ALKALINE PROTEASE PRODUCTION BY MARINE FUNGUS ENGYODONTIUM ALBUM BTMF S10

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Alkaline Protease Production by Marine Fungus *Engyodontium album* BTMF S10**" is based on the original research work carried out by Miss. Sreeja Chellappan under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree.

M. CHANDRASEKARAN

ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
µ	-	micron
µg	-	microgram
µl	-	microlitre
µM	-	micro Molar
A_{280}	-	Absorbance at 280 nm
AIDS	-	Acquired Immuno Deficiency Syndrome
Asn	-	Asparagine
Asp	-	Aspartic acid
ATP	-	Adenosine triphosphate
BOD	-	Biological Oxygen Demand
BSA	-	Bovine Serum Albumin
cfu	-	colony forming unit
CM Cellulose	-	Carboxymethyl cellulose
cm	-	centimetre
Cys	-	Cysteine
Da	-	Dalton
DEAE Cellulose	-	Diethyl amino ethyl cellulose
DFP	-	di-isopropylfluorophosphate
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic Acid
DW	-	Distilled water
E_a	-	Energy of Activation
EC	-	Enzyme Classification
EDTA	-	Ethylene diamine tetra acetic acid
$[E]_T$	-	Total enzyme concentration

FPLC	-	Fast Protein Liquid Chromatography
g	-	grams
Glu	-	Glutamic acid
Gly	-	Glycine
Gppp	-	Guanosine triphosphate
GTP	-	Guanosine triphosphate
His	-	Histidine
hrs	-	hours
Kcal/mol	-	kilo calories/ mole
K_{cat}	-	Turn over number
kDa	-	kilo Dalton
K_m	-	substrate concentration at which the reaction velocity is half maximum
M	-	Molar
(M_r)	-	Relative molecular weight
MALDI	-	Matrix Assisted Laser Desorption Ionization
mg	-	milligram
ml	-	millilitre
mm	-	millimetre
mM	-	milli Molar
mRNA	-	messenger Ribonucleic Acid
MW	-	molecular weight
nm	-	nanometer
O D	-	optical density
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PEG	-	Polyethylene glycol
pI	-	Isoelectric point

PVDF	-	Polyvinylidene flouride
rDNA	-	recombinant Deoxy ribonucleic acid
RNA	-	Ribonucleic Acid
rpm	-	rotations per minute
SDS	-	Sodium dodecyl sulphate
Ser	-	Serine
SmF	-	Submerged Fermentation
sp.	-	Species
SSF	-	Solid State Fermentation
TCA	-	Trichloro acetic acid
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine
Thr	-	Threonine
U/gIDS	-	Units/ gram Initial Dry Substrate
U/mg	-	Units/ milligram
U/ml	-	Units / millilitre
V_{max}	-	maximal velocity
WB	-	Wheat Bran

CONTENTS

Chapter 1

INTRODUCTION

Enzymes are the focal point of biotechnological process since they are involved in all aspects of biochemical conversion, from the simple fermentation conversion to the complex techniques in genetic engineering and molecular biology. They are used as cost-effective and ecofriendly substitutes for chemical processing in several industries, including pharmaceutical, food and beverages, starch, laundry detergents; in the processing of textiles, leather, wood, pulp and paper; in the production of fine and specialty chemicals, in organic synthesis and transformation of compounds; bioremediation and waste treatment.

The world market for industrial enzymes is worth $1000 million with an average growth rate of 10 percent per annum (Biospectrum, June 2005). Proteases are the single class of enzymes, among all the enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. They are hydrolytic enzymes, that catalyze the cleavage of peptide bonds in other proteins. Today, proteases account for approximately 60% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management, and silver recovery (Gupta et al., 2002b; Rao et al., 1998). This dominance of proteases in the industrial market is expected to increase further in the coming years.

Among the source of enzymes i.e., animals, plants and microbes, enzymes from microorganisms have become the choice for industrial production. The current trend is to use microbial enzymes since they provide a greater diversity of catalytic activities and can be produced more economically. Microorganisms have qualified as apt source of industrial enzymes owing to their consistency, ease of

process optimization and modification, and the possibility of enzyme production using recombinant strains with enhanced yield.

Microorganisms elaborate a large array of intracellular and/or extracellular proteases. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz, 1988).

The technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzymes insufficient and the search for new microbial sources is continued. In fact only 2% of the world's microorganisms have been tested as enzyme sources (Wiseman, 1985), although microorganisms from diverse and exotic environments, and extremophiles are considered an important source of enzymes, and their specific properties are expected to result in novel process applications (Govardhan and Margolin, 1995; Robertson et al., 1996). Looking into the depth of microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties that are suitable for commercial exploitation. The multitude of physicochemically diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products and processes.

About 80% of the commercial enzymes are produced using microorganisms, and they are of terrestrial origin. Although the oceans cover more than two third of the earth's surface, the knowledge on marine microbes as source of enzymes is still very limited and they remain as untapped sources of many metabolites and biomolecules with novel properties.

For the last two decades the common conclusions of international flora considering strategic challenges in science have uniformly identified the marine

biotope as a large and untapped area for exploration (ESF-MarineBoard, 2001). The rich diversity of marine form and function, and its unique physiological adaptations to the harsh marine environment coupled with new developments in biotechnology, has opened up a new and exciting vista for the extraction of bioactive products of use in medicine, novel industrial processes and environmental monitoring.

Marine organisms represent a dramatically different environment for biosynthesis than do terrestrial organisms and therefore represent a vast untapped resource with potential benefits in many different areas such as medicine, aquaculture and fisheries, industry, research tools and environmental applications. Bioscreening of microorganisms, plants or animals select out those with the most desirable characteristics.

The potential applications offered by the screening of marine substances extend to pharmacology, agrochemistry and the environment. Moreover, the use of combined approaches enhances these possibilities because marine molecules often belong to new classes without terrestrial counterparts. High throughput screening techniques are particularly suitable for such combined approaches. In addition, marine microorganisms are a source of new genes, the exploitation of which is likely to lead to the discovery of new drugs and targets. Secondary metabolites produced by marine bacteria and invertebrates have yielded pharmaceutical products such as novel anti-inflammatory agents (e.g. pseudopterosins, topsentins, scytonemin, manoalide), anti-cancer agents (e.g. bryostatins, discodermolide, eleutherobin and sarcodictyin) and antibiotics (e.g. marinone). Melanins have a range of chromophoric properties that can be exploited for sunscreens, dyes and colouring. They also sequester different kinds of organic compounds, inducing fungicides and antibiotics, which may allow them to act as slow-release agents.

Extracts, hydrolysates and enzymes from seawater fish species and marine invertebrates have revealed interesting characteristics. In particular, many marine

enzymes have characteristics deviating significantly from their mammalian counterparts. Marine microorganisms have a diverse range of enzymatic activity including minerlisation process and cycling of elements in various environments and are capable of catalyzing various biochemical reactions with novel enzymes (Chandrasekaran, 1997). Most important is the activity-temperature relationship; high activities are obtained at 5-12°C for enzymes of marine origin, instead of 30-35°C for classical mesophilic systems. Hence, these enzymes are suitable for optimal processing at low temperatures.

Since marine microorganisms are salt tolerant and have the potential to produce several novel metabolites, it is assumed that among them, there may be potential protease producers also. The marine microorganisms, which have immense potential as source of exoenzymes are yet to be tapped. Though few reports were made on the production of protease from marine bacterial sources (Estrada-Badillo et al., 2003; Kumar et al., 2004; Salamanca et al., 2002), hardly any report is available on the production of protease at an industrial scale using marine fungus (Chandrasekaran and Kumar, 2002).

In recent years, solid state fermentation (SSF) has gained importance in the production of microbial enzymes as a result of search for cheap alternative production system which utilizes simpler agro-industrial wastes as substrates. SSF has several economic advantages over conventional submerged fermentation such as minimal requirement of water, production of metabolites in a more concentrated form and making the downstream processing less time consuming and less expensive (Hesseltine, 1972; Lonsane et al., 1985). Among the various groups of microorganisms used in SSF, filamentous fungi are the most widely exploited owing to their ability to grow and produce a wide range of extracellular enzymes on complex solid substrates (Moo-young et al., 1983). It is reported that the amount of enzymes secreted in solid state cultures are large and frequently exceed the amounts secreted in submerged culture, and solid state specific gene expression appears to be responsible for this increased production (Iwashita, 2002). In recent

years, few reports are available on the production of protease from fungal cultures using solid state fermentation but none of them are from a marine source (Germano et al., 2003; Sandhya et al., 2005; Tunga et al., 2003).

Since 1914, proteases are being widely used as detergent additive and over the past 30 years, the importance of proteases in detergents has changed from being the minor additives to key ingredients and now the use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. In spite of this fact, all detergent proteases currently used in the market are serine proteases produced by *Bacillus* sp. (Rao et al., 1998). Fungi as enzyme producers, have many advantages such as the produced enzymes are normally extracellular, making ease of downstream processing to prepare cell free enzyme etc. Available reports on protease production by fungi such as *Metarhizium anisopliae* (St.Lagers et al., 1986) and *Beauveria bassiana* (Bidochka and Khachatourians, 1987), mainly deals with the virulence factors that are contributed by these proteases in entomopathogenic fungal infection. A few fungal sources such as *Conidiobolus coronatus* (Phadatare et al., 1993), *Penicillium* sp. (Germano et al., 2003) and *Aspergillus parasiticus* (Tunga et al., 2003) were studied for their application in commercial detergent industry.

During the course of an earlier investigation in this laboratory, it was observed that an alkalophilic and salt tolerant fungus, isolated from marine sediment of Cochin coast and identified as *Engyodontium album*, secretes extracellular protease, which shows greater activity in alkaline pH (Beena, 1999; Suresh and Chandrasekaran, 1998). In this context, the present study mainly deals with the production of this fungal protease through solid state fermentation towards consequent development of ideal bioprocess for industrial production, isolation, characterisation and evaluation of the potential of the protease enzyme for various industrial applications.

OBJECTIVES OF THE PRESENT STUDY

A cursory glance of the review of literature would indicate that in spite of the fact that 80% of the commercial enzymes produced using microorganisms are of terrestrial origin, hardly any information is available about marine microbial sources. Hence it was desired to pursue some exploratory studies on the alkalophilic and salt tolerant fungus, isolated from the marine sediments of Cochin coast, that secretes an extracellular protease which shows greater activity in alkaline pH, towards exploiting the fungus as a potential source of alkaline protease for commercial application. Thus, the primary objectives of this study include the evaluation of the potential of this marine *Engyodontium album*, for protease production through solid state fermentation, purification, characterisation of this enzyme and assessment of its potential applications.

Specific objectives of the present study include

1. Optimization of various physicochemical factors in solid state fermentation for the production of alkaline protease by *Engyodontium album*
2. Purification of the proteases enzyme
3. Characterisation of the enzyme
4. Evaluation of the enzyme for various industrial applications

Chapter 2

REVIEW OF LITERATURE

2.1 Proteases

Proteases, which can hydrolyse the peptide bonds in other proteins, constitute a very large and complex group of enzymes, and differ in properties such as substrate specificity, active site, catalytic mechanism, pH and temperature of activity and stability profiles (Ward, 1985). They execute a large variety of functions, extending from the cellular level to the organ and organism level, and to produce cascade systems in an organism to maintain homeostasis.

Extracellular microbial proteases contribute to the nutritional well being of the organism by hydrolyzing large peptide substrates into smaller molecules that the cell can absorb. Mammalian pancreatic proteases and intestinal and stomach peptidases generally perform a similar nutritional role in the digestion and absorption process of these species. Proteolytic enzymes are also involved in the regulation of biological metabolic processes such as spore formation (Kornberg et al., 1968; North, 1982), spore germination (Jackson and Cotter, 1984), protein maturation in viral assembly, activation of certain viruses of importance for pathogenicity (Katoh et al., 1985), various stages of the mammalian fertilization process (Honda et al., 2002), blood coagulation, fibrinolysis, complement activation (Chambers and Laurent, 2001; Sim and Tsiftsoglou, 2004), phagocytosis and blood pressure control (Li et al., 2004).

In all cell systems there is a balance between metabolic processes involving protein synthesis and breakdown, and intracellular proteinases play a vital role in these protein turnover processes, which is essential for the adaptation of microbial and other cells to the new environmental conditions, particularly in

environments lacking in amino acids, as it generates amino acid pool for synthesis of newly required enzymes and other proteins (Hershko et al., 1984). Proteases are also important for cell differentiation, translation, modulation of gene expression, and in enzyme modification and secretion (Roberts et al., 1977). Proteases convert inactive enzymes and other biologically inactive protein molecules into their active forms and during extracellular enzyme secretion, the hydrophobic peptide extension which facilitates the passage of the enzyme through the cell membrane is cleaved by proteolytic action (Smeekens, 1993).

2.2 Classification of Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified into subgroup 4 of group 3 (hydrolases) (IUBMB, 1992). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Barrett, 2001).

Depending on their site of action, proteases are broadly classified into two major groups, i.e., exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960). But, there are a few miscellaneous proteases, which do not precisely fit into the standard classification. Based on the pH of their optimal activity, proteases are also referred to as acidic, neutral or alkaline proteases. In the EC list, peptidases are divided among 13 sub-classes comprising exopeptidases (3.4.11-19) and endopeptidases (3.4.21-24 together with 3.4.99) as given in Table 2.1.

According to the widely used and most comprehensive database of proteases (MEROPS; http://www.merops.sanger.ac.uk), enzymes of each catalytic type are classified into evolutionarily distinct "clans" and each clan is subdivided into "families" (Rawlings et al., 2004). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, T, A, M or U for serine, cysteine, threonine, aspartic, metallo or unknown type, respectively (Rawlings and Barrett, 1993). Threonine type peptidases are the most recently discovered catalytic type (Seemuller et al., 1995).

Table 2.1 The EC system of classification of peptidases

Sub-subclass	Type of peptidase
	Exopeptidases
3.4.11	Amino peptidases
3.4.13	Dipeptidases
3.4.14	Dipeptidyl-peptidases
3.4.15	Peptidyl-dipeptidases
3.4.16	Serine-type carboxypeptidases
3.4.17	Metallocarboxypeptidases
3.4.18	Cysteine-type carboxypeptidases
3.4.19	Omega peptidases
	Endopeptidases
3.4.21	Serine endopeptidases
3.4.22	Cysteine endopeptidases
3.4.23	Aspartic endopeptidases
3.4.24	Metalloendopeptidases
3.4.99	Endopeptidases of unknown type

2.2.1 Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino and carboxypeptidases, respectively.

2.2.1.1 Aminopeptidases

Aminopeptidases act at a free N-terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide or a tripeptide. They are known to remove the N-terminal 'Met' that may be found in heterologously expressed proteins but not in many naturally occurring mature proteins. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi (Watson, 1976). In general, aminopeptidases are intracellular enzymes, but there has been a single report on an extracellular aminopeptidase produced by *Aspergillus oryzae* (Labbe et al., 1974). The substrate specificities of the enzymes from bacteria and fungi are distinctly different in that the organisms can be differentiated on the basis of the product profiles of enzyme hydrolysis (Cerny, 1978). Aminopeptidase I from *Escherichia coli* is a large protease (4,00,000 Da). It has a broad pH optimum of 7.5 to 10.5 and requires Mg^{2+} or Mn^{2+} for optimal activity (Matco and Dick, 1978). The *Bacillus licheniformis* aminopeptidase has a molecular weight of 34,000 Da. It contains 1 g-atom of Zn^{2+} per mol, and its activity is enhanced by Co^{2+} ions. On the other hand, aminopeptidase II from *Bacillus stearothermophilus* is a dimer with a molecular weight of 80,000 to 100,000 Da and is activated by Zn^{2+}, Mn^{2+} or Co^{2+} ions (Stoll et al., 1976).

2.2.1.2 Carboxypeptidases

The carboxypeptidases act at C-terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, namely serine carboxypeptidases, metallocarboxypeptidases and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes. The serine carboxypeptidases isolated from *Penicillium* spp., *Saccharomyces* spp. and *Aspergillus* spp. are similar in their substrate specificities but differ slightly in other properties such as pH optimum, stability, molecular weight and effect of inhibitors. Metallocarboxypeptidases from *Saccharomyces* spp. (Felix and Brouillet, 1966) and *Pseudomonas* spp. require

Zn^{2+} or Co^{2+} for their activity (Lu et al., 1969). The enzymes can also hydrolyze the peptides in which the peptidyl group is replaced by a pteroyl moiety or by acyl groups.

2.2.2 Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N- and C-termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism: (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases and (iv) metalloproteases.

2.2.2.1 Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase and endopeptidase groups. Based on their structural similarities, serine proteases have been grouped into 40 families, which have been further subdivided into 10 clans with common ancestors (Barrett, 1994). The primary structures of the members of four clans, chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia* D-Ala–D-Ala peptidase A (SE) are totally unrelated, suggesting that there are at least four separate evolutionary origins for serine proteases. Clans SA, SB and SC have a common reaction mechanism consisting of a common catalytic triad of the three amino acids, serine (nucleophile), aspartate (electrophile) and histidine (base). Although the geometric orientations of these residues are similar, the protein folds are quite different, forming a typical example of a convergent evolution. The catalytic mechanisms of clans SE and SF are distinctly different from those of clans SA, SB and SE, since they lack the classical Ser-His-Asp triad. Another interesting feature of the serine

proteases is the conservation of glycine residues in the vicinity of the catalytic serine residue to form the motif Gly-Xaa-Ser-Yaa-Gly (Brenner, 1988).

Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxy trans-2,3-epoxypropyl-leucylamido (4-guanidine), butane (E-64), di-isopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), and tosyl-L-lysine chloromethyl ketone (TLCK) (Barrett, 2001). Some of the serine proteases are inhibited by thiol reagents such as *p*-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7.0 and 11.0. They have broad substrate specificities including esterolytic and amidase activity. Their molecular mass range between 18 and 35kDa, and the serine protease from *Blakeslea trispora* has a molecular mass of 126kDa (Govind et al., 1981). The isoelectric points of serine proteases are generally between pI 4.0 and 6.0. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases.

2.2.2.1.1 Serine alkaline proteases

Serine alkaline proteases are produced by several bacteria, molds, yeasts and fungi. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or tosyl-L-lysine chloromethyl ketone (TLCK). Their substrate specificity is similar to, but less stringent than that of chymotrypsin. They hydrolyze a peptide bond, which has tyrosine, phenylalanine or leucine at the carboxyl side of the splitting bond. The optimal pH of alkaline proteases is around pH 10.0, and their isoelectric point is around pH 9.0. Their molecular mass is in the range of 15 to 30kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces* and *Flavobacterium* spp. (Boguslawski et al., 1983), subtilisins produced by *Bacillus* spp. are the best known. Alkaline proteases are also produced by *S. cerevisiae*

(Mizuno and Matsuo, 1984) and filamentous fungi such as *Conidiobolus* spp (Phadatare et al., 1993), *Aspergillus* and *Neurospora* spp. (Lindberg et al., 1981).

2.2.2.1.2 Subtilisins

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protease Nagarase (BPN9) have been identified. Subtilisin Carlsberg produced by *Bacillus licheniformis* was discovered in 1947 by Linderstrom, Lang and Ottesen at the Carlsberg laboratory. Subtilisin Novo or BPN9 is produced by *Bacillus amyloliquefaciens*. Subtilisin Carlsberg is widely used in detergents. Its annual production amounts to about 500 tons of pure enzyme protein. Subtilisin BPN9 is less commercially important. Both subtilisins have a molecular mass of 27.5kDa but differ from each other by 58 amino acids. They have similar properties such as an optimal temperature of 60°C and an optimal pH of 10.0. Both enzymes exhibit broad substrate specificity and have an active-site triad made up of Ser221, His64 and Asp32. The Carlsberg enzyme has broader substrate specificity and does not depend on Ca^{2+} for its stability. The active-site conformation of subtilisins is similar to that of trypsin and chymotrypsin despite the dissimilarity in their overall molecular arrangements. The serine alkaline protease from the fungus *Conidiobolus coronatus* was shown to possess a distinctly different structure from subtilisin Carlsberg in spite of their functional similarities (Phadatare et al., 1997).

2.2.2.2 Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families namely, pepsin (A1), retropepsin (A2) and enzymes from pararetroviruses (A3) and have been placed in clan AA (Barett, 1995). The members of families A1 and A2 are known to be

related to each other, while those of family A3 show some relatedness to A1 and A2. Most aspartic proteases show maximal activity at low pH (3.0 to 4.0) and have isoelectric points in the range of pH 3.0 to 4.5. Their molecular mass is in the range of 30 to 45kDa. The members of the pepsin family have a bilobal structure with the active-site cleft located between the lobes (Sielecki et al., 1991). The active-site aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be 'Ser' or 'Thr'. The aspartic proteases are inhibited by pepstatin (Fitzgerald et al., 1990). They are also sensitive to diazoketone compounds such as di-azoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3- (*p*-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Microbial aspartic proteases can be broadly divided into two groups: (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus* and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* spp.

2.2.2.3 Cysteine/thiol proteases

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of 'Cys' and 'His' (Cys-His or His-Cys) residues differs among the families (Barrett, 1994). Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB, but are unaffected by DFP and metal-chelating agents. Clostripain,

produced by the anaerobic bacterium *Clostridium histolyticum*, exhibits a stringent specificity for arginyl residues at the carboxyl side of the splitting bond and differs from papain in its obligate requirement for calcium. Streptopain, the cysteine protease produced by *Streptococcus* spp., shows a broader specificity, including oxidized insulin B chain and other synthetic substrates. Clostripain has an isoelectric point of pH 4.9 and a molecular mass of 50kDa, whereas the isoelectric point and molecular mass of streptopain are pI 8.4 and 32kDa, respectively

2.2.2.4 Metalloproteases

Metalloproteases are the most diverse of the catalytic type of proteases (Barett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms and thermolysin from bacteria (Hibbs et al., 1985; Okada et al., 1986; Shannon et al., 1989; Weaver et al., 1977). At present, metalloproteases have been classified into 54 families (http://www.merops.sanger.ac.uk), which have been grouped into 16 clans based on the nature of the amino acid that completes the metal-binding site; e.g., clan MA has the sequence HEXXH-E and clan MB corresponds to the motif HEXXH-H. In one of the groups, the metal atom binds at a motif other than the usual motif

Based on the specificity of their action, metalloproteases can be divided into four groups: (i) neutral, (ii) alkaline, (iii) *Myxobacter* I and (iv) *Myxobacter* II. The neutral proteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity. *Myxobacter* protease I is specific for small amino acid residues on either side of the cleavage bond, whereas *Myxobacter* protease II is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP.

Thermolysin, a neutral protease, is the most thoroughly characterized member of clan MA. Histidine residues from the HEXXH motif serve as Zn ligands and Glu has a catalytic function (Weaver et al., 1977). Thermolysin produced by *B. stearothermophilus* is a single peptide without disulfide bridges and has a molecular mass of 34kDa. It contains an essential Zn atom embedded in a cleft formed between two folded lobes of the protein and four Ca atoms, which impart thermostability to the protein. Thermolysin is a very stable protease, with a half-life of 1 h at 80°C.

Collagenase, another important metalloprotease, was first discovered in the culture broth of the anaerobic bacterium *Clostridium histolyticum* as a component of toxic products. Later, it was found to be produced by the aerobic bacterium *Achromobacter iophagus* and other microorganisms including fungi (Demina and Lysenko, 1996). The action of collagenase is very specific; i.e., it acts only on collagen and gelatin and not on any of the other usual protein substrates. Elastase produced by *Pseudomonas aeruginosa* is another important member of the neutral metalloprotease family.

The alkaline metalloproteases produced by *Pseudomonas aeruginosa* and *Serrana* spp. are active in the pH range from 7.0 to 9.0 and have molecular mass in the region of 48 to 60kDa. *Myxobacter* protease I has a pH optimum of 9.0 and a molecular mass of 14kDa and can lyse cell walls of *Arthrobacter crystallopoites*, whereas protease II cannot lyse the bacterial cells. Matrix metalloproteases play a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation and wound healing and may be useful in the treatment of diseases such as cancer and arthritis (Browner et al., 1995).

2.3 Mechanism of Action of Proteases

The mechanism of action of proteases has been a subject of great interest to researchers and several reports are available in literature for serine proteases, aspartic proteases, metalloproteases and cysteine proteases.

2.3.1 Serine Proteases

Serine proteases usually follow a two-step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragment (Fastrez and Fersht, 1973). This acylation step is followed by a deacylation process, which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide (Fig. 2.1). Serine endopeptidases can be classified into three groups mainly based on their primary substrate preference: (i) trypsin-like, which cleave after positively charged residues; (ii) chymotrypsin- like, which cleaves after large hydrophobic residues; and (iii) elastase-like, which cleaves after small hydrophobic residues. The P1 residue exclusively dictates the site of peptide bond cleavage.

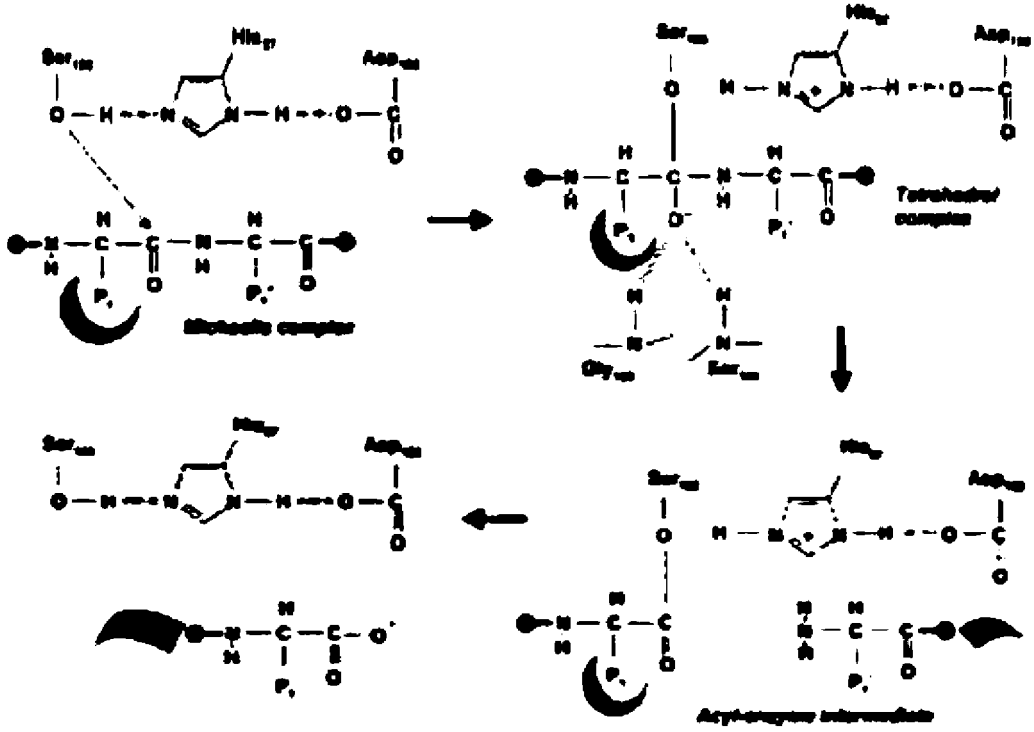


Fig. 2.1 Schematic representation of the steps involved in catalysis by the serine protease type of enzyme.

The primary specificity is affected only by the P1 residues; the residues at other positions affect the rate of cleavage. The sub site interactions are localized to specific amino acids around the P1 residue to a unique set of sequences on the enzyme

Some of the serine peptidases from *Achromobacter* spp. are lysine-specific enzymes (Masaki et al., 1978), whereas those from *Clostridium* spp are arginine specific (clostripain) (Gilles et al., 1979), and those from *Flavobacterium* spp are post proline-specific (Yoshimoto et al., 1980). Endopeptidases that are specific to glutamic acid and aspartic acid residues have also been found in *B. licheniformis* and *Staphylococcus aureus* (Drapeau et al., 1972)

The recent studies based on the three-dimensional structures of proteases and comparisons of amino acid sequences near the primary substrate-binding site in trypsin-like proteases of viral and bacterial origin suggest a putative general substrate binding scheme for proteases with specificity towards glutamic acid involving a histidine residue and a hydroxyl function. However, a few other serine proteases such as peptidase A from *E. coli* and the repressor LexA show distinctly different mechanism of action without the classic Ser-His-Asp triad (Barett, 1994). Some of the glycine residues are conserved in the vicinity of the catalytic serine residue, but their exact positions are variable (Brenner, 1988). The chymotrypsin-like enzymes are confined almost entirely to animals, the exceptions being trypsin-like enzymes from actinomycetes and *Saccharopolyspora* spp and from the fungus *Fusarium oxysporum*.

A few of the serine proteases belonging to the subtilisin family show a catalytic triad composed of the same residues as in the chymotrypsin family; however, the residues occur in a different order (Asp-His-Ser). Some members of the subtilisin family from the *Tritirachium* and *Metarhizium* spp require thiol for their activity. The thiol dependance is attributable to Cys173 near the active-site histidine (Jany et al., 1986)

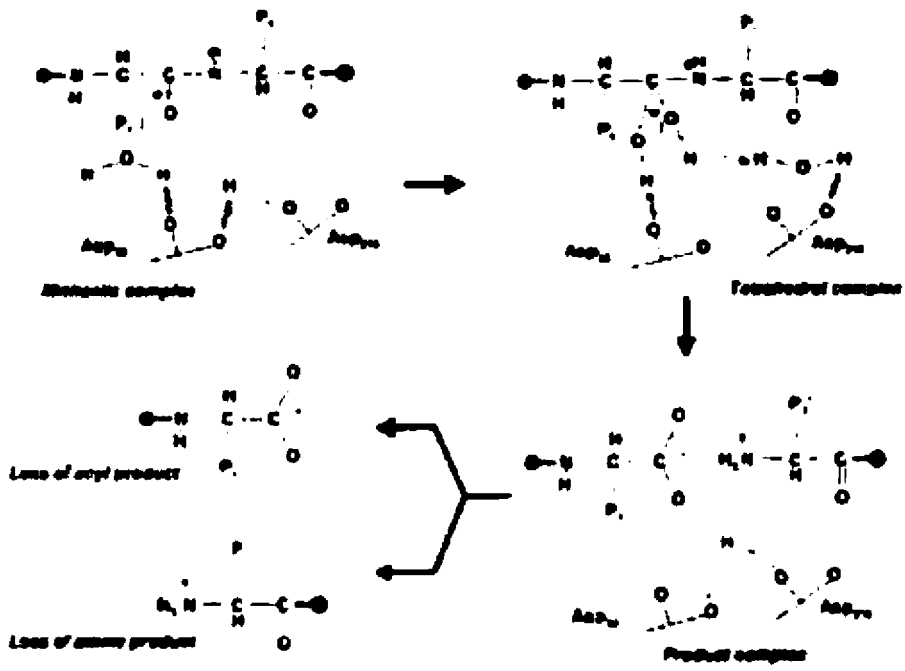
The carboxypeptidases are unusual among the serine-dependent enzymes in that they are maximally active at acidic pH. These enzymes are known to possess a 'Glu' residue preceding the catalytic 'Ser', which is believed to be responsible for their acidic pH optimum. Although the majority of the serine proteases contain the catalytic triad Ser-His-Asp, a few use the Ser-base catalytic dyad. The Glu-specific proteases display a pronounced preference for Glu-Xaa bonds over Asp-Xaa bonds (Austew and Smith, 1976).

2.3.2 Aspartic Proteases

Aspartic endopeptidases depend on the aspartic acid residues for their catalytic activity. A general base catalytic mechanism has been proposed for the hydrolysis of proteins by aspartic proteases such as penicillopepsin (James et al., 1977) and endothiapepsin (Pearl, 1987). Crystallographic studies have shown that the enzymes of the pepsin family are bilobed molecules with the active site cleft located between the lobes and each lobe contributing one of the pair of aspartic acid residues that is essential for the catalytic activity (Blundell et al., 1991; Sielecki et al., 1991). The lobes are homologous to one another, having arisen by gene duplication. The retropepsin molecule has only one lobe, which carries only one aspartic residue and the activity requires the formation of a noncovalent homodimer (Miller et al., 1989). In most of the enzymes from the pepsin family, the catalytic 'Asp' residues are contained in an Asp-Thr-Gly-Xaa motif in both the N- and C-terminal lobes of the enzyme, where Xaa is 'Ser' or 'Thr', whose side chains can form hydrogen bond to 'Asp'. However, Xaa is 'Ala' in most of the retropepsins. A marked conservation of cysteine residue is also evident in aspartic proteases. The pepsins and the majority of other members of the family show specificity for the cleavage of bonds in peptides of at least six residues with hydrophobic amino acids in both the P1 and P19 positions (Keil, 1992). The specificity of the catalysis has been explained on the basis of available crystal structures (Liu et al., 1996). The structural and kinetic studies also have suggested

that the mechanism involves general acid-base catalysis with lytic water molecule that directly participates in the reaction (Fig. 2.2). This is supported by the crystal structures of various aspartic protease-inhibitor complexes and by the thiol inhibitors mimicking a tetrahedral intermediate formed after the attack by the lytic water molecule (James et al., 1992).

