to obtain good signal/noise ratio, 512 scans were recorded.

X-ray Photoelectron Spectrometer (XPS)

Chemical analysis of a drop-coated film of the gold and silver nanoparticles on a silicon wafers was carried out on a ESCALAB MK II set-up X-ray photoelectron spectrometer (V. G. Scientific, UK) with A1 kα as the exiting source (hγ = 1486.6 eV) operating at an accelerating voltage of 10 kV and 20 mA at a pressure of about 10^{-8} Pa.

RESULTS AND DISCUSSION

Characterization of Nanoparticles

UV –Visible Spectroscopy

Figure 5.2a & b shows the UV-vis spectra recorded from the reaction vessel at different times of reaction for synthesis of gold and silver nanoparticles. The time at which the aliquots were removed for analysis is indicated. The strong surface plasmon resonance centered at nearly 520 nm (Figure 5.2a) indicates the formation of gold nanoparticles (Luo, 2006) whereas at nearly 400 nm (Figure 5.2b) indicates the formation of silver nanoparticle (Kumar et al., 2007). The intensity of peaks was increased with time and stabilized after 24 h of reaction. An absorption band at nearly 280 nm is clearly visible and is attributed to aromatic amino acids (tyrosine and tryptophan) of proteins. It is well known that the absorption band at nearly 270 nm arises due to electronic excitations in tryptophan and tyrosine residues in the proteins (Eftink and Ghiron, 1981). This observation indicates the presence of proteins in the solution of gold and silver nanoparticles. In case of silver nanoparticle release of proteins into solution by the action of protease on gelatin layer and suggests a possible mechanism for removal of silver from waste X-ray film and reduction of the silver ions present in the film.

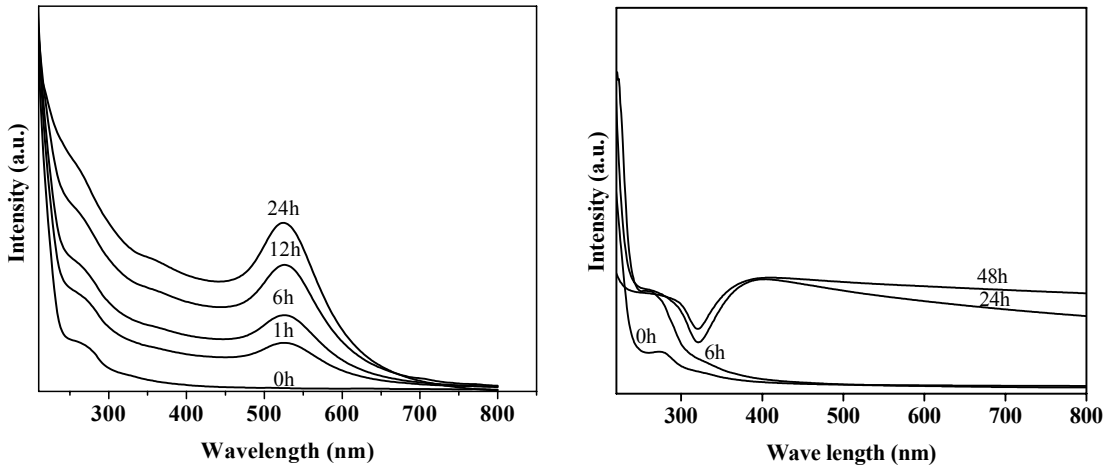
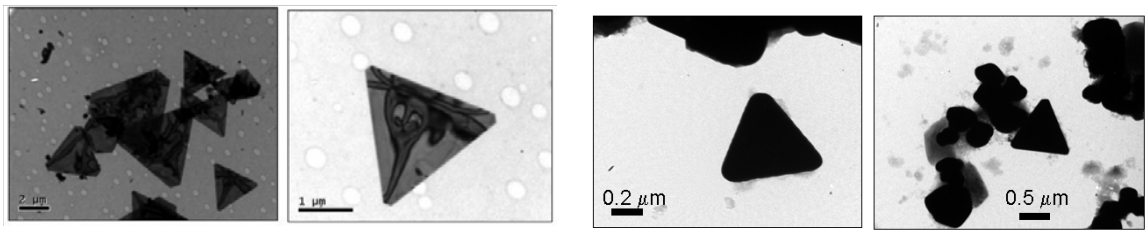


Figure 5.2: UV-Visible spectrum of (a) gold nanoparticle (b) silver nanoparticle at different time interval.

It was observed that film was initially black in color and aqueous solution was colourless which was changed to dark black colour within 24 h of reaction and the film was stripped completely due to the action of protease. Silver nanoparticle was not formed when X-ray film was incubated with denatured protease (boiled protease).

Transmission Electron Microscopy

The objects having nanometer size dimensions can be easily seen under TEM. A representative TEM picture of the gold and silver nanoparticle film (synthesized after 24 h of reaction) deposited on a carbon coated copper TEM grid is shown (Figure 5.3a, b, c & d). This picture showed individual gold and silver nanoparticles as well as a number of aggregates. By observing this image in an optical microscope, the aggregates of gold nanoparticle and silver nanoparticles found in the size range 1-1.5 μm and 200–600 nm respectively. Both gold and silver nanoparticles formed are of different shape including triangular. The size of individual gold and silver nanoparticle was 1.5 μm and 600 nm respectively. The nanoparticles were not in direct contact even within the aggregates, indicating the stabilization of the nanoparticles by proteins. The separation between the gold and silver nanoparticles seen in the TEM image could be due to capping/stabilizing by biomolecules, mainly proteins, present on their surface. The gold and silver particles are crystalline as observed from the selected area electron diffraction (SAED) pattern recorded from one of the nanoparticles in the aggregates (Figure 5.4a & b).



(a) (b) (c) (d)

Figure 5.3: TEM of (a) and (b) gold nanoparticle (c) and (d) silver nanoparticle.

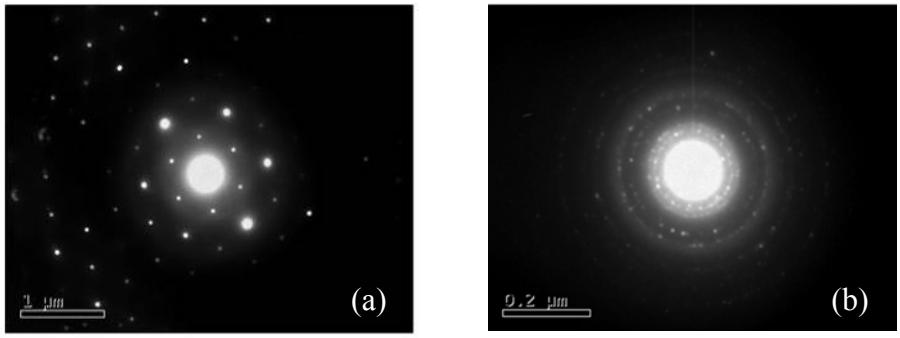


Figure 5.4: Selected area electron diffraction (SAED) of (a) gold nanoparticle (b) silver nanoparticle

X-ray Diffraction

To further find out the crystallinity of gold and silver nanoparticles, powder X-ray diffraction was carried out. Figure 5.5a & b represents the XRD patterns of the drop cast film of gold and silver nanoparticle on a glass substrate. The XRD patterns show the characteristic number of Bragg reflections of gold and silver nanoparticles. The XRD pattern from synthesized nanoparticles indexed on the basis of the structure of gold and silver. The XRD pattern of gold nanoparticle exhibits only one peaks corresponding to the (111) diffraction peaks of metal gold, indicating that the nanoparticle is composed of pure crystalline gold (Sun et al., 2005). These observations confirm that synthesized gold nanoparticles were primarily dominated by (111) facets. The XRD pattern of silver nanoparticle exhibits four peaks corresponding to the (111), (200), (220) and (311) diffraction peaks of silver metal, indicating that the solution is composed of pure crystalline silver (Kumar et al., 2007). These observations confirm that synthesized silver nanoparticles were primarily dominated by (111) facets followed by (200), (220) and (311). The XRD pattern thus clearly shows that the gold and silver nanoparticles formed are crystalline in nature and well matched with PCPDF # (04-0783).