A



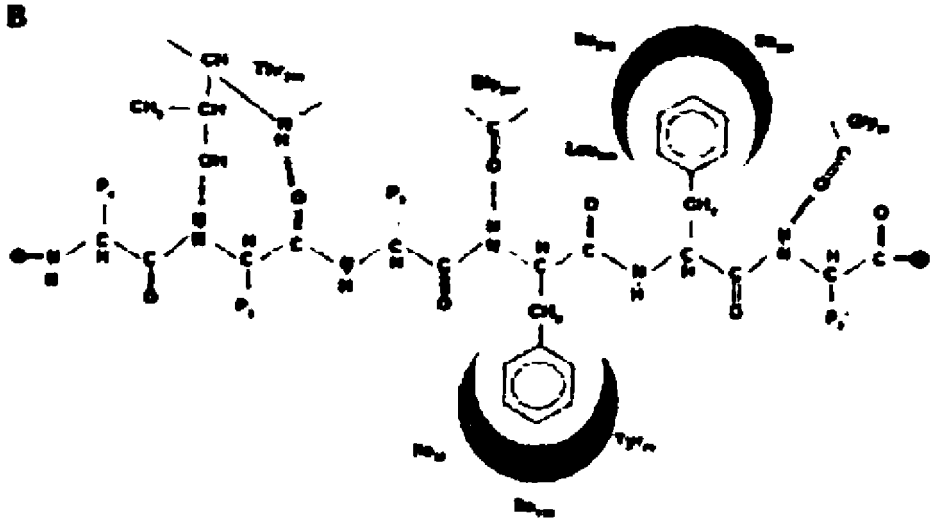


Fig. 2.2

(A) Schematic representation of the general acid-base catalytic mechanism of the aspartic protease type of enzyme.

(B) Schematic representation of the multitude of hydrogen bonding interactions available in the active site cleft of a typical aspartic protease.

2.3.3 Cysteine Proteases

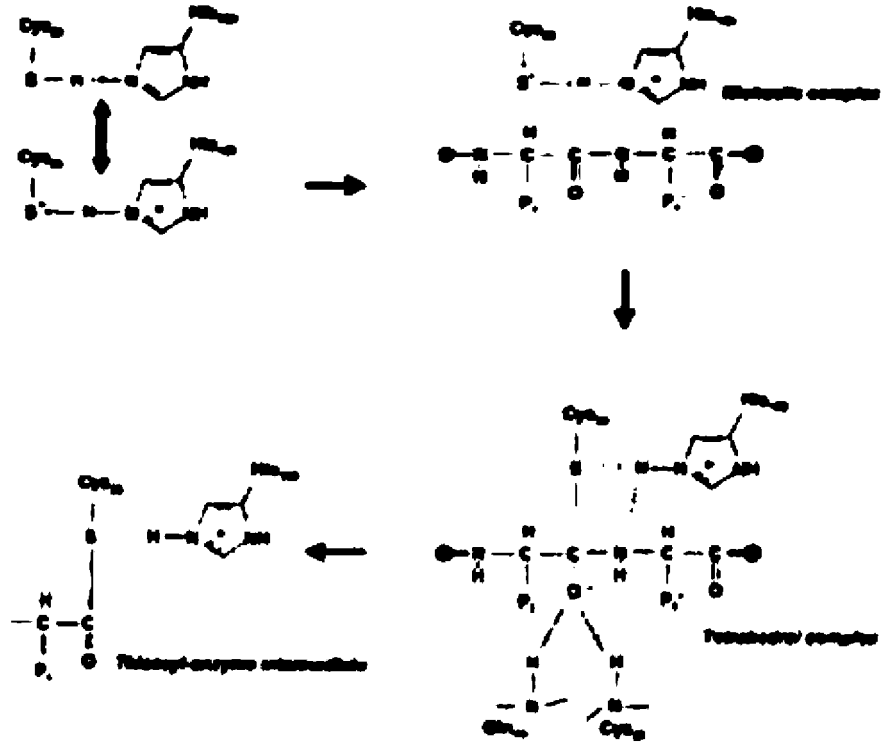


Fig. 2.3 Schematic representation of the steps involved in catalysis by the cysteine protease type of enzyme.

Cysteine proteases catalyse the hydrolysis of carboxylic acid derivatives through a double displacement pathway involving general acid base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of serine proteases. A striking similarity is also observed in the reaction mechanism for several peptidases of different evolutionary origin. The plant peptidase papain can be considered the archetype of cysteine peptidases and constitutes a good model for this family of enzymes. They catalyze the hydrolysis of peptide, amide ester, thiol ester and thiono ester bonds (Polgar, 1990). The initial step in the catalytic process (Fig. 2.3) involves the noncovalent

binding of the free enzyme and the substrate to form the complex. This is followed by the acylation of the enzyme, with the formation and release of the first product, the amine R9-NH_2. In the next deacylation step, the acyl-enzyme reacts with a water molecule to release the second product, with the regeneration of free enzyme. The enzyme papain consists of a single protein chain folded to form two domains containing a cleft for the substrate to bind. The crystal structure of papain confirmed the Cys25- His159 pairing (Baker and Drenth, 1987). The presence of a conserved aspargine residue (Asn175) in the proximity of catalytic histidine (His159) creating a Cys-His-Asn triad in cysteine peptidases is considered analogous to the Ser-His-Asp arrangement found in serine proteases.

2.3.4 Metalloproteases

The mechanism of action of metalloproteases (Fig. 2.4) is slightly different from that of the above described proteases. These enzymes depend on the presence of bound divalent cations and can be inactivated by dialysis or by the addition of chelating agents. For thermolysin, based on the X-ray studies of the complex with a hydroxamic acid inhibitor, it has been proposed that Glu143 assists the nucleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond, which is polarized by the Zn^{2+} ion (Holm and Matthews, 1981). Most of the metalloproteases are enzymes containing the His-Glu-Xaa-Xaa-His (HEXXH) motif, which has been shown by X-ray crystallography to form a part of the site for binding of the metal, usually zinc.

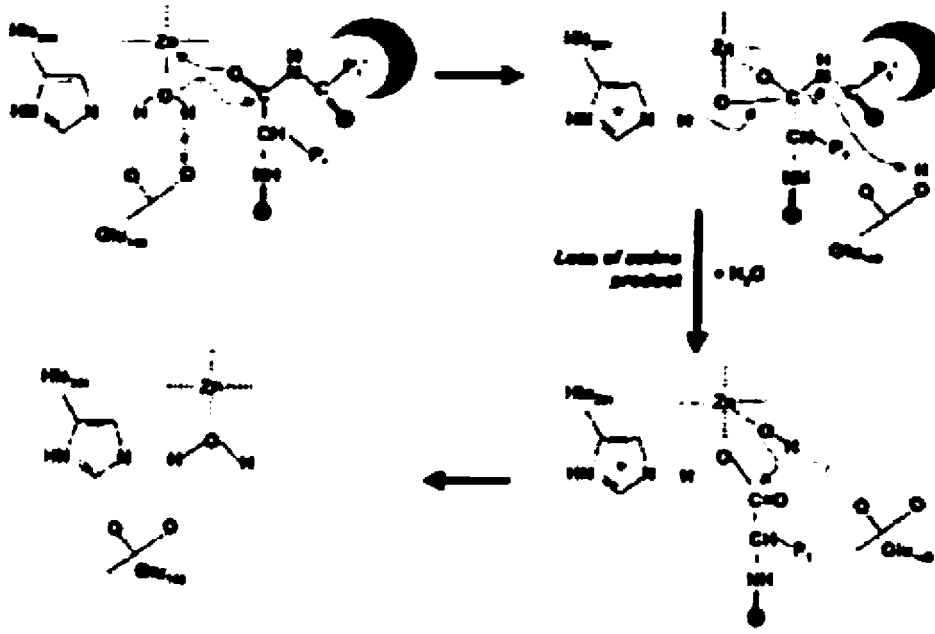


Fig. 2.4 Schematic representation of the catalysis of peptide bond cleavage carried out by a member of the metalloprotease class of enzyme.

In general, studies of the mechanism of action of proteases have revealed that they exhibit different types of mechanism based on their active site configuration. The serine proteases contain a Ser-His-Asp catalytic triad, and the hydrolysis of the peptide bond involves an acylation step followed by a deacylation step. Aspartic proteases are characterized by an Asp-Thr-Gly motif in their active site and by an acid-base catalysis as their mechanisms of action. Cysteine proteases adopt a hydrolysis mechanism involving a general acid-base formation followed by hydrolysis of an acyl-thiol intermediate. The activity of metalloproteases depends on the binding of a divalent metal ion to a His-Glu-Xaa-Xaa-His motif.

2.4 Microbes as source of protease

Proteases are known to be produced by plants, animals and microbes. However, in this study, a review on plant and animal sources is out of scope and the review is restricted only to microorganisms.

Microbes have drawn the attention as ideal source of proteases and account for approximately 40% of the total worldwide enzyme sales. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. They represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods, and they produce an abundant, regular supply of the desired product. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals. They have a longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity.

Despite the long list of protease-producing microorganisms, only a few are considered as appropriate producers for commercial exploitation, being 'genetically regarded as safe' (GRAS), non-toxic and non-pathogenic. A large number of microbes belonging to bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases of the serine type (Kumar and Takagi, 1999). Some of the reported proteases are listed in the Table 2.2.

Table 2.2 Optimised production conditions for alkaline protease producing microorganisms

Microorganism	pH	Temp (°C)	Agitation (rpm)	Incubation period(h)	Preferred /optimised nitrogen sources	Preferred/ optimised carbon sources	References
Bacteria							
Alcaligenes faecalis	8.0	30	200	48	Soybean meal	None[a]	Thangam and Rajkumar, 2000
Bacillus sp. IS-3	10.5	37	200	72	Soybean meal	Glucose	Purva et al., 1998
Bacillus sp. JB99	10.0	55	180	24	$NaNO_3$	Citric acid	Johnvesly and Naik, 2001
Bacillus sp. K2	7.0	37	300–500	60–72	Casein hydrolyzate, gelatine	Glycerol	Hameed et al., 1999
Bacillus sphaericus	10.0	30	300	24			Singh et al., 2004
Bacillus circuli	9.8	42	400	40	Casein	Corn starch	Kumar et al., 2004
Bacillus sp. P-2	9.5	30	-	24	Peptone, yeast extract	Glucose	Kaur et al., 2001
Bacillus sp. RGR-14	7.0	37	200	72–96	Soybean meal; peptone	Starch	Oberoi et al., 2001; Puri et al., 2002
Bacillus sp. SSR-1	10.0	40	150	18	Biopeptone, yeast extract	Beef extract, lactose	Singh et al., 2001a
B. brevis MTCC H0016	10.5	37	200	96	Soybean meal	Lactose	Banerjee et al., 1999
B. licheniformis ATCC 21415	7.0	30	250–400	48	Soybean, $(NH_4)_2PO_3$	Lactose, glucose	Mabrouk et al., 1999
B. mojavensis	7.0	50	200–250	24	Casein or casamino acids	Glucose	Beg et al., 2002a
B. pumilis MK6-5	9.6	35	250	60	Cornsteep liquor, tryptone	Glucose, sodium citrate	Kumar, 2002
B. sphaericus	n.s.[b]	30	300	n.s.	Biopeptone, yeast extract	Glucose	Singh et al., 2001b
B. subtilis 168	n.s.	36	250	6–8	Nutrient broth; yeast extract	Glucose, yeast extract	Lange et al., 1999
Flavobacterium balustinum P104	7.4	10	150	72	Polypeptone, yeast extract, casein	None[a]	Morita et al., 1998
Pseudomonas aeruginosa	7.0	37	static	24–48	-	-	Rahman et al., 2005
Serratia marcescens ATCC 25419	n.s.	30, 36	250	24, 16–18	Yeast extract, tryptone, asparagine, NH_4Cl	Whey; sucrose	Ramesh et al., 2001; Lange et al., 1999
Serratia marcescens	7.6	30	200	48	-	-	Ustariz et al., 2004
Vibrio harveyi	7.2	30	700	7.5			Estrada-Badillo et al., 2003

Fungi							
Aspergillus oryzae	7.0	33	250	-			Wang et al., 2005
Aspergillus oryzae	7.5	30	180	72	-	-	Sandhya et al., 2005
" (SSF)	-	30	-	72	-	-	
Coniothelium corronatus (NCL 86.8.20)	7–7.5	28	220	48	Ammonium nitrate, tryptone, casein	Sucrose	Bhosale et al., 1995
Ophiostoma piceae	n.a	23	250	9 days	Soydrink from soybean meal	Starch	Abraham and Hroudl, 1996
Tolypocladium album	5.9	28	200	24–120	Peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$	Glucose	Petrellius and Markkanen, 2000
Actinomycetes							
Streptomyces sp. NCIB 10070	7	n.a.	n.a	24	Rapemeal	None[a]	Yeoman and Edwards, 1997
Thermoactinomyces sp. E79	7.2	50	250	16	Soytone	Starch	Lee et al., 1996

a No carbon source was present in the medium and the major organic nitrogen source supplied the required carbon
b Not specified

Bacteria are the most dominant group of most commercial proteases, mainly neutral and alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B licheniformis*, *B subtilis*, *B amyloliquifaciens*, and *B mojavensis* (Gupta et al., 2002b; Kalisz, 1988; Kumar and Takagi, 1999, Rao et al., 1998) Another bacterial source, known as a potential producer, is *Pseudomonas* sp. (Rajoudh et al., 2000; Ogino et al., 1999). The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases, which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10.0, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (http www.novozymesbiotech.com)

Fungi elaborate a wide variety of enzymes than do bacteria For example, *Aspergillus oryzae* produces acid, neutral and alkaline proteases. Fungal enzymes can be conveniently produced in a solid-state fermentation process. The fungal proteases are active over a wide pH range (4.0 to 11.0) and exhibit broad substrate specificity. However, they have a lower reaction rate and worse heat tolerance than do the bacterial enzymes. Fungal acid proteases have an optimal pH between 4.0 and 4.5 and are stable between pH 2.5 and 6.0. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents. In fungi, *Aspergilli* (Chaicrabarti et al., 2000; Rajamani and Hilda, 1987; Sandhya et al., 2005) is the most exploited group, and *Conidioholus sp.* (Bhosale et al., 1995), *Penicillium* sp. (Germano et al., 2003) and *Rhizopus sp.* (Banerjee and Bhattacharyya,

1993) produce protease. Among actinomycetes, strains of *Streptomyces* are the preferred source (Petinate et al., 1999). Among yeasts, *Candida* sp. has been studied in detail as a potential alkaline protease producer (Poza et al., 2001).

Viral proteases have gained importance due to their functional involvement in the processing of proteins of viruses that cause certain fatal diseases such as AIDS and cancer. Serine, aspartic and cysteine peptidases are found in various viruses (Rawlings and Barrett, 1993). All of the virus encoded peptidases are endopeptidases; there are no metallopeptidases. Retroviral aspartyl proteases that are required for viral assembly and replication are homodimers and are expressed as a part of the polyprotein precursor. The mature protease is released by autolysis of the precursor. An extensive literature is available on the expression, purification, and enzymatic analysis of retroviral aspartic protease and its mutants (Kuo and Shafer., 1994). Extensive research has focused on the three-dimensional structure of viral proteases and their interaction with synthetic inhibitors with a view to designing potent inhibitors that can combat the relentlessly spreading and devastating epidemic of AIDS (Patick and Potts, 1998).

2.4.1 Enzymes From Marine Microbial Sources

The marine environment, which encompasses about 71 percent of the Earth's surface, is potentially a vast resource for useful enzymes. Microbes live in various habitats in the marine environment, including neuston, plankton, nekton, seston and epibiotic, endobiotic, pelagic and benthic environments. These habitats harbor a diverse range of microbes including archaebacteria, cyanobacteria, actinomycetes, yeasts, filamentous fungi, microalgae, algae and protozoa. Almost all these groups are potential sources of useful enzymes. The ecological role of these microorganisms is in the mineralization and recycling of complex organic matter through degradative pathways and thus also contribute to the secondary production in the sea. Bacteria and fungi secrete different enzymes such as protease, amylase, lipase, chitinase, cellulase, ligninase, pectinase, xylanase,

nucleases (DNases, RNases, restriction enzymes etc.) etc. based on their habitat and ecological functions. Harsh marine environments, such as deep ocean, hydrothermal vents, polar oceans and extremely saline bodies of water, have yielded valuable extremophilic microorganisms, which are the primary source of enzymes that are active at extreme conditions. An extensive review of marine microbial enzymes is presented by Chandrasekaran and Kumar (2002). Marine microbial enzymes have become the focal point of interest and several enzymes have drawn the attention of microbial prospectors. A few enzymes have been isolated from seawater and marine sediments and have been purified and characterized for their properties and applications.

2.4.1.1 Protease From Marine Microbes

The marine microorganisms which have immense potential as source of exoenzymes are yet to be tapped in spite of few reports on the production of protease from marine bacterial sources like *Vibrio* sp., *Bacillus* sp., *Pseudomonas* sp. etc (Croucker et al., 1999; Estrada-Badillo et al., 2003; Farrel and Crosa, 1991; Kumar et al., 2004; Makino et al., 1981; Salamanca et al., 2002). Nevertheless, the production of protease at an industrial scale using marine fungus is yet to be attempted (Chandrasekaran and Kumar, 2002).

A halotolerent strain of *Bacillus licheniformis*, isolated from marine sediments produced high protease activity during the early stationary phase of growth (Manachini and Fortina, 1998). The use of sea water in the fermentation medium enhanced the production of this activity to 150%. After partial purification, three different proteolytic enzymes could be detected which were alkaline serine proteases, exhibiting optimal activity at pH 9.0 and at 70°C. Proteases were activated by NaCl, with a three- fold increase in activity and were stable in the presence of 0.7% $NaBO_3$, 0.5% NaClO and 3% H_2O_2.

Intracellular proteases from a hyper thermophilic archaeon *Pyrococcus furiosus* was purified by Halio et al. (1997). Proteases Pfpl has a molecular mass of

19kDa and occurs in at least two conformational forms, i.e., one as a hexamer with molecular mass 12446 kDa comprising about 90% of the total activity and the second one occurring as a trimer with a molecular mass of 59±3 kDa. The temperature optimum for the normal Pfpl was - 85°C where as, the enzyme prepared by incubating the cell extract of *P. furiosus* at 98°C in 1% SDS for 24h, was 100°C. Moreover, the half life was increased from less than 30 minutes to 33h. The enzyme appears to be a predominant serine- type protease in cell extract, but is converted *in vitro*, probably in part, by deamidation of 'Asn' and 'Gln' residues, to a more thermally stable form by prolonged heat treatment.

A hyperthermophilic and barophilic protease was isolated from *Methanococcus jannaschii*, an extremely thermophilic deep-sea methanogen. This enzyme is the first protease isolated from an organism adapted to a high pressure and high temperature environment. The enzyme has a molecular mass of 29kDa and narrow substrate specificity with strong preference for leucine at the P1 site of polypeptide substrates. Enzyme activity was measured up to 130°C. Enzyme activity and thermal stability increased with pressure. Raise in the pressure to 500 atm, led to increased reaction rate at 125°C to 3.4 fold and the thermostability to 2.7 fold (Michels and Clark, 1997).

An alkaline protease isolated from a symbiotic bacterium found in the gland of marine shipworm was evaluated as a cleansing additive. The addition of this protease significantly improved the cleaning power of non-phosphate detergents. The protease was stable in sodium perborate, as well as hydrogen peroxide and retained good activity in the presence of sodium hypochloride (Greene et al., 1996).

Two extra cellular protease from crude oil degrading marine *Nocardiopsis* was isolated (Dixit and Pant, 2000). The two proteases, Protease I and Protease II, belonging to alkaline serine endopeptidase family, have a molecular weight of 21 kDa and 23 kDa and PI of 8.3 and 7, with pH and temperature optima for activity between 10.0 and 11.0 and about 60°C respectively.

A 5 fold increase in enzyme production in marine bacterium *Vibrio harveyi* by the addition of skimmed milk which has a molecular weight of 34kDa has been reported (Estrada-Badillo et al., 2003).

An oxidative and SDS stable alkaline protease was reported to be secreted by marine halophilic *Bacillus clausii* isolated from the tidal mud flats of Korean Yellow Sea (Kumar et al., 2004). The protease showed extreme stability towards SDS and oxidizing agents, retaining its activity above 96 and 75% on treatment for 72 hrs with 5% SDS and 5% H_2O_2. The enzyme belongs to the family serine protease with optimal pH and temperature of activity at 11.5 and 80°C respectively.

2.5 Protease Production in Microorganisms

Protease production is an inherent property of all organisms and these enzymes are generally constitutive; however, at times, they are partially inducible (Beg et al., 2002a; Kalisz, 1988). Proteases are largely secreted during stationary phase and thus are generally regulated by carbon and nitrogen stress. They are known to be associated with the onset of stationary phase, which is marked by the transition from vegetative growth to sporulation stage in spore-formers. Therefore, protease production is often related to the sporulation stage in many bacilli, such as *B. subtilis* (O'Hara and Hageman, 1990) and *B. licheniformis* (Hanlon and Hodges, 1981). In contrast, a few reports also suggest that sporulation and protease production, although co-occurring, are not related, as spore-deficient strains of *B. licheniformis* were not protease deficient (Fleming et al., 1995). It was also established that protease production and sporulation are two independent events in stationary phase by analysis of nucleotide pools (GTP and ATP) in the cells (Bierbaum et al., 1991). These observations strongly suggest that protease production is under stringent control responsive to amino acid deficiency and is related to the Gppp ratio in the cell. The transitions between different growth phases or different nutritional limitations were easily discerned by the alterations in the nucleotide pool. A marked decrease in the GTP content of the cells (after

addition of mycophenolic acid in the exponential phase) increased protease production during stationary phase. Hence, it is conclusively suggested that extracellular protease production is a manifestation of nutrient limitation at the onset of stationary phase (Bierbaum et al., 1991).

2.6 Fermentation production of protease

Proteases are generally produced by submerged fermentation (SmF) and on a commercial scale, this is preferred over solid state fermentation (SSF). Recently SSF has generated much interest, because of lower manufacturing costs by utilizing unprocessed or moderately processed raw materials. Further, the less initial capital cost, superior productivity, low water output and improved product recovery are other advantages of SSF. Different methods in submerged and solid state fermentations have been used to regulate protease synthesis with strategies combining fed-batch, continuous, chemostat cultures etc. (Gupta et al., 2002b; Hameed et al., 1999; Sandhya et al., 2005; Uyar and Baysal, 2004). Such strategies can achieve high yields of alkaline protease in the fermentation medium over a longer period of incubation during prolonged stationary state. Since the final protease yield during this phase is also determined by the biomass produced during exponential phase, medium manipulation is needed to maximize growth and hence protease yield. Optimization of the medium is associated with a large number of physiological and nutritional parameters that effect protease production, viz., pH, temperature, incubation period and agitation, effect of carbon and nitrogen and divalent cations. Although a large array of factors influences protease production, there is a complex interaction among these parameters that can be studied by following response surface methods. A comprehensive account of culture conditions for protease production from various microorganisms is listed in Table 2.2

2.6.1 Solid State Fermentation (SSF)

Solid state fermentation processes, which involve the growth of microorganism on moist solid substrates in the absence of free-flowing water, have considerable economical potential in producing products for the food, feed, pharmaceuticals and agricultural industries. However, owing to the great success of large-scale submerged fermentation (SmF) processes, SSF was almost completely neglected. However, over the past 15-20 years, SSF has gained renewed interest because it has certain advantages over SmF (Barrios-Gonzalez and Mejia, 1996).

SSF technology has been known for centuries from approximately 2600 BC. It was used by the Egyptians for making bread, and information on the "Koji process" dates back to 1000 BC. SSF processes that have existed for centuries include fermented foods (e.g., tempeh, miso and pozol), mold ripened cheese (e.g., Roquefort), starter cultures for fermented brews and ensiling and composting. More recent applications of SSF include the protein enrichment of agro-industrial residues, the production of enzymes, organic acids and other fungal metabolites and spore production (Rambault, 1998).

As part of the search for a cheap alternative production system which utilizes simpler agro industrial wastes as substrates, solid state fermentation (SSF) has gained importance in the production of microbial enzymes. SSF has several economic advantages over conventional submerged fermentation such as minimal requirement of water, production of metabolites in a more concentrated form and making the downstream processing less time consuming and less expensive (Hesseltine, 1972, Lonsane et al., 1985). Among the various groups of microorganisms used in SSF, filamentous fungi are the most widely exploited owing to their ability to grow and produce a wide range of extracellular enzymes on complex solid substrates (Moo-young et al., 1983).

In SSF, microorganisms grow on the moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and

energy source. The fermentation takes place in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted. Free water does not appear to be the natural milieu for the majority of microorganisms. Not even marine microorganisms prefer swimming in free seawater since more than 98% of isolates from the marine environment have been obtained from the underwater surfaces of solid substrates, and less than 1% of all known fungi have been found in marine habitats (Kelecom, 2002). The evolution of higher fungi took place on solid growth substrates. Ascomycetes and Basidiomycetes spent their evolutionary history as terrestrials, with only some species adapting to water, later in their evolution. Fungal products of biotechnological interest, i.e., enzymes, secondary metabolites and spores, were developed for use in moist solid substrates but not in liquids. Consequently, the cultivation of microorganisms in aqueous suspension may rather impair their metabolic efficiency. In this respect, submerged fermentation technology (SmF) may be considered as a kind of violation of the natural habitats of wild-type microorganisms. However, solid state (substrate) fermentation (SSF) is currently used only to a small extent for enzyme and secondary metabolite production because of severe process engineering problems.

Approximately 90% of all industrial enzymes are produced in SmF, frequently using specifically optimized, genetically manipulated microorganisms. In this respect SmF processing offers an insurmountable advantage over SSF. On the other hand, almost all these enzymes could also be produced in SSF using wild-type microorganisms (Hölker et al., 2004). Interestingly, fungi, yeasts and bacteria that were tested in SSF in recent decades exhibited different metabolic strategies under conditions of solid state and submerged fermentation. Advantages of SSF over SmF are listed in the Table 2.3.

Table 2.3 Biotechnological advantages of solid state fermentation (SSF) against submerged fermentation (SmF)

Advantages	Consequences
Biological advantages	
Low water demand	Less waste water
High concentration of the end product	Lower downstream costs
Catabolite repression significantly lower or missing	Fermentation in the presence of glucose
Utilisation of solid substrate	High concentration of the growth substrates
Lower sterility demands	Mixed cultures of fermenting microorganisms
Solid support for microorganism Simulation of the natural environment	Better performance of cultivated microorganisms
Fermentation of water-insoluble solid substrates Mixed culture of microorganisms	Synergism of metabolic performance
Processing advantages	
High-volume productivity	Smaller fermenter volumes
Low energy demand for heating	Easy aeration
Utilisation of otherwise unusable carbon sources	Cheap and abundant carbon sources
No anti-foam chemicals	No loss of microorganisms during fermentation

It has also become clear that the cost-factor for the production of "bulk-ware" enzymes in most cases favours SSF over SmF. Tengerdy (1996), estimated fermentation costs of cellulase production at US $ 0.2 kg^{-1} in an in-situ SSF, in contrast to US $20 kg^{-1} in a stirred tank reactor. One important biological factor in favour of SSF was the low catabolite repression, which appeared to be limiting enzyme production by *Aspergillus niger* in SmF (Nandakumar et al., 1999). The lack of catabolite repression also allowed for fast growth of the fungus in the presence of high sugar concentrations (Favela-Torres et al., 1998).

Several fungi need a solid substrate as an anchor for optimal growth and productivity. Therefore, genetically modified organisms, which were optimised for

liquid cultivation conditions, have often been used in SmF. Whereas, natural isolates have played a major role in SSF.

Another problem that could not be satisfactorily solved in SmF is the change in oxygen supply due to changes in the growth medium during the course of fermentation. In many cases, fungi need a highly viscous medium for secretion of the required metabolites. This viscosity is achieved through secretion of polymeric substances during fungal growth. In such cases, SSF appears to be the better alternative since stirrer speed and oxygen supply play no role (Elibol and Muvituna, 1997).

Although a direct comparison between SSF and SmF is very difficult due to the different consistencies of the microbial cultures used in the two technologies, microorganisms involved in SSF have a higher metabolic potential since they proliferate in an almost natural environment, i.e., under conditions of limited free water and with a solid support for growth. Whereas, there has been significant development in SSF processing, regarding both biochemical engineering and reactor design with the goal of scaling up the process, very little, if any work has been done, as yet to elucidate the molecular and physiological background of the different behavior of individual microorganisms when cultivated on solids or in liquids. The physiological and molecular biological aspects of microbial cultivation can thus be regarded as the current "black box" of SSF biotechnology (teBiesebeke et al., 2002). This may, and will, be changed by more focused consideration of the biological parameters applicable to SSF and SmF. Thus, the perspective that SSF will gain prevailing significance in the industrial production of enzymes, secondary metabolites and spores by wild type microorganisms is warranted since, compared with SmF, it is more effective in several aspects including lower energy and sterility demands as well as higher stability of products and variability of microorganisms used, especially the use of mixed cultures.

2.6.1.1 Substrates For Solid State Fermentation

Two types of SSF systems can be distinguished depending on the nature of the solid phase used. The first, and the most commonly used system involves cultivation on a natural material. The second system, which is not as frequently used, involves cultivation on an inert support impregnated with a liquid medium (Barrios-Gonzalez and Mejia, 1996).

SSF cultivation on natural substrates uses natural materials that serve both as support and a nutrient source. These materials are typically starch or lignocellulose based agricultural products or agro-industrial sources such as grains and grain byproducts like cassava, potato, sugar beet pulp, beans, wheat bran and rice bran. The solid support of the second type include inert materials which serves only as an anchor point for the microorganism. Materials proposed include: hemp, perlite, polyurethane foam (PUF) and vermiculate (Barrios-Gonzalez and Mejia, 1996; Larroche and Gros, 1992; Larroche and Gros, 1997). The materials used in SSF are listed in the Table 2.4.

Table 2.4 Some examples of substrates and applications of SSF

Substrate/support	Applications	References
Ligno cellulosic		
Wheat straw	Protein enrichment	Viersturs et al., 1981
	Lignin degradation for ruminant feedstock	Zadrazil, 1982
	Mushroom cultivation	Calzada et al., 1987
Wheat bran	Enzyme production	Silman, 1980
	Protease production	Villegas et al., 1993
	Flavour production	Christen et al., 1997
	L-Glutaminase	Sabu et al., 2000
Sugar cane bagasse	Protein enrichment	Gonzalez-Blanco et al., 1990
	Penicillin production	Barrios-Gonzalez et al., 1988
	L-lactic acid production	Soccol et al., 1994b
Sugar beet pulp	Protein enrichment for feed	Durand et al., 1988
Coffee pulp	Pectinase production	Boccas et al., 1994
Coffee husk	Citric acid production	Shankaranand and Lonsane, 1994
	Flavour production	Soares et al., 2000

Starchy		
Rice bran	Aroma production	Yamauchi et al., 1989
	Protease production	Hasan and Mitchell, 1994
	Pigment production	Rosenblut et al., 2000
Cassava bagasse	Growth studies	Soccol et al., 1994a
	Flavour production	Bramorski et al., 1998; Christen et al., 2000
Buck wheat seeds	Fungal spore production	Larroche et al., 1988
Soybean	Fermented food production	Hachmeister and Fung, 1993
Banana wastes	Protein enrichment for feed	Baldensperger et al., 1985
Banana stem	Amylase production	Krishna and Chandrasekaran, 1996
Synthetic supports		
Polyurethane foam	Enzyme production	Zhu et al., 1994
Polymeric resin	Growth studies	Amin et al., 1990
	Lipase production	Christen et al., 1995
	Gibberellic acid production	Gelmi et al., 2000
Polystyrene	L-Glutaminase	Prabhu and Chandrasekaran, 1996
Others		
Prawn waste	Chitinase	Suresh and Chandrasekaran, 1998
Citrus peel	Growth studies	Leon et al., 1985
Apple pomace	Ethanol production	Ngadi and Correia, 1992
Kiwifruit peel	Citric acid production	Hang et al., 1987
Amaranth grain	Volatile compound production	Bramorski et al., 1998

2.6.1.2 Solid State Fermentation Systems

A considerable amount of work has been done in recent years to understand the biochemical and engineering aspects of SSF processing (Mitchell et al., 2000a; Mitchell et al., 2000b). It is rather surprising that the technical problems of SSF have not yet been solved as SSF is one of the oldest biotechnological processes known. A comparison of the reactor systems used in SSF is listed in the Table 2.5.

Table 2. 5 Main reactors used in SSF

Reactor	Brief description	Advantages/draw backs	References
Bench scale reactors			
Erlenmeyer flask	Flask stopped with cotton wool or gauze layers	Very simple, low cost, no control, allows numerous runs/no regulation, passive aeration	(Christen et al., 1997)
Packed bed reactor	Column reactor with air prehumidification	Low cost, easy regulation of temperature and airflow rate, probes compatible/poor heat removal	(Fasidi et al., 1996) (Framorski et al., 1998) (Silman et al., 1979) (Durand et al., 1996; Raimbault and Alazard, 1980)
Pilot plant and large scale reactors			
Tray reactor	Perforated plates in a moistening chamber, covered with a thin layer of substrate	Good aeration, no heat build up/no stirring, labor intensive	(Fasidi et al., 1996) (Ghildyal et al., 1981) (Durand et al., 1996) (Daubresse et al., 1987) (Zadrazil et al , 1996)
Drum reactor	Horizontal or inclined cylindrical drum. Agitation by a rotating system around the central axis or by paddles within the container	Possible aseptic conditions, gentle mixing, possible automatization./ clumping of the solid substrate, poor heat removal.	(Han and Anderson, 1975) (Hesseltine, 1977) (Lonsane et al., 1985)
Fluidized bed reactor	Column reactor with a perforated base through which air is blown with force to suspend the substrate particles	Excellent aeration, effective removal of metabolic heat/high cost	(Hong et al., 1988)

Prototype reactors			
INRA-Dijon reactor	More elaborated version of a stirred tank reactor (working volume =3.6m^3), equipped with an endless screw for mixing	Accurate control of airflow, humidity and temperature, good mixing/high investment and maintenance costs, economically effective only for high added value products	(Durand and Che'reau, 1988) (Durand et al., 1993)
Disk fermentor	Cylindrical device with disks fixed on a rotative horizontal axis. Each disk consists in 2 steel grids maintaining a synthetic support soaked with a nutritive solution	Good homogenization of the medium and heat transfer, effective for spore production/ hard to scale up, uneasy product recovery	(Raimbault and Roussos, 1985) (Roussos et al., 1991)
Zymotis	Cubic reactor with heat exchanger sheets (with water recycling)	Good heat removal and aeration, easy scaling up/no stirring	(Roussos et al., 1993) (Deschamps et al., 1985)
Semi-automated Reactor (from the University of Santiago, Chile)	Packed bed reactor with periodic agitation, sophisticated control of temperature, water content, bed agitation	Bioreactor operation considerably simplified, efficient control of key parameters/expensive for building	(Fernandez et al., 1996) (Fernandez et al., 1997)

2.7 Protease Purification Methods

Purification of proteases to homogeneity is a prerequisite for studying their mechanism of action. Vast numbers of purification procedures for proteases, involving affinity chromatography, ion-exchange chromatography, gel filtration techniques and preparative polyacrylamide gel electrophoresis have been used for the purification.

A number of alkaline proteases from different sources have been purified and characterized and a summary of various purification strategies adopted for purification of microbial proteases is presented in Table 2.6. There are no set rules for the purification of proteases. After separating the culture from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by means of ultrafiltration (Kang et al., 1999; Smacchi et al., 1999), salting out by solid ammonium sulfate (Hutadilok-Towatana et al., 1999; Kumar, 2002), or solvent extraction methods using acetone (Kumar and Takagi, 1999; Thangam and Rajkumar, 2002) and ethanol (El-Shanshoury et al., 1995). In addition, other methods, such as use of PEG-35,000 (Larcher et al., 1996), activated charcoal (Aikat et al., 2001), temperature-sensitive hydrogel (Han et al., 1995), heat treatment of enzyme (Rahman et al., 1994) and lyophilization (Manonmani and Joseph, 1993) are also used for concentration of alkaline proteases.

To further purify the enzyme, a combination of one or more techniques is applied, viz., affinity chromatography (AC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and gel filtration chromatography. Other methods of choice, including aqueous two-phase systems (Sinha et al., 1996), dye ligand chromatography (Cowan and Daniel, 1996) and foam fractionation (Banerjee et al., 1993), have also been employed on a small scale and yet await commercial exploitation.

2.7.1 Affinity chromatography (AC)

The most common affinity adsorbents used for alkaline proteases are hydroxyapatite (Kobayashi et al., 1996), immobilized N-benzoyloxycarbonyl phenylalanine agarose (Larcher et al., 1996), immobilized casein glutamic acid (Manonmani and Joseph, 1993), aprotinin-agarose (Petinate et al., 1999) and casein-agarose (Hutadilok-Towatana et al., 1999). Although AC is one of the most successful purification techniques, a major limitation is the high cost of enzyme supports and the labile nature of the affinity ligands, which lowers their use at process scale.

2.7.2 Ion exchange chromatography (IEC)

The matrices for IEC contain ionizable functional groups such as diethyl amino ethyl (DEAE) and carboxy methyl (CM), which get associated with the charged protein molecules, thereby adsorbing the protein to the matrices. The adsorbed protein molecule is eluted by a gradient change in the pH or ionic strength of the eluting buffer or solution.

2.7.3 Hydrophobic interaction chromatography (HIC)

HIC exploits the variability of external hydrophobic amino acid residues on different proteins, leading to protein interaction by virtue of the fact that in aqueous solvents, hydrophobic patches on proteins preferentially seek out other hydrophobic surfaces. These hydrophobic interactions are strengthened by high salt concentrations and higher temperatures and are weakened by the presence of detergents or miscible organic solvents. The extent of binding of a hydrophobic protein depends on the type and density of substitution of the matrix, as well as on the nature of buffer conditions. Hydrophobic interactions are much more variable in behavior than ion exchangers and, because of this, resolution is generally poorer than IEC. HIC has been extensively used in FPLC in various columns, such as Mono-Q HR 5/5 (Rattray et al., 1995; Smacchi et al., 1999) Econo-pac Q (Yeoman and Edwards, 1997) and Mono S 5/10 (Yum et al., 1994). The most commonly used hydrophobic adsorbents are octyl- (C8-) and phenyl-substituted matrices.

Table 2.6 Combination of purification techniques applied to purification of various proteases

Microorganism	Concentration method	Column matrices	Reference
Bacteria			
Alcaligenes faecalis	Acetone	DEAE cellulose, Sephadex G-100	Thangam and Rajkumar, 2002
Arthrobacter nicotianae 9458	Ultrafiltration	DEAE Sephacyl, Sephacryl 200, Phenyl Sepharose, FPLC Mono Q HR 5/5	Smacchi et al., 1999
Bacillus sp. PS719	$(NH_4)_2SO_4$	DEAE cellulose, α-casein agarose	Hutadilok-Towatana et al., 1999
Bacillus sp. NCDC 180	Acetone, $(NH_4)_2SO_4$	DEAE Sepharose CL-6B, CM Sepharose CL-6B, Sephacryl S-200	Kumar et al., 1999
Bacillus sp. SSR1	$(NH_4)_2SO_4$	DEAE Sephadex A-50, Sepharose 6B	Singh et al., 2001a
B. pumilis MK6–5	$(NH_4)_2SO_4$	DEAE Sepharose CL-6B, CM Sepharose CL-6B, Sephacryl S-200	Kumar, 2002
B. pumilis	Ultrafiltration	CM Sepharose fast flow, DEAE Sepharose fast flow, Sephacryl S-100, S-200, Hydrophobic interaction	Huang et al., 2003
B. sphaericus MTCC B-0014	Ultrafiltration, $(NH_4)_2SO_4$	Phenyl agarose, Q-Sepharose	Singh et al., 2001b
B. subtilis PE11	$(NH_4)_2SO_4$	Sephadex G-200	Adinarayana et al., 2003
Oligotropha carboxydovorans DSM 1227	Ultrafiltration	Sephadex G-75, CM-cellulose	Kang et al., 1999
Pimelobacter sp. *T*-483	$(NH_4)_2SO_4$	Butyl-Toyopearl 650C, Butyl Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-50F	Oyama et al., 1997
Pseudomonas aeruginosa MN1	$(NH_4)_2SO_4$	Sephadex G-100, DEAE cellulose	Bayoudh et al., 2000
P. aeruginosa PST-01	$(NH_4)_2SO_4$	Butyl-Toyopearl 650C, Butyl Toyopearl 650 M	Ogino et al., 1999

Pseudomonas sp.	Ultrafiltration	S- Sepharose fast flow PD 10	Vazquez et al., 2004
Serratia marcescens ATCC 25419	$(NH_4)_2SO_4$	Q-Sepharose, Sephacryl S-200	Romero et al., 2001
Fungi			
Aspergillus terreus (UIRA 6.2)	Acetone, $(NH_4)_2SO_4$	DEAE Sephadex A25, SDS-PAGE, electroelution	Chakrabarti et al., 2000
Aspergillus fumigatus	$(NH_4)_2SO_4$	Sephadex G-75	Davies et al., 2005
Scedosporium apiospermum	$(NH_4)_2SO_4$, PEG 35,000	Sephadex G-75, immobilized phenylalanine-agarose	Larcher et al., 1996
Actinomycetes			
Oerskovia xanthineolytica TK-1	Ultrafiltration, $(NH_4)_2SO_4$	Phenyl-Sepharose CL 4B, DEAE Sephacel	Saeki et al., 1994
Streptomyces cyaneus	-	Aprotinin-agarose	Petrasic et al., 1999
Streptomyces thermovulgaris	Ultrafiltration	FPLC Enono-pac Q, Superose 12	Yeoman and Edwards, 1997
Thermoactinomyces sp. E79	$(NH_4)_2SO_4$	DEAE Sepharose CL-6B, Butyl-toyopearl 650 M	Lee et al., 1996
Moulds			
Aureobasidium pullulans	Ultrafiltration	Sephadex G-75	Donaghy and McKay, 1993
Candida caseinolytica		Sephacryl S-200, DEAE Biogel	Poza et al., 2001

DEAE-Diethyl amino ethyl, CM-carboxy methyl

2.7.4 Affinity precipitation

Affinity precipitation is a function of a soluble macromolecule (ligand polymer and macroligand) that has two functions. (1) it contains an affinity ligand (preferably more polyvalent macromolecule) and (2) it can be precipitated in many ways, i.e., by change in pH, temperature or ionic strength. With this technique, the ligand polymer is added to the enzyme solution under conditions favoring binding of the protein of interest. The ligand polymer is then precipitated, and the supernatant is removed. The protein of interest is then eluted from the polymer under suitable conditions and the polymer can be recycled. An alkaline protease (Maxatase from *B. licheniformis*), used as a washing powder additive, has been purified by affinity precipitation (Pees et al., 1991).

In addition to the above chromatographic techniques, gel filtration is used for rapid separation of macromolecules based on size. Recently, many new agarose based and more rigid and cross-linked gels, such as Sephacryl, Superose, Superdex and Toyopearl are also being used for purification purposes. They are generally used either in the early-to-middle stage of purification (Chakrabarti et al., 2000) or in the final stages of purification (El-Shanshoury et al., 1995; Lee et al., 1996). Major disadvantages of this method are the lower capacity for loading proteins and that the desired protein gets too diluted.

2.8 Characteristics of Protease

Proteases from several microorganisms have been studied extensively and based on their properties they are used in various industries. The important properties are summarized in Table 2.7. However, a brief account of individual properties is presented in the following section.

Table 2.7 Properties of some proteases from different microbial sources

Microorganism	pH optima	Temp. optima (°C)	Substrate specificity	MW (kDa)	pI	Other properties	Reference
Bacteria							
Alcaligenes faecalis	9.0	55	Casein, BSA, gelatin, azocoll, azocasein	67	n.s[a]	–	Thangam and Rajkumar, 2002
Arthrobacter viscosus 9458	9.0; 9.5	55–60; 37	α_{S1}- and β-casein	55; 70–72	n.s.	–	Smacchi et al., 1999
Bacillus sp. 1899	11.0	70	Casein	29	n.s.	Metal ions enhance thermostability	Johnvesly and Naik, 2001
Bacillus sp. NO 27	9.2	40	Casein	n.s.	n.s.	Half life of 55 min at 90°C	Sumandeep et al., 1999
Bacillus sp. KSM-KP43	11.0	70	Casein	n.s.	n.s.	Oxidation-resistant	Saeki et al., 2002
Bacillus sp. NCDC-180	11.0, 12.0	50; 55	Casein, synthetic p-nitroanilides	28; 29	n.s.	Stable up to pH 13	Kumar et al., 1999
Bacillus sp. PS179	9.0	75	Azocasein	42	4.8	Ca^{2+} enhances thermostability	Hutadilok-Towatana et al., 1999
Bacillus sp. SSR1	10.0	40	Azocasein	29	n.s.	Ca^{2+} enhances thermostability	Singh et al., 2001a
B. brevis MTCC 8001G	10.5	37	Azocasein	n.s.	n.s.	Detergent compatible	Banerjee et al., 1999
B. mojavensis	10.5	60	Casein	30	n.s.	Bleach- and SDS -stable, detergent compatible	Ileg et al., 2002a; Gupta et al., 1999
B. pumilus MK6 5	11.5	50–55	p-Nitroanilides	28	n.s.	Ca^{2+}-independent	Kumar, 2002
B. pumilus	10.0	55	Casein	32	9	Dehairing activity	Huang et al., 2003
B. subtilis Pb-11	10.0	60	Casein	15	n.s.	Strongly activated by Ca^{2+}, Mg^{2+}, Mn^{2+}	Adinarayana et al., 2003
Oligotropha carboxydovorans DSM 1227	9.0	60; 50	Casein, azocasein, azocoll, carbon monoxide dehydrogenase	23	n.s.	–	Kang et al., 1999
Pimelobacter sp. Z-483	9.0	50	Casein	23	n.s.	EDTA-resistant	Oyama et al., 1997

Pseudomonas aeruginosa PST-01	8.5	55	Casein	38	n.s.	Organic solvent stable	Ogino et al., 1999
Serratia marcescens ATCC 25419	9.5	48	Azocasein	66.5	n.s.	–	Romero et al., 2001
Fungi							
A. terreus (UTRA 6.2)	8.5	37	Na-caseinate, synthetic substrates (p-nitroanilidines)	37	n.s.	-	Chakrabarti et al., 2000
Beauveria bassiana	7.5–9.5	25	Azocoll, elastase	31.5	7.5	Elastase and cuticle degradation activity	Urtz and Rice, 2000
Actinomycetes							
Oerskovia xanthineolytica TK-1	9.5–11.0	50	Synthetic esters	20	n.s.	Yeast-lytic activity	Saeki et al., 1994
Streptomyces cyaneus	9.0	25	α-N-*p*-Tosyl-L-arginine methyl ester	30, 120	n.a.		Petinate et al., 1999
Streptomyces tendae	6.0	70	Azocasein	21	n.s.	Resistant to organic solvents and neutral detergents	Seong et al., 2004
Thermoactinomyces sp. E79	11.0	85	Casein	31		Calcium enhanced thermostability, broad pH stability (5–12)	Lee et al., 1996
Moulds							
Aureobasidium pullulans	9.5–10.5	41	Azocoll, α–casein	27	n.s.		Donaghy and McKay, 1993
Candida caseinolytica	4.5–11.0	37	Casein	30	4.7	-	Poza et al., 2001

a Not specified

2.8.1 pH and temperature kinetics

In general, all currently used detergent-compatible proteases are alkaline and thermostable in nature with a high pH optimum (the pH of laundry detergents is generally in the range of 8.0 to 12.0) and have varying thermostabilities at laundry temperatures (50–70°C). Therefore, most of the commercially available subtilisin-type proteases are also active in the pH and temperature ranges 8–12 and 50–70°C, respectively (Table 2.7). In addition, a recent trend in the detergent industry is a requirement for alkaline protease active at low washing temperatures; for example, Kannase - marketed by Novozymes - is active even at temperatures as low as 10-20°C.

2.8.2 Effect of stabilizers/additives and metal ions

Some of the major commercial uses of alkaline proteases necessitate high temperatures and hence, improving the thermal stability of the enzyme is distinctly advantageous. Thermostability can be enhanced either by adding certain stabilizers (PEG, polyhydric alcohols, starch etc.) to the reaction mixture or by manipulating the tertiary structure of the enzyme by protein engineering. A thermostabilization effect of up to a 2-fold increase in the half-life of *Cucurbita ficifolia* protease at 65°C has been reported by using polyhydric alcohols, PEG and casein (Gonzalez et al., 1992). The ion Ca^{2+} is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline protease at higher temperatures (Kumar, 2002; Lee et al., 1996). Other metal ions such as Ba^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+} and Zn^{2+} are also used for stabilizing proteases (Johnvesly and Naik, 2001; Rattray et al., 1995). 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.

2.8.3 Substrate specificity

Alkaline proteases have broad substrate specificity and are active against a number of synthetic substrates and natural proteins. However, the literature conclusively suggests that they are more active against casein than against azocasein, hemoglobin or BSA (Table 2.7). Moreover, there are specific types of alkaline proteases, viz., collagenase, elastase, keratinase (Friedrich et al., 1999) and insect cuticle-degrading protease (Uruz and Rice, 2000), which are active against specific protein substrates such as collagen, elastin, keratin and cuticle. Alkaline proteases are also specific against aromatic or hydrophobic amino acid residues such as tyrosine, phenylalanine or leucine at the carboxylic side of the cleavage site (Barrett, 2001).

2.8.4 Kinetic parameters

To develop an enzyme-based process, prior information about kinetic parameters of the enzyme in question is of utmost importance. To be precise, kinetic properties like V_{max}, K_m, K_{cat} and E_a are important, being not only enzyme-specific, but also substrate and environment specific, and knowledge of these is essential for designing enzyme reactors or quantifying the applications of the enzyme under different conditions. Various complexes, viz., casein, azocasein etc., and synthetic substrates, viz., p-nitroanilide esters are used for determining kinetic parameters for alkaline proteases. The synthetic substrates are much more popular than complex substrates for defining K_m and V_{max} as they are convenient (Kumar, 2002, Larcher et al., 1996). For an alkaline protease from *B. megaterium*, the K_m for casein decreased with corresponding increase in V_{max}, as the reaction temperature was raised from 45 to 60°C (Beg et al., 2002a). In contrast, the K_m and V_{max} for an alkaline protease from *Rhizopus oryzae* increased with an increase in temperature from 37°C to 70°C (Banerjee and Bhattacharyya, 1993).

2.9 Application of Proteases

Proteases constitute a complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature of activity and stability profiles. Commercial applications of these enzymes in a range of processes take the advantage of the unique physical and catalytic properties of individual proteolytic enzyme types. They represent one of the three largest group of industrial enzymes and account for about 60% of the total worldwide sale of enzymes. Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing ecofriendly technologies, they are envisaged to have extensive applications in leather treatment and in several bioremediation processes. The worldwide requirement of enzymes for individual applications varies considerably. Proteases are used extensively in the pharmaceutical industry for preparation of medicines like ointments for debridement of wounds, in the food and detergent industries, prepared in bulk quantities and used as crude preparations. Whereas, those that are used in medicine are produced in small amounts but require extensive purification before they can be used. Some of the commercial proteases are listed in the Table 2.8.

2.9.1 Detergents

In spite of the fact that the detergent industry is the largest single market for enzymes at 25 - 30% of total sales, details of the enzymes used and the ways in which they are used, have rarely been published. There are three basic types of enzymes used in detergents: proteases, amylases and lipases. The use of enzymes in detergent formulations is now common in developed countries, with over half of all detergents, presently available contain enzymes. Over the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients. Until today, the largest share of the enzyme market has been held by detergent alkaline proteases, active and stable in the alkaline pH range (Gupta et al., 2002b).

The preparation of the first enzymatic detergent, "Burnus," dates back to 1913, which consisted of sodium carbonate and a crude pancreatic extract. The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name BIO-40. In 1960, Novo Industry A/S introduced Alcalase®, produced by *Bacillus licheniformis*; its commercial name was BIOTEX. This was followed by Maxatase, a detergent made by Gist-Brocades. Unfortunately, detergent proteases faced a setback in the early 1970s, due to unfavorable publicity when some workers developed an allergic reaction during the handling of these enzymes. This problem was solved by the introduction of dust-free encapsulated products. Today, detergent enzymes account for 89% of the total protease sales in the world; and a significant share of the market is captured by subtilisins and/or alkaline proteases from many *Bacillus* species. The detergent enzyme market has grown nearly 10-fold during the past 20 years. In the 1980s and early 1990s, the major market share (>55%) of the detergent enzyme was held by Gist-Brocades in The Netherlands, Genencor International in the United States, Solvay in Belgium and Showa-Kenko inJapan. Beginning in 1995, however, there was considerable need for rationalization in the detergent enzyme industry, owing to the relatively high cost of manufacturing, coupled with increased pressure from detergent manufacturers to drive down raw material costs. Genencor International purchased the detergent enzyme businesses of Gist-Brocades and Solvay; and Novo Nordisk acquired Showa-Denko's detergent enzyme business. Today, Novo Nordisk and Genencor International are the major suppliers of detergent enzymes, supplying up to 95% of the global market of proteases.

Table 2.8 Commercial producers of proteases with their trade name

Trade names	Organism	Characteristic	Manufacturer
Laundary detergents			
Alcalase	*Bacillus licheniformis*	World's first mass produced enzyme for detergent industry pH –Neutral & mildly alkaline (pH 7-10), used in Detergents, milk protein modification & silk degumming	Novo Nordisk, Denmark
Savinase	Alkalophilic *Bacillus* sp.	pH optima 8-11, Works well under most washing conditions Used for wool finishing	"
Esperase	Alkalophilic *Bacillus* sp	High pH & temperature optima (pH up to 12) used in Detergents & wool finishing	"
Durazym	Protein engineered variant of Savinase®		"
Everlase	Protein engineered variant of Savinase®	Strong stability in bleach containing detergents Used in detergents & cleaning dentures	"
Lipolase		Active at low temperatures used in Detergents of low washing (down to 10-20°C)	"
Polarzyme		next generation protease, works at 5°C & at higher temperatures	"
Godo-Bap	*B. licheniformis*	Detergent	Godo Shusei, Japan
Bioprase	*B. subtilis*	Detergent, cleaning	Nagase Biochemicals, Japan
Wuxi	*Bacillus* sp	Detergent	Wuxi Synder Bioproducts China
Food Industry			
Neutrase	Bacterial	used in brewery, In alcohol industry Preparation of biscuits, crackers & cookies	Novo Nordisk, Denmark
Flavourzyme	Fungal	Complex of exo & endopeptidase Used for extensive hydrolysis of proteins	"
Protamex	Bacterial	Used in food industry (meat processing)	"
Godo Bap	*B. licheniformis*	Food	Godo Shusei, Japan
Corolase 7089	*B. subtilis*	Food	Rohm, Germany
Fuzeo alkaline Protease L FG	*B. licheniformis*	Food	Enzyme Development, USA

Bioprase SP-10	*B. subtilis*	Food	Nagase Biochemicals, Japan
Leather processing			
Novocor S	Microbial	Used for soaking	Novo Nordisk, Denmark
Novo Unhairing Enzyme (NUE)		Used for unhairing of hides & skins	"
NovoLime		Protease Lipase mix for small skins	"
NovoRate 160	Trypsin & genetically modified *Bacillus*	Serine protease. Used for bating hides & skin	"
NovoCor AB		Used for acid bating hides & skin	"
NovoCor WB		Used for re-bating wet blue before re-tanning at neutral pH	"
Maxacal, Maxatase	Alkalophilic *Bacillus* sp.		Gist-brocades, The Netherlands
Purafect	Genetic engineered Donor *B. lentus* Expressed in *Bacillus* sp.	High alkaline protease for detergents	Genencor International Inc., USA
Purafect OX		Oxidatively stable detergent enzyme	"
Properase		High alkaline protease for low temperature washing	"
Genencor Protease 899	Bacterial	Alkaline protease for industrial Proteolysis	"
Multifect Neutral	Bacterial	Neutral protease for functional peptidase (eg: Soy, meat etc)	"
Multifect P-3000	Bacterial	Mild alkaline protease for protein digestion	"
Protex 6L	Bacterial	Alkaline protease for protein digestion (eg: Pet foods)	"
Opticlean, Optimase	Alkalophilic *Bacillus* sp.		Solvay Enzymes GmbH, Germany
Maxapem	Protein engineered variant of alkalophilic *Bacillus* sp.		"
Proleather	Alkalophilic *Bacillus* sp.		Amano Pharmaceuticals, Japan
Protease P	*Aspergillus* sp.		"
Bioprase concentrate	*B. subtilis*	Cosmetic, pharmaceuticals	Nagase Biochemicals Japan
Enzeco alkaline protease	*B. licheniformis*	Industrial	Enzyme Development, USA

Enzeco high alkaline protease	*Bacillus* sp	Industrial	"
Research purpose			
Ps. protease	*Pseudomonas aeruginosa*	Research	Nagase Biochemicals, Japan
Ps. elastase	*Pseudomonas aeruginosa*	Research	"
Cryst. protease	*B. subtilis* (K2)	Research	"
Cryst. protease	*B. subtilis* (*bioteus*)	Research	"
ProteaseK	*Tritirachium album*	Research	Sigma Aldrich

Current market trends and consumer needs are influencing the development of enzymes for detergent applications, with the emphasis on enzymes that have improved performance/cost ratios, increased activity and improved compatibility with other detergent ingredients. In addition, enzyme suppliers and detergent manufacturers are actively pursuing the development of new enzyme activities that address the consumer-expressed need for improved cleaning, fabric care and antimicrobial benefits. However, apart from their use in laundry detergents, they are also popular in the formulation of household dishwashing detergents and both industrial and institutional cleaning detergents (Godfrey and West, 1996; Showell, 1999).

Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of alkaline proteases, which are effective over a wide temperature range. In addition, the current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the use of low washing temperatures. This has pushed enzyme manufacturers to look for novel enzyme that can act under low temperatures. Novo Nordisk Bioindustry in Japan has developed a detergent protease called Kannase, which keeps its high efficiency, even at very low temperatures (10–20°C).

There are many parameters involved in the selection of a good detergent protease, such as compatibility with detergent components, e.g., surfactants, perfumes, oxidizing agents and bleaches (Gupta et al., 1999; Kumar et al., 1998), good activity at relevant washing pH and temperature, compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and shelf life (Gupta et al., 2002b). In general, the majority of the commercially available enzymes are not stable in the presence of bleaching/oxidizing agents. Hence, the latest trend in enzyme-based detergents is the use of recombinant DNA technology to produce bioengineered enzymes with better stability. Bleach and

oxidation stability has been introduced through site directed mutagenesis and protein engineering by the replacement of certain amino acid residues (Bech et al., 1993; Estell et al., 1985; Wolff et al., 1996; Yang et al., 2000).

2.9.2 Food Industry

Traditionally, microbial proteases have been exploited in the food industries in many ways. The use of proteases in the food industry dates back to antiquity. They have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates and meat tenderization.

2.9.2.1 Dairy industry

The major application of proteases in the dairy industry is in the manufacture of cheese. The milk-coagulating enzymes fall into three main categories: (i) animal rennets, (ii) microbial milk coagulants and (iii) genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have molecular weights between 30,000 to 40,000 Da. Rennet extracted from the fourth stomach of unweaned calves contains the highest ratio of chymosin (EC 3.4.23.4) to pepsin activity. A world shortage of calf rennet due to the increased demand for cheese production has intensified the search for alternative microbial milk coagulants. Extensive research in this area has resulted in the production of enzymes that are completely inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases. In cheese making, the primary function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate *p*-k-casein and macro peptides. Chymosin is preferred due to its high specificity for casein, which is responsible for its excellent performance in cheese making. The proteases produced by GRAS (Genetically Regarded As Safe)-cleared microbes such as *Mucor michei*, *Bacillus subtilis* and *Endothia parasitica* are gradually

replacing chymosin in cheese making. In 1988, chymosin produced through recombinant DNA technology was first introduced to cheesemakers for evaluation.

2.9.2.2 Baking industry

Wheat flour is a major component of baking processes. It contains an insoluble protein called gluten, which determines the properties of the bakery doughs. Endo and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. Enzymatic treatment of the dough facilitates its handling and machining and permits the production of a wider range of products. The addition of proteases reduces the mixing time and results in increased loaf volumes (Rao et al., 1998).

2.9.2.3 Manufacture of soy products

Soybeans serve as a rich source of food, due to their high content of good-quality protein. Proteases have been used from ancient times to prepare soy sauce and other soy products. The alkaline and neutral proteases of fungal origin play an important role in the processing of soy sauce.

2.9.2.4 Debittering of protein hydrolysates

Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates commonly generated from casein, whey protein and soyprotein find major application in hypoallergenic infant food formulations (American Academy of pediatric committe 1989), fortification of fruit juices or soft drinks, in manufacturing protein-rich therapeutic diets and health products and as flavoring agents. The bitter taste of protein hydrolysates is a major barrier to their use in food and health care products. The intensity of the bitterness is proportional to the number of hydrophobic amino acids in the hydrolysate. The presence of a proline residue in the center of the peptide also contributes to the bitterness. The peptidases that can

cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolysates.

Aminopeptidases from lactic acid bacteria are available under the trade name Debitrase. Carboxypeptidase A has a high specificity for hydrophobic amino acids and hence, has a great potential for debittering. A careful combination of an endoprotease for the primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness.

Bacterial neutral proteases are active in a narrow pH range (pH 5.0 to 8.0) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant proteinase inhibitors and is therefore useful in the brewing industry (Rao et al., 1998).

2.9.2.5 Meat tenderization

Proteases play a prominent role in meat tenderization, especially of beef. An alkaline elastase (Takagi et al., 1992) and thermophilic alkaline protease (Wilson et al., 1992) have proved to be successful and promising meat tenderizing enzymes, as they possess the ability to hydrolyze connective tissue proteins as well as muscle fibre proteins. The tenderization process can be achieved by sprinkling the powdered enzyme preparation or by immersion in an enzyme solution and/or by injecting the concentrated protease preparation into the blood stream or meat.

Soluble meat hydrolysates can also be derived from lean meat wastes and from bone residues after mechanical deboning by solubilization with proteolytic enzymes. Alcalase® has been found to be the most appropriate enzyme in terms of cost, solubilization and other relevant factors. In an optimized process with Alcalase® at a pH of 8.5 and temperature of 55–60°C, a solubilization of 94% was

achieved (O'Meara and Munro, 1984a;b). The resulting meat slurry is further pasteurized to inactivate the enzyme and finds wide application in canned meat products, soups and seasonings. The cleaned bones may also be used as an excellent raw material for the production of gelatin.

Alkaline proteases can hydrolyze proteins from plants, fish or animals to produce hydrolysates of well-defined peptide profile. In addition, protein hydrolysates having angiotensin I-converting enzyme inhibitory activity were produced from sardine muscle by treatment with a *B. licheniformis* alkaline protease. These protein hydrolysates could be used effectively as a physiologically functional food that plays an important role in blood pressure regulation (Matsui et al., 1993).

Keratinolytic activity of alkaline protease has also been exploited in the production of proteinaceous fodder from waste feathers or keratin-containing materials. Alkaline proteases (B72) from *B. subtilis* and *B. licheniformis* PWD-1 was used for the hydrolysis of feather keratin, to obtain a protein concentrate for fodder production (Cheng et al., 1995; Dalev, 1990;1994).

2.9.3 Leather Industry

Alkaline proteases with elastolytic and keratinolytic activity can be used in leather-processing industries. Proteases are used for selective hydrolysis of noncollagenous constituents of the skin and for removal of nonfibrillar proteins such as albumins and globulins. The conventional methods of leather processing involve hazardous chemicals such as sodium sulfide, which create problems of pollution and effluent disposal. Thus, for environmental reasons, the biotreament of leather using an enzymatic approach is preferable as it offers several advantages like easy control, speedy waste reduction and ecofriendly (Andersen, 1998).

Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. At present, alkaline proteases with hydrated lime and

sodium chloride are used for dehairing, because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair, resulting in a significant reduction in the amount of wastewater generated. Currently, trypsin is used in combination with other *Bacillus* and *Aspergillus* proteases for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin, and the amount of enzyme needed depends on the type of leather (soft or hard). Novo Nordisk manufactures different proteases and protease mix like Aquaderm, Novocor S, NUE, NovoLime, Pyrase etc. for use in different stages of leather processing.

2.9.4 Textile industry

Protease enzymes are used for wool processing and degumming of silk for producing sand washed effects on silk garments. 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 (Kanehisa, 2000). 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. Treatment of Silk-Cellulosic blend is claimed to produce some unique effects. Proteases are also being used to wash down printing screens after use in order to remove the proteinaceous gums, which are used for thickening of printing pastes. Bio-stoning and the closely related process of bio-polishing are perhaps attracting most current attention in the area of enzyme processing (Ramachandran and Karthik, 2004).

2.9.5 Pharmaceutical Industry

The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Oral administration of proteases from

Aspergillus oryzae (Luizym and Nortase) has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. Clostridial collagenase or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. An asparginase isolated from *E. coli* is used to eliminate aspargine from the bloodstream in the various forms of lymphocytic leukemia. Collagenases with alkaline protease activity are increasingly used for therapeutic applications in the preparation of slow-release dosage forms. A new semi-alkaline protease with high collagenolytic activity was produced by *Aspergillus niger* 1.CF9. The enzyme hydrolyzed various collagen types without amino acid release and liberated low molecular weight peptides of potential therapeutic use (Barthomeuf et al., 1992). Similarly, *Elastoterase*, a preparation with high elastolytic activity from *Bacillus subtilis* 316M, was immobilized on a bandage for therapeutic application in the treatment of burns and purulent wounds, carbuncles, furuncles and deep abscesses (Kudrya and Simonenko, 1994). Furthermore, *Bacillus* spp. have been recognized as being safe to humans (deBoer and Diderichsen, 1991) and an alkaline protease having fibrinolytic activity has been used as a thrombolytic agent (Kim et al., 1996).