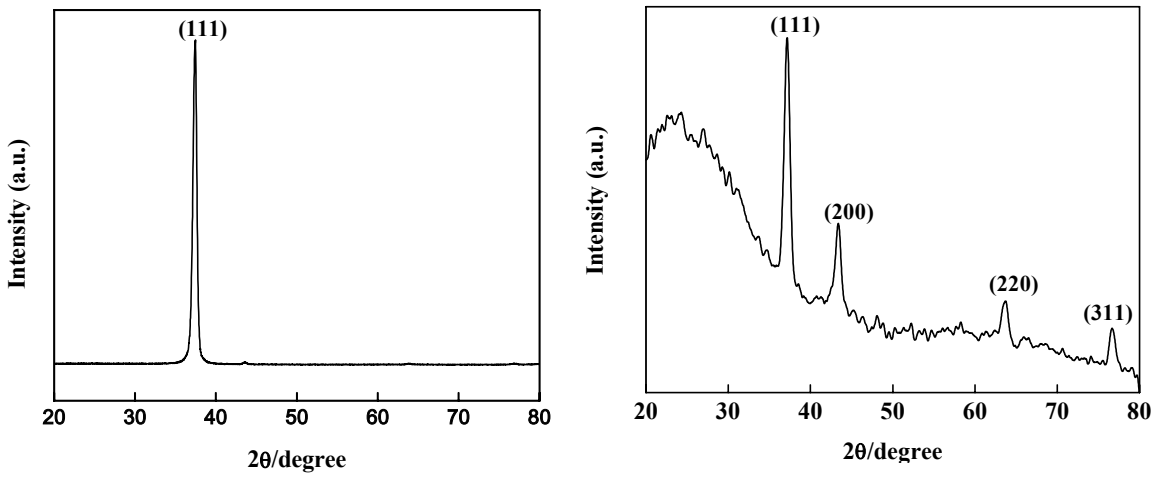


Figure 5.5: XRD of (a) gold and (b) silver nanoparticle

Fourier Transform Infrared Spectroscopy

Figure 5.6a & b shows the FTIR spectrum recorded from the sample after 24 h of reaction. These nanoparticles are capped with robust layer of protein/biomolecules. The presence of proteins on nanoparticle surface was also confirmed by FTIR. The band around 1540 and 1650 cm^{-1} is due to C-N (amide II) and C=N (amide I) bond respectively which arise due to the carbonyl stretch and –N-H stretch vibrations respectively in the amide linkages of the proteins. This indicates the presence of capped protein in nanoparticles.

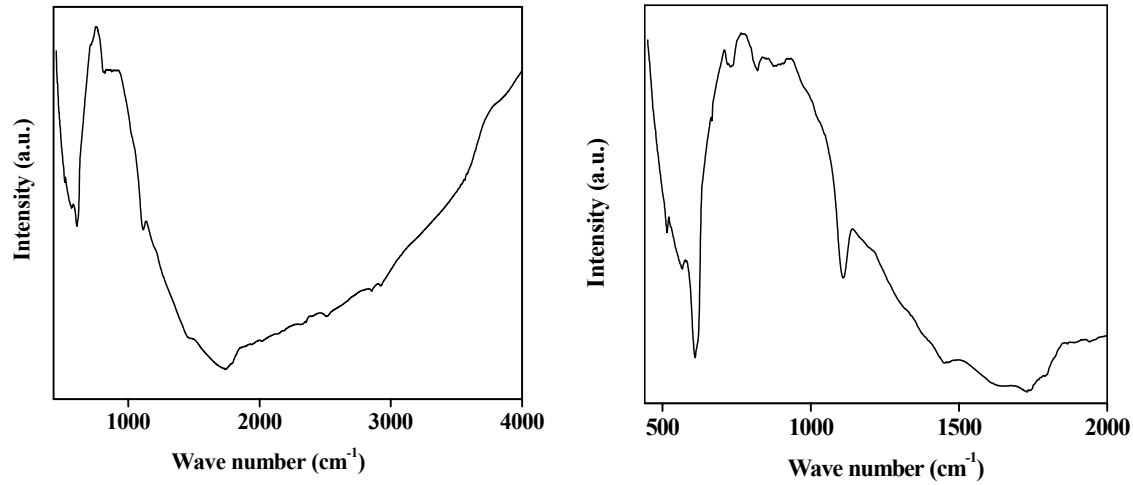


Figure 5.6: FTIR spectra of (a) gold nanoparticles and (b) silver nanoparticles.

X-ray Photoelectron Spectrometer

A chemical analysis of the gold nanoparticles synthesized was investigated by X-ray photoemission spectroscopy (XPS) measurement. The Au 4f spectrum could be resolved into two spin-orbit components. The Au 4f7/2 and 4f5/2 peaks occurred at a binding energy of 81 eV and 85 eV respectively which are characteristic of metallic Au and agree with the reported core level binding energies (Figure 5.7a). The absence of higher binding energy for Au 4f component shows that all the chloroaurate ions are fully reduced and are in metallic form.

Figure 5.7b, presented the background corrected XPS results of the metallic silver nanoparticles. It shows Ag3d spectrum which could be resolved into two peaks (3d 5/2 and 3d 3/2) due to the spin-orbit coupling with binding energies 366 and 372 eV respectively which are characteristic of metallic Ag.The absence of a higher binding energy Ag3d clearly indicates that all the silver ions are fully reduced and are in metallic form.

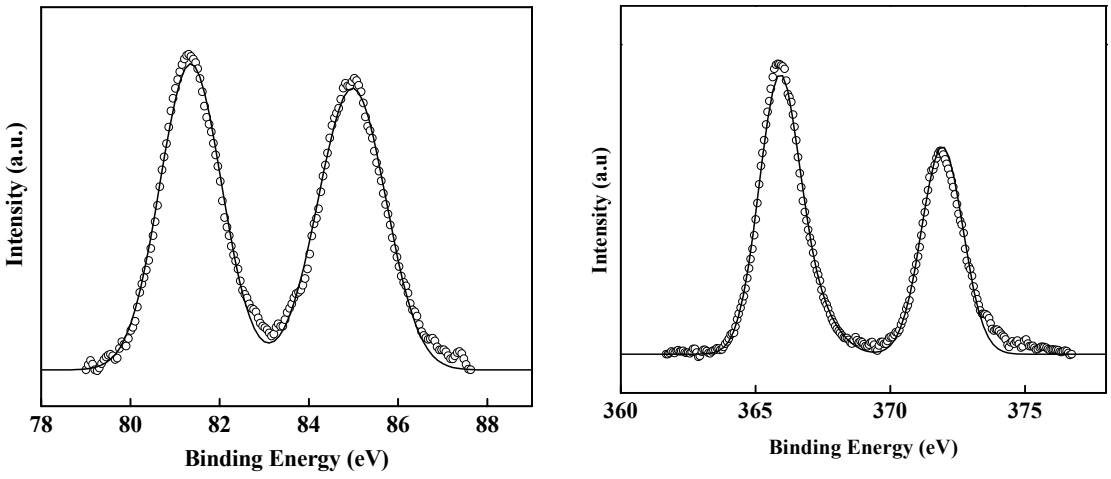


Figure 5.7: XPS of (a) gold nanoparticles and (b) silver nanoparticles.

CONCLUSION

The gold and silver nanoparticles synthesized are of various size and shape and are crystalline in nature. The UV-Vis and FTIR spectrum data showed the presence of protein in the nanoparticle solution. SAED and XRD pattern proved the nanoparticles formed are crystalline in nature. In case of gold nanoparticle XRD pattern exhibited single diffraction peak (111) whereas in case of silver nanoparticle four peaks were observed in which peak (111) was dominant. XPS data showed the nanoparticles formed are metallic in nature.

REFERENCES

- Ahmad, A., Mukherjee, P., Senapati, S., Mandal, D., Khan, M. I., Kumar, R. and Sastry, M. (2003). *Colloid Surfaces B: Biointerfaces*, 28 : 313-318.
- Baglioni, P. and Giorgi, R. (2006). *Soft Matter,* 2: 293-303.
- Bharde, A., Rautaray, D., Bansal, V., Ahmad, A., Sarkar, I., Yusuf, S. M., Sanyal, M., and Sastry, M. (2006). *Small*, 2: 135-141.
- Caski, A., Maubach, G., Born, D., Reichert, J. and Fritzsche, W. (2002). *Single Mol,* 3: 275-280.
- Cengiz, E., Wissing, S. A., Muoller, R. H. and Yazan, Y. (2006). *Intl J Cosmetic Sci,* 28: 371-378.
- Chandrasekharan, N. and Kamat, P. V. (2000). *J Phys Chem B*, 104: 10851–10857.
- Doudna, C. M., Bertino, M. F., Blum, F. D., Tokuhiro, A. T., Lahiri-Dey, D., Chattopadhyay, S. and Terry, J. (2003). *J Phys Chem B,* 107: 2966-2970.
- Eftink, M. R. and Ghiron, C. A. (1981). *Anal Biochem,* 114 : 199-227.
- Fichtner, M. (2005). *Adv Engg Mater,* 7: 443-455.
- Hou, Y., Kondoh, H., Kogure, T. and Ohta, T. (2004). *Chem Mater,* 16: 5149-5152.
- Klabunde, K. J. (2000). Nanoscale Materials in Chemistry. *John Wiley*, New York, pp. 18-22
- Krolikowska, A., Kudelski, A., Michota, A. and Bukowska, J. (2003). *Surf Sci,* 532: 227–232.
- Kumar, A., Mandal, S., Selvakannan, P. R., Parischa, R., Mandale, A. B. and Sastry, M. (2003). *Langmuir,* 19: 6277–6282.
- Kumar, S. A., Abyaneh, M. K., Gosavi, S. W., Kulkarni, S. K., Pasricha, R., Ahmad, A. and Khan, M. I. (2007). *Biotechnol Lett*, 29: 439–445.
- Lawrie, G., Grøndahl, L., Battersby, B., Keen, I., Lorentzen, M., Surawski P. and Trau, M. (2006). *Langmuir,* 22: 497-505.
- Li, X., Xu, W., Zhang, J., Jia, H., Yang, B., Zhao, B., Li, B. and Ozaki, Y. (2004). *Langmuir*, 20: 1298-1304.
- Liu, G., Garrett, M. R., Men, P., Zhu, X., Perry, G. and Smith, M. A. (2005). *Biochim Biophysic Acta,* 1741: 246-252.
- Luo, Y. (2006). *Materials Letters*, 60: 3361–3363.