2.9.6 Peptide synthesis

Since the first report on protease-catalyzed peptide synthesis using the reverse-enzymatic reaction of hydrolysis (Bergmann and Frankel-Conrat, 1937), the proteases have frequently been used for peptide synthesis (Clapes et al., 1997; Isono and Nakajima, 2000; Kise et al., 1990; Morihara, 1987). Enzymatic peptide synthesis offers several advantages over chemical methods, e.g., reactions can be performed stereospecifically and reactants do not require side-chain protection, increased solubility of non-polar substrates or shifting thermodynamic equilibria to favor synthesis over hydrolysis. There is less need for expensive protecting-groups, organic solvents or hazardous chemicals, resulting in production costs competitive with those of chemical methods (Morihara, 1987). However, the major limitation

for the use of protease in synthetic chemistry is the strongly reduced activity of the enzyme under anhydrous conditions. Proteases have been used successfully for the synthesis of dipeptides (Barros et al., 1999) and tripeptide (So et al., 2000), regioselective sugar esterification (Riva et al., 1988) and dia-stereoselective hydrolysis of peptide esters (Chen et al., 1991b). A number of reports are available on the use of alkaline protease in peptide synthesis and the resolution of racemates of amino acids. The nature and type of organic solvent have a strong effect on protease activity in organic solvents (Kawashiro et al., 1997). Subtilisin showed 500-fold higher activity in glycerol compared with ethylene glycol, *N*-methylformamide, 1,2- and 1,3-propanediol (Castro, 1999). The effect of enzyme, solvent, medium and substrate reactions, using α-chymotrypsin, subtilisin BPN' and subtilisin Carlsberg from *B. subtilis* strain 72 on peptide synthesis in organic solvents was studied by Nagashima et al. (1992) and Gololobov et al. (1994). An industrial protease, Neutrase (co-deposited with sorbitol on to polyamide) for the synthesis of several N^{α}-protected dipeptide derivatives in acetonitrile was used (Clapes et al., 1997). The kinetic resolution of N-protected amino acid esters in organic solvents catalyzed by an industrial alkaline protease, Alcalase® was reported (Chen et al., 1991a). Alkaline protease was used for the resolution of DL-phenylalanine and DL-phenylglycine (Sutarli et al., 1992). Proteinase from an extremophile, *Thermus* R41A, immobilized on controlled pore glass beads, was used for peptide synthesis, using the synthesis of Bz-Ala-Tyr-NH_2 as a model system. The use of a surfactant–protease complex as a novel biocatalyst for peptide synthesis in hydrophilic organic solvents was described by Okazaki et al. (2000).

2.9.7 Silver Recovery

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. These waste films contain 1.5–2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films,

which causes undesirable environmental pollution. Furthermore, base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the recycling of polyester film. Alkaline protease from *B. subtilis* decomposed the gelatin layer within 15 -30 min at 50–60°C and released the silver (Fujiwara et al., 1989; Nakiboglu et al., 2001). Alkaline protease of *Bacillus* sp. B21-2 was used for the enzymatic hydrolysis of gelatin layers of X-ray films to release silver particles (Ishikawa et al., 1993). The alkaline proteases of *Bacillus* sp. B18 (Fujiwara et al., 1991) and *B. coagulans* PB-77 were also efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered (Gajju et al., 1996).

2.9.8 Waste Treatment

Proteases solubilize proteinaceous waste and thus help to lower the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities has opened up a new era in the use of proteases in waste management. The use of keratinolytic protease for food and feed industry waste, for degrading waste keratinous material from poultry refuse (Ichida et al., 2001) and as depilatory agent to remove hair from the drains (Takami et al., 1992) have been reported. A formulation containing proteolytic enzymes from *B. subtilis*, *B. amyloliquefaciens* and *Streptomyces* sp. and a disulfide reducing agent (thioglycolate), that enhances hair degradation and helps in clearing pipes clogged with hair-containing deposits, is currently available in the market. It was prepared and patented by Clenex (Jacobson et al., 1985).

2.9.9 Other Applications

Besides their industrial and medicinal applications, proteases play an important role in basic research. 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. In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in the food, detergent, leather, and pharmaceutical industries and in the structural elucidation of proteins. Whereas, their synthetic capacities are used for the synthesis of proteins.

Chapter 3

MATERIALS AND METHODS

3.1 Microorganism

Engyodontium album BTMF S10, isolated from marine sediment of Cochin (Suresh and Chandrasekaran, 1999), available as stock culture at Microbial Technology Laboratory, Department of Biotechnology, Cochin University of Science and Technology was used in the study.

The culture was maintained on Bennet's agar slants prepared in 50% aged seawater. The fungus was subcultured periodically, grown at 28°C for 14 days and stored at 4°C. A set of stock culture was also maintained under sterile mineral oil at room temperature.

Bennet's Agar Composition

Casein enzymatic hydrolysate	-	2g
Beef Extract	-	1g
Yeast Extract	-	1g
Dextrose	-	10g
Distilled water	-	1L
pH	-	7.3 ± 0.2
Agar	-	20g

3.2 SOLID STATE FERMENTATION (SSF)

Bioprocess for production of protease by *Engyodontium album* under Solid State Fermentation was optimised using wheat bran as solid substrate as described below.

3.2.1 Solid substrate medium

Commercially available wheat bran (WB) was used as the solid substrate medium for protease production. Wheat bran, sorted into varying particle sizes by sieving through mechanical sieve of different mesh size, was dried in sunlight for one day and stored in airtight containers.

Ten grams of WB, with particle size <425 μ, taken in 250ml Erlenmeyer flasks was moistened with aged seawater, so that the final moisture content was 60% (w/v) after inoculation (Suresh and Chandrasekaran, 1999). The contents were mixed thoroughly, autoclaved at 121°C for 30 minutes and cooled to room temperature. This was the general procedure followed for the solid substrate preparation for SSF studies unless otherwise specified.

3.2.2 Inoculum Preparation

Fungal inoculum for SSF studies was prepared using *E. album* (Suresh and Chandrasekaran, 1999). Conidial inoculum was prepared from a freshly raised 14-day-old Bennet's agar slant by dispersing the spores in 0.1% Tween 80 prepared in distilled water. One milliliter of this inoculum (2×10^8 cfu/ml) was used for inoculating each flask unless otherwise mentioned.

3.2.3 Inoculation and Incubation

Sterile WB prepared as mentioned earlier under section 3.2.1 was inoculated with 1ml of conidial inoculum, mixed thoroughly and incubated in a slanting position in the BOD incubator at 28 ± 2°C. The humidity inside the incubator was maintained using distilled water. At intervals of 24hrs, the contents inside the flask were mixed thoroughly by mechanical shaking. After the desired period of incubation (120 hrs arbitrarily selected), the enzyme was extracted from

the moldy wheat bran (MWB). This was the general procedure followed for solid state fermentation unless mentioned otherwise.

3.2.4 Extraction and recovery of enzyme

Protease from the MWB was extracted by the simple contact method of extraction using distilled water as extractant. Ten volumes of distilled water per gram MWB (based on initial dry weight of the substrate) was added to the flask and the extraction was performed by agitation at room temperature in a rotary shaker for 30 minutes at 150 rpm. The slurry was then squeezed through cheese cloth (Prabhu and Chandrasekaran, 1996) and clarified by centrifugation at 10,000 rpm at 4°C for 15 minutes. The clear supernatant, used as crude enzyme, was assayed for protease activity and protein content as mentioned under section 3.2.5.2 and 3.2.5.3 respectively.

3.2.5 Analytical Methods**

3.2.5.1 Estimation of Dry Weight of the Substrate (wheat bran)

One gram of moistened wheat bran was dried in oven at 105°C for 2hrs and weighed. The drying was continued until a constant weight was obtained for WB. Dry weight of the substrate was calculated by subtracting the moisture content from the wet weight.

3.2.5.2 Enzyme Assay

Protease activity was determined by caseinolytic method of (Kunitz, 1947) with minor modification. In this method, the TCA soluble fractions formed by the action of protease enzyme on the protein substrate Hammerstein casein was measured by the increase in absorbance at 280 nm. The method is as described below

- 2 ml of 1% (w/v) Hammerstein casein prepared in 0.05 M carbonate-bicarbonate buffer (pH 10.0) and 0.5ml of the same buffer were preincubated at 40°C for 10 minutes.
- To the above solution, 0.5 ml of diluted enzyme solution was added and incubated at 40°C for 30 minutes.
- The reaction was arrested with 2.5 ml of 0.44M trichloroacetic acid (TCA) solution. (To the control, TCA was added before the addition of enzyme sample).
- The reaction mixture was transferred to centrifuge tubes and the precipitated protein was removed by centrifugation at 10,000rpm for 15 minutes (Kubota, Japan).
- The absorbance of the clear supernatant was measured at 280nm in UV-Visible spectrophotometer (Shimadzu, Japan) against suitable blanks. The TCA soluble fractions of protein formed were quantified by comparison with a standard graph plotted with tyrosine as standard.
- One unit of protease activity was defined as the amount of enzyme that liberated 1µg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions.
- Enzyme activity was expressed as Units per gram Initial Dry Substrate (U/gIDS).

3.2.5.3 Protein Estimation

Protein content was determined according to the method of Lowry et al., (1951) using Bovine Serum Albumin (BSA) as the standard and was expressed in milligram per gram Initial Dry Substrate (mg/gIDS).

Reagent

(a) 2% solution of sodium carbonate in 0.1 N sodium hyrdoxide

(b) 0.5% solution of cupric sulphate in distilled water

(c) 1% solution of sodium potassium tartrate in distilled water

(d) *Working reagent- To 100 ml of solution (a), add 1ml each of solution (b) and solution (c).

(e) *1:1 Folin and Ciocalteau's phenol reagent diluted with distilled water

*Prepared fresh before use

Estimation

Two hundred microlitre of sample was made up to 2 ml with distilled water and 5 ml freshly prepared working reagent (d) was added, mixed thoroughly, and incubated for 10 minutes. 0.5ml of solution (e) was added and incubated for 30 minutes followed by measuring the absorbance at 750 nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

3.2.5.4 Specific Activity

Specific activity of the sample was calculated by dividing the enzyme units with the protein content and was expressed as U/ mg protein

$$\text{Specific activity} = \frac{\text{Enzyme activity (U gIDS)}}{\text{Protein (mg gIDS)}}$$

** All the experimental data were statistically analysed using Microsoft Excel.

3.2.6 Optimisation of Bioprocess variables for protease production by *E. album*

Various physico-chemical and bioprocess parameters affecting protease production by fungus under SSF were optimised towards maximal enzyme production. Strategy adopted for the optimisation was to evaluate individually the

effect of different parameters on protease production under SSF and perform finally a time course experiment under optimised condition. The parameters optimised included initial moisture content of the solid substrate medium, incubation time, particle size, incubation temperature, initial pH of the medium, proteinaceous substrates that induce enzyme production, additional carbon and nitrogen sources, sodium chloride concentration and inoculum concentration. Solid substrate medium preparation, inoculum preparation, inoculation and incubation, and extraction of enzyme were done as described under sections 3.2.1, 3.2.2, 3.2.3 and 3.2.4 respectively unless mentioned otherwise. In each case, samples were assayed for enzyme activity, protein content and specific activity as detailed under sections 3.2.5.2, 3.2.5.3 and 3.2.5.4, respectively unless otherwise described.

3.2.6.1 Initial moisture content

Impact of initial moisture content on protease production was evaluated by adjusting the moisture content of the WB to various levels ranging from 30% to 90% and assaying the enzyme yield. Moisture content of the WB medium was adjusted with varying volume of aged sea water such that after autoclaving, the initial moisture content after inoculation was 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% (v/w). Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.2 Incubation time

Optimal incubation time for maximal enzyme production was determined by incubating the inoculated media for a total period of 144 hrs and analyzing the samples at a regular interval of 24hrs for enzyme activity. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.3 Particle size of the Substrate

Optimal particle size of WB that support maximal enzyme activity was evaluated using the culture grown in WB medium of varying particle size i.e., <425µ, 425µ-600µ, 600µ-1000µ and WB without sieving. Irrespective of the size of WB, 60% initial moisture content (after inoculation) was employed arbitrarily. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.4 Incubation Temperature

Optimal incubation temperature for maximal enzyme production was evaluated by incubating the inoculated WB media at the following temperatures 15°C, 20°C, 25°C, 30°C and 35°C and determining the enzyme activity. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.5 Initial pH of the Medium for Enzyme Production

Suitable initial pH of the WB medium that support maximal enzyme production was determined by adjusting the pH of the moistening medium (aged sea water) to various levels i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 with 1N HCl or 1N NaOH. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.6 Additional Proteinaceous substrates

Requirement for an additional proteinaceous substrate, besides WB, as inducer for enzyme production was evaluated using casein and gelatin. While

preparing the WB medium, casein and gelatin were incorporated at 1% w/w level to the aged seawater. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.7 Additional Carbon Sources

Need for additional carbon sources for maximal enzyme production, was evaluated using various sugars which included arabinose, dextrose, fructose, galactose, maltose, mannose, mannitol, lactose, ribose, sucrose, sorbitol and xylose. While preparing the WB medium, the sugars were incorporated into the aged seawater so that the final concentration of the sugar in the WB medium was 0.1M. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.8 Additional Nitrogen sources

Effect of additional nitrogen sources on protease production was evaluated using organic nitrogen sources, inorganic nitrogen sources and different amino acids as detailed below.

3.2.6.8.1 Organic Nitrogen Sources

Effect of organic nitrogen sources on enzyme production was studied using yeast extract, beef extract, peptone, soyabean meal, tryptone and urea individually at 0.5% (w/w) level added to aged seawater while moistening the WB. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.8.2 Inorganic Nitrogen sources

Effect of addition of inorganic nitrogen sources on enzyme production was studied by the addition of ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium hydrogen carbonate, ammonium acetate, ammonium oxalate, ammonium hydrogen phosphate, ammonium iron sulphate, sodium nitrate and potassium nitrate at 0.1M level to WB medium. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.8.3 Amino acids

Requirement for addition of amino acids for maximal enzyme production was studied by preparing WB media added with each of the amino acids viz., alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at 1% level (w/w). Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.9 Inoculum Concentration

Optimal inoculum concentration that supports maximal enzyme production was evaluated using different concentrations of conidial inoculum. WB medium prepared as described in section 3.2.1 was inoculated with 2×10^4, 2×10^5, 2×10^6, 2×10^7, 2×10^8, 4×10^8 and 6×10^8 spores. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.10 Sodium Chloride Concentration

Effect of ionic strength on enzyme production was studied by the addition of sodium chloride to the WB media moistened with aged seawater. Medium was prepared as described in section 3.2.1 with 1%, 4%, 8%, 12%, 16% and 20% of final NaCl concentration. WB media was also prepared using distilled water as moistening medium incorporated with 1%, 2.5%, 5%, 7.5%, 10%, 12.5% and 15% of final NaCl concentration. Besides these, WB medium was also prepared with different dilutions of aged seawater i.e., 75%, 50% and 25%. Media moistened to 60% moisture content with 100% aged sea water and 100% distilled water were used as control. Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.2.6.11 Time Course Study Under Optimal Condition

Time course experiment was conducted with the optimised condition determined after optimisation of various variables. The conditions selected include the following

- Wheat Bran with <425 µ particle size
- 60% moisture content
- 0.1M Sucrose
- 0.1M ammonium hydrogen carbonate
- pH 10.0
- 2ml inoculum with spore count of 2×10^6 cfu/ml (i.e., 4×10^6 spores)
- Incubation temperature of 25°C

Solid substrate medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.2.1, 3.2.3 and 3.2.4 respectively.

3.3 ENZYME PURIFICATION

Protease produced by *E. album* under SSF was purified employing standard protein purification procedures which included ammonium sulphate precipitation, followed by dialysis, ion exchange chromatography and preparative polyacrylamide gel electrophoresis as detailed below. All the operations were done at 4°C unless otherwise specified.

3.3.1 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was done according to Englard and Seifter (1990). Ammonium sulphate (Sisco Research Laboratories Pvt. Ltd., India) required to precipitate protease enzyme was optimised by its addition at varying levels of concentrations (20%, 40%, 60%, 80% and 90% saturation) to the crude extract.

(i) To precipitate the protein, ammonium sulphate was slowly added initially at 20% saturation to the crude extract while keeping in ice with gentle stirring.

(ii) After complete dissolution of ammonium sulphate, the solution was kept at 4°C for over night.

(iii) Protein precipitated was collected by centrifugation at 10,000 rpm for 15 minutes at 4°C.

(iv) To the supernatant, ammonium sulphate required for next level of saturation was added and the procedure as mentioned above was repeated. This exercise was continued upto 90% of ammonium sulphate saturation.

3.3.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialysed against the buffer in order to remove the ammonium sulphate from the precipitate, as detailed below.

(i) The precipitated protein was resuspended in minimum quantity of 0.1M phosphate buffer (pH 7.0)

(ii) Dialysed in the pretreated dialysis tube (section 3.3.2.1) (cut off value 12kDa) against 0.01M solution of phosphate buffer of pH 7.0 for 24 hrs, at 4°C with 6 changes of buffer and assayed for protease activity, protein content and specific activity as described in section 3.3.5. Yield and fold of purification were calculated as described in section 3.3.5.1.

3.3.2.1 Pretreatment of Dialysis Tube

Dialysis tube (Sigma-Aldrich) is treated to remove the humectants and protectants like glycerin and sulfur compounds present in it, and to make the pores of the tube more clear. The treated tube retain most of the proteins of molecular weight 12kDa or greater. The method followed for the treatment of the dialysis tube was as follows.

(a) Washed the tube in running water for 3-4 hrs

(b) Dipped in 0.3% (w/v) solution of sodium sulfide, at 80°C for 1 minute

(c) Washed with hot water (60°C) for 2 minutes

(d) Acidified with 0.2% (v/v) sulphuric acid

(e) Rinsed with hot water (60°C)

3.3.3 Ion Exchange Chromatography

Ion exchange chromatography was done according to Rossomando (1990). Active fraction obtained after ammonium sulphate fractionation followed by

dialysis was further purified by ion exchange chromatography using the anion exchanger DEAE cellulose as the column material.

3.3.3.1 Standardisation of binding pH of protease to DEAE Cellulose

The pH at which the enzyme binds at its maximum to the anion exchanger was standardised by eluting the enzyme solution after incubating with DEAE Cellulose equilibrated to each pH. DEAE Cellulose was activated by following the method described in section 3.3.3.2, suspended in distilled water and equilibrated to each pH using 0.01M buffers of HCl-KCl buffer (pH 2.0), Glycine-HCl (pH 2.5), Citrate-Phosphate buffer (pH 3.0 to 6.5), Phosphate buffer (pH 7.0 & 7.5), Tris-HCl buffer (pH 8.0-9.0) and Carbonate-bicarbonate buffer (pH 9.5 & 10.0). One milliliter of diluted sample of 40-90% ammonium sulphate precipitated fraction was mixed with 2ml slurry of DEAE Cellulose equilibrated to each pH, incubated at 4°C for overnight, and the supernatant was collected by decanting without disturbing the suspension. Added 2ml of 0.4M NaCl and incubated for 2hrs to elute the bound protein from the DEAE Cellulose. Supernatant collected was centrifuged at 10,000rpm for 10 minutes to remove fine particles and assayed for protease activity and protein content as described in section 3.3.5.

3.3.3.2 Activation of DEAE Cellulose

The following method was adopted for the activation of DEAE Cellulose

(a) Ten gram of DEAE Cellulose (Sisco Research Laboratories Pvt. Ltd., India) was soacked in Phosphate buffer (pH 7.0, 0.01M) and fine particles were removed by decanting.

(b) It was then suspended in 1M NaCl solution for overnight.

(c) Decanted sodium chloride solution and washed several times with distilled water in sintered glass funnel using vacuum filtration, until the pH of washings became neutral.

(d) It was equilibrated in Phosphate buffer of pH 7.0 (0.01M) by repeated washing with the same.

3.3.3.3 Purification Using DEAE Cellulose column

DEAE Cellulose activated as described in section 3.3.3.2 was carefully packed in XK16/26 column (Amersham Biosciences) without trapping any air bubble. The column was equilibrated with Phosphate buffer of pH 7.0 (0.01M) for overnight.

Sixty millilitre of dialysed sample prepared as in section 3 3.2 with protein content of 1.94 mg/ml was applied to the pre-equilibrated DEAE Cellulose column with height 30 cm. After the complete entry of sample to the column the unbound proteins were washed with Phosphate buffer of pH 7.0 (0.01M) until the OD_{280} reached near zero. Stepwise elution was done at a flow rate of 2ml/minute using 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl in the same buffer. Five milliliter fractions were collected and protein content was estimated by measuring the absorbance at 280nm. Peak fractions from the column were pooled and assayed for protease activity, protein content and specific activity as described in section 3.3.5. Yield and fold of purification was calculated as described in section 3.3.5.1.

3.3.4 Preparative Polyacrylamide Gel Electrophoresis (Preparative-PAGE)

Active fractions pooled from ion exchange chromatography was lyophilized in 1ml aliquot and resuspended in 0 1ml of sample buffer (0.0625M Tris-HCl, 2% SDS, 10% sucrose, 0.01% bromophenol blue, pH 6.8). Aliquots of three tubes were loaded on to a gel prepared as described in section 3.4.1.3.2 and subjected to electrophoresis (Hoefer Mini electrophoresis apparatus). Low molecular weight marker of Amersham Pharmacia was used as standard. After electrophoresis, a portion of the gel with the marker was stained, destained, matched with the original gel, and the portion of the gel containing protease band

was cut out, chopped into pieces and transferred to dialysis tube. Three milliliter of reservoir buffer for Native-PAGE (3.4.1.1) was added and dialysis tube was closed with closure clips. Protein was eluted by applying 80V current for overnight at 4°C and, just before completing elution the current was reversed for 30 seconds to facilitate the detachment of the protein bound to the dialysis bag. The eluted protein was dialysed in phosphate buffer (pH 7.0, 0.01M) and lyophilized in aliquots.

3.3.5 Analytical Methods*

Protease activity, protein content and specific activity were determined as described earlier in sections 3.2.5.2, 3.2.5.3 and 3.2.5.4 and expressed as U/ml, mg/ml and U/mg protein respectively.

3.3.5.1 Calculation of Yield of Protein, Yield of Enzyme Activity and Fold of Purification

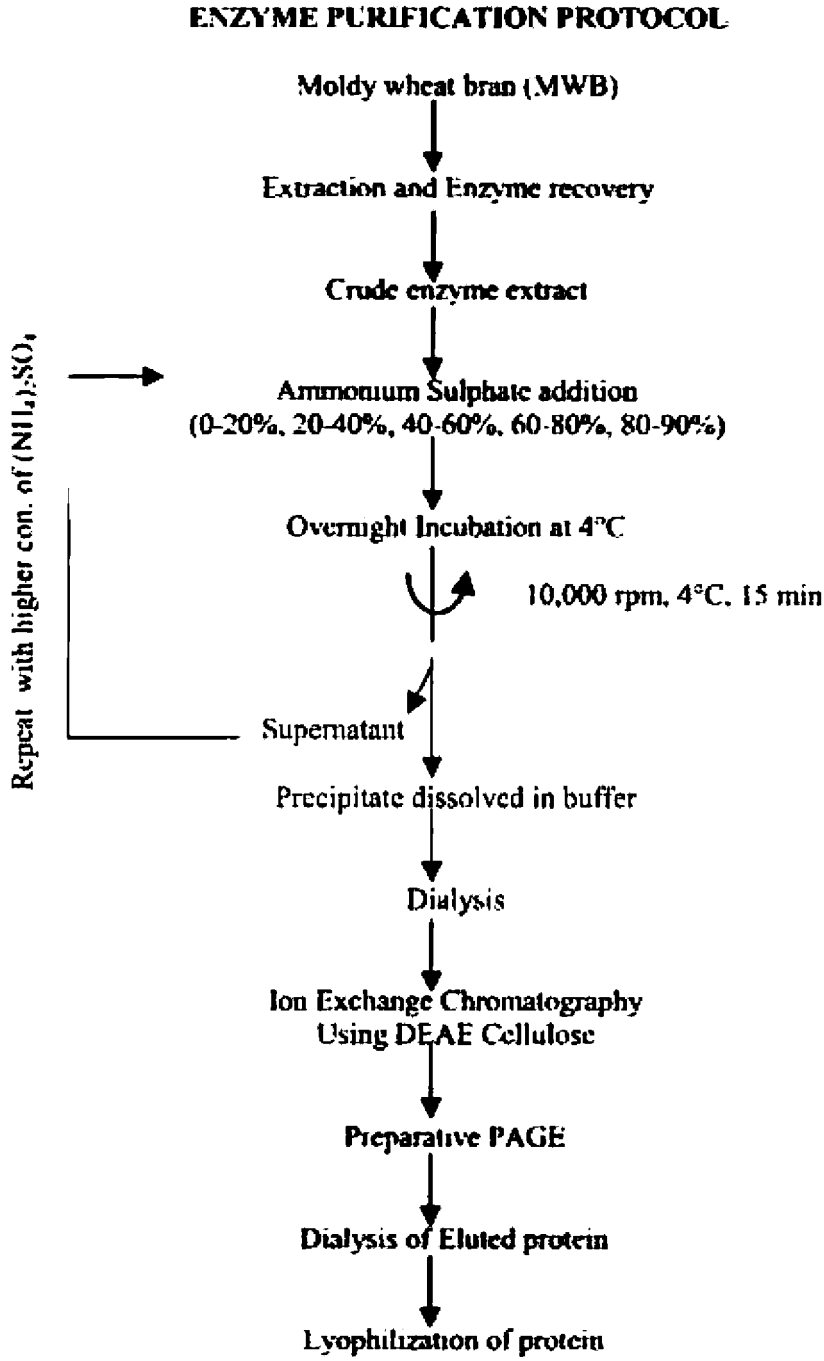
Yield of protein and enzyme activity of each fraction during purification is the percentage activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract as the case may be. Fold of purification in each step was calculated by dividing the specific activity of the respective fraction with that of the crude extract.

$$\text{Yield of Protein} = \frac{\text{Total Protein content of the fraction} \times 100}{\text{Total Protein content of the crude extract}}$$

$$\text{Yield of activity} = \frac{\text{Total activity of the fraction} \times 100}{\text{Total activity of the crude extract}}$$

$$\text{Fold of Purification} = \frac{\text{Specific activity of the fraction}}{\text{Specific activity of the crude extract}}$$

*All the experimental data were statistically analysed using Microsoft Excel.


ENZYME PURIFICATION PROTOCOL
Moldy wheat bran (MWB)
Extraction and Enzyme recovery
Crude enzyme extract
Repeat with higher con. of $(NH_4)_2SO_4$
Ammonium Sulphate addition
(0-20%, 20-40%, 40-60%, 60-80%, 80-90%)
Overnight Incubation at 4°C
10,000 rpm, 4°C, 15 min.
Supernatant
Precipitate dissolved in buffer
Dialysis
Ion Exchange Chromatography
Using DEAE Cellulose
Preparative PAGE
Dialysis of Eluted protein
Lyophilization of protein

3.4 CHARACTERISATION OF PURIFIED ENZYME

Purified protein after chromatography was further characterised for their biophysical and biochemical properties like molecular mass determination, zymogram profile, isoelectric point, amino acid analysis, N-terminal sequence, MALDI, enzyme kinetics etc. as described in the following sections.

3.4.1 Electrophoretic Methods

Ammonium sulphate precipitated sample and active fractions collected after ion exchange chromatography were electrophoresed by Native-PAGE and SDS-PAGE in a 10% polyacrylamide gel according to the method of Laemmli (1970). SDS-PAGE of purified enzyme was carried out under reductive and non-reductive conditions, i.e., with and without β-mercaptoethanol respectively.

3.4.1.1 Reagents for Polyacrylamide Gel Electrophoresis

1) ***Stock acrylamide solution*** **(30:0.8)**

Acrylamide (30%)	-	60.0 g
Bis-acrylamide (0.8%)	-	1.6 g
Distilled water (DW)	-	200.0 ml

Stored at 4°C in amber coloured bottle

2) ***Stacking gel buffer stock*** **(0.5 M Tris-HCl, pH 6.8)**

Tris buffer - 6 g in 40 ml DW
Titrated to pH 6.8 with 1M HCl (~48 ml) and made up to 100ml with DW
Filtered with Whatman No:1 filter paper and stored at 4°C

3) ***Resolving Gel buffer stock*** **(3M Tris-HCl, pH 8.8)**

Tris buffer - 36.3 g
Titrated to pH 8.8 with 1M HCl (~48 ml) and made up to 100ml with DW
Filtered with Whatman No:1 filter paper and stored at 4°C

4) ***Reservoir buffer for Native-PAGE*** **(pH 8.3)**

Tris buffer	-	3.0 g
Glycine	-	14.4 g

Dissolved and made up to 1L with DW
Prepared in 10X concentration and stored at 4°C

5) ***Reservoir buffer for SDS-PAGE*** **(pH 8.3)**

Tris buffer	-	3.0 g
Glycine	-	14.4 g
SDS	-	1.0 g

Dissolved and made up to 1L with DW
Prepared in 10X concentration and stored at 4°C

6) ***Sample buffer for Native-PAGE***

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C

7) ***Sample buffer for Reductive SDS-PAGE***

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Dithiothreitol	-	0.1M
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C

8) ***Sample buffer for Non-Reductive SDS PAGE***

Tris HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C

9) ***SDS*** **(10%)** - 1g in 10ml DW

10) ***Sucrose*** **(50%)** - 5g in 10ml DW (autoclaved at 121°C for 15 minutes and stored at 4°C)

11) *Protein Staining solution*

Coomassie brilliant blue (0.1%)	-	100mg
Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

12) *Destaining Solution*

Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

13) *Protein Marker for Native – PAGE*

Separate markers from Sigma-Aldrich were used.

Components		Volume	MW(M_r)
Bovine Serum Albumin-	-	10 µl	66,000
Chickalbumin	-	10 µl	45,000
Carbonic anhydrase	-	5 µl	29,000
Lactalbumin	-	10 µl	14,200

Markers were mixed with 65µl of Native 1X sample buffer, and 30µl of marker mix was loaded on to the gel.

14) *Protein Marker for SDS–PAGE*

Low molecular weight marker mix of Amersham Pharmacia was used. Lyophilized marker mix was reconstituted in 1X sample buffer for reductive SDS-PAGE, boiled for 5 minutes, and 5µl of marker was loaded on to the gel. The composition of the marker mix is as given below.

Components		MW(M_r)
Phosphorylase b	-	97,000
Bovine Serum Albumin	-	66,000
Ovalbumin	-	45,000
Carbonic anhydrase	-	30,000
Trypsin inhibitor	-	20,100
α-Lactalbumin	-	14,400

3.4.1.2 Native- Polyacrylamide Gel Electrophoresis (Native-PAGE)

3.4.1.2.1 Gel Preparation

Resolving gel (10%)

Acrylamide : bis-acrylamide (30: 0.8)	-	10.0 ml
Resolving gel buffer stock	-	3.75ml
Ammonium persulphate (APS)	-	a pinch
Water	-	16.25ml
TEMED	-	15.0µl

Stacking gel (2.5%)

Acrylamide : bis- acrylamide (30: 0.8)	-	2.5 ml
Stacking gel buffer stock	-	5.0 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	12.5 ml
TEMED	-	15.0 µl

Sample buffer (1X)

Native-PAGE sample buffer (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

3.4.1.2.2 Sample preparation

Added 100µl of 1X sample buffer to lyophilized sample or 20µl of 2X sample buffer and 10µl of 50% sucrose to 30µl liquid sample, mixed well and 30µl sample and 5µl marker mix was loaded on to the gel.

3.4.1.2.3 Procedure

(a) Cleaned and assembled the gel plates

(b) **Resolving gel** - Added all the components except APS in to a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and poured a layer of butanol over the gel and allowed to solidify at least for 1hr.

(c) **Stacking gel-** Added the components of stacking gel except APS into a beaker, mixed gently and finally added APS. Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed it to solidify at least for 30 minutes.

(d) Gel was placed in the electrophoresis apparatus, and upper and lower reservoir was filled with reservoir buffer for Native-PAGE.

(e) The gel was pre run for 1hr at 80V.

(f) Loaded the gel with the protein sample.

(g) The gel was run at 80 V till the sample entered the resolving gel.

(h) When the dye front entered the resolving gel increased the current to 100 V.

(i) Stopped the current when the dye front reached 1cm above the lower end of the glass plate.

(j) Removed the gel from the cast and stained for at least 1hr in the staining solution.

(k) Destained till the bands became clear and observed under a transilluminator.

3.4.1.3 Sodium Dodecyl Sulphate- Polyacrylamide Gel electrophoresis (SDS-PAGE)

Purified protein was subjected to reductive or non-reductive SDS-PAGE i.e., with or without β-mercaptoethanol. Low molecular weight marker of Amersham Pharmacia was used as standard and molecular weight of protease was determined using Quantity One Software of Biorad.

3.4.1.3.1 Reductive SDS-PAGE

3.4.1.3.1.1 Gel Preparation

Resolving gel (10%)

Acrylamide : bis - acrylamide (30: 0.8)	-	10.0 ml
Resolving gel buffer stock	-	3.75ml
10% SDS	-	0.3ml
Ammonium persulphate (APS)	-	a pinch
Water	-	15.95 ml
TEMED	-	15.00 µl

Stacking gel (2.5%)

Acrylamide : bis – acrylamide (30: 0.8)	-	2.5 ml
Stacking gel buffer stock	-	5.0 ml
10% SDS	-	0.2ml
Ammonium persulphate (APS)	-	a pinch
Water	-	12.3 ml
TEMED	-	15.0 µl

Sample buffer (1X)

SDS-PAGE sample buffer (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

3.4.1.3.1.2 Sample preparation

Added 100µl of 1X sample buffer to purified and lyophilized protease sample, mixed well, boiled for 5 minutes in a water bath, cooled to room temperature, and 30µl sample and 5µl low molecular weight markers were loaded on to the gel.

3.4.1.3.1.3 Procedure

Procedure followed for electrophoresis and staining was essentially same as described in section 3.4.1.2.3 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.4.1.3.2 Non-reductive SDS- PAGE

3.4.1.3.2.1 Gel preparation

Resolving and Stacking gel was prepared as described in section 3.4.1.3.1.1

Sample buffer (1X)

Sample buffer for Non-reductive SDS-PAGE (2X)	-	1ml
50% Sucrose	-	0.4 ml
DW	-	0.6ml

3.4.1.3.2.2 Sample preparation

Added 100µl of 1X sample buffer to lyophilized sample or 20µl of 2X sample buffer and 10µl of 50% sucrose to 30µl liquid sample, mixed well, and 30µl sample and 5µl low molecular weight markers were loaded on to the gel.

3.4.1.3.2.3 Procedure

Procedure followed for electrophoresis and staining was essentially the same as described in section 3.4.1.2.3 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.4.1.4 Zymogram

Proteolytic activity of enzyme protein band was confirmed by zymogram analysis on X-ray film according to the method of Cheung et al (1991). 40-90% ammonium sulphate precipitated sample and active fractions pooled from ion exchange chromatography were lyophilized in 1ml aliquots, resuspended in 0.1ml of sample buffer under non-reducing condition (0.0625M Tris-HCl, 2% SDS, 10% sucrose, 0.01% bromophenol blue, pH 6.8) and subjected to electrophoresis in a 10% polyacrylamide gel at 4°C as described in section 3.4.1.3.2. After electrophoresis, the gel was washed with 2.5% (v/v) Triton X-100 for 30 minutes

followed by rinsing with carbonate-bicarbonate buffer (0.05M, pH 10.0) and incubated on a fresh X-ray film for 10 minutes at 40°C. After incubation, the film was rinsed with distilled water. The clear zones on X-ray film indicated the presence of protease bands.

3.4.1.5 Isoelectric focusing

Isoelectric focusing of the purified protein sample, prepared as described in section 3.3.4 was done following the method of O'Farrells (1975) at Centre for Cellular and Molecular Biology (CCMB), Hyderabad.

3.4.2 Molecular Weight determination by Gel Filtration Chromatography

Gel filtration chromatography was performed for the 40-90% precipitate of ammonium sulphate fractionation using Sephadex G75 (Sigma-Aldrich) in order to determine the molecular weight of protease.

3.4.2.1 Preparation of Column

(a) 23g of Sephadex G75 (Sigma-Aldrich) was suspended in distilled water and allowed to hydrate for 3hrs at 100°C in a water bath, and fine particles were removed by decantation.

(b) Hydrated gel suspension was degassed under vacuum to remove the air bubbles.

(c) Gel suspension was carefully poured into the column (Amersham Biosciences XK26/70 column) without trapping air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column.

(d) Column was stabilized by allowing two times the bed volumes of eluent (0.1M Phosphate buffer, pH 7.0) to pass through the column bed in descending eluent flow.

3.4.2.2 Running the Column

Two milliliter of dialysed sample, prepared as in section 3.3.2, with protein content of 1.94 mg/ml was applied to the column. After the complete entry of sample to the column the proteins were eluted using 0.1M Phosphate buffer of pH 7.0, with a flow rate of 1ml/minute. One milliliter fractions were collected and protein content was estimated by measuring the absorbance at 280nm in a UV-Visible Spectrophotometer (Shimadzu, Japan). Peak fractions from the column were pooled and assayed for protease activity and protein content as described in sections 3.4.24.

3.4.2.3 Calculation of molecular weight of the Protease Enzyme

The molecular weight of the eluted protein was determined by calibrating the column with low molecular weight gel filtration markers from Amersham Biosciences.

The markers used included the following:

Components		MW(M_r)
Ribonuclease A	-	13,700
Bovine Serum Albumin	-	67,000
Ovalbumin	-	45,000
Chymotrypsinogen A	-	25,000

K_{av} (partition coefficient) of each protein was calculated by the formula

$$K_{av} = \frac{V_e}{V_o}$$

Where V_e is the elution volume of each protein and V_o is the void volume of the column, which was calculated by running Blue Dextran 2000.

Molecular weight of protease enzyme was calculated from the semilogarithmic graph plotted for the K_{av} v/s molecular weight of the standard proteins.

3.4.3 Aminoacid analysis

Aminoacid analysis of the purified protein sample prepared as described in section 3.3.4 was done using Shimadzu High-Performance Liquid Chromatograph (LC-4A) "Amino Acid Analysis System" (Ammu et al., 1994) at Central Institute of Fisheries Technology (CIFT), Kochi.

3.4.4 Determination of the N-terminal sequence of the enzyme

Purified enzyme sample (section 3.3.4) was subjected to electrophoresis (section 3.4.1.3.2). Protein band was blotted on to a PVDF membrane by electrotransfer at 4°C for overnight in a Genei protein-blotting unit (transfer buffer- 30mM Glycine, 48mM Tris base, 0.037% SDS, 20% Methanol, pH 8.3). After blotting, the membrane was air dried and N- terminal sequencing was done at Indian Institute of Science (IISc), Bangalore.

3.4.5 Matrix-Assisted Laser Desorption Ionization - (MALDI)

An attempt was made to determine the molecular weight and peptide fingerprinting of the purified sample using MALDI Analysis. Protease enzyme purified by ion exchange chromatography was electrophoresed (section 3.4.1.3.2) and the stained protease band was used for MALDI analysis using Applied Biosystems-Voyager System 4263 at CCMB, Hyderabad.

MALDI-MS analysis of the purified protein was done to determine the molecular weight. For peptide fingerprint, protein bands digested with trypsin were

extracted, desalted and Mass spectrum was generated. Mass peak list obtained was submitted to Profound and Mascot database for protein identification.

3.4.6 Antibody production

Antibodies were raised against electrophoretically pure protease in New Zealand white rabbits by intramuscular injection of 1ml (100µg) of protein emulsified with an equal volume of Freund's complete adjuvant. Three booster injections were given at an interval of 15 days with 2ml (200µg) of protein emulsified with an equal volume of Freund's incomplete adjuvant (Harlow and Lane, 1988).

One week after the third booster dose, the blood was collected from the marginal ear vein and allowed to clot at 37°C for 30-60 minutes. The clot was retracted from the sides of the test tube with the help of a glass rod and after complete clotting, the serum was harvested by centrifugation at 4,000rpm for 15 minutes at room temperature.

3.4.6.1 Immunodiffusion test for antibody reaction

Antiserum was detected by simple immunodiffusion (Harlow and Lane, 1988). One percent agarose containing 0.02% sodium azide was prepared in phosphate buffered saline (PBS - 0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, pH 7.4). Melted contents were poured on a glass plate to prepare a 0.2cm thick gel. After solidification, 2mm wells were cut using gel cutter and 25µl of serum was loaded in succession to peripheral wells and antigen (purified by ion exchange chromatography) in the central well. Slide was kept in a humid environment for 24hrs. After incubation the slide was washed in PBS for 30minutes in a rotary shaker. Washed slide was dried by keeping blotting paper over it and incubating it at 37°C overnight followed by staining in Coomassie Blue

and destaining. Antigen antibody specificity was detected by the formation of a precipitation arc between the protein samples and antibody applied to the wells.

3.4.7 Optimal pH for Protease Activity

Optimum pH for maximal activity of the purified enzyme was determined by conducting enzyme assay at various levels of pH in the range of 2-13. The enzyme assay was essentially the same as described in section 3.2.5.2 with the following modification. The enzyme solution used was 0.2 ml of diluted sample and the substrate casein, was prepared in the respective buffer of each pH. The buffer systems used included, HCl-KCl buffer (pH 2.0), Citrate–Phosphate buffer (pH 3 to 6), Phosphate buffer (pH 7.0), Tris-HCl buffer (pH 8.0), Glycine-NaOH buffer (pH 10.0), Carbonate-bicarbonate buffer (pH 10.0), Boric acid/ potassium chloride/ sodium hydroxide (pH 11.0), Disodium hydrogen phosphate/sodium hydroxide (pH 12.0) and KCl/NaOH (pH 13.0). Enzyme activity and relative activity were calculated as described in section 3.4.24 and 3.4.24.2 respectively.

3.4.8 Stability of Protease at different pH

Stability of the purified enzyme over a range of pH was determined by measuring the residual activity at pH 10.0 after incubating the enzyme in different buffer systems of pH 2.0-13.0 for 24hrs. at 4°C. Purified enzyme as 0.2 ml aliquot was incubated in 3.8 ml of different buffer systems, which included, HCl-KCl buffer (pH 2.0), Citrate–Phosphate buffer (pH 3.0 to 6.0), Phosphate buffer (pH 7.0), Tris-HCl buffer (pH 8.0), Glycine-NaOH buffer (pH 10.0), Carbonate-bicarbonate buffer (pH 10.0), Boric acid /potassium chloride/sodium hydroxide (pH 11.0), disodium hydrogen phosphate/sodium hydroxide (pH 12.0) and KCl/NaOH (pH 13.0). After incubation 0.2ml sample was assayed for protease activity as described in section 3.4.24. Enzyme activity was expressed as U /ml.

3.4.9 Optimal Temperature for Protease Activity

Temperature optimum for enzyme activity was determined by incubating 0.2ml of purified enzyme essentially following the method described in section 3.4.24 at the temperature range from 5 to 100°C. Relative activity was calculated as described in section 3.4.24.2.

3.4.10 Enzyme stability at different temperatures

Temperature stability of purified enzyme was determined by incubating the enzyme sample at various temperatures ranging from 30-80°C and the enzyme assay was conducted at 30 minutes, 1hr, 2hr, 4hr, 6hr, 8hr, 10hr, 12hr and 24hr of incubation as described in section 3.4.24. Enzyme activity of the sample kept at 4°C was taken as control. Residual activity of the enzyme was calculated as described in section 3.4.24.1.

3.4.11 Effect of stabilizers on thermal stability of protease

Effect of stabilizers on enzyme activity at higher temperature was studied by incubating the enzyme solution added with various reported thermal stabilizers at 65°C and 70°C for 3hrs and estimating the residual activity at regular time intervals. Stabilizers studied include $CaCl_2$ (1, 5 and 10mM), $CoCl_2$ (1mM), PEG 6000, Glycerol, Sucrose, Mannitol, Sorbitol, Starch, Glycine and Bovine Serum Albumin (at 1% level). Enzyme assay was carried out as described in section 3.4.24 and the residual activity was calculated as described in section 3.4.24.1.

3.4.12 Effect of Inhibitors on Protease Activity

Effect of various protease inhibitors on the purified enzyme sample was done in order to classify the enzyme depending on the inhibition pattern. The following inhibitors, i.e., 20 to 50 mM Phenylmethylsulphonyl fluoride (PMSF),

0.1 to 1μM Aprotinin, 20 to 50 mM Ethylene diamine tetra acetic acid (EDTA), 10mM 1.10- Phenanthroline, 10 to 50μM L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, N-[N'-(L-3-trans- carboxyirane - 2-carbonly)-L-leucyl]-agmatine (E-64), 1 to 50μM Iodoacetamide and 5 to 100μM Pepstatin were added to the purified enzyme and incubated at room temperature for 30 minutes. After incubation, 1 ml of 1% casein was added to each enzyme reaction mixture and residual enzyme activity was measured as described in section 3.4.24.1. Residual enzyme activity was expressed in percentage.

3.4.13 Substrate specificity

Ability to hydrolyse various proteinaceous substrates by the purified enzyme was evaluated by conducting enzyme assay with Casein, Gelatin, haemoglobin and Bovine Serum Albumin, as described in section 3.4.24 with 1% solution of respective substrates prepared in carbonate-bicarbonate buffer of pH 10.0. TCA soluble fractions were measured at 280 nm and enzyme activity was expressed in U/ml.

3.4.14 Kinetic studies

Purified enzyme was subjected to kinetic studies towards determining the K_m and V_{max}. K_m, the substrate concentration at which the reaction velocity is half maximum and V_{max}, the velocity maximum of the enzyme reaction was determined by incubating 0.2ml of purified enzyme in different concentrations of casein (0.01mg to 20mg) at pH 10.0 for 30 minutes at 40°C. Enzyme assay was done as described in section 3.4.24.

The initial velocity data was plotted as the function of the concentration of substrate by the linear transformation of the Michaelis-Menten equation and usual non-linear curve fitting of the Michaelis-Menten equation for the calculation of K_m and V_{max} of the reaction.

Catalytic constant of the reaction or Turnover number (K_{cat}) of the enzyme was calculated by the equation

$$K_{cat} = \frac{V_{max}}{[E]_T}$$

Where V_{max} is the Maximal Velocity and $[E]_T$ is the total enzyme concentration.

Enzyme's catalytic efficiency was calculated by the equation,

$$\frac{K_{cat}}{K_m}$$

3.4.15 Effect of various metal ions on enzyme activity

Effect of various metal ions on enzyme activity, was evaluated by incubating the enzyme along with different concentrations of various metal ions in the enzyme reaction mixture for 30 minutes followed by measuring the residual enzyme activity (section 3.4.24.1). The metals studied included 1, 5, 10, 15 and 20mM final concentrations of sodium chloride, calcium chloride, magnesium sulphate, zinc sulphate, potassium sulphate, cupric sulphate, ferric chloride, manganese chloride, nickel chloride, cobalt chloride, mercury chloride, barium chloride, cadmium sulphate, lithium chloride, sodium molybdate, lead acetate, aluminium sulphate and chromium nitrate which contribute the metal ions, Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Hg^{2+}, Ba^{2+}, Cd^{2+}, Li^{+}, Mo^{6+}, Pb^{2+}, Al^{3+} and Cr^{3+} respectively.

3.4.16 Effect of various Detergents on Enzyme Activity

Effect of various non-ionic and ionic detergents such as Triton X-100, SDS, Tween-80, Tween-20, and Brij-35 (w/v) on enzyme activity was determined by conducting enzyme assay in the presence of each detergent and residual activity

was calculated. After incubation of the enzyme in different concentrations of each detergent viz., 0.2, 0.4, 0.6, 0.8, 1 and 5% for 30 minutes, the residual enzyme activity was measured as described in section 3.4.24.1.

3.4.17 Effect of Hydrogen Peroxide (as oxidizing agent) on enzyme activity

Activity and stability of the enzyme in the presence of oxidizing agent, hydrogen peroxide, was studied by measuring the residual activity after 30 minutes of incubation of the enzyme in different concentrations of H_2O_2 viz., 1, 2, 3, 4, 5 and 8% (v/v). The residual enzyme activity was assayed as described in section 3.4.24.1.

3.4.18 Effect of Reducing agents on enzyme activity

Activity and stability in the presence of reducing agents were studied by incubating enzyme solution with 0.2, 0.4, 0.6, 0.8, 1 and 5% (v/v) of dithiothreitol, β- mercaptoethanol and sodium thioglycolate for 30 minutes and measuring the residual activity as described in section 3.4.24.1.

3.4.19 Effect of Ionic strength on protease activity

Effect of ionic strength on enzyme activity was tested by measuring the enzyme activity after incubating the enzyme assay mixture added with various ionic concentration of sodium chloride (1, 2, 3, 4 and 4.5 M). The residual activity after 30 minutes of incubation was measured as described in section 3.4.24.1.

3.4.20 Effect of organic solvents on protease activity

Impact of various organic solvents on enzyme activity was evaluated by incubating the enzyme with each organic solvent for 30 minutes and assaying the residual activity as described in section 3.4.24.1. Organic solvents studied

included, dimethyl sulfoxide, isopropanol, acetonitrile, (1, 2, 3, 4, 6, 9, 12 and 12% (v/v)), ethanol (1, 2, 3, 4, 5, 10, 15 and 20% (v/v)), phenol, petroleum ether, acetone and ethyl ether (0.2, 0.5, 0.8, 1, 4, 6, 8 and 10% (v/v)).

3.4.21 Storage stability of the Protease

Lyophilized and liquid samples of partially purified enzyme (i.e., 40-60% ammonium sulphate precipitate) were stored at room temperature, 4°C and 20°C for a period of one year. Periodically, samples were taken, properly diluted and enzyme activity, protein content and specific activity were tested by the method described in section 3.4.24.

3.4.22 Stability of the Enzyme in the presence of Hydrocarbons

Stability of the enzyme in the presence of hydrocarbons was evaluated by incubating the enzyme in various hydrocarbons for 30 minutes and assaying the residual activity as described in section 3.4.24.1. Hydrocarbons studied included petrol, kerosene, diesel, grease, used machine oil and used engine oil, at a concentration of 1 and 5%.

3.4.23 Stability of the Enzyme in the presence of Natural Oils

Stability of the enzyme in the presence of natural oils, was determined using Coconut oil, Gingelly oil, Palm oil, Mustard oil, Sun flower oil, Vegetable oil, Dalda, Olive oil, Castor oil and Ghee each at 1 and 5% concentration. The enzyme was incubated for 30 minutes in various natural oils and the residual activity was estimated as described in section 3.4.24.1.

3.4.24 Analytical Methods*

Protease activity, protein content and specific activity were determined as described earlier in sections 3.2.5.2, 3.2.5.3 and 3.2.5.4 and were expressed as U/ml, mg/ml and U/mg protein respectively.

3.4.24.1 Residual Activity

Residual activity is the percent enzyme activity of the sample with respect to the enzyme activity of the control sample.

$$\text{Residual activity} = \frac{\text{Activity of sample (U/ml) x 100}}{\text{Activity of the Control (U/ml)}}$$

3.4.24.2 Relative Activity

Relative activity is the percent enzyme activity of the sample with respect to the sample for which maximum activity was obtained

$$\text{Relative activity} = \frac{\text{Activity of sample (U/ml) x 100}}{\text{Activity of the maximal enzyme activity obtained sample (U/ml)}}$$

* All the experimental data were statistically analysed using Microsoft Excel.

3.5 APPLICATION STUDIES

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing ecofriendly technologies, they are envisaged to have extensive applications in leather treatment, silver recovery from X-ray films and in several bioremediation processes. The worldwide requirement of enzymes for individual applications varies considerably. Proteases are used extensively in the food and detergent industries, prepared in bulk

quantities and used as crude preparations. The present study evaluated the potential of the protease from marine *E. album* in industrial application

3.5.1 Commercial detergent compatibility of enzyme

The stability of the enzyme in the presence of commercial detergents were determined using various detergents which included Ujala washing powder, Surf Multi Action with Kids Stain Formula, Surf Excel, Surf Excel Automatic, Ariel Compact, Henko Stain Champion, Henko Power Pearls, Tide, Rin Shakti, Sunlight Extrabright with Colourlock, Wheel, Mr White, Speed, Godrej dish wash liquid and Harpic Power toilet cleaner at concentration 7mg/ml and liquid soap 1% (w/v). Enzymes already present in the detergent and soap solutions were first heat inactivated by boiling for 10 minutes and to the 50ml solution, 2ml of purified enzyme sample was added and incubated for 3 hrs at room temperature. Samples were taken out at intervals of 0hr, 30 minutes, 1hr, 1.5hr, 2hr, 2.5hr and 3hr and the residual activity was determined as described in section 3.5.6.4.

3.5.2 Comparison of performance of *E. album* protease with different Commercial proteases in the presence of detergents at 60°C

A comparative evaluation on the performance of *E album* protease and commercially available proteases was performed by determining the residual enzyme activity after incubation with commercial detergent Surf Excel Automatic (7mg/ml) for 3hrs at 60°C. Initially, enzymes already present in the detergent were first heat inactivated by boiling for 10 minutes and to the 50ml of detergent solution, properly diluted enzyme solution was added and incubated. Samples were taken at intervals of 0hr, 30 minutes, 1hr, 1.5hr, 2hr, 2.5hr and 3hr and the residual activity was determined as described in section 3.5.6.4.

3.5.3 Wash performance studies

Wash performance analysis of purified protease (40-90% ammonium sulphate precipitate with enzyme activity of 480 U/ml) was studied on white cotton cloth piece (5 x 5 cm) stained with human blood. The stained cloth pieces were taken in separate flasks and subjected to the following wash treatment studies.

1. 100ml heat inactivated detergent (7mg/ml) + stained cloth piece
2. 100ml heat inactivated detergent (7mg/ml) + 1ml enzyme solution + stained cloth piece
3. 100ml distilled water – 1ml enzyme solution + stained cloth piece
4. 100ml distilled water + stained cloth piece

After 30 minutes of incubation at 50°C in a water bath shaker, the cloth pieces were taken out, rinsed with tap water, dried and visual examination was done to check the effectiveness of stain removal.

3.5.4 Esterase activity of the Protease Enzyme

Esterase activity of purified protease enzyme was determined using different *p*-nitrophenyl derivatives according to the method of Prim et al. (2003) with some modification as described in section 3.5.6.6. It was confirmed by activity staining which releases the fluorescent 4-methylumbelliferone (MUF) from methylumbelliferyl butyrate (Prim et al., 2003) as described in section 3.5.6.7.

3.5.5 Decomposition of gelatin layer of X-ray film

Ability of the enzyme to hydrolyse the gelatin layer of the X-ray film for the recovery of silver was studied by incubating 2g of X-ray film in enzyme solution (enzyme purified by 40-90% ammonium sulphate precipitate with activity of 3,300 U/ml). Following sets were prepared in flasks and studied.

1. 19 ml carbonate-bicarbonate buffer (pH 10.0) – 1 ml enzyme solution + 2g X-ray film
2. 20 ml carbonate- bicarbonate buffer (pH 10.0) – 2g X-ray film
3. 19 ml distilled water – 1 ml enzyme solution + 2g X-ray film
4. 20 ml distilled water + 2g X-ray film

The flasks were kept on a rotary shaker with 120 rpm at room temperature. After 90 minutes of incubation, X-ray film was taken out, rinsed with tap water, dried and visual examination was done. Protein stripped to the supernatant by the action of the enzyme was estimated by the method of Lowry et al. (1951) as described in section 3.5.6.2.

3.5.6 Analytical Methods*

3.5.6.1 Enzyme Assay

Protease activity was determined by caseinolytic method of Kunitz (1947) as described in section 3.2.5.2 and expressed in U/ml.

3.5.6.2 Protein Estimation

Protein content was determined according to the method of Lowry et al (1951) as described in section 3.2.5.3 and was expressed in mg/ml.

3.5.6.3 Specific Activity

Specific activity of the sample was calculated by dividing the enzyme units with the protein content and was expressed as U/ mg protein.

3.5.6.4 Residual Activity

Residual activity is the percent enzyme activity of the sample with respect to the activity of the control sample.

$$\text{Residual activity} = \frac{\text{Activity of sample (U/ml) X 100}}{\text{Activity of the Control (U/ml)}}$$

3.5.6.5 Relative Activity

Relative activity is the percent enzyme activity of the sample with respect to the sample for which maximum activity was obtained.

$$\text{Relative activity} = \frac{\text{Activity of sample (U/ml) X 100}}{\text{Activity of the maximal enzyme activity obtained sample (U/ml)}}$$

* All the experimental data were statistically analysed using Microsoft Excel.

3.5.6.6 Assay for Esterase activity

Lipase activity was determined by measuring the release of *p*-nitrophenol (*p*NP) from different *p*NP derivatives according to modified method of (Prim et al., 2003) in microtitre plate. *p*NP Acetate (Sigma and Mooser), *p*NP Butyrate (Sigma and Mooser), *p*NP Caprylate (Fluka), *p*NP Laurate (Fluka) and *p*NP Palmitate (Sigma and Mooser) were used as substrates.

Substrate preparation

Solution A (0.15% stock solution of each substrate in Isopropanol)

Substrates were dissolved in isopropanol and sonicated for 6 minutes in a continuous mode for proper emulsification.

Solution B

50mM Tris buffer (pH 8.0) containing 0.1% gum arabic and 0.4% Triton X-100

Buffered substrate (1:10 dilution of the substrate stock solution A in Solution B)

To 9 ml of continuously stirred solution of A, 1 ml of solution B was added drop wise.

Procedure

(a) An aliquot of 230µl of buffered substrate was incubated at 40°C for 10 minutes in the ELISA plate reader (Bio-Rad).
(b) To the preincubated buffered substrate, 20µl of properly diluted enzyme solution was added.
(c) Incubated at 40°C for 30 minutes and the released *p*NP was determined by immediate measurement of the absorbance at 415 nm against suitable blanks.
(d) One unit of activity was defined as the amount of enzyme that released 1 µmol of *p*NP per minute under the assay conditions described.

3.5.6.7 Activity Staining for Detection of Esterase Activity

Electrophoresis of the purified protease was conducted as described in section 3.4.1.3.2. For activity staining, after the run, the gel was soaked for 30 minutes in 2.5% TritonX-100 at room temperature, briefly washed in 50mM Tris buffer, pH 8.0, and covered by a solution of 100µM methylumbelliferyl butyrate (diluted with 50mM Tris buffer from a stock of 25mM solution in Methyl cellosolve) and incubated for a short period at room temperature. Activity bands were observed under UV illumination. Following zymogram analysis gel was stained with Coomassie Brilliant Blue R-250 for visualizing protein bands.

Chapter 4

RESULTS

4.1 Microorganism-Culture identification

The fungus used in the present study was identified as *Engyodontium album* by the Microbial Technology Culture Collection (MTCC) of Institute of Microbial Technology (IMTECH), CSIR, Govt. of India, Chandigarh. Infact, this fungus was identified as *Beauveria bassiana* (Suresh, 1996) earlier. But, when submitted to MTCC, it was confirmed as *E. album*, a close relative of *B. bassiana*.

4.2 SOLID STATE FERMENTATION (SSF)

4.2.1 Optimisation of Bioprocess variables for protease production by *E. album*

4.2.1.1 Optimisation of moisture content

Results presented in Fig. 4.1 clearly evidence the impact of moisture content on the extracellular enzyme production by *E. album*. The fungus required moisture content above 50% to have enhanced level of protease production and thus a maximal of 4.351 U/gIDS could be recorded at 60% moisture level. However, further increase in moisture content resulted in a decline. While 70% moisture content (3.754 U/gIDS) supported considerable level of enzyme activity, further increase led to rapid decline in enzyme level. Thus, 60% moisture content could be considered as optimal level of moisture requirement, irrespective of incubation period. Protein content and specific activity showed positive correlation

with the enzyme activity recorded. In general, the protein varied from 10 to 25.3 mg/gIDS, irrespective of the level of moisture content of the wheat bran.

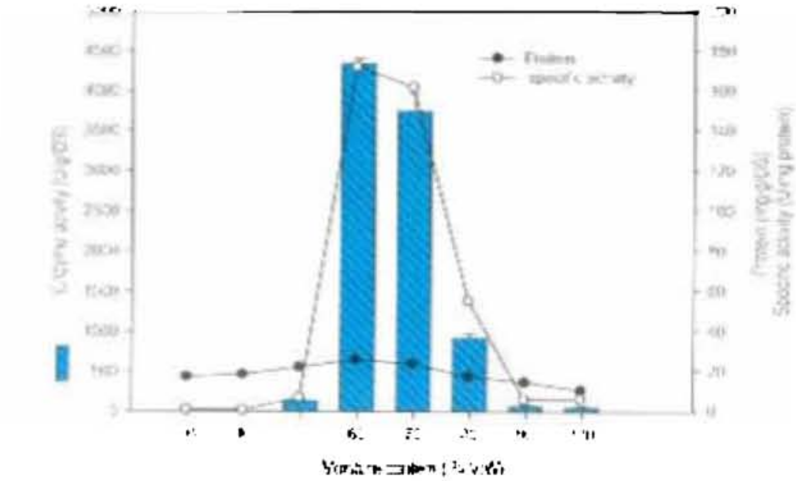


Fig. 4.1 Optimisation of moisture content for Protease production by *E. album*: SSF was conducted using WB [illegible]

4.2.1.2 Optimisation of Incubation period

In general, irrespective of the level of moisture content of the WB, protease production was observed only after 72 hrs of incubation and maximal enzyme activity could be recorded after 120 hrs for both 60% (4,351 U/gIDS) and 70% (3,754 U/gIDS) moisture content (Fig. 4.2 A). Whereas, maximum specific activity was observed for both the moisture content at 144 hrs (341 U/mg protein and 276 U/mg protein respectively) (Fig. 4.2 B). Incubation period beyond 120 hrs led to a decline in enzyme level. As observed with moisture content experiment, low levels of moisture content and high levels of moisture content did not support enzyme production.

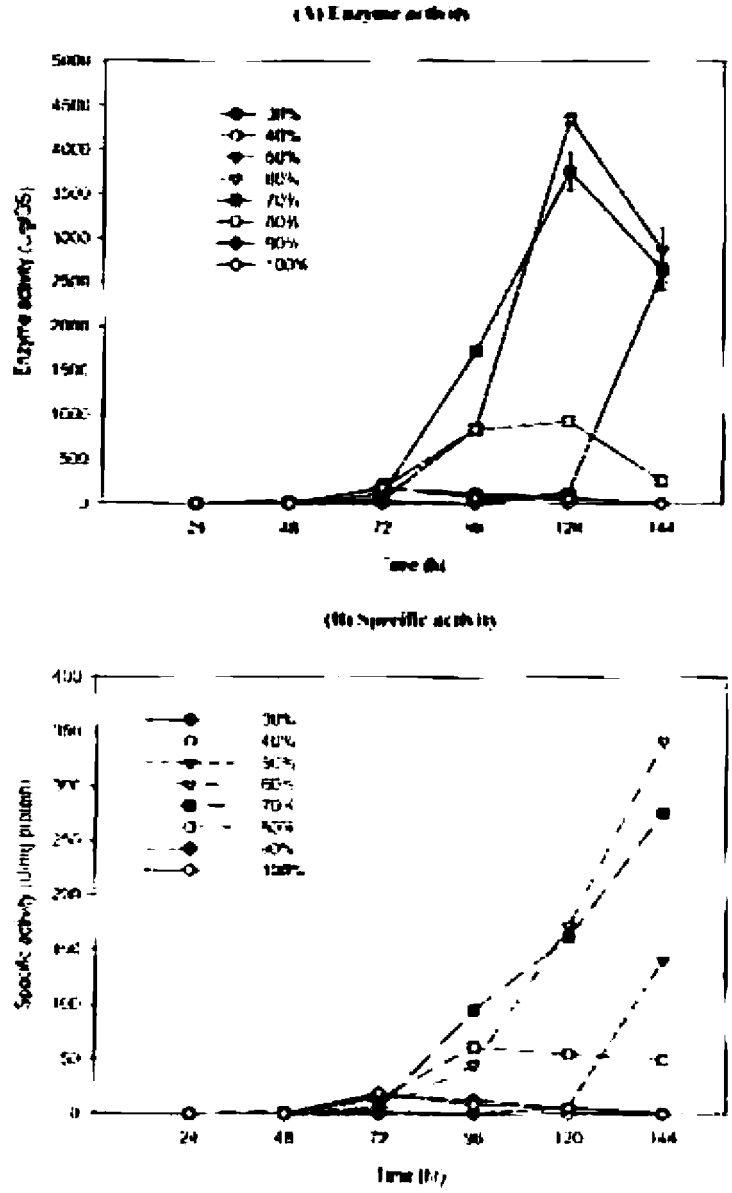


Fig 4.2 (A & B) Optimisation of incubation period for Protease production by *P. album* under SSF using WB at different levels of moisture content. SSF was conducted using WB of particle size < 425µ moistened to varying levels with seawater and incubated at 28°C

4.2.1.3 Optimisation of particle size of the Substrate

Particle size of commercial WB used in the present study significantly influenced enzyme production by *T. album* during SSF as it could be inferred from the data presented in Fig. 4.3. WB particles lesser than 425µ alone could support maximal enzyme synthesis (12,089 U/gIDS), followed by particles with mixed size, which was used as control (10,267 U/gIDS). Whereas, particles with 425µ and above did not support enhanced levels of enzyme and instead led to decline in enzyme activity (about 50% reduction). Specific activity recorded for the various particle sizes also present a similar scenario, while protein content did not show any notable variation in response to difference in particle size.

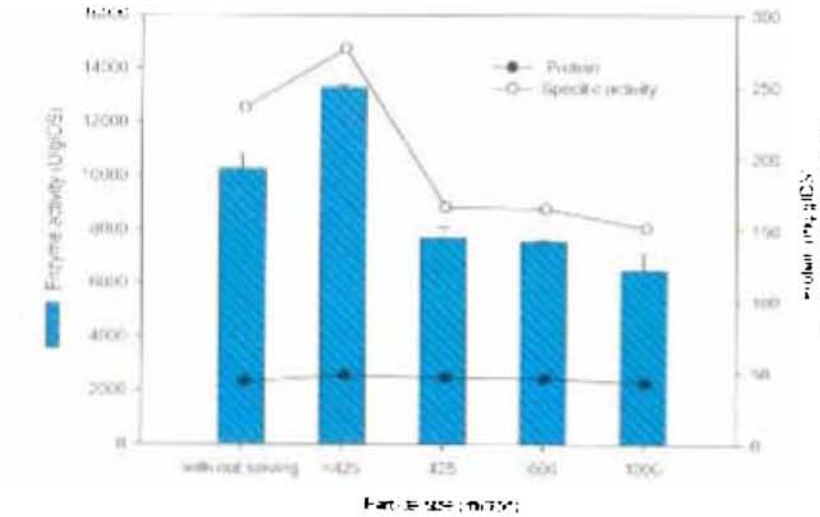


Fig. 4.3 Optimisation of particle size of the substrate (WB) for protease production by *T.album* under SSF. [illegible]

4.2.1.4 Optimisation of Temperature

Results presented in Fig. 4.4 clearly evidence the optimal requirement of 25°C as incubation temperature for maximal enzyme production by *F. album* (5,106 U/gIDS) and maximal specific activity (82.45 U/mg protein). Incubation at temperatures above 25°C did not support protease production by *F. album* under SSF, although the fungus could record some amount of enzyme activity at 20°C. Interestingly, the protein level also showed a similar trend for the various temperatures tested. Of course, the protein levels recorded were higher than the levels observed for the experiments with particle size and incubation period, and thus the specific activity was relatively less compared to the above said experiments.