- Maier, S. A., Brongersma, M. L., Kik, P. G., Meltzer, S., Requicha, A. A. G. and Atwater, H. A. (2001). *Adv Mater,* 13: 1501-1505.
- Maier, S. A., Friedman, M. D., Barclay, P. E. and Painter, O. (2005). *Appl Phys Lett,* 86: 1-3.
- Mukherjee, P., Senapati, S., Mandal, D., Ahmad, A., Khan, M. I., Kumar, R. and Sastry, M. (2002). *Chembiochem,* 3: 461-463.
- Otsuka, H., Nagasaki, Y. and Kataoka, K. (2003). *Adv Drug Delivery Rev,* 55: 403-419.
- Perekrestov, V. I. (2005). *Tech Phys Lett,* 31: 830-832.
- Peto, G., Molnar, G. L., Paszti, Z., Geszti, O., Beck, A. and Guczi, L. (2002). *Mater Sci Eng* C, 19: 95–99.
- Pol, V. G., Grisaru, H. and Gedanken, A. (2005). *Langmuir,* 21: 3635-3640.
- Roucoux, A., Schulz, J., Patin, H. (2002). *Chem Rev*, 102: 3757.
- Sakamoto, M., Tachikawa, T., Fujitsuka, M. and Majima, T. (2006). *Langmuir,* 22: 6361-6366.
- Shankar, S. S., Ahmad, A. and Sastry, M. (2003). *Biotechnol Prog,* 19: 1627-1631.
- Shankar, S. S., Rai, A., Ahmad, A. and Sastry, M. (2005). *Chem Mater,* 17: 566-572.
- Shankar, S. S., Rai, A., Ankamwar, B., Singh, A., Ahmad, A. and Sastry, M. (2004). *Nat Mater,* 3: 482-488.
- Silva, G. A. (2004). *Surg Neurol*, 61: 216-220.
- Stephen, J. R. and Maenaughton, S. J. (1999). *Curr Opin Biotechnol,* 10: 230-233.
- Stoeva, S., Klabunde, K. J., Sorensen, C. M. and Dragieva, I. (2002). *J Am Chem Soc,* 124: 2305-2311.
- Suh, W. H. and Suslick, K. S. (2005). *J Am Chem Soc,* 127: 12007-12010.
- Sun, X., Dong, S. and Wang, E. (2005). *Langmuir,* 21: 4710-4712.
- Yae, S., Kobayashi, T., Abe, M., Nasu, N., Fukumuro, N., Ogawa, S., Yoshida, N., Nonomura, S., Nakato, Y. and Matsuda, H. (2007). *Solar Energy Mater Solar Cells,* 91: 224- 229.
- Zhu, X. P., Suzuki, T., Nakayama, T., Suematsu, H., Jiang, W. and Niihara, K. (2006). *Chem Phys Lett,* 427: 127-131.

CHAPTER 6

STUDIES ON *CONIDIOBOLUS CORONATUS* PROTEASE

SECTION A

IMMOBILIZATION OF *CONIDIOBOLUS CORONATUS* ALKALINE PROTEASE ON FUNGAL BIOMASS

ABSTRACT

The strain in the present study was classified as *Conidiobolus coronatus* on the basis of 18S rDNA sequence which showed 99% homology with *Conidiobolus coronatus* strain NRRL1912 18S ribosomal RNA gene. The 18S rDNA sequence of *C. coronatus* was assigned accession number FJ895303 in NCBI GenBank database. An alkaline protease from *C. coronatus* was chosen for immobilization studies as the enzyme was optimally active at pH 10 and 40°C and found to be suitable for soaking, dehairing and bating operations in leather industry. The protease was immobilized in 2% Ca-alginate, polyacrylamide gel and alkali treated fungal biomass (ATFB). ATFB was found to be most suitable among the matrices tested. ATFB with 2% glutaraldehyde had marginal benefit as extent of binding was similar (58-65%). The enzyme immobilized on ATFB may be by simple adsorption confirmed by FTIR spectra. Temperature optima of free and immobilized protease were similar however the optimum pH of the immobilized enzyme shifted to higher pH of 11 as compared with the free enzyme (10). The pH stability did not change whereas temperature stability increased after immobilization.

INTRODUCTION

The genera *Conidiobolus coronatus* belongs to order of entemoptherales, characterized by its propensity to infect insects (Hoogendijk et al., 2006). There are various hydrolytic enzymes involved in cuticle degradation like chitinase, lipases and proteases, among which proteases are believed to play major role in fungal virulence (Bania et al., 2006). Therefore apart from the exploitation of such fungi as biocontrol agent, it can be interested to utilize its potential to produce enzymes at large scale and explore applications in various areas. In such quest, a fungal strain, *Conidiobolus* sp has been isolated from plant detritus and found to secret high level of alkaline protease extracellularly. *Conidiobolus coronatus* secretes high levels of alkaline protease in short fermentation cycles. This enzyme has been scaled up in 100 L bioreactor and has been evaluated for its applications in pre–tanning operations in the leather manufacture (Laxman et al., 2004; 2005).

Recent awareness of environmental pollution caused by chemical-based industries has necessitated the development of enzyme-based processes. In this regard, proteases have potential application in industry for partial or total replacement of currently employed toxic chemical processes. Increased stability under operating conditions is an important factor for the suitability of an enzyme for industrial applications. Various strategies used to improve the stability of proteins include use of additives, introduction of disulfide bonds, site-specific mutagenesis and chemical modification or crosslinking. However, in all these cases recovery of free enzyme and reusability as industrial catalysts are quite limited (Tanksale et al., 2001). Immobilization of enzymes on water insoluble supports facilitates product and enzyme separation, improves biocatalysts stability and paves the way for their reuse and application in continuous operations, with a positive consequence on the process economy (Kumar et al., 2009). Proteases have been immobilized on various supports such as chitin (Han and Shahidi, 1995), porous glass (Parrado et al., 1995), vermiculite (Chellapandian, 1998), polyamide (Tanksale et al., 2001), chelating sepharose (Afaq and Iqbal, 2001), silica support (Ferreira et al., 2003), magnetic composite microspheres (Lei et al., 2004), entrapment in Ca.-alginate beads (Mittal et al., 2005; Sharma et al., 2006), Eudragit S-100, a reversible soluble–insoluble polymer (Kumar and Gupta, 1998; Silva et al., 2006), activated glyoxyl-agarose (Yust et al., 2007) and chitosan beads (Altun and Cetinus, 2007; Bhandari et al., 2009). Immobilization of enzymes can be carried out by entrapment, ionic interaction,

covalent attachment, encapsulation and adsorption onto hydrophobic or hydrophilic surfaces. Among these methods, adsorption has been considered as a simple and an economical mode for immobilization with the benefit of wide applicability and also provides relatively small perturbation of the enzymes native structure and function contributing to the maintenance of enzyme activity (Kumar et al., 2009). Immobilization of various enzymes including protease on chitin and chitosan-based materials have been reviewed (Krajewska, 2004).

The present section of this chapter describes the identification of an organism, *Conidiobolus coronatus* on the basis of molecular characteristic. The work has also been carried out to evaluate the potential of different matrixes including alkali treated fungal biomass (ATFB) for immobilization of *C. coronatus* protease with a view to increase the efficiency for its application and to study the change in properties of immobilized enzyme.

MATERIALS AND METHODS

Na-alginate from Sigma and glutaraldehyde from Fluka were used. Malt extract, yeast extract and peptone were obtained from Hi-media chemicals. Hammerstein casein was obtained from M/s Sisco Research Laboratories, India. Acrylamide and bis-acrylamide were of Qualigens make and all other chemicals were of analytical grade.

Microorganism and Protease Production

C. coronatus strain ATCC PTA- 4132 was isolated as described earlier (Laxman et al., 2005). The fungus was periodically sub-cultured and maintained on MGYP agar slant (g/L)- glucose-10; malt extract-.3; yeast extract-3; bacteriological peptone-5; agar- 20 adjusted to pH-7. Protease production and protease assay was carried out as described earlier (Laxman et al., 2005).

Identification of the Microorganism

PCR amplification and sequencing of 18S rDNA gene for identification of the strain was done according to method described in chapter 2.

Protease Production

The organism was routinely sub-cultured and maintained on MGYP agar slants and preserved. Enzyme production was carried out in 500 ml Erlenmeyer flasks containing 100 ml medium. After 72 h of growth in MGYP broth containing 2% (w/v) soyabean meal, the cell-free supernatant (obtained by centrifugation at 10000 rpm, 4˚C, 20 min) was used as source of enzyme for immobilization. Protease activity was determined at 40°C, pH 10 by method described by Laxman et al. (2005). The protein content was estimated according to (Lowry et al., 1951) with bovine serum albumin (BSA) as the standard. The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein from the protein originally added.

Preparation of Fungal Biomass as Matrix

The waste fungal biomass of *C. coronatus* obtained after fermentation was washed several times with double distilled water to remove the media constituents. Fifty gram wet biomass was boiled with 500 ml of 1 N NaOH for 2 h to remove alkali soluble fraction. The alkali insoluble material was separated by centrifugation at 10000 rpm, 20 min and washed with double distilled water till pH of the washings became

neutral. 15 g (wet weight) ATFB was washed with 0.1 M phosphate buffer pH 7 and resuspended in 150 ml same buffer and stored at 4°C for further use.