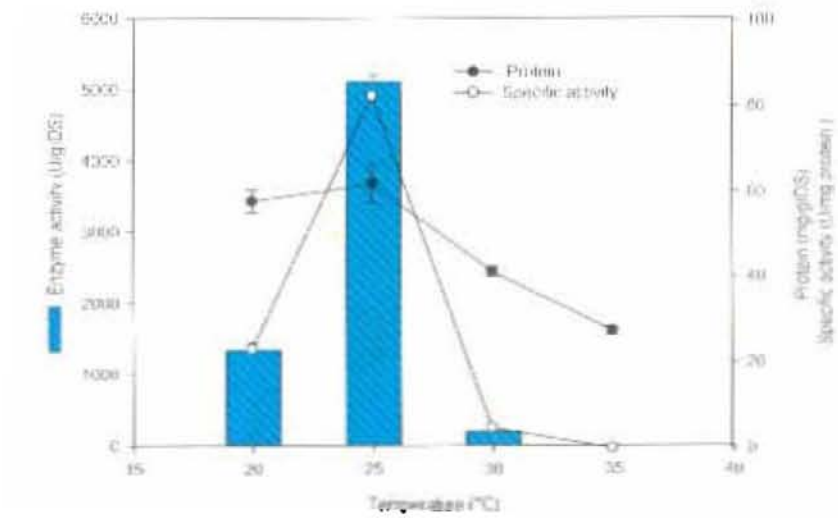


Fig. 4.4 Optimisation of incubation temperature for Protease production by *F. album*: SSF was conducted using Wheat [illegible]

4.2.1.5 Optimisation of pH

Results presented in Fig 4.5 indicate that the fungus *T. album* is capable of producing protease over a broad pH range from pH 2.0 to pH 12.0, eventhough the enzyme activity levels varied considerably in the range of 3,833 U/gIDS to 9,437 U/gIDS. Apparently, it seems that this fungus has two pH optima, one at pH 4.0-5.0 (9,437 U/gIDS) and another at pH 10.0 (7,999 U/gIDS) for maximal protease production. There was an increase in enzyme activity along with increase in pH from 2.0 to 5.0 and a sudden decline in enzyme activity followed by a gradual increase when the pH was raised above 5.0. Similarly, when pH was raised above 10.0 to 13.0 there was proportionate decrease in enzyme activity. The trend recorded for specific activity was identical to enzyme activity, while protein levels recorded for various pH did not show any correlation with the enzyme activity and remained in the range of 31.9 mg/gIDS to 39.9 mg/gIDS.

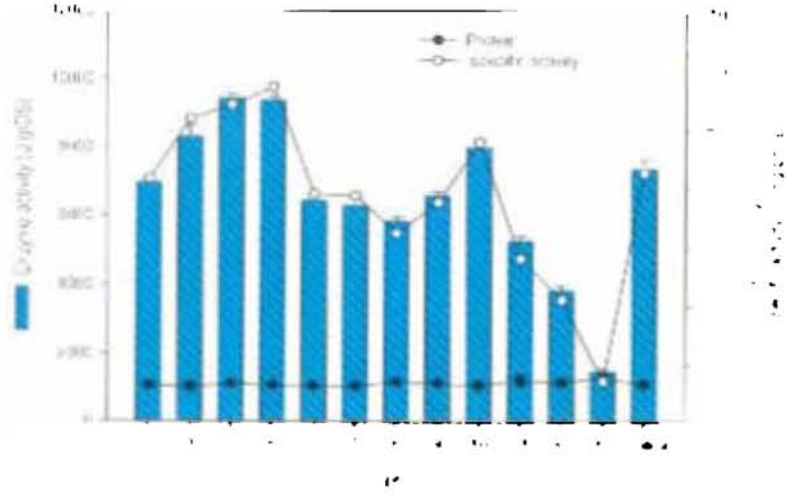


Fig. 4.5 Optimisation of pH for Protease production by *T. album*

4.2.1.6 Additional Proteinaceous substrates

None of the additional proteinaceous substrates tested have a profound effect on enzyme production (Fig. 4.6). Both casein and gelatin caused a decreased level of enzyme production compared to control medium. Wheat bran without any proteinaceous substrates added additionally (control) gave maximal enzyme production and specific activity (13,317 U/gDS and 276.6 U/mg protein respectively).

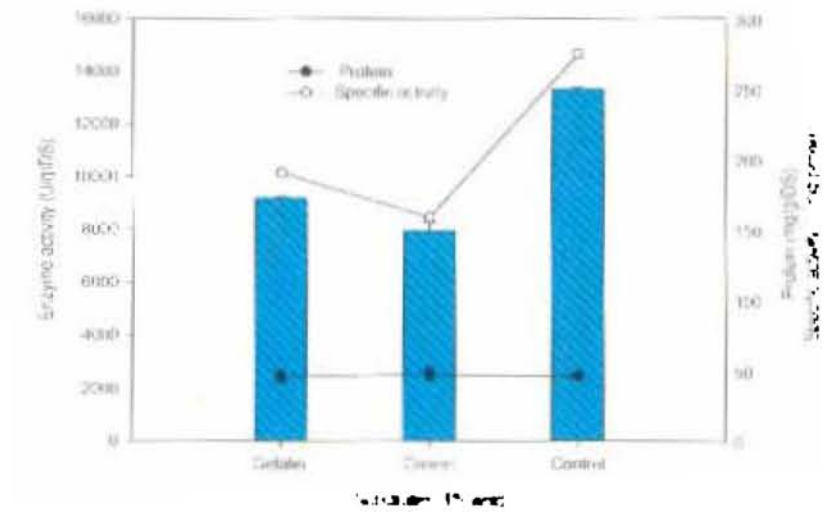


Fig. 4.6 Effect of additional Proteinaceous substrates on Protease production by *F. album*

4.2.1.7 Additional Carbon Source

Data presented in Fig. 4.7 suggests that the different carbon sources tested had varied impact on enzyme activity by *F. album*. Thus, few of them promoted a positive effect, few inhibited, and some totally inhibited the enzyme production. When compared to control (i.e., without any additional carbon source), except sucrose, mannitol and maltose, all other sugars led to a decrease in enzyme

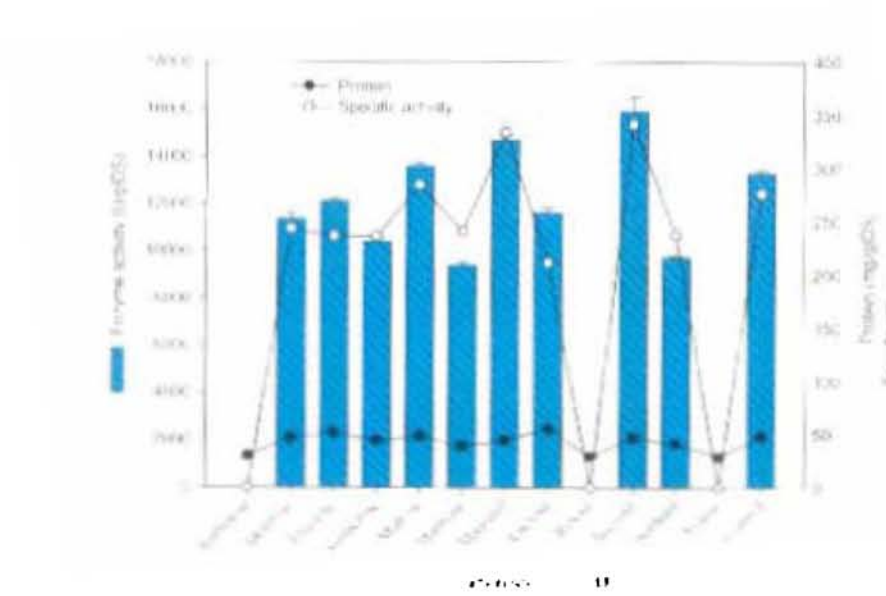


Fig. 4.1 Effect of different additional Carbon sources on Protease production [illegible]

4.2.1.8 Organic Nitrogen Sources

All the organic nitrogen sources tested, except meat supported enhanced protease production when compared to control (Fig 4.8). Malt extract, soyabean meal and tryptone recorded similar levels of enzyme activity leading to a 17-19% increase in enzyme activity. Of these, malt extract recorded a maximum of 19% increase in enzyme activity (11,348 U/gDS and specific activity of 318.5 U/mg protein). In fact, both soyabean meal and malt extract recorded maximal specific activity compared to tryptone inspite of similar levels of enzyme activity for all the three. Nevertheless peptone, beef extract and yeast extract also led to a 38.1%, 26.5% and 17.6% increase in enzyme activity compared to control. In the case of meat, a 20.3% reduction in enzyme activity was recorded while the extracellular protein content was maximum as compared to others.

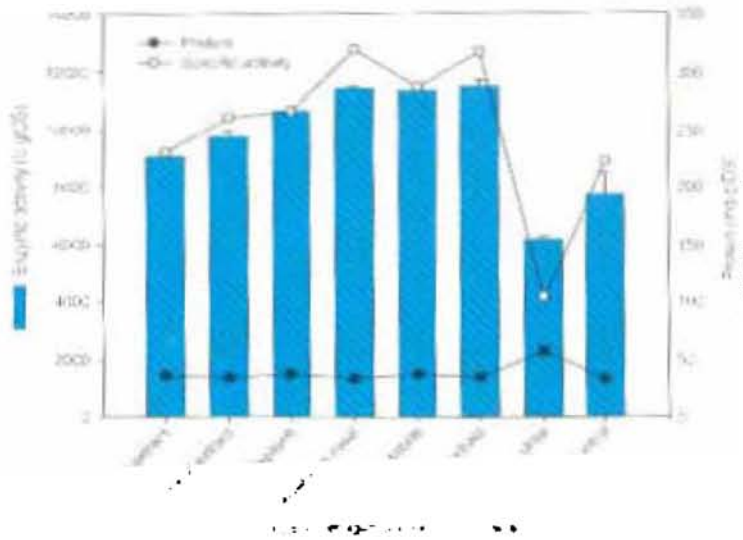


Fig. 4.8 Effect of different additional organic nitrogen sources on Protease production:

4.2.1.9 Inorganic Nitrogen sources

Among the inorganic nitrogen sources tested only ammonium hydrogen carbonate, ammonium nitrate and ammonium hydrogen phosphate have a positive effect on enzyme production (Fig. 4.9). When compared to control, 94% increase in activity was observed with the addition of ammonium hydrogen carbonate, which yielded an enzyme activity of 15,187 U/gIDS and specific activity of 380.4 U/mg protein. A 22.9% and 8.3% increase in activity was observed in the case of ammonium nitrate and ammonium hydrogen phosphate respectively. Ammonium oxalate and ammonium iron sulphate as additional nitrogen sources, totally inhibited enzyme production. Maximal extracellular protein content was recorded with ammonium hydrogen phosphate (46.3 mg/gIDS), although it did not enhance enzyme activity. In fact the protein content in the enzyme extract did not show any correlation with enzyme activity. However, specific activity showed a similar trend very much like that of enzyme activity.

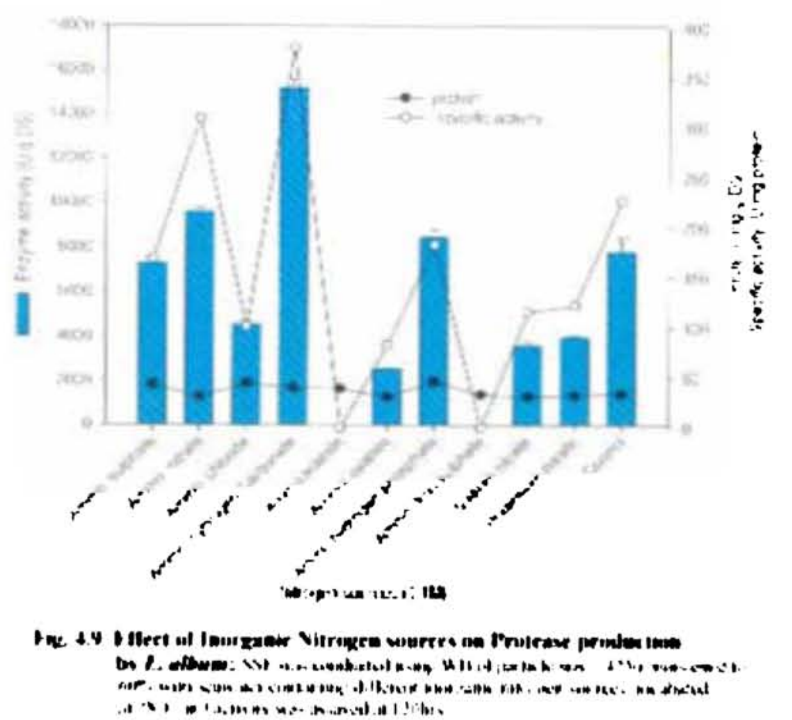


Fig. 4.9 Effect of Inorganic Nitrogen sources on Protease production by *E. album*: [illegible]

4.2.1.10 Amino acids

Results presented in Fig. 4.10 indicate that all the amino acids tested do not have similar impact on enhancing protease production by *I. albus*. Among the 19 different amino acids tested, only 9 amino acids favored enhanced enzyme production. Of them, leucine, histidine and lysine supported 44.9%, 32.2% and 30.2% increase in enzyme activity compared to control respectively. On the other hand, methionine and tryptophan caused a 92.2% and 89.6% reduction in enzyme activity respectively when compared to the control. Thus leucine could be considered as optimal amino acid as additional nitrogen source for protease production. Specific activity also presented a similar trend very much like that of enzyme activity, while protein levels did not show any specific trend.

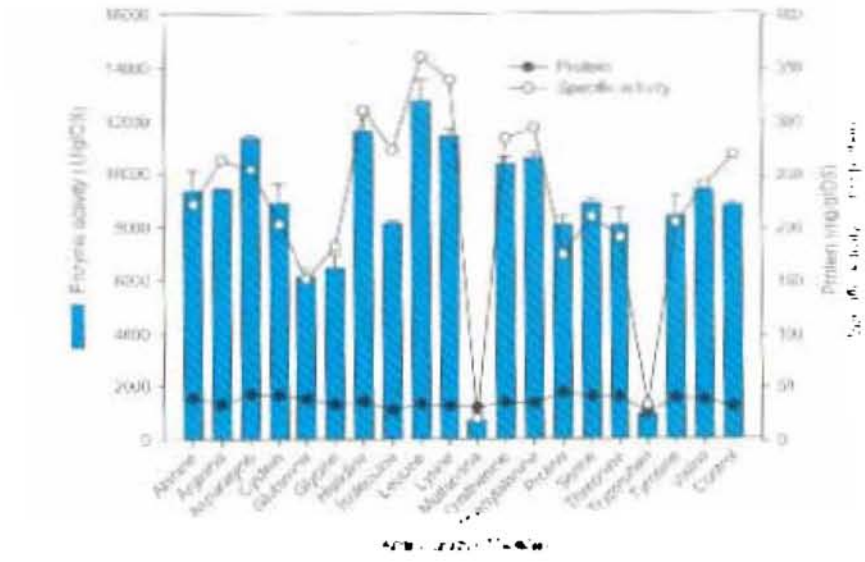


Fig. 4.10 Effect of Amino acids on Protease production by *I. albus*

4.2.1.11 Optimisation of inoculum size

Number of spores used for inoculation had a linear effect on the enzyme production (Fig. 4.11). Data presented suggest that 4×10^5 cfu ml was found to be optimal for maximal enzyme production, yielding an enzyme activity and specific activity of 8830.1 U/gDS and 198.2 U/mg protein respectively. Practically, enzyme activity was not recorded for the inoculum with a spore count less than 2×10^5 spores. Even though the extracellular protein content increased for the inoculum with spore count of 6×10^5 spores, there was decline in enzyme production. Results also indicate that though protein could be recorded in crude extract for all the levels of inoculum tested, protease activity could not be detected. There was no direct relation between protein content and enzyme activity.

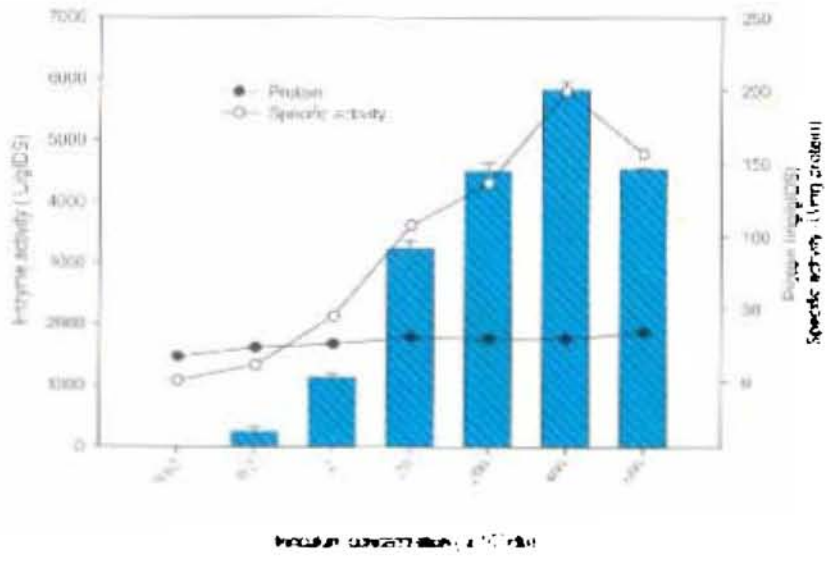


Fig. 4.11 Optimisation of inoculum concentration for Protease production by *P. album*:

4.2.1.12 Sodium Chloride Concentration

Results presented in Fig.4.12 A & B clearly evidence that sodium chloride significantly affects protease production by *E. album* under SSF. Protease production in distilled water based medium (14.779 U/gIDS) was almost double fold compared to the seawater based medium (6.953 U/ gIDS). Sodium chloride showed an inverse effect on enzyme production both in seawater and distilled water based media. In the presence of seawater, addition of even 1% sodium chloride caused a 13% decrease in enzyme production. Further, addition of sodium chloride above 1% to seawater based medium and above 5% in distilled water based medium totally inhibited protease production. However, the trend shown by protein content in the enzyme extract was not significant. In the case of distilled water added with sodium chloride, the protein level declined for sodium chloride addition above 5%, while in the case of seawater, sodium chloride concentration above 1% added to seawater resulted in decrease in protease level. Nevertheless, there was no direct relation between protein and enzyme activity, though specific activity showed a similar trend very much like that of enzyme activity.

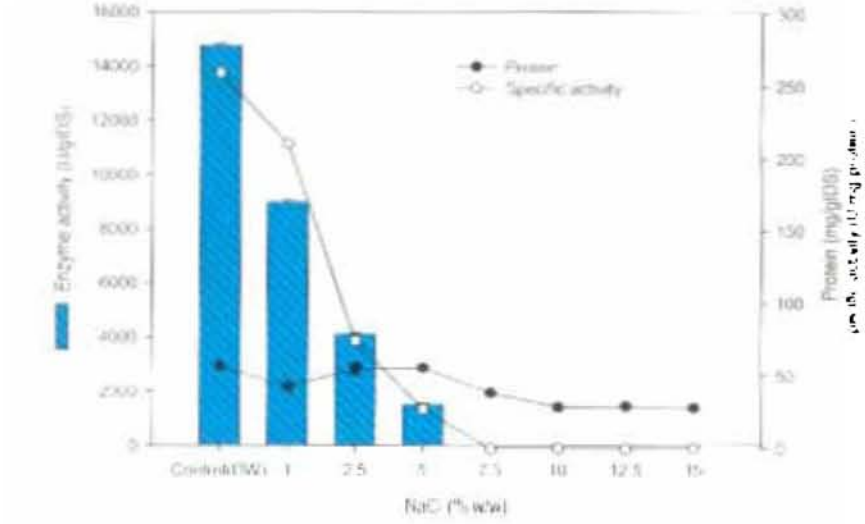


Fig. 4.12(A) Effect of NaCl on Protease production by *E. album* in WB medium moistened with Distilled Water (DW)

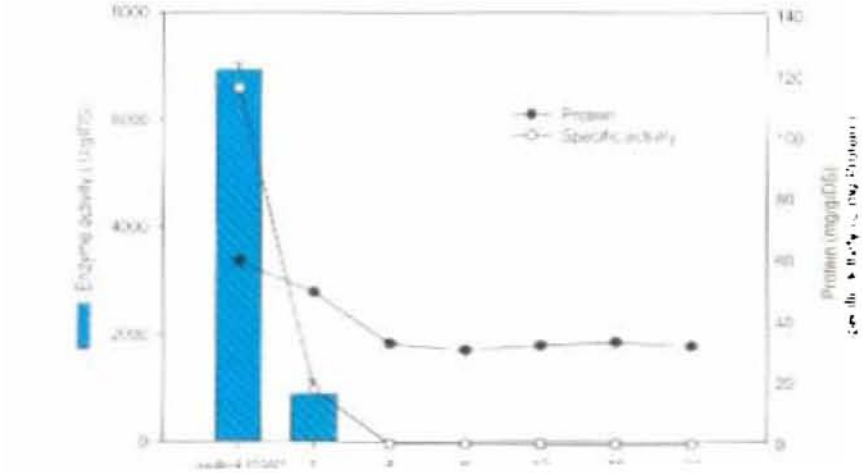


Fig. 4.12 B Effect of additional NaCl on Protease production by *E. album* in WB medium moistened with sea water (SW)

4.2.1.13 Impact of different dilutions of seawater on protease production by *E. alsum* under SSF

Results presented in Fig. 4.13 for the study conducted using different dilutions of sea water with distilled water on protease production by *E. alsum* suggest that this fungus can produce double fold protease under SSF using WB in distilled water compared to that in sea water. Further, even addition of 25% distilled water to seawater (75% sea water) could induce significant levels of protease (11,170 U/gDBS). The enzyme activity increased along with increase in dilution of seawater with distilled water. Specific activity also showed a similar trend. In fact a maximal specific activity for protease could be observed with [illegible] seawater + 75% distilled water. Nevertheless, the protein content in the enzyme extract recorded almost identical level and was independent of enzyme activity observed.

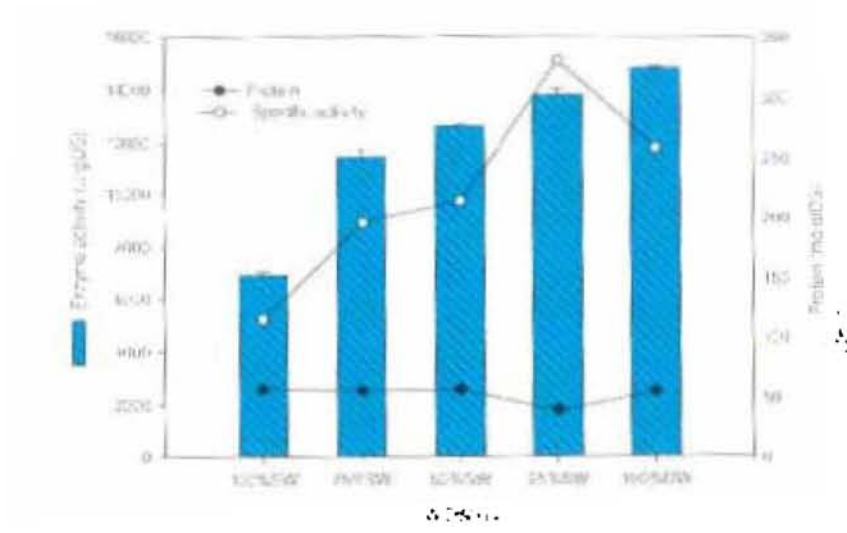


Fig. 4.13 Protease production by *E. alsum* on WB medium moistened with different ratio of Sea Water (SW) and Distilled Water (DW)

4.2.1.14 Time Course Study Under Optimal Conditions

Data obtained for the time course experiment conducted over a period of 216 hrs under optimised condition is depicted in the Fig. 4.14. It is inferred from the figure that enzyme production commenced on 2nd day (i.e., 48hrs) and reached a peak after 120 hrs (11,540 U/gIDS). Further incubation beyond 120 hrs did not favour enhanced enzyme activity and instead resulted in a decline. Rapid decline in enzyme activity occurred after 168 hrs. However, maximal specific activity was recorded at 168 hrs (3991 U/mg protein).

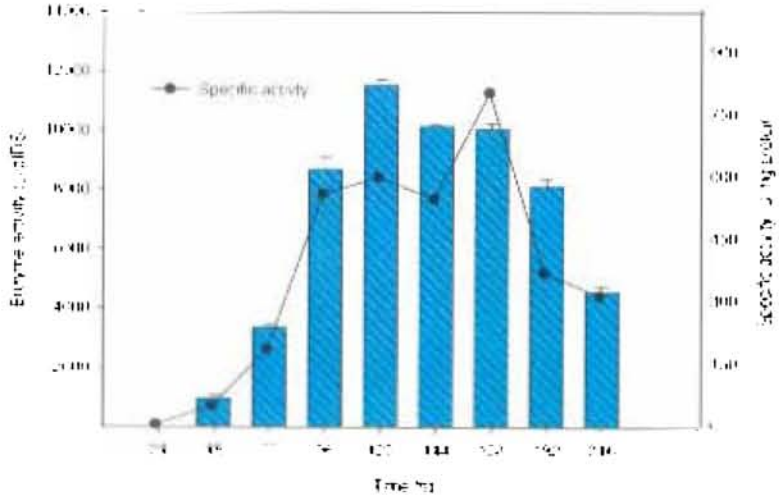


Fig. 4.14 Time course study under optimized conditions

4.3 ENZYME PURIFICATION

Protease was purified employing standard protein purification procedures, which included ammonium sulphate fractionation followed by dialysis, ion exchange chromatography and electrophoresis. Results obtained for purification of crude enzyme is summarised in Table 4.1

Table 4.1 Yield and fold of purification

Sample	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude extract	[illegible]	[illegible]	[illegible]	23	100	100	1
Ammonium sulphate fractionation (10-90%)	[illegible]	[illegible]	[illegible]	286	[illegible]	82	[illegible]
Ion Exchange chromatography (DEAE)	150	7	3148	438	[illegible]	[illegible]	16
PAGE	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Ammonium sulphate required to precipitate the protease enzyme, from the enzyme extract obtained from molds WB was standardised. Protease could be precipitated with 40%–90% ammonium sulphate saturation, though protease with maximal specific activity was precipitated at 90% saturation (Table 4.1). The precipitate formed at 10-90% saturation of ammonium sulphate, which showed a ten fold increase in specific activity, compared to the crude sample, was used for further purification employing Ion exchange chromatography (Table 4.1). Preparative PAGE yielded 45 fold of purification and 1.5% yield (Table 4.1).

4.3.1 Standardisation of binding pH of protease to DEAE-Cellulose

The binding affinity slowly increased from pH 4.0 and showed a maximum at pH 7.0. The enzyme eluted from the DEAE cellulose equilibrated to pH 7.0 showed maximum activity and the binding pH to DEAE cellulose was standardised to pH 7.0 (Fig. 4.15).

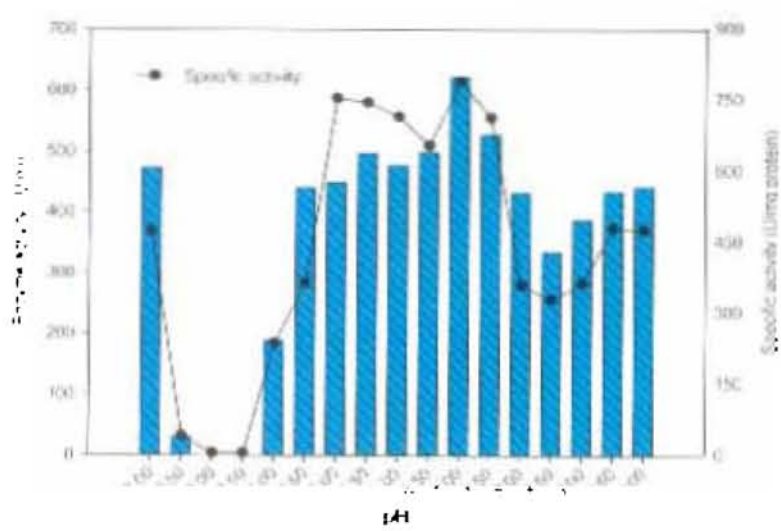


Fig 4.15 Optimisation of pH for the binding of protease to the anion exchange resin DEAE Cellulose

Elution profile (Fig. 4.16) from the DEAE Cellulose column furnished a single peak with protease activity, which could be eluted with buffer containing 0.2 M NaCl. This step resulted in 8.5% protease recovery (16 fold of purification) with a specific activity of 438.01 U/mg protein.

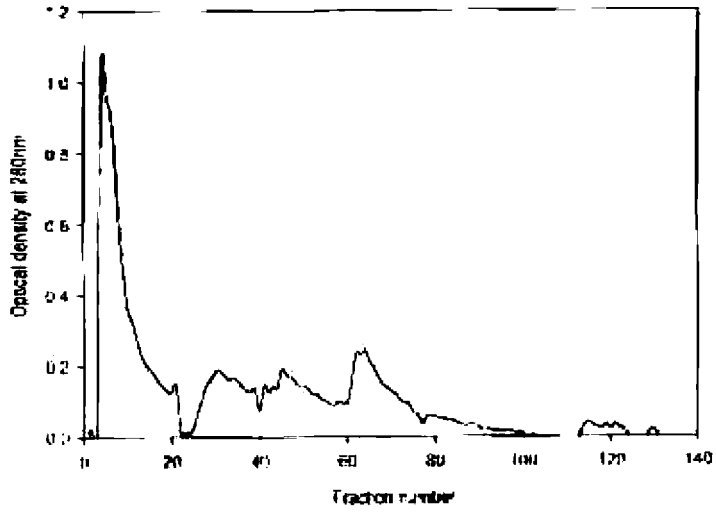


Fig 4.16 Ion-exchange chromatography profile for alkaline protease

4.4 CHARACTERISATION OF PURIFIED ENZYME

4.4.1 Native-Polyacrylamide Gel Electrophoresis (Native-PAGE)

All the fractions, which had significant protease activity, obtained after ion exchange chromatography, were pooled and lyophilized. They were subjected to Native polyacrylamide gel electrophoresis and confirmed their homogenity. Enzyme protein, which was eluted with 0.2 M NaCl, gave a single band in Native PAGE (Fig. 4.17).

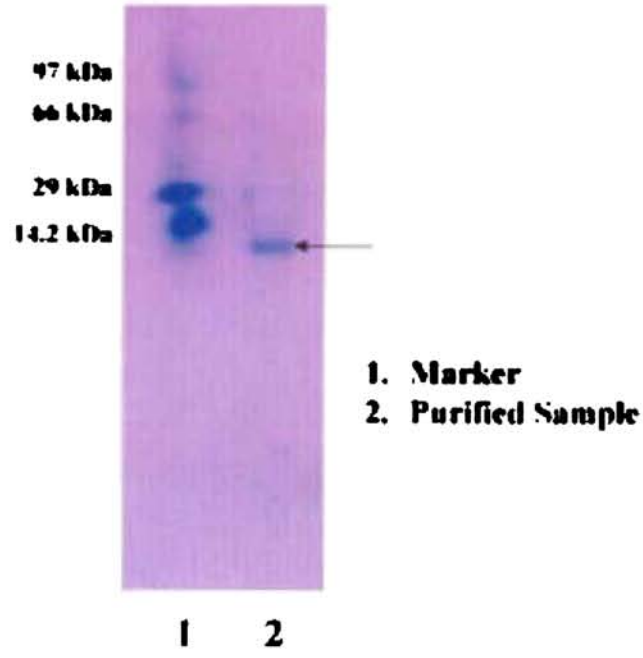


Fig. 4.17 Native PAGE of purified sample

4.4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE)

SDS-PAGE performed under non-reducing and reducing conditions also yielded a single band, evidencing the single polypeptide nature of the enzyme (Fig. 4.18). The molecular mass of protease, estimated by comparison with the electrophoretic mobility of marker protein, indicated that the *F. album* protease has an apparent molecular mass of 38kDa.

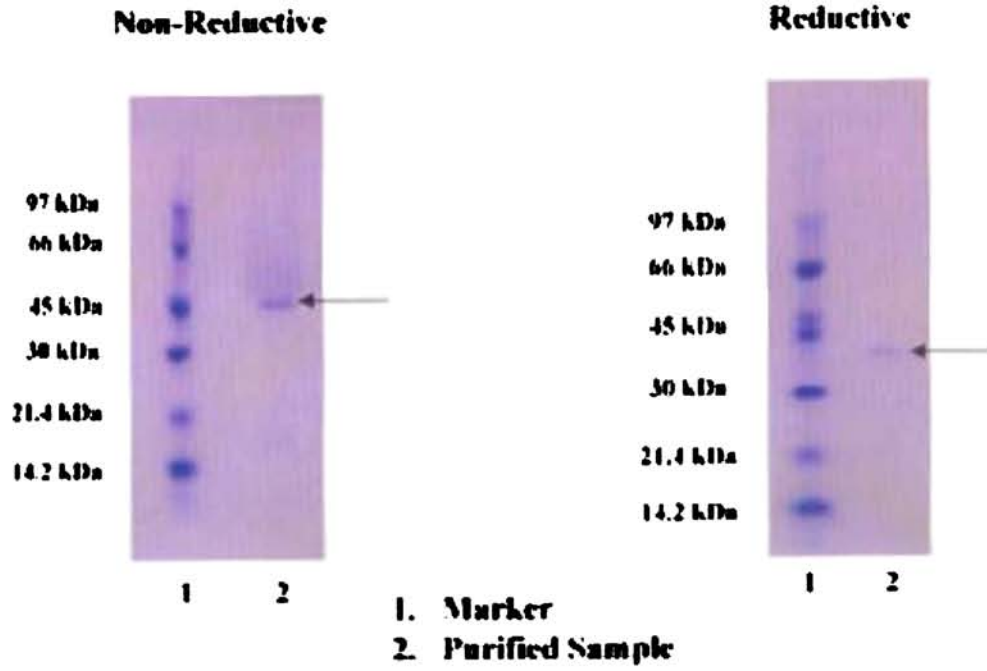
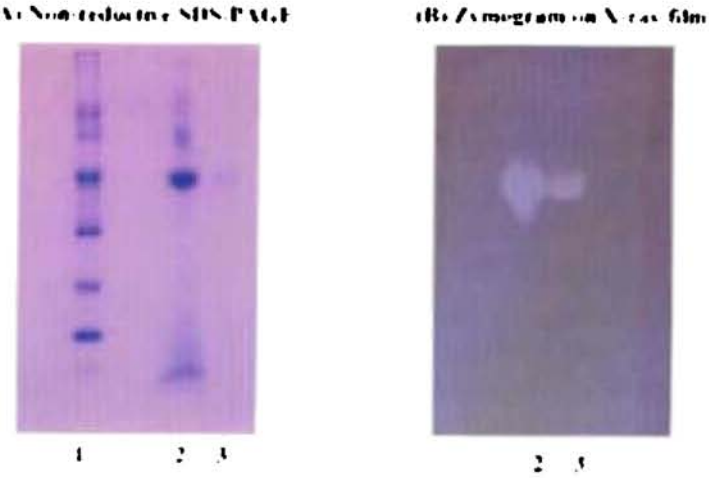


Fig. 4.18 SDS-PAGE of purified sample

4.4.3. Zymogram

The proteolytic activity of the purified enzyme protein was confirmed by zymogram analysis on X-ray film (Fig. 4.19 B). There was only one clearing zone visible even in the case of ammonium sulphate precipitated sample, which indicates a single type of extracellular protease (Fig. 4.19 A)



Lane 1- Marker

Lane 2- Sample after Ammonium sulphate fractionation

Lane 3- Sample after Ion exchange chromatography

Fig. 4.19 Zymographic analysis of protease

4.4.4 Isoelectric focusing

In Isoelectric focusing, the enzyme appeared as a single band testifying the purity of protease with a pI value between 3 and 4 (Fig. 4.20).

Fig. 4.20 Isoelectric focusing of purified enzyme

4.4.5 Gel Filtration Chromatography for Estimation of Molecular Weight of Protease Enzyme

Gel filtration chromatography of the purified enzyme protein yielded a single peak with protease activity (Fig. 4.21). From the K_{av} value obtained for the protease, molecular weight was calculated to be 30kDa.

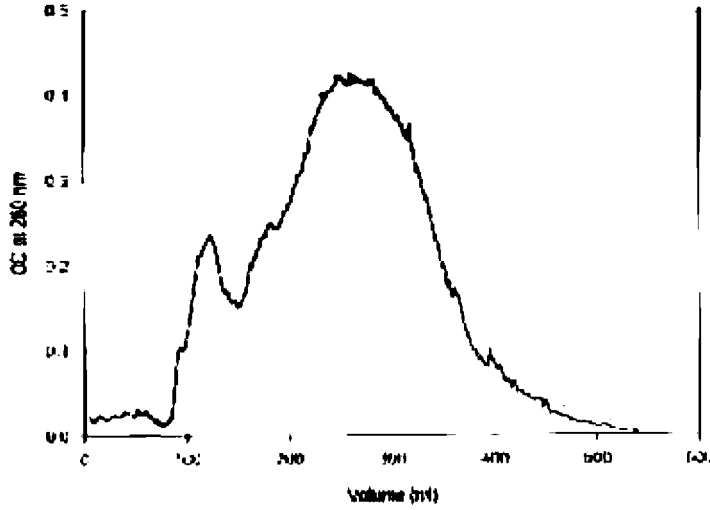


Fig. 4.21 Elution profile of protein in Gel Filtration chromatography

4.4.6 Aminoacid analysis

Aminoacid analysis profile of purified enzyme protein sample is presented in Table 4.2. The highest amount of amino acid represented in the protein was glycine (0.670μ mol per sample) and the lowest one was cysteine (0.013μ mol per sample) Based on the amino acid composition, it is inferred that the enzyme protein contain approximately 255 amino acids and the molecular weight calculated for this protein is 24,803 Daltons.

Table 4.2 Amino acid composition of *E. album* Protease

Amino acid	µ mol in the sample
Glycine	0.670
Alanine	0.507
Glutamic acid	0.442
Leucine	0.229
Proline	0.212
Aspartic acid	0.200
Valine	0.191
Serine	0.156
Threonine	0.147
Isoleucine	0.147
Lysine	0.128
Arginine	0.076
Histidine	0.075
Phenylalanine	0.068
Methionine	0.027
Tyrosine	0.027
Cysteine	0.013

4.4.7 Matrix-Assisted Laser Desorption Ionization-Mass spectroscopy (MALDI-MS)

MALDI-MS spectrum obtained for the purified protease enzyme presented in the Fig. 4.22, testify that the protease is pure with a single peak having the molecular weight of 28,627.09 Daltons.

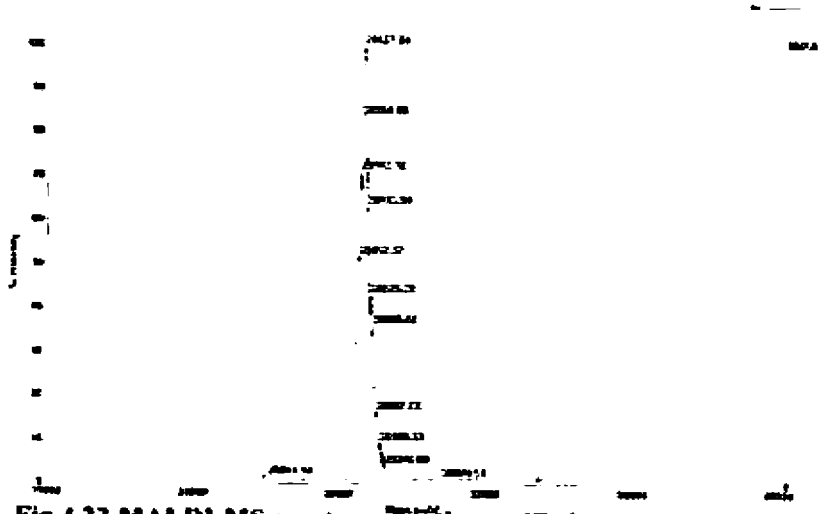


Fig 4.22 MALDI-MS spectrum for the purified protease enzyme

4.4.8 Determination of the N-terminal sequence of the enzyme

The N-terminal sequencing of the *E. album* protease was carried out at IISc, Bangalore. They reported a probability of N-terminal blocking since sequence analysis got terminated after 3 cycles. Hence, MALDI analysis was done to get a peptide fingerprint.

4.4.9 Matrix-Assisted Laser Desorption Ionization- (MALDI)

Peptide fingerprint profile using MALDI Analysis for the purified protease enzyme protein is given in the Fig. 4.23.

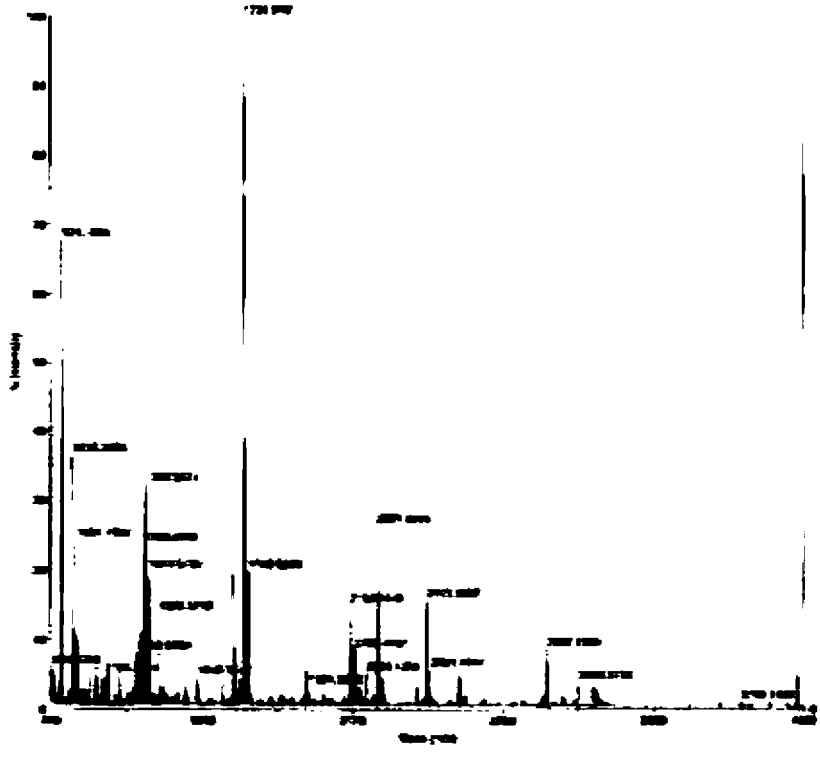


Fig. 4.23 Protein identification by MALDI-Peptide mass mapping

MALDI analysis of the *T. album* enzyme protein identified three internal peptide sequences which showed homology to *Tritirachium album* precursor Protease T and of these, one showed homology to the conserved stretch assigned to Subtilisin class Serine protease.

Tritirachium album precursor protease T with matched sequences in red is given below

```
  1 EFIEQDAVVT [illegible]ATQEDAPW GLARISSQEP GGTTYTYDDS AGTGTCAYII
 51 DTGIYTNHTD FEGRAKFLKN FAGDGQDTDG NGHGTHVAGT VGSTTYGVAK
101 KTSLFAVKVL DANGQGSNSG VIAGMDFVTK DASSQNCPKG VVVNMSLGGP
151 [illegible]SA[illegible]NRAAA [illegible]A[illegible]FLA VAA[illegible]NEATDA SSSSPASEPS ATTVGATDKT
201 [illegible]TLAEYSNF[illegible] [illegible]LLAPGT DIESTWNDGP TKSISGTSMA SPHVAGLGAY
251 FLGLGQKVQG LCDYMVEKGL EDVIQSVPSD TANVLINNGE GSA
```

4.4.10 Antibody production and Immunodiffusion

Antibodies were raised in rabbit against electrophoretically pure protease. The raised antibodies showed a single band in the immunodiffusion experiment (Fig. 4.24), testifying the purity of the protease.

Fig. 4.24 Immunodiffusion experiment using antibodies raised against purified protease

4.4.11 Determination of Optimal pH for Protease Activity

Results depicted in Fig. 4.25 indicate that the protease has an optimum pH between 10.0 and 11.0 for maximal activity. In general, the protease was active over a pH range of 6.0-12.0 and increase in pH from 6.0 to 11.0 recorded proportionate increase in activity, whereas, enzyme is totally inactive in the pH below 6.0. Considering pH 11.0 as optimal for maximal activity, it was observed

that the activity declined significantly along with decrease in pH. More than 80% of maximal activity was measured in the pH range between 9.0-12.0 (Table 4.3) The results suggest that this is an alkaline protease.

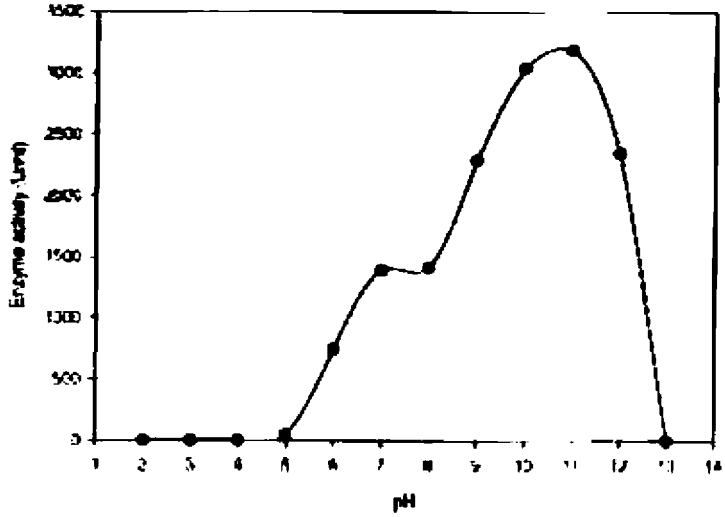


Fig 4.25 Activity profile of the enzyme at different pH

Table 4.3 Relative activity of protease enzyme at different pH

pH	Relative Activity (%)
2	0
3	0
4	0.059
5	1.466
6	23.28
7	43.69
8	44.37
9	71.78
10	95.27
11	100
12	73.70

4.4.12 Determination of pH Stability of Protease Enzyme

From the data obtained for the pH stability studies of the protease (Fig. 4.26), it is inferred that the enzyme is stable over a wide range of pH from 5.0-12.0. However, maximal residual enzyme activity was recorded with the sample incubated in the buffer having pH 9.0. Nevertheless, when compared to other levels of pH tested, the difference was only marginal.

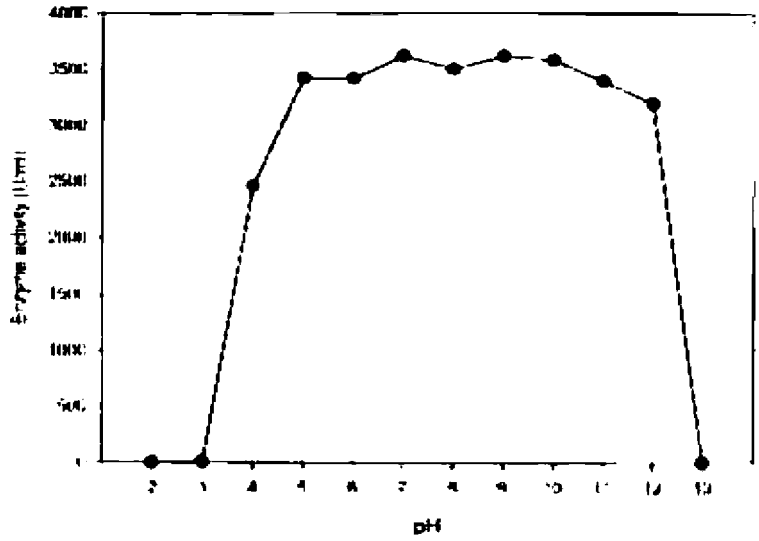


Fig 4.26 Stability profile of enzyme in different pH

4.4.13 Determination of Optimal Temperature for Protease Activity

Results presented in the Fig. 4.27 indicated that enzyme was active over a broad range of incubation temperature with maximal activity at 60°C. Temperatures above 60°C led to a sharp decline in enzyme activity and was totally nil above 85°C. Nevertheless, the enzyme showed activity even at 5°C (67.9 U/ml) even though it is negligible considering the activity at 60°C (4,658 U/ml). In fact, the protease activity showed a linear increase along with increase in temperature and particularly, the increase was rapid during 40°C - 60°C. Data documented in Table 4.4 on relative activity of protease indicated clearly the preference for a

higher temperature of 50°C-60°C for maximal activity. Rise in temperature above 60°C also showed rapid decrease in enzyme activity.

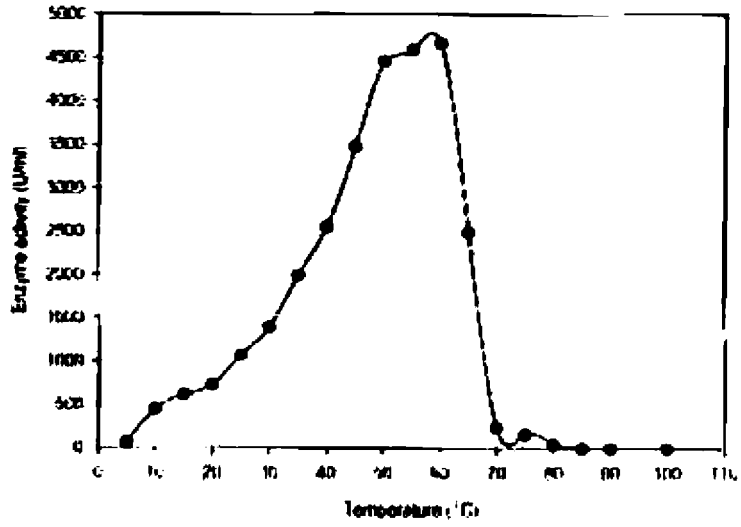


Fig 4.27 Effect of Temperature on Protease Activity

Table 4.4 Relative activity of protease enzyme at different temperatures

Temperature (°C)	Relative Activity (%)
5	1.45
10	9.54
15	13.27
20	15.75
25	22.92
30	29.77
35	42.56
40	54.78
45	74.69
50	95.59
55	98.43
60	100
65	53.26
70	5.06
75	3.37
80	0.92
85	0
90	0
100	0

4.4.14 Determination of Temperature stability of Protease Enzyme

From the results presented in Fig. 4.28 for the temperature stability studies conducted using protease, it is concluded that at 30–40°C and at 50°C, the enzyme could retain 100% and 95% of residual activity respectively even after 24 hrs. Whereas, at 60°C, the optimal temperature for maximal activity, the enzyme retained 66% of activity after 12 hrs of incubation. Results suggest that at high temperatures above 60°C the enzyme had denatured and lost activity within 1 hour.

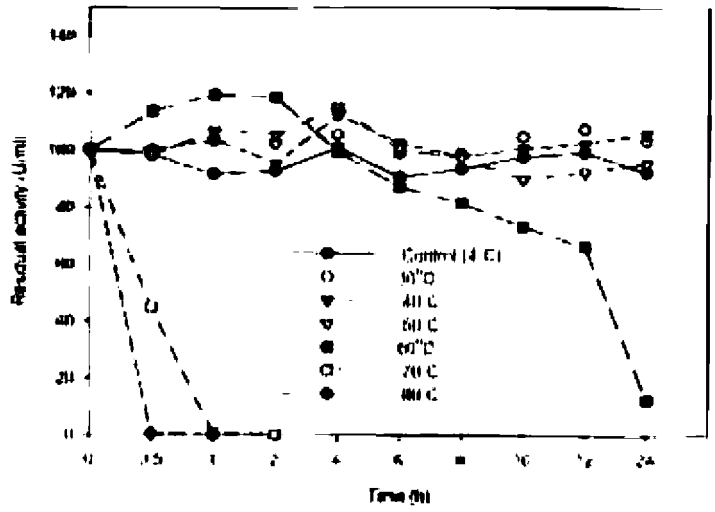


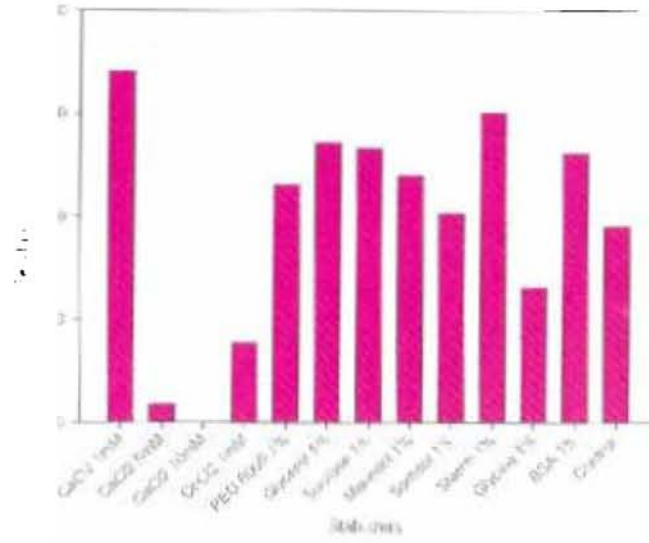
Fig 4.28 Thermostability of alkaline protease at various temperatures

4.4.15 Effect of stabilizers on thermal stability

Effect of stabilizers on thermal stability was studied by the addition of different reported stabilizers and incubating at 65°C and 70°C. In general, of the ten substances tested as stabilizers to promote enzyme activity at higher temperatures, starch, glycerol, sucrose, BSA, mannitol and sorbitol enabled enhanced thermal stability of the protease at both the temperatures (Fig. 4.29 A & B). Besides these, $CaCl_2$ (1mM) and PEG 6000 supported thermal stability of protease at 65°C. On the other hand, glycine and CoCl$_2$ did not enhance thermal stability at both the temperatures. Eventhough $CaCl_2$ at a concentration of 1 mM showed a maximal residual activity at 65°C, it is inferred from the results that starch (1%)

could play the role of stabilizer promoting thermal stability at both the higher temperatures compared to other substances tested

(A) Incubation at 65 °C



(B) Incubation at 70°C

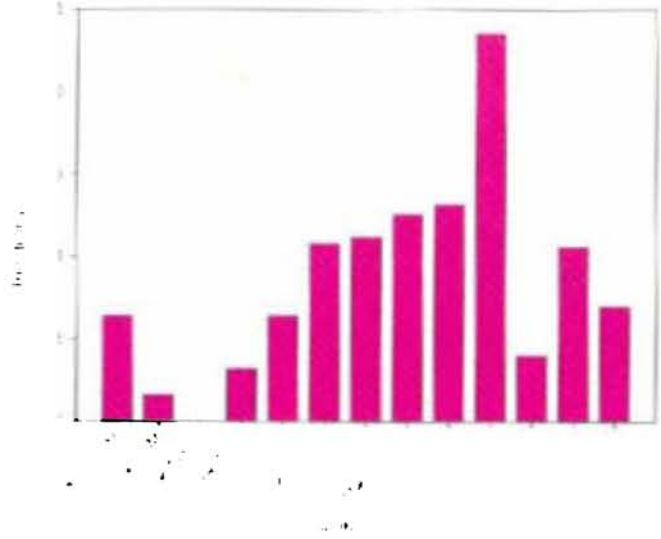


Fig. 4.29 (A&B) Enhancement of Thermal Stability of Protease by different stabilizers after 2hrs of incubation at two different temperatures

4.4.16 Protease Enzyme Inhibition Studies Using Various Inhibitors

Results given in Table 4.5 suggest that, of all the inhibitors tested, PMSF, an irreversible inhibitor of serine protease, significantly inhibited protease activity at all the concentrations tried, and at 50mM concentration the residual activity was almost nil (98% inhibition). A reduced level of inhibition was detected in the presence of aprotinin (a reversible serine protease inhibitor), EDTA (metallo-protease inhibitor) and E-64 (Cysteine protease inhibitor). The activity was not inhibited by 1, 10-Phenanthroline (metallo-protease inhibitor) or Pepstatin (Aspartic protease inhibitor). Iodoacetamide, another cysteine protease inhibitor at its higher concentration caused a slight inhibition on enzyme activity. These observations strongly suggest that this fungal protease belong to the serine protease family.

Table 4.5 Effect of Protease Inhibitors on activity

Inhibitor & Class	Concentration	Residual activity (%)
PMSF (Serine protease)	20mM	8.9
	40mM	3.7
	50mM	2.5
Aprotinin (Serine protease)	0.1μM	88.9
	0.5 μM	89.8
	1 μM	89.2
EDTA (Metallo-protease)	20mM	89.5
	40mM	90.5
	50mM	80
1,10-Phenanthroline (Metallo-protease)	10mM	107.9
E-64 (Cysteine protease)	10 μM	90.8
	30 μM	96.3
	50 μM	93.5
Iodoacetamide (Cysteine protease)	1mM	104.6
	10mM	108.9
	50mM	76.9
Pepstatin (Aspartic protease)	5 μM	106.1
	50 μM	115.7
	100 μM	115.0

4.4.17 Substrate specificity

Substrate specificity of protease was evaluated using various proteinaceous substrates by incubating the enzyme in 1% solution of respective substrates. Results depicted in Fig. 4.30 testify that the enzyme has highest affinity for casein followed by Hemoglobin. Gelatin and BSA were less preferred by the enzyme.

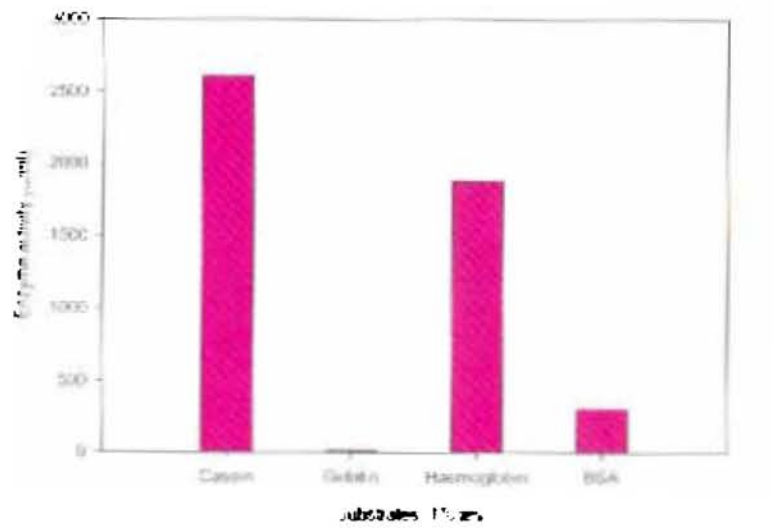


Fig 4. 30 Substrate specificity of *E.albus* Protease tested with different natural substrates

4.4.18 Kinetic studies

Protease kinetic studies were conducted using casein as the substrate and the data obtained is presented in Fig. 4.31. K_m and V_{max} were estimated by plotting the initial velocity data as the function of the concentration of substrate, by the linear transformation of the Michaelis-Menten equation and non-linear curve fitting of the Michaelis-Menten equation (Fig. 4.31). K_m and V_{max} were recorded as 4.327×10^{-3} and 394.7 U respectively. K_{cat} of the enzyme was estimated to be 4.2175×10^{-1} s.

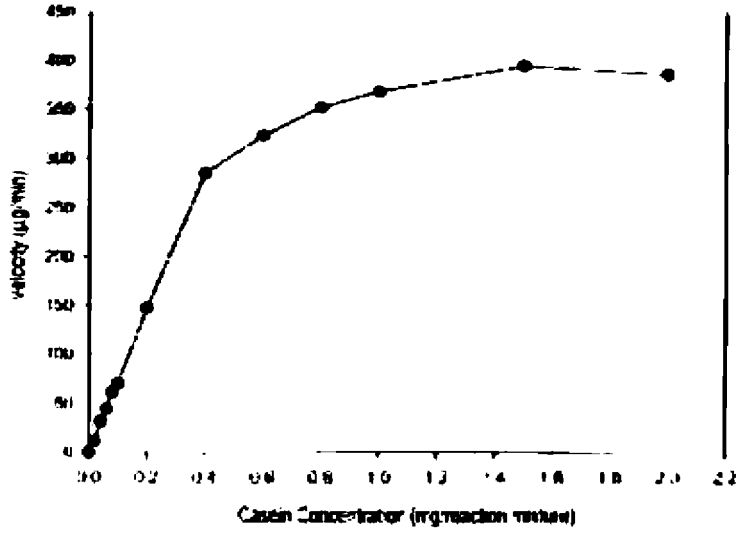


Fig. 4.31 Kinetic studies using Casein as substrate

4.4.19 Effect of various metal ions on enzyme activity

The result on the study of the effect of metal ions on enzyme activity is depicted in the Table 4.6. Of the various metals evaluated for their effect on protease activity, K^+ followed by Li^+, Pb^{2+}, Mg^{2+}, Ca^{2+}, Na^+ and Mn^{2+} did not have any negative effect and could effect marginal enhancement of enzyme activity at all the concentrations tested. On the other hand, Cu^{2+}, Fe^{2+} and Hg^{2+} showed a significant negative effect. Whereas Zn^{2+}, Cd^{2+}, Mo^{6+} and Al^{3+} showed negative effect at concentration 5 mM and above. Cr^{3+} showed positive effect at 1 mM and 5mM only. Ni^{2+} and Co^{2+} showed negative effect only at 15mM and 20mM concentration.

In fact, Co^{2+} effected 45% and 26% enhancement of residual enzyme activity at 1mM and 5mM concentration respectively compared to any other metals

tested. Next to Ca^{2+}, K^{+} effected 9-20% enhancement in residual activity although 20% and 18% enhancement was effected at 15mM and 20mM concentrations respectively.

Table 4.6 Effect of various metal ions on alkaline protease activity (Residual activity in %)

Metal ions	Concentration (mM)				
	1	5	10	15	20
Aluminium sulphate (Al^{3+})	111	89	22	10	4
Barium chloride (Ba^{2+})	110	105	103	102	86
Cadmium sulphate (Cd^{2+})	99	89	57	8	5
Calcium chloride (Ca^{2+})	106	106	102	103	103
Chromium nitrate (Cr^{3+})	100	101	85	96	76
Cobalt chloride (Co^{2+})	145	126	106	52	34
Cupric sulphate (Cu^{2+})	22	9	21	39	19
Ferric chloride (Fe^{3+})	52	0	0	0	0
Lead acetate (Pb^{2+})	108	108	106	106	104
Lithium chloride (Li^{+})	105	112	109	111	111
Magnesium sulphate (Mg^{2+})	101	104	107	106	105
Manganese chloride (Mn^{2+})	99	99	98	106	102
Mercury chloride (Hg^{2+})	2	0	0	0	0
Nickel chloride (Ni^{2+})	111	111	107	92	38
Potassium chloride (K^{+})	109	114	111	120	118
Sodium chloride (Na^{+})	103	104	104	105	104
Sodium molybdate (Mo^{6+})	108	66	0	0	0
Zinc sulphate (Zn^{2+})	101	87	84	67	24

4.4.20 Effect of various Detergents on Enzyme Activity

Effect of various detergents on enzyme activity presented in Table 4.7 and Fig. 4.32 shows that most of the detergents studied did not affect enzyme activity at its lower concentrations. However, in all cases, there was a slight decrease in activity at lower detergent concentrations, which further enhanced the activity along with increasing concentrations. Whereas, in the presence of SDS, at lower concentration enzyme is inactive and at 0.6% it regained more than 85% relative activity, but at higher concentration the enzyme looses its activity. In the case of Brij 35 and Tween 20, more than 70% and 80% activity is conserved even at 5% concentration respectively.

Table 4.7 Relative activity of protease enzyme in the presence of different detergents

Detergent	Concentration (%)	Residual activity (%)
Triton X-100	0.2	96.80
	0.4	138.43
	0.6	111.16
	0.8	105.34
	1	94.03
	5	8.90
Tween 80	0.2	94.80
	0.4	94.66
	0.6	92.16
	0.8	101.66
	1	90.80
	5	90.30
Tween 20	0.2	91.07
	0.4	91.74
	0.6	89.4
	0.8	89.61
	1	89.82
	4	87.86
SDS	0.2	0
	0.4	18.29
	0.6	28.41
	0.8	14.31
	1	0.85
	5	0
Brij 35	0.2	84.98
	0.4	77.33
	0.6	71.17
	0.8	72.03
	1	68.9
	5	72.53

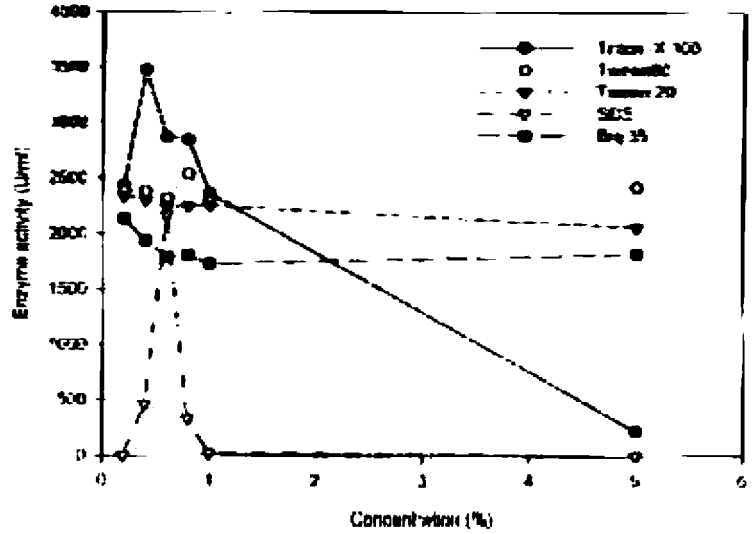


Fig. 4.32 Effect of detergents on enzyme activity

4.4.21 Effect of Hydrogen Peroxide (as oxidizing agent) on enzyme activity

Results documented in Table 4.8, indicate that in the presence of oxidizing agent H_2O_2 even at lowest concentration studied (1%), only 57% of activity was retained and above 2%, the enzyme is inactive. Thus, the results suggest that H_2O_2 has a drastic inhibitory effect on protease.

Table 4.8 Effect of oxidizing agents on enzyme activity

Concentration of Hydrogen peroxide(%)	Residual activity (%)
1	57.4
2	18.7
3	0
4	0
5	0
8	0

4.4.22 Effect of Reducing agents on enzyme activity

Results documented in Table 4.9. indicates that the reducing agents have a positive effect on enzyme activity except at highest concentration tried. β-mercaptoethanol and sodium thioglycolate at its highest concentration (5%) studied caused the reduction in residual activity to 72.2% and 40% respectively. Whereas, upto a concentration of 1%, all the reducing agents enhanced enzyme activity compared to the control.