Immobilization of the Protease on ATFB

All the steps were carried out between 4 to 10 °C. Glutaraldehyde was added to 10 ml of ATFB suspension to a final concentration of 2% (v/v) and stirred for 2 h. Excess of glutaraldehyde was removed by repeated washing with 0.1 M phosphate buffer pH 7 before the addition of protease. Two ml crude enzyme (108 U and 7.2 mg protein) was added drop-wise with constant stirring to the mixture. After 1 h the mixture was centrifuged at 10000 rpm, 10 min. ATFB immobilized enzyme was washed twice with the same buffer and resuspended to a final volume of 10 ml in 0.1 M phosphate buffer at pH 7. The protease was also immobilized on ATFB without glutaraldehyde under above conditions. Protease activity in bound and unbound fractions were measured.

Immobilization of the Protease in Ca-alginate Beads

Alkaline protease was immobilized in Ca-alginate beads by entrapment. 100 mg Na-alginate was suspended in 3 ml of double distilled water and warmed to dissolve. Two ml protease was added to the above mixture and stirred gently. The entrapment was carried out by dropping the mixture through 10 ml disposable plastic syringe into 25 ml of chilled 2% (w/v) $CaCl_2$ solution. After 20 min, the beads formed were washed with 0.1 M phosphate buffer pH 7 and stored at 4°C till further use.

Immobilization of the Protease in Polyacrylamide Gel

Alkaline protease was immobilized in 12% polyacrylamide gel. The gel was prepared according to Laemmli (1970) with slight modifications. Two ml of protease was mixed with 2 ml of acrylamide-bisacrylamide solution (30:0.8). One ml of 1 M Tris-HCl buffer pH 6.8 was then added followed by addition of 100 µl of 10% (w/v) ammonium per sulphate. The mixture was stirred for 30 min at 4°C after which 5 µl of TEMED was added and allowed to polymerize in glass tubes. The gel was taken out from glass tubes after polymerization and cut into small pieces. The gel pieces were washed with double distilled water and were stored at 4°C.

Properties of Free and Immobilized Protease

Optimum pH and temperature of the free and immobilized protease were investigated in the pH range of 6 to 12 at 40°C and temperature range of 25 to 60°C at pH 10 respectively. The pH stability was determined by incubating the free as well as immobilized protease with 0.1 M buffer of desired pH for 2 h at 28°C. Temperature stability of free and immobilized protease was determined by incubating at 40°C up to 90 min at three pH values (5, 8 and 11). Residual activity was expressed as percentage of initial activity taken as 100%. The *K*m of free and immobilized protease was determined from the Lineweaver-Burk plots using casein in the range of 1 to 10 mg. For reuse, immobilized enzyme was incubated with casein at 40°C for 10 min and the immobilized enzyme was recovered by centrifugation and used for next cycle after washing with buffer.

FTIR Spectra Analysis

For FTIR studies, ATFB and ATFB-protease were grounded in KBr and dried. FTIR spectroscopy measurements were carried out on a Perkin-Elmer Spectrum One instrument at a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION

Identification of Microorganism and Phylogenetic Analysis

In order to identify the fungus, 18S rDNA (1.8 kb) region was amplified, sequenced and analyzed in the NCBI database using BLAST program. The 18S rDNA sequence showed 99% homology with *Conidiobolus coronatus* strain NRRL1912 18S ribosomal RNA gene (Voigt et al., 1999), *Conidiobolus firmipilleus* small subunit ribosomal RNA gene (Huang et al., 2001), *Conidiobolus coronatus* strain ARSEF 206 18S ribosomal RNA gene (Huang et al., 2007); 98% with *Conidiobolus coronatus* strain NRRL28638 18S ribosomal RNA gene (Voigt et al., 1999), *Conidiobolus brefeldianus* small subunit ribosomal RNA gene (Huang et al., 2001); 96% with *Conidiobolus incongruus* 18S ribosomal RNA gene (Voigt et al., 1999); 90% with *Conidiobolus pumilus* small subunit ribosomal RNA gene; 86% with *Conidiobolus lamprauges* 18S ribosomal RNA gene (Vigot et al., 1999) and 82% with *Conidiobolus rhysosporus* small subunit ribosomal RNA gene (Huang et al., 2001).

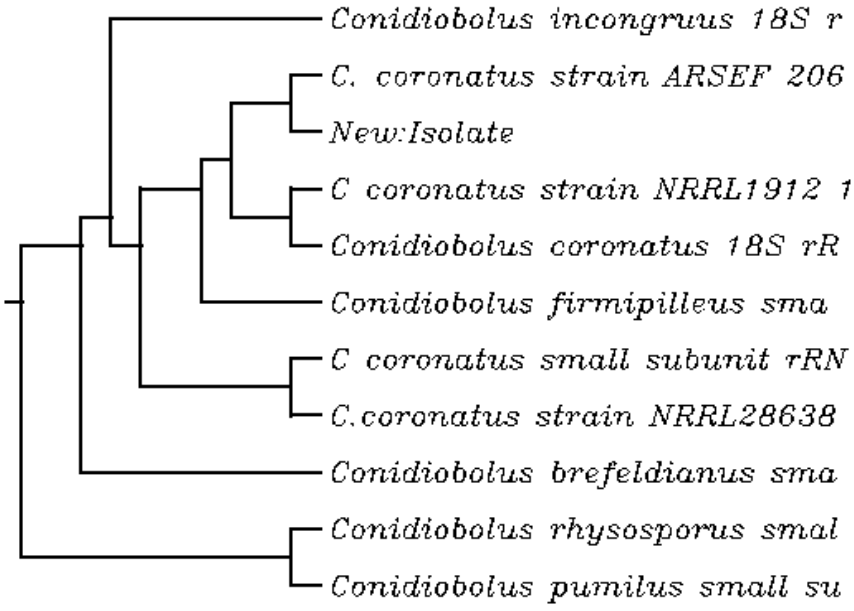


Figure 6.1: Phylogenetic tree of *Conidiobolus coronatus* associated with the other members of the *Conidiobolus* genus

Multiple sequence alignment was performed with Clustal W programme. The phylogenetic tree was constructed by using neighbour-joining method (Figure 6.1). Multiple sequence alignment and phylogenetic tree showed that the fungal strain is

Conidiobolus coronatus. The nucleotide sequence of 18S rDNA from isolate of *Conidiobolus* sp was assigned accession number FJ895303 in NCBI GenBank database.

Immobilization of Protease

The extent of binding of *Conidiobolus coronatus* protease in terms of protein to polyacrylamide, Ca-alginate, ATFB with and without glutaraldehyde was 90, 55, 28 and 22 %, while in terms of activity was 15.5, 7.3, 65.3 and 57.7 % respectively (Table 6.1). Although more than 90% protein was bound to polyacrylamide, the activity expressed was only 15%. This may be due to poor accessibility of enzyme entrapped in the gel to high molecular weight substrate (casein). In case of Ca-alginate around 55% protein was immobilized. High percentage of unbound protein (45%) might be due to leakage of enzyme at 2% concentration owing to large pores size of the beads. However, in spite of significant protein in the Ca-alginate beads, protease activity in the beads was very low (7.3%) which may be due to poor accessibility of enzyme to casein or leakage of small molecular weight protease from the bead and retention of other high molecular weight protein inside the bead. Sharma et al. (2006) have studied the various concentrations of alginate for immobilization of alkaline protease from *Aspergillus oryzae* AWT-20. Two percent alginate was found to be optimum which showed 47% binding of protease whereas there was no effect of different concentrations of $CaCl_2$ when concentration of Na-alginate was 2%. Ahmed et al. (2007) have immobilized protease from *Bacillus Licheniformis* ATCC 21415 by physical adsorption on loofa and chitosan had immobilization yield of 70.5% and 36.4% respectively.

Table 6.1: Immobilization of protease on various matrices

Matrix	**Immobilization (%)**	
	Protein	**Activity**
polyacrylamide	90	15
Ca-alginate	55	7
ATFB with glutaraldehyde	28	65
ATFB without glutaraldehyde	22	58

Among the matrices tested, ATFB was found to be the best with a immobilized protease activity of 58-65%. There was marginal increase in protease immobilization when glutaraldehyde was added. The further studies were carried out with ATFB immobilized in presence of glutaraldehyde. Since no significant difference was observed it was suggested that immobilization did not involve covalent bonding between biomass, glutaraldehyde and protease. The protease immobilized on biomass by simple adsorption. Glutaraldehyde acts as a linker molecule between matrix and enzyme for covalent bonding.

Ahmed et al. (2007) have found good loading efficiency for the immobilization by covalent binding might be due to the formation of stable cross linking between the matrix, glutaraldehyde and the enzyme. In addition, the covalent binding through glutaraldehyde probably increased the surface area of matrix and consequently reduced the stearic hindrance around the active site of the enzyme molecule.

Effect of Temperature on Protease Activity and Stability

The most important criteria of enzyme for the industrial applications are thermal stability and reuse. In general, an increase in temperature results in increase in enzyme activity up to a certain point (optimum temperature) beyond which there was decrease in activity which may be due to denaturation of proteins. The optimum temperature for free as well as immobilized alkaline protease was 40°C and no shift was observed (Figure 6.2). Similar results of identical temperature optima for free and immobilized systems were reported for alkaline protease immobilized on vermiculite (Chellapandian, 1998), dipeptidylpeptidase IV immobilized in Ca-alginate beads (Mittal et al., 2005), highly porous activated carbon (Kumar et al., 2009) and magnetic nanoparticle (Jin et al., 2010). Contrary to the above results, a shift in optimum temperature on immobilization was reported for protease immobilized on porous glass (Parrado et al., 1995) and chitosan beads (Benkhelifa et al., 2005). Tanaksal et al. (2001) have reported that the optimum temperature for *Conidiobolus macrosporus* alkaline protease was increased from 40 to 50°C after immobilization. Ahmed et al. (2007) have reported that the optimum temperature for *Bacillus Licheniformis* alkaline protease was increased from 70 to 80°C after immobilization.

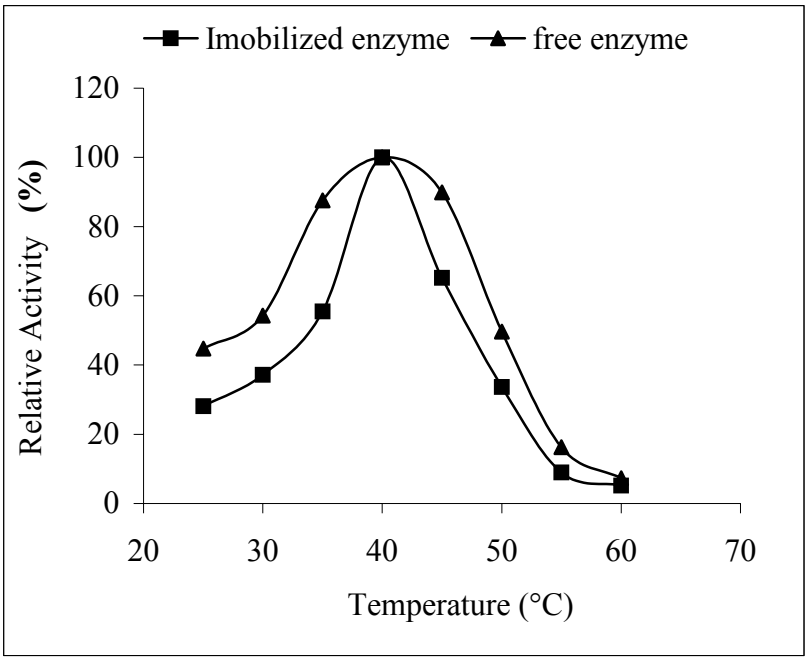


Figure 6.2: Optimum temperature of free and immobilized protease.

Thermal stability of the immobilized protease was marginally increased as compared to free enzyme at all the pH values examined (Figure 6.3). The immobilized protease showed higher thermo stability at 40°C as compared to free enzyme. At pH 5 and pH 8, immobilized protease retained 75-80% and 85-90% of residual activity, whereas free enzyme has residual acivity of 60-65% and 55-60% respectively after 90 min of incubation. Increased thermal stability of immobilized alkaline protease may be due to attachment of enzyme to the biomass thereby offering protection against auto degradation. Ahmed et al. (2007) have reported the increased thermal stability of the immobilized protease compared to the free enzyme. The immobilized enzymes retained 59.9% of the original activity after heating at 60°C for 1 h however the free enzyme retained only 19.4%. Kumar et al. (2009) and Jin et al. (2010) have reported the higher storage ability of protease after immobilization as compare to free enzyme.

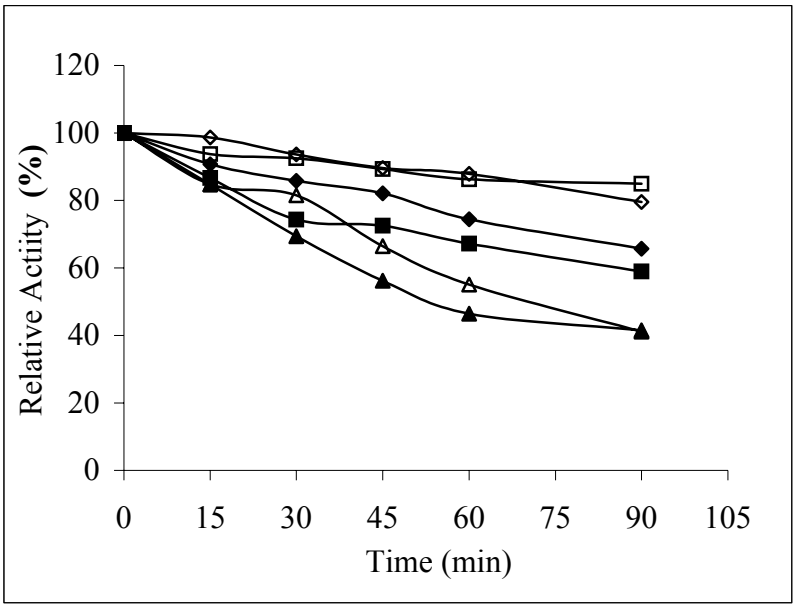


Figure 6.3: Temperature stability of free and immobilized protease at 40°C, (◆◇) pH 5; (■□) pH 8 and (▲△) pH 11. Closed symbols for free enzyme and opened symbols for immobilized enzyme.

Effect of pH on Protease Activity and Stability

The optimum pH of immobilized protease was shifted towards alkaline side with optimum pH of 11 whereas free protease has pH 10 (Figure 6.4).

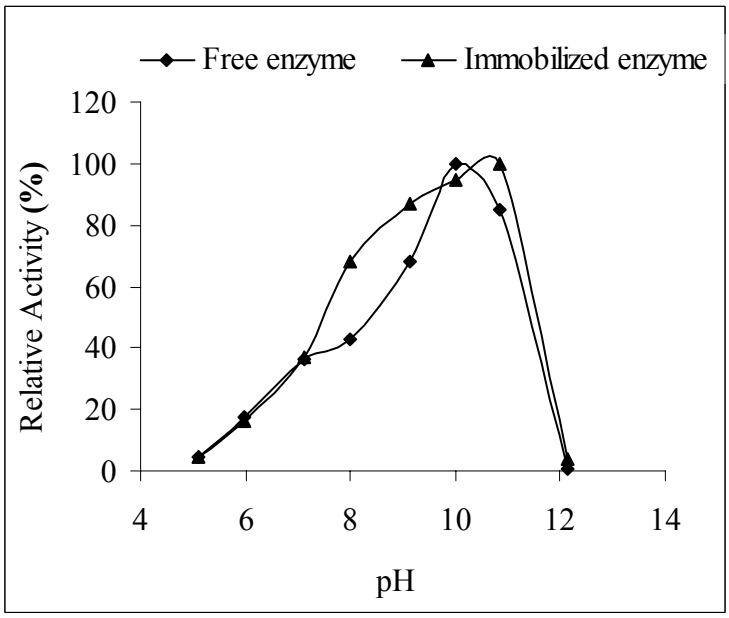


Figure 6.4: Optimum pH of free and immobilized protease.

This shift could be due to change in the microenvironment of the immobilized protease. Similar results of shift in optimum pH towards alkalinity were reported when protease was immobilized in Ca-alginate beads (Sharma et al., 2006), Eudragit S-100 (Silva et al., 2006) and Ahmed et al. (2007).

The stability of immobilized protease was compared with free protease in the pH range of 5-12 (Figure 6.5). Free as well as immobilized protease was stable over a wide range of pH from 5 to 12 and retained 80-90% of original activity after incubation at room temperature for 2 h. Sharma et al. (2006) have reported that immobilized enzyme was stable in wider rage of pH (5.5-10) than free enzyme which was stable in pH (7-9). Immobilized enzyme retained ~80% activity at pH 10.5 whereas free enzyme was inactivated.

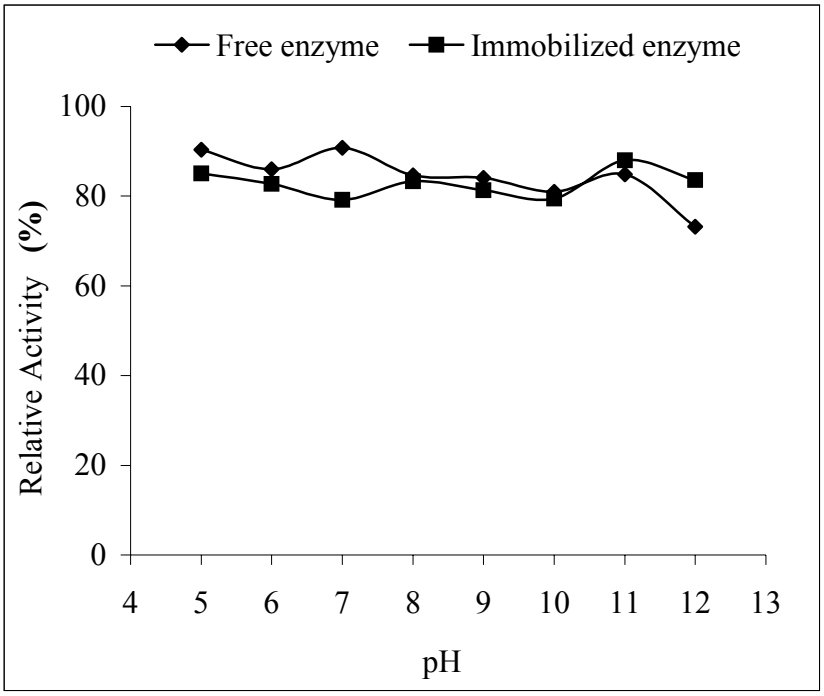


Figure 6.5: pH stability of free and immobilized protease.

Km Studies

To determine the *K*m, the activity of the free and immobilized protease was assayed at 40°C, pH 10 with casein as substrate from 1-10 mg. The *K*m values from Lineweaver-Burk plot for immobilized and free proteases were found to be approximately 1.96 mg/ml and 2.56 mg/ml whereas the V_{max} values were 27.10 U/ml and 40 U/ml respectively (Figure 6.6). The decrease in K*m* after immobilization indicates the

increase in affinity of protease for substrate which may be due to decrease in mass transfer resistance of the substrate accessibility to active site of the enzyme. Changes in *K*m on immobilization were also reported for other proteases (Tanksale et al., 2001; Silva et al., 2006; Altun and Cetinus, 2007; Ahmed et al., 2007).

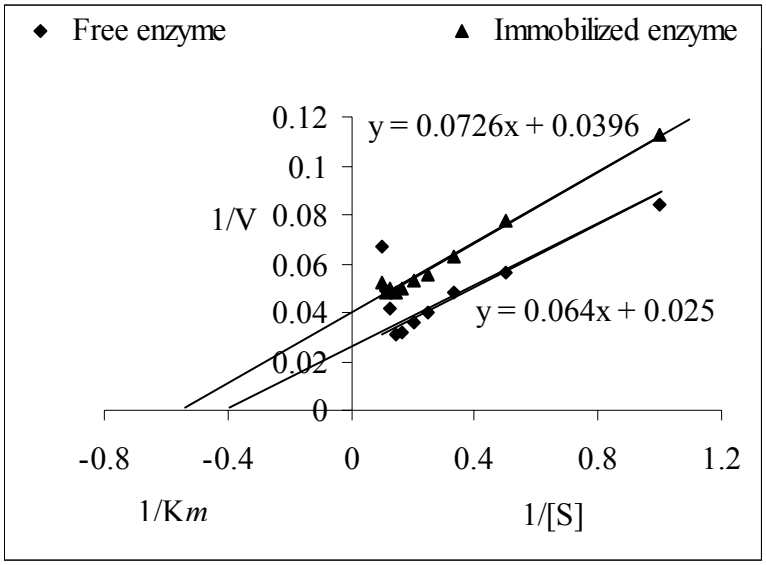


Figure 6.6: Lineweaver-Burk plot of free and immobilized protease.

Reuse of Immobilized Protease

The reusability of ATFB immobilized protease was investigated with casein hydrolysis (Table 6.2). The immobilized protease retained more than 70% of its original activity after three cycles of repeated use. There was 10-12% loss in activity in each cycle which could be due to handling losses. The residual activity was abruptly decreased after 4^{th} reuse.

Immobilized trypsin on Eudragit S-100 polymer was reused for 3 times with 12% loss in activity (Kumar and Gupta, 1998). Tanksale et al. (2001) have reported reuse of protease immobilized on polyamide up to 22 times. The vermiculite bound alkaline protease retained 78% of its original activity after five cycle of reuse (Chellapandian, 1998).

Table 6.2: Reuse of immobilized protease

No. of cycle	Residual activity (%)
1	100
2	85.43
3	73.52
4	47.15

FTIR Spectra Analysis

Figure 6.7 shows FTIR spectra of the ATFB with glutaraldehyde before and after immobilization of protease. The peak around 1150 cm^{-1} assigned to saccharide structure (Altun and Cetinus, 2007). The bands around 1550 and 1620 cm^{-1} are due to C-N and C=N bond respectively. There were no changes in the peak before and after immobilization indicates that enzyme immobilized on biomass by simple adsorption and not by covalent bonding. Due to simple adsorption enzyme can not be reused for several times and there was loss in activity after every use.

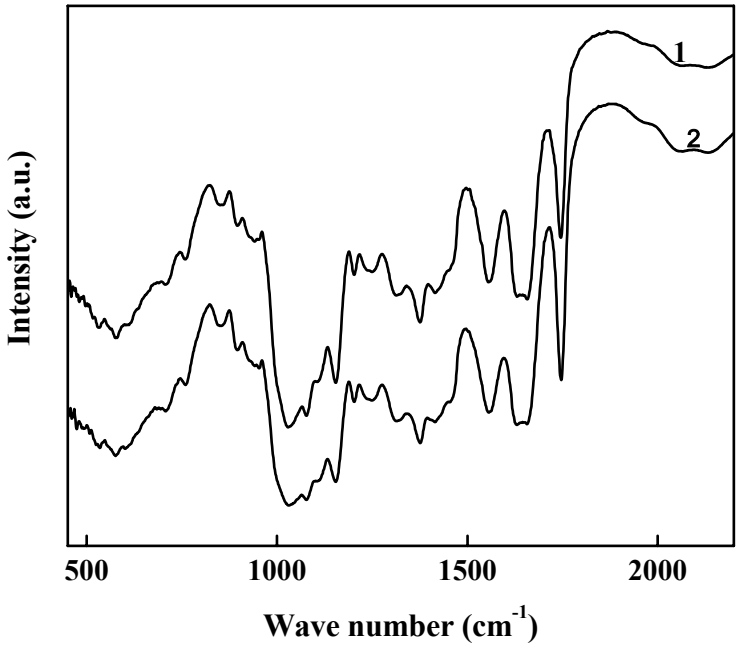


Figure 6.7: The FTIR spectra of (1) ATFB, (2) protease immobilized on ATFB

CONCLUSIONS

Reports on use of variety of matrixes such as calcium alginate beads, polyacrylamide gel, porous glass, vermiculite, chelating sepharose, polyamide, silica support, magnetic composite microspheres, Eudragit S-100, activated glyoxyl-agarose, chitin, chitosan beads for the immobilization of protease are available in literature. However, there are no reports on use of fungal biomass as a matrix. Since fungal biomass contains chitin and chitosan, we felt it appropriate to investigate its suitability as a matrix. The protease immobilized on ATFB by simple adsorption since no significant difference was observed when glutaraldehye was used. This was also confirmed by FTIR spectra. Around 60% binding of protease activity, shift in optimum pH, good thermal stability with significant retention of activity after 3 reuses and cheap cost of the waste biomass make it attractive.

REFERENCES

- Afaq, S. and Iqbal, J. (2001). *Elect J Biotechnol,* 4: 120-124.
- Ahmed, S. A., Saleh, S. A. and Abdel-Fattah, A. F. (2007). *Aust J Basic App Sci,* 1: 313-322.
- Altun, G. D. and Cetinus, S. A. (2007). *Food Chem*, 100: 964-971.
- Bania, J., Samborski, J., Bogus, M. and Polanowski, A. (2006). *Arch Insect Biochem Biophysiol,* 62: 186-196.
- Benkhelifa, H., Bengoa, C., Larre, C., Guibal, E., Popineau, Y. and Legrand, J. (2005). *Proc Biochem,* 40: 461-467.
- Bhandari, S., Gupta, V. K. and Singh, H. (2009). *Biocatal Biotransform,* 27: 71-77.
- Chellapandian, M. (1998). *Proc Biochem*, 33: 169-173.
- Ferreira, L., Ramos, M. A., Dordick, J. S. and Gil, M. H. (2003). *J Mol Catal B: Enz,* 21: 189-199.
- Han, X. Q. and Shahidi, F. (1995). *Food Chem,* 52: 71-76.
- Hoogendijk, C. F., Pretorius, E., Marx, J., Heerden, W. E. P. V., Imhof, A. and Schneemann, M. (2006). *Ultrastructural pathol,* 30: 51-58.
- Huang, B., Li, Z. Z. and Humber, R. A. (2007). Molecular phylogeny of the entomophthoralean fungi on small subunit ribosomal DNA sequences (Unpublished).
- Ingale, S. S., Rele, M. V. and Srinivasan, M. C. (2002). *World J Microbiol Biotechnol,* 18: 403-408.
- Jin, X., Li, J. F., Huang, P. Y., Dong, X. Y., Guo, L. L., Yang, L., Cao, Y. C., Wei, F., Zhao, Y. D. and Chen, H. (2010). *J Magnetism Magnetic Materials*, In press
- Krajewska, B. (2004). *Enz Microb Technol*, 35: 126-139.
- Kumar. A. and Gupta, M. N. (1998). *J Mol Catal B: Enz,* 5: 289-294.
- Kumar, A. G., Swarnalatha, S., Kamatchi, P., Kirubagaran, R., Perinmbam, K. and Sekaran, G. (2009). *J Porous Mater,* 16: 439–445.
- Laemmli, U. K. (1970). *Nature,* 227: 680-685
- Laxman, R. S., More, SV, Rele, MV, Rao, BS, Jogdand, VV, Rao, MB, et al., Process for the preparation of alkaline protease. US Patent No. 6,777,129; 2004.

- Laxman, R. S., Sonawane, A. P., More, S. V., Rao, B. S., Rele, M. V., Jogdand, V. V., Deshpande, V. V. and Rao, M. B. (2005). *Proc Biochem,* 40: 3152-3158.
- Lei, H., Wang, W., Chen, L. L., Li, X. C., Yi, B. and Deng, L. (2004). *Enz Microb Technol*, 35: 15-21.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). *J Biol Chem,* 193: 265-275.
- Mittal, A., Khurana, S., Singh, H. and Kamboj, R. C. (2005). *Enz Microb Technol*, 37: 318-323.
- Parrado, J., Millan, F. and Bautista, J. (1995). *Proc Biochem*, 30: 735-741.
- Sharma, J., Singh, A., Kumar, R., Mittal, A. (2006). *The Internet J Microbiol*, 2: 1-16.
- Silva, C. J. S. M., Zhang, Q., Shen, J. and Cavaco-Paulo, A. (2006). *Enz Microb Technol*, 39: 634-640.
- Tanksale, A., Chandra, P. M., Rao, M. and Deshpande, V. (2001). *Biotechnol Lett*, 23: 51-54.
- Voigt, K., Cigelnik, E. and O'donnell, K. (1999). *J Clin Microbiol,* 37: 3957-3964.
- Yust, M. D. M., Pedroche, J., Alaiz, M., Calle, J. G., Vioque, J., Mateo, C., Guisaan, J. M., Millaan, F. and Lafuente, R. F. (2007). *J Agric Food Chem,* 55: 6503-6508.

SECTION B

RECOVERY OF SILVER FROM WASTE X-RAY FILM BY ALKALINE PROTEASE FROM *CONIDIOBOLUS CORONATUS*

ABSTRACT

The waste X-ray/ photographic films contain 1.5 - 2 % (w/w) black metallic silver which is recovered and reused. Around 18-20% of the world's silver needs are supplied by recycling photographic waste. Since silver is linked to gelatin in the emulsion layer, it is possible to break the same and release the silver using proteolytic enzymes. Alkaline protease from *Conidiobolus coronatus* was investigated for enzymatic hydrolysis of gelatin from waste X-ray films. At the end of the treatment, gelatin layer was completely removed leaving the polyester film clean while the silver was recovered from the hydrolysate and both can be reused. Silver in hydolysate was around 3.87 % (w/w) based on total weight of sludge. Gelatin hydrolysis was monitored by measuring the increase in the turbidity which was accompanied by release of protein and hydroxyproline in the hydrolysate. Gelatin layer was stripped completely within 6 min with 1.35 U/ml of protease at 40˚C, pH 10 and the rate of hydrolysis increased with increase in concentration of protease. The enzyme could be effectively reused for four cycles.

INTRODUCTION

Silver is one of the precious and noble metals used in large quantities for many purposes, particularly in the photographic industry. The waste X-ray/photographic films containing black metallic silver spread in gelatin are very good source for silver recovery compared to other types of film (Shankar et al., 2010). With an increasing demand for silver in the world, recent attention is focused on X-ray/photographic films as one of the secondary sources of silver owing to the considerable amount of silver present in them. Various studies have been carried out to recover the silver from photographic/X-ray film wastes and following methods are reported in literature: (a) burning the films directly (b) oxidation of the metallic silver following electrolysis (c) stripping the gelatin-silver layer using different chemical solutions (d) enzymatic hydrolysis of gelatin (Nakiboglu et al., 2003). Recovery of silver by burning the films directly, a conventional method used at present is the most primitive method and generates undesirable foul smell. The method causes environmental pollution and polyester film on which emulsion of silver and gelatin is coated cannot be recovered. Stripping the gelatin-silver layer by chemical methods using ammonium thiosulphate, sodium thiosulphate, nitric acid or reagents such as sodium cyanide, NaOH, nitric acid or organic compounds cause environmental hazards and are either time consuming or very expensive while the use of NaOH at high temperatures poses a serious industrial safety problem. For this reason, the methods applied to recover silver from X-ray/ photographic waste should be cost effective and have minimal impact on environment. Enzyme based methods can be an alternative option. Gelatin is a protein from animal collagen which contains a large number of glycine, proline and 4- hydroxyproline residues. Since the emulsion layer on X-ray film contains silver and gelatin, it is possible to break down the gelatin layer using proteases and release the silver (Nakiboglu et al., 2001). The enzymatic hydrolysis of the gelatin layers on the X-ray film enables not only the recovery of the silver, but also the polyester base which can be recycled. Hence in recent years, enzymatic methods using 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., 2003; Masui et al., 2004). Basically enzymatic processes are more specific and remove gelatin layer from X-ray film in few minutes without damaging the polyester film base. Gelatin molecules are cross linked with hardners and it is difficult for the usual proteases to degrade it in a short

time. Most of the proteases used so far for silver recovery are of bacterial origin and there are only few reports on use of fungal alkaline protease.

The objective of the present work is to remove the gelatin layer from waste X-ray film and recover both silver as well as the polyester film in an eco-friendly manner. The present section of this chapter describes the use of alkaline protease from *Conidiobolus coronatus* for silver removal from X-rays films and its recovery for reuse.

MATERIALS AND METHODS

Malt extract, yeast extract and peptone were obtained from Hi Media Chemicals, India. Hammerstein casein was obtained from Sisco Research Laboratories, India. All other chemicals were of analytical grade. X-ray films were obtained from local medical center.

Protease Production and Activity

As described in section A of chapter 6.

Hydrolysis of Gelatin and Release of Silver

Used X-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in an oven at 40˚C for 30 min. One g of X-ray film (cut into 2 x 2 cm pieces) was then incubated with 10 ml of crude protease (such that the film was completely immersed in the solution) at 40˚C, pH 10 in a water bath with continuous shaking. Turbidity of the reaction mixture (hydrolysate) increased with time (as the hydrolysis progressed) and no further increase in turbidity was observed when hydrolysis was complete. Hence, progress of hydrolysis i.e. turbidity was monitored by measuring the absorbance at 660 nm. Samples were removed at 1 min intervals and time required for complete removal of gelatin layer was noted.

Effect of Enzyme Concentration & Time Course of Hydrolysis

Effect of enzyme concentration on hydrolysis of gelatin was measured by incubating 1 g film (2 x 2 cm pieces) with 10 ml of enzyme at 40˚C and pH 10 and protease activities ranging from 0 to 2.7 U/ml. Samples were removed at an interval of 5 min until the gelatin layer was completely stripped off and time required for complete removal was noted. Extent of hydrolysis was expressed as percentage compared to the highest absorbance which was taken as 100%. For time course of gelatin removal, the film was incubated with 10 ml of 0.9 U/ml of protease at 40˚C and pH 10. Samples were removed at 1 min intervals and increase in turbidity due to gelatin removal was measured at 660 nm.

Release of Protein and Hydroxyproline during Hydrolysis

Hydroxyproline and protein released during gelatin hydrolysis was monitored. Protein was estimated according to Lowry et al. (1951) with bovine serum albumin (BSA) as the standard. Hydroxyproline was estimated by the method of Neuman and Logan (1950). The purple colour formed was measured at 540 nm and hydroxyproline content was calculated from a standard curve.

Reuse of Enzyme for Hydrolysis

Reuse of enzyme for gelatin hydrolysis and silver removal was carried out at three enzyme concentrations. One g of X-ray film (2 x 2 cm pieces) was incubated at 40˚C, pH 10 with 10 ml of protease having 0.18, 0.45 and 0.9 U/ml. After complete removal of gelatin, old X-ray film was removed and fresh film (1 g) was added to the same enzyme solution and incubation continued till complete removal of gelatin was observed. The process was repeated till gelatin hydrolysis stopped. Time required for complete gelatin removal in each case was noted.

Recovery of Silver

Five grams of X-ray film was incubated with 50 ml of 1.35 U/ml of protease at 40˚C, pH 10, and 200 rpm on a rotary shaker. After complete gelatin removal, reaction slurry containing gelatin and silver was acid digested and used for analysis of metals by AAS-Chemito-201.

RESULTS AND DISCUSSION

Effect of Enzyme Concentration

Effect of protease concentration on hydrolysis of gelatin was studied at 40˚C and pH 10 since protease from *Conidiobolus coronatus* was optimally active at above conditions. Higher the enzyme concentration, greater was the hydrolysis rate. It was observed that gelatin layer was stripped completely within 10 min when enzyme concentration ranged from 0.81 to 2.7 U/ml (Figure 6.8). Hydrolysis percentages were 13, 70 and 90 for 0.81, 1.35 and 2.7 U/ml respectively at the end of 5 min. (Hydrolysis would have been complete in 6 minutes itself with 1.35 and 2.7 U/ml but not recorded as samples were removed at 5 min intervals). Longer incubations were required for complete removal of gelatin with lower enzyme concentrations. Complete gelatin removal occurred only after 35 min with the lowest enzyme concentration of 0.16 U/ml. Ingale et al. (2002) have reported the use of 50 U/ml of protease from *Basidiobolus* for complete hydrolysis. Lower enzymes concentrations of 10 U/ml required 12 min while degradation was complete within 5 min with higher enzyme concentration of 90 U/ml. Masui et al. (2004) have reported that the time required for complete gelatin hydrolysis from X-ray film by *Bacillus* B21-2 protease (at the enzyme to film ratio of 5.6 x 10^{-7} g/cm^3) to be temperature dependent and was between 8-10 min at 50˚C, pH 10.5. Singh et al. (1999) have reported complete gelatin degradation in 24 min by 10 U/ml of alkaline protease while 8-12 min was required when the enzyme concentration was increased to 25-100 U/ml.

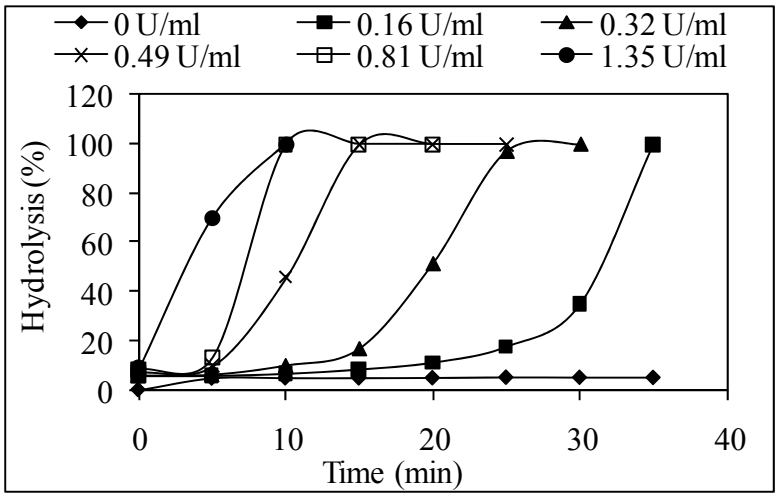


Figure 6.8: Effect of enzyme concentration on gelatin hydrolysis

Time Course of Hydrolysis and Release of Protein and Hydroxyproline

Nearly more than 80-90% of stripping off of gelatin from X-ray film was achieved in 6-7 min at pH 10 and 40˚C. Protein and hydroxyproline in the hydrolysate increased with progress of gelatin removal and the ratio of hydroxyproline to protein was nearly constant (Figure 6.9a). After complete stripping off of gelatin, hydroxyproline and protein concentrations ranged between 40-45 μg/ml and 160-170 μg/ml respectively. In all the experimental runs except in case of high enzyme concentrations, lag periods were observed when the hydrolysis was very slow. This could be due to slow adsorption of protease on to the surface. With time, the hydrolysis rates increased as a result of the increase in the amount of the enzyme adsorbed on the gelatin surface (Figure 6.9b). Masui et al. (1999) have studied the decomposition of gelatin by proteases from alkalophilic *Bacillus* and its three mutants. They have reported 0-17% gelatin degradation in 30 min depending on the enzyme (wild or mutants) while complete degradation was achieved in 45 min for all the enzymes tested at concentration of 5 U/ml and 50˚C, pH 10.5. They observed that swelling of gelatin on the X-ray film takes place during initial 30 min after which hydrolysis proceeds rapidly from 30-45 min. Fujiwara et al. (1989) have 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 while all the alkaline proteases from the neutrophile *Bacillus subtilis* took more than 20 min to act. Subtilisin BPN, took 30 min to decompose the gelatin layer at 50-60˚C while treatment time increased to 120-180 min at 30˚C.

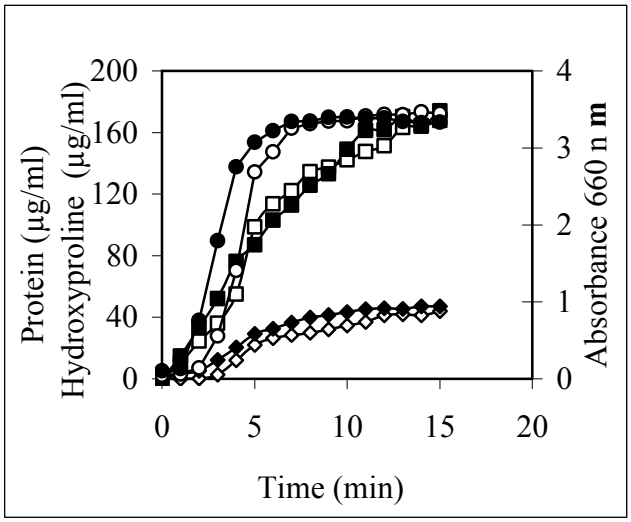


Figure 6.9a: Time course of gelatin hydrolysis: hydrolysis (● ○), protein (■ □) and hydroxyproline (◆ ◇) released. Closed symbols- 2.7 U/ml; opened symbols-1.35 U/ml.

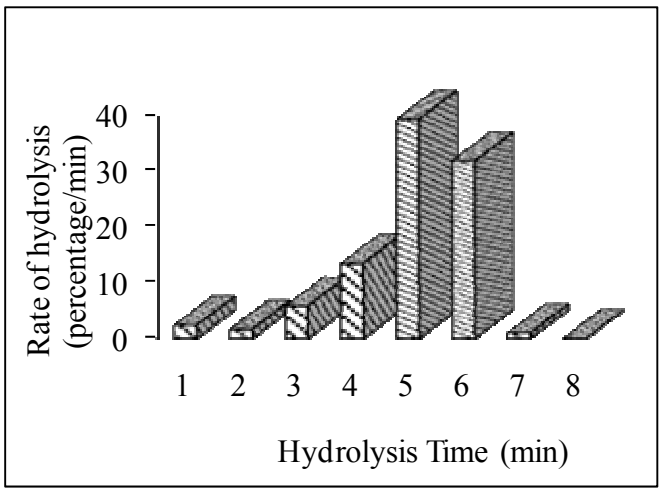


Figure 6.9b: Rate of gelatin hydrolysis during the time course

Reuse of Protease

The reuse of protease for gelatin hydrolysis was evaluated at 40˚C, pH 10 and with protease concentrations varying from 0.18 to 0.9 U/ml in a water bath with continuous shaking until the gelatin layer was completely removed. With 0.9 U/ml, protease could be reused up to 4 times (Table 6.2). Lowering the enzyme concentration resulted in longer reaction time for complete gelatin removal and also reduced the number of recycles. Repeated treatment of X-ray films for every 60 min was carried

out at 50˚C with 5-20 U/ml of protease using wild type and A187P enzyme (Masui et al., 1999). They have reported that the treatment time increased after every reuse of enzyme. At an enzyme concentration of 5 U/ml, first decomposition was complete in 60 min while 2nd use required more than 2 h. Increasing the enzyme concentration, resulted in increased number of reuses (Masui et al., 1999).

Table 6.2: Reuse of enzyme for gelatin hydrolysis from waste X-ray film

Protease (U/ml)	Time required for complete gelatin removal (min)			
	Cycle 1	Cycle 2	Cycle 3	Cycle 4
0.18	40	70	-	-
0.45	8	17	20	-
0.90	6	10	14	20

- incomplete removal

Recovery of Silver

Treatment of X-ray films with protease resulted in the sliver bound with gelatin being stripped off into the reaction mixture and clean plastic film was recovered. The loss in weight after the treatment was around 5% (w/w) based on initial weight of the film. The silver content in the hydrolysate was determined by atomic absorption and corresponded to 3.87% (w/w) of the solid sludge and 0.2% (w/w) based on the weight of the X-ray film. Ingale et al. (2002) have reported 0.1% (w/w) of silver recovery from photographic film. Apart from Ag, other metals in the slurry were also analyzed and only Mg was detected in trace amounts while metals like Fe, Cr, Cu, Al, Pb and Ni were not detected. Silver from the hydrolysate was recovered either as metallic silver or as silver chloride. Silver chloride can be used to make photographic paper, as pottery glazes, in photochromic lenses, in stained glass manufacture, in bandages and wound healing products.

CONCLUSION

Complete stripping off of gelatin in 6-7 min at pH 10 and 40°C with 13.5 U of protease. Gelatin removal, release of total protein, hydroxyproline follow similar trend and increased with time. Rate of gelatin hydrolysis increased up to certain limit then after decreased. Enzyme can be reused for 4 times. Silver recovered- 0.2% (w/w) based on the weight of the X-ray film.

REFERENCES

- Fujiwara, N., Tsumiya, T., Katada, T., Hosobuchi, T. and Yamamoto, K. (1989). *Proc Biochem*, 24: 155-156.
- Ingale, S. S., Rele, M. V. and Srinivasan, M. C. (2002). *World J Microbiol Biotechnol*, 18: 403-408.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). *J Biol Chem*, 193: 265-275.
- Masui, A., Fujiwara, N., Takagi, M. and Imanaka, T. (1999). *Biotechnol Techniq*, 13: 813-815.
- Masui, A., Yasuda, M., Fujiwara, N. and Ishikawa, H. (2004). *Biotechnol Prog*, 20: 1267-1269.
- Nakiboglu, N., Toscali, D. and Nisli, G. (2003). *Turk J Chem*, 27: 127-133.
- Nakiboglu, N., Toscali, D. and Yasa, I. (2001). *Turk J Chem*, 25: 349-353.
- Neuman, R. E. and Logan, M. (1950). *J Biol Chem*, 184: 299-306.
- Singh, J., Vohra, R. M. and Sahoo, D. K. (1999). *Biotechnol Lett*, 21: 921-924.