Table 4.9 Relative activity of protease enzyme with different reducing agents

Reducing agent	Concentration (%)	Residual activity (%)
Dithiothreitol	0.2	136.4
	0.4	131.0
	0.6	131.0
	0.8	136.0
	1	126.0
β-Mercaptoethanol	0.2	118.5
	0.4	126.0
	0.6	125.0
	0.8	125.0
	1	115.0
	5	72.1
Sodium thioglycolate	0.2	117.7
	0.4	126.6
	0.6	130.1
	0.8	135.2
	1	144.7
	5	40.0

4.4.23 Effect of ionic strength on protease activity

The effect of ionic strength on protease activity was evaluated by incubating purified enzyme in various sodium chloride concentrations (1 to 4 SM) and calculating the residual activity. From the data shown in Fig. 4.33, it is evident that increase in the ionic strength of NaCl led to decrease in enzyme activity. Thus, at 1M concentration enzyme retained 78% of activity and on increase in NaCl concentration activity decreased and reached to 7% at 4 SM concentration.

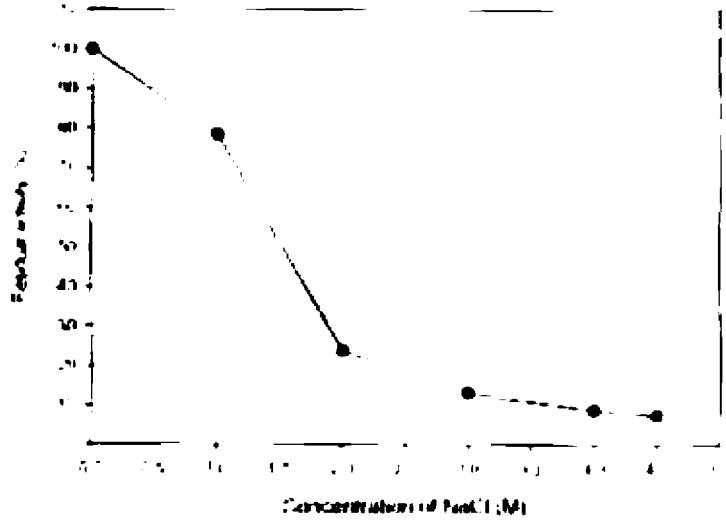


Fig. 4.33 Effect of NaCl concentration on Enzyme activity

4.4.24 Effect of organic solvents on protease activity

Effect of organic solvents on protease activity was tested using DMSO, isopropanol, acetonitrile, ethanol, glycol, petroleum ether, acetone and ethyl ether. Results obtained for the study indicate that the enzyme retained considerable amount of activity in the presence of most of the organic solvents tested (Fig. 4.34 A & B). Residual activity above 80% was retained for the concentration upto 60% in the case of DMSO, petroleum ether and acetone. Whereas, in the case of ethyl ether more than 90% of activity was retained at the highest concentration used

(10%) and in petroleum ether and DMSO the enzyme showed more than 65% of activity at its highest concentration (10% and 15% concentration respectively showing 69% and 73% of residual activity). Enzyme incubated in isopropanol and acetonitrile showed 7% and 32% residual activity at the highest concentrations studied (15%). However, enzyme was totally inactive at concentration above 0.2% and 6% of phenol and acetone respectively. Protease retained 8% of its initial activity in the presence of 20% ethanol. In general, protease activity was affected by the organic solvents to a marked extent.

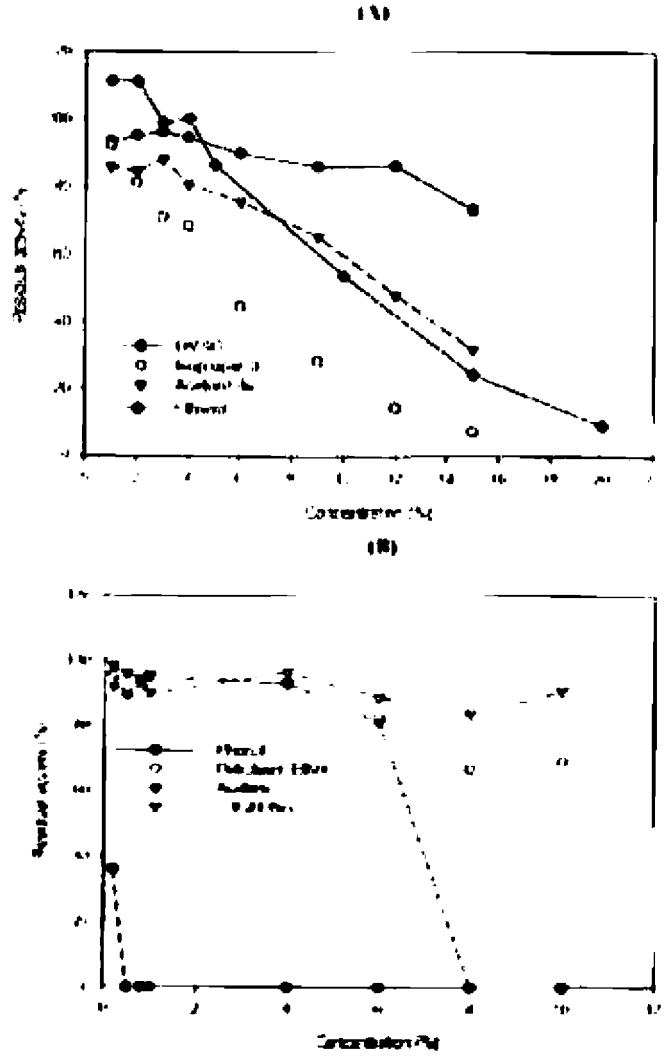


Fig. 4.34 (A & B) Effect of different organic solvents on Protease activity

4.4.25 Storage stability studies of the Protease enzyme

Storage stability of purified protease was evaluated by storing the enzyme at various conditions over a period of one year and estimating residual activity periodically. Lyophilized and liquid samples of partially purified enzyme (i.e. by ammonium sulphate precipitation) was stored at room temperature, 4°C and at -20°C. Results obtained for the storage stability studies of the enzyme are presented in Fig. 4.35. It was observed that the enzyme was relatively stable for the observed period.

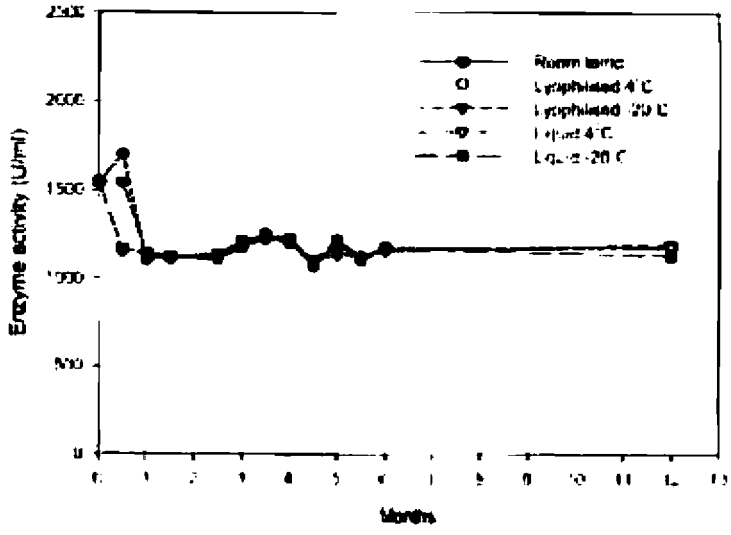


Fig. 4.35 Enzyme activity profile of protease enzyme showing the stability of the enzyme

4.4.26 Stability of the Enzyme in the presence of Hydrocarbons

Effect of hydrocarbons including petrol, kerosene, used engine oil, used lubricant oil, diesel and grease on protease activity was evaluated and the results are presented in Fig. 4.36. It was observed that, there was no marked inhibition of enzyme activity in general by the various hydrocarbons, even though there was decrease in residual enzyme activity (≤ 80%) in the case of petrol, kerosene and grease at 5% concentration. In the presence of engine oil, used engine oil, used

lubricant oil and diesel, the enzyme showed marginal increase in activity compared to the control even at the highest concentration tested (5%)

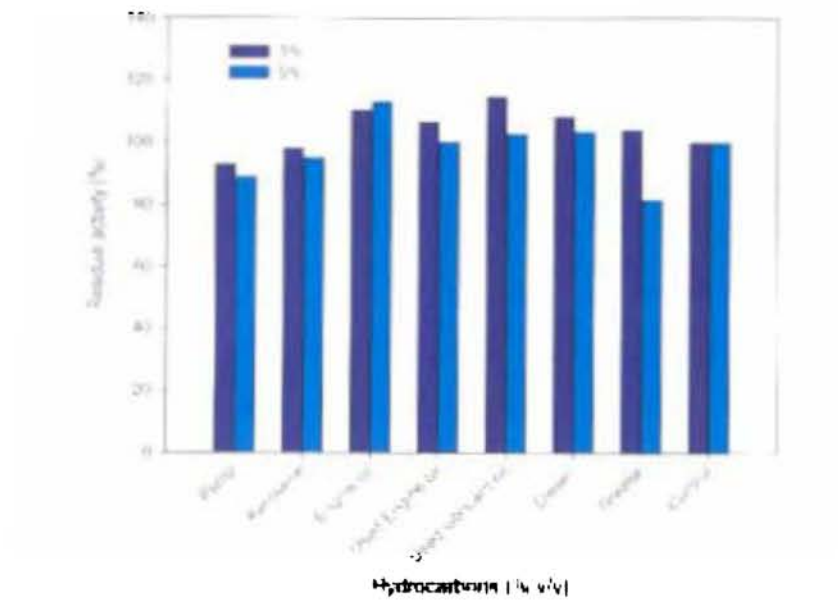


Fig. 4.36 Protease activity in the presence of Hydrocarbons

4.4.27 Stability of the Enzyme in the presence of Natural Oils

Effect of natural oils on protease activity was evaluated using coconut oil, gingelly oil, palm oil, mustard oil, sun flower oil, vegetable oil, dalda, olive oil, ghee and castor oil and the data obtained for the residual activity of enzyme is presented in Fig. 4.37. Results indicated that except in the case of coconut oil, gingelly oil, dalda and castor oil where there was marginal decrease in enzyme activity, in all the oils tested enzyme retained more than 95% of its initial activity even at the highest concentration. Sun flower oil, vegetable oil, olive oil and ghee in fact led to a marginal increase in enzyme activity. Gingelly oil and castor oil at 5% caused depletion in enzyme activity to 86% and 89% respectively. It may be said that all the natural oils did not have any drastic effect on protease activity when they co-exist in a reaction mixture.

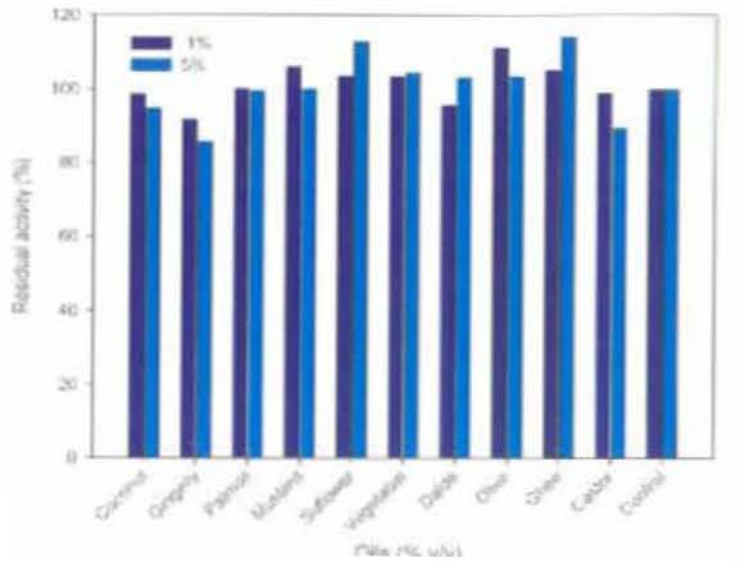


Fig. 4.5: Activity of Alkaline Protease in the presence of Natural oils

4.5 APPLICATION STUDIES

4.5.1 Commercial detergent compatibility of the enzyme

Commercial detergent compatibility of the enzyme was tested by incubating protease in detergent solutions for a period of 3 hrs and evaluating the residual enzyme activity at intervals of thirty minutes. Result depicted in Fig. 4.6 indicated that in all the detergent except Harpic toilet cleaner more than 90% of activity was retained even after 3hrs of incubation. In fact the protease is observed to be 100% compatible with Surf Excel, Surf Excel Automatic, Ariel, Rin, Wheel, Speed and Godrej Dish Wash since there was no detergent stimulation activity. This result can be explained from the pH values of each solution (Table 4.10) where all the detergents except Harpic have an alkaline or near neutral pH. Harpic have a pH of 2.15 and may contain certain strong bleaches. It is also

interesting to note that the enzyme incubated in Godrej dish wash having a pH o
9.72 also retained 100% of its activity even after 3hrs of incubation.

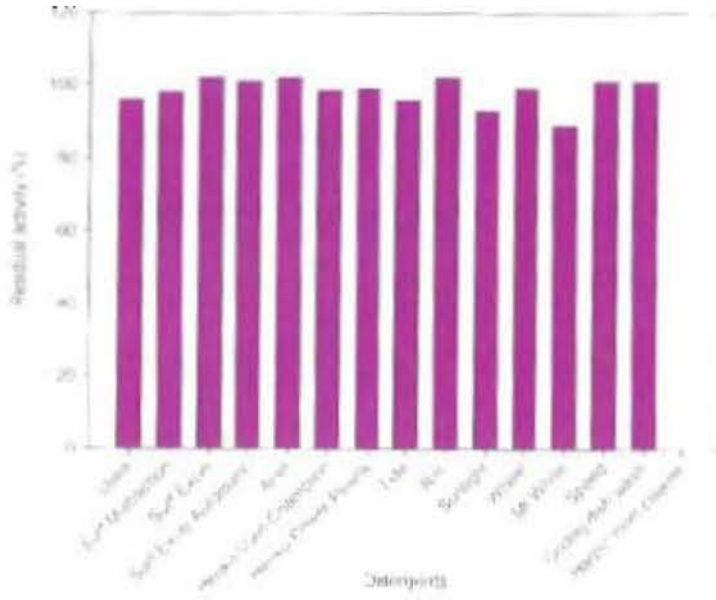


Fig. 4.38 Stability of alkaline protease in the presence of commercial detergents

Table 4.10 pH of the detergent solution

Detergent	pH of 7 mg/ml solution
Ujala washing powder	9.79
Surf Multiaction	9.55
Surf Excel	9.70
Surf Excel Automatic	9.98
Ariel Compact	9.92
Henko Stain Champion	10.12
Henko Power Pearls	9.73
Tide	9.89
Rin Shakti	9.93
Sunlight Extra Bright with Color Lock	10.00
Wheel	10.03
Mr.White	9.90
Speed	10.02
Godrej dish wash liquid	9.72
Harpic toilet cleaner	2.13

4.5.2 Comparison of performance of *E. album* protease with different Commercial proteases in the presence of detergents at 60°C

Stability of *E. album* protease and other proteases in the presence of commercial detergent at higher temperature was studied by incubating various proteases in Surf Excel Automatic (7mg/ml) for 3hrs at 60 °C and residual activity was calculated at every 30 minutes. Specific activity determined after 30 minutes of incubation showed that *E. album* protease retained the maximal specific activity (Fig. 4.39). Well known protease-Esperase, retained less specific activity compared to *E. album* protease and the protease subtilisin Calsberg was totally inactive after 30 minutes of incubation at 60°C. However, in the presence of commercial detergent, except Esperase, Pronase E and *B. licheniformis* proteases, all the others were totally inactive after 30 minutes of incubation

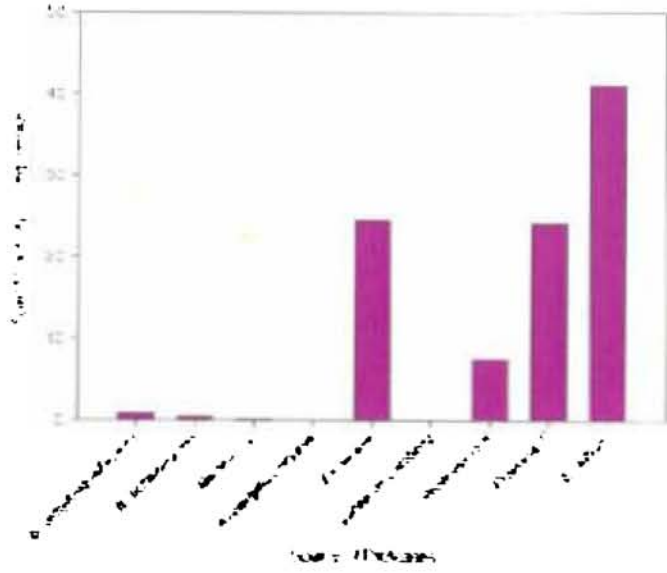


Fig. 4.39 Comparison of activity of *E. album* protease with different commercial proteases in the presence of detergent at 60°C

4.5.3 Wash performance studies

Wash performance analysis of protease was studied on white cotton cloth piece stained with human blood. Visual examination of the stained cloth pieces subjected to wash treatment exhibited the effectiveness of *E. album* protease in removal of stains (Fig. 4.40)

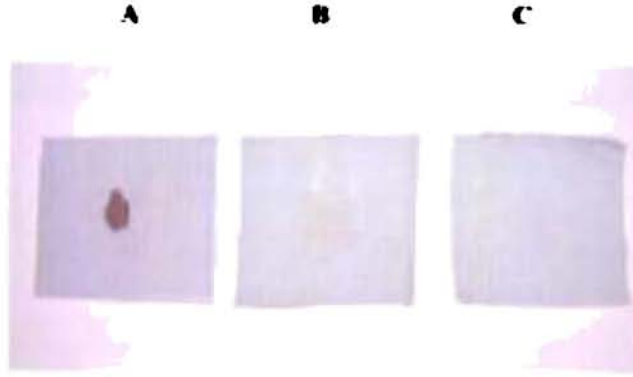


Fig. 4. 40 Wash performance studies

A- Cotton cloth stained with blood (Before washing)
B- Stained cloth washed in commercial detergent
C- Stained cloth washed with commercial detergent + protease

4.5.4 Esterase activity of the Protease Enzyme

Esterase activity of protease enzyme was determined using different *p*-nitrophenyl derivatives and the data obtained is depicted in Fig. 4.41. Results indicate that the enzyme has a higher affinity towards the short chain fatty acid derivatives like *p*-nitrophenyl butyrate (four carbon containing fatty acid) followed by *p*-nitrophenyl caprylate (eight carbon containing fatty acid). The enzyme is not able to cleave *p*-nitrophenyl laurate and *p*-nitrophenyl palmitate, which are the ideal substrates for lipase. This clearly indicates the esterase activity of the *E. album* protease. It was also confirmed by activity staining which released the

fluorescent 5-methylumbelliferone (MU) which is measured [illegible] (Fig. 4.42)

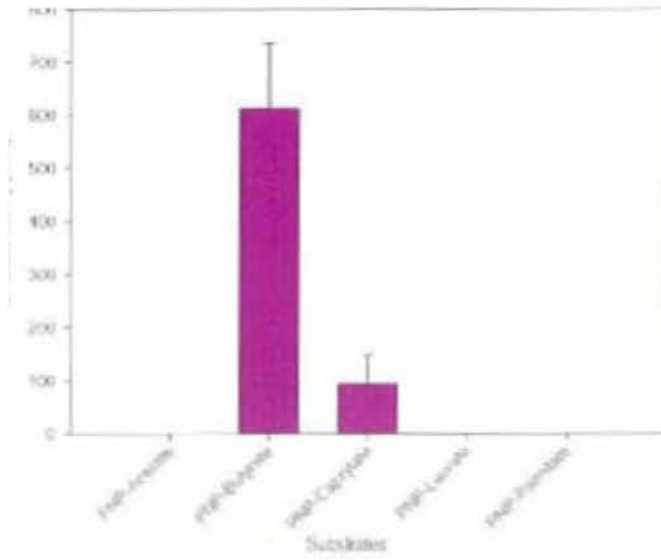


Fig. 4.41 Determination of Esterase activity of *E. album* Protease using different PNP substrates

Fig 4.42 Activity staining for esterase showing fluorescent protease band

4.5.5 Decomposition of gelatin layer of X-ray film

Hydrolytic activity of protease on the gelatin layer of the X-ray film was evaluated by incubating the X-ray film in enzyme solution. Protein content of the supernatant estimated after incubation clearly evidence the ability of the enzyme to degrade the protein layer of the X-ray film. From the Fig. 4.43, it is inferred that the X-ray film incubated in enzyme solution with pH 10.0 yielded higher protein content (13.6 mg/ml) compared to the X-ray film in enzyme solution diluted in distilled water (11.9 mg/ml). In the control (with out enzyme), protein content of the supernatant is very less (1.46 mg/ml). The result clearly suggests that the enzyme was able to degrade the protein layer of the X-ray film even in distilled water. Visual observation of the film (Fig. 4.44) conclusively testify the ability of the enzyme to decompose the gelatin layer of the X-ray film.

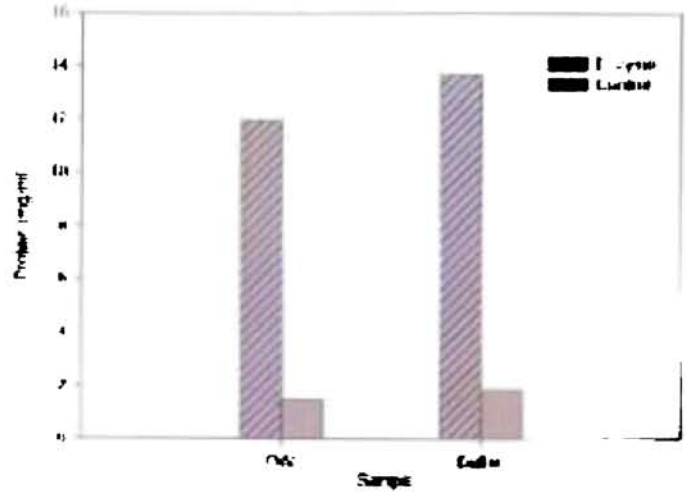
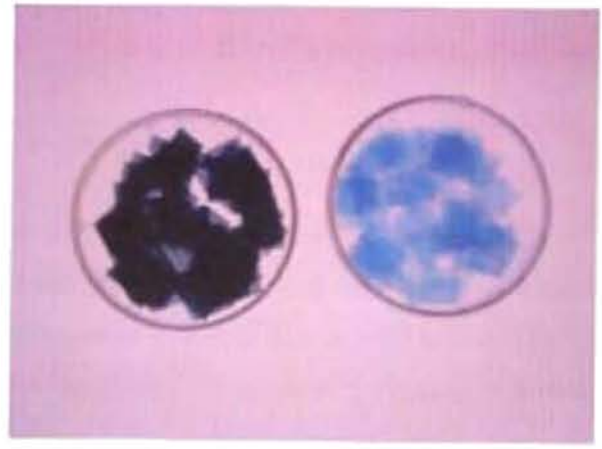


Fig. 4.43 Protein content of supernatant estimated after the treatment of X-ray film with *E. alkaw* protease

A B



A- Control
B- Treated with Protease

Fig. 4.44 Degradation of Gelatin layer of X-ray film for the recovery of silver

Chapter 5

DISCUSSION

5.1 Solid State Fermentation (SSF)

Alkaline protease production by *E. album* BTMF S10, under SSF using WB as solid substrate was optimised for various physicochemical parameters that influence the protease production. The fungus required moisture content above 50% to have enhanced level of protease production, and the maximal protease activity was recorded at 60% moisture level. However, further increase in moisture content resulted in a decline in enzyme production. Protein content and specific activity also recorded a similar trend. In SSF, the initial moisture content significantly affect hydrolytic enzyme production since the moisture content of the medium is a critical factor that determines microbial growth and product yield (Lonsane et al., 1985; Ramesh and Lonsane, 1990). Moisture is reported to cause swelling and there by facilitating better utilization of the substrate by the organisms (Kim et al., 1985) which in turn resulted in enhanced level of metabolic activity by the organism and increased level of proteins including secretory proteins. Thus, a higher level of protein content and enzyme level was obtained in the solid substrate moistened to the optimal level of moisture content for the organism. At lower and higher initial moisture levels, the metabolic activities of the culture and consequently, product synthesis were variously affected (Ramesh and Lonsane, 1990). In fungal and bacterial SSF, lower moisture content was reported to lead to reduced solubility of the nutrients present in the solid substrate, a lower degree of substrate swelling and higher water tension (Zandrazil and Brunert, 1981). Similarly, higher moisture content may cause decreased porosity, loss of particle structure, development of stickiness, reduction in gas volume, decreased exchange

and enhanced formation of aerial mycelia (Lonsane et al., 1985; Nishio et al., 1979). In the present study, a similar observation was made at moisture levels above 70%.

The incubation time for enzyme production is governed by the characteristics of the culture and is based on growth rate. *F. album* showed an optimal incubation of 120 hrs for maximal enzyme production and irrespective of the level of moisture content of the WB, protease production was observed only after 72 hrs of incubation. Early hours of incubation could have played the role of probable logarithmic period for mycelial growth. Similarly a reduction in enzyme activity beyond 120 hrs of incubation could have contributed by the next lag phase of the mycelium and protease degradation in the fermented medium. The fungal growth takes place on the terminal end of the mycelia and there may be a lag period in between the production phase, since the matured hyphae produces the enzyme. Considering the economic aspects of fermentation, the initial stage with considerable level of enzyme production can be taken as the optimal production period. Although many reports on protein secretion by the yeast *Saccharomyces cerevisiae* are available (Cleves and Bankaitis, 1991; Reid, 1991), only few are available for the filamentous fungi.

Particle size of commercial WB used in the present study significantly influenced enzyme production by *F. album* during SSF and particles <425µ supported maximal enzyme synthesis. In SSF, particle size of the substrate used has a profound effect on enzyme production. The small particles have more surface area for growth but reduced porosity, leading to lowering of gas diffusion and heat transfer, while the big particles absorb less moisture, swell less and by drying rapidly support only a sub-optimal growth of fungi (Ramesh and Lonsane, 1987; Zadrazil and Puniya, 1995). The available surface area will decrease with an increase in particle size of the substrate, leading to a poor hydrolytic activity caused by limited contact with the hydrolysing agent. Similarly, lower particle size

fraction may contain more nutritionally rich and readily soluble nutrients. This may be the primary reason for the higher enzyme productivity observed with the lower particle size substrate, in the present study.

The incubation temperature has a significant effect on the enzyme yield and duration of enzyme synthesis phase (Ramesh and Lonsane, 1987). In the present study, the organism required 25°C as optimal temperature for maximal enzyme production. Incubation at temperatures above 25°C did not support protease production by *E. album* under SSF, although the fungus could record some amount of enzyme activity at 20°C. Most of the marine fungi investigated showed optimum growth in the range of 10-20°C and none appeared to require a temperature above 30°C (Jones and Byrne, 1983). Protein level also showed a similar trend for the various temperatures tested indicating that the fungus was active at all the temperatures studied.

Temperature is a critical parameter that has to be controlled and it varies from organism to organism. Temperature strongly affects the synthesis of protease either nonspecifically, influencing the rate of biochemical reactions, or specifically inducing or repressing their production. Temperature can regulate the synthesis and the secretion of extracellular proteases by microorganisms (Ray et al., 1992). It was reported that a link existed between enzyme synthesis and energy metabolism in bacteria, which was controlled by temperature and oxygen uptake (Frankena et al., 1986). Temperature was also proved to regulate enzyme synthesis at mRNA transcription and probably translation levels (Votruba et al., 1991). Temperature regulates the synthesis of several enzymes, including intracellular and extracellular enzymes. For extracellular enzymes, temperature influences their secretion, possibly by changing the physical properties of the cell membrane.

E. album is capable of producing protease over a broad pH range from pH 2.0 to pH 12.0. Apparently, it seems that this fungus has two pH optima - one at pH

4.0-5.0 and another at pH 10.0 for maximal protease production. This dual optimum for growth is characteristic of most marine fungi (Jones and Byrne, 1983; Suresh and Chandrasekaran, 1999).

In a previous communication, it was reported that this fungus has an optimal pH of 6.0 for chitinase production and maximal production was given in the alkaline pH of 9.0 to 10.0 (Suresh and Chandrasekaran, 1999). In the present study, the time course experiment conducted with this fungus for protease production also showed that, when interacting with other factors, this fungus prefers an alkaline pH 10.0 instead of an acidic pH as optimal for maximal enzyme activity.

Wheat bran without any additional proteinaceous substrates (control) yielded maximal enzyme production and specific activity. Casein and gelatin caused a decreased level of enzyme production compared to the control medium. Protease production is an inherent property of all organisms and these enzymes are generally constitutive, although, at times, they are partially inducible (Beg et al., 2002a; Kalisz, 1988). In insect pathogenic fungus *Metarhizium anisopliae* produce an extracellular protease PR 1 which is induced specifically by insect cuticle, but not by other soluble or insoluble proteinaceous substrates (Paterson et al., 1994).

Since, WB is a complex substrate with high protein content, the protease production by *E. album* under SSF can be considered as an inducible one. In submerged fermentation, without any proteinaceous substrate, the enzyme productivity was nil, which supports this view. High substrate concentration may cause substrate inhibition or repression of protease production as is evident from the result when casein or gelatin was supplemented to WB. Similar observations were reported earlier by Joo et al. (2002) and Brandelli and Riffel (2005). Additional proteinaceous substrates like gelatin and casein cause depletion in enzyme activity, which may be due to the release of free amino acids by hydrolysis

of these proteins, which may cause enzyme repression. Individual amino acids were observed to curtail protease production by various fungi such as *Trichophyton rubrum* (Meevootisom and Niederpruem, 1979), *Neurospora crassa* (Cohen and Drucker, 1977) and *Rhizopus oligosporus* (Farley and Ikasari, 1992).

Proteases are largely produced during stationary phase and thus are generally regulated by carbon and nitrogen stress (Hölker et al., 2004). In the present study, except sucrose, mannitol and maltose, all other sugars led to a decrease in enzyme production when compared to control. Sucrose supported maximal enzyme activity and specific activity followed by mannitol. Arabinose, ribose and xylose totally inhibited enzyme production. Even though lactose caused depletion in enzyme production, maximum extracellular protein was observed for this. Besides these, all the other sugars tested caused depletion in enzyme activity compared to the control. In bacterial system, carbohydrate inhibition of protease production, i.e., a catabolite repression regulatory mechanism, which is a common control mechanism for biosynthesis of protease is known (Brandelli and Riffel, 2005).

One important biological factor in favour of SSF is the low catabolite repression, which appeared to be a limiting factor of enzyme production in SmF (Nandakumar et al., 1999). The lack of catabolite repression also allowed fast growth of the fungus in the presence of high sugar concentrations (Favela-Torres et al., 1998). Perhaps, in *E. album*, the depletion in protease production by some of the sugars is due to the mechanism of catabolite repression is to be further investigated.

All the organic nitrogen sources tested, except urea, supported enhanced protease production when compared to control. Malt extract, soyabean meal and tryptone recorded similar levels of enzyme activity leading to a 47 - 49% increase in enzyme activity. Of these, malt extract recorded a maximum of 49% increase in enzyme activity. Nevertheless, peptone, beef extract and yeast extract also led to a

38.4%, 26.5% and 17.6% increase in enzyme activity compared to control. In fact, both soyabean meal and malt extract recorded maximal specific activity compared to tryptone, in spite of similar levels of enzyme activity for all the three.

Since the final protease yield is dependent on the biomass produced during exponential phase, medium manipulation is needed to maximize growth. In submerged fermentation, soyabean meal was reported to be a preferred organic nitrogen source in *Bacillus* sp. (Banerjee et al., 1999; Fujiwara and Yamamoto, 1987; Janssen et al., 1994, Kalisz, 1988).

Catabolic enzymes respond to both carbon control and nitrogen control. Production of extracellular protease has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Haulon et al., 1982; Levisohn and Aronson, 1967). Citric acid and $NaNO_3$ are reported to be most effective carbon and nitrogen sources for the production of a thermostable alkaline protease from *Bacillus* sp. JB-99 under submerged fermentation and the inorganic nitrogen source contributed more enzyme production than the organic nitrogen source (Johnvesly and Naik, 2001). Observations made in the present study is in agreement with the observation made with *Bacillus* sp. JB99

Results obtained in the present study indicate that all the amino acids tested do not have similar impact on enhancing protease production by *E. album*. Among the 19 different amino acids tested, only 9 amino acids favored enhanced enzyme production. Of them, leucine, histidine and lysine supported 44.9%, 32.2% and 30.2% increase in enzyme activity compared to control respectively. On the other hand, methionine and tryptophan caused a 92.2% and 89.6% reduction in enzyme activity respectively when compared to the control. Thus leucine could be considered as optimal amino acid as additional nitrogen source for protease production. Specific activity also presented a similar trend very much like that of enzyme activity, while protein levels did not show any specific trend. In literature,

It is reported that amino acids inhibit protease production by various fungi such as *Trichophyton rubrum* (Moevootisom and Niederpruem, 1979), *Neurospora crassa* (Cohen and Drucker, 1977) and *Rhizopus oligosporus* (Farley and Ikawari, 1992). This may be due to the repression caused by amino acid for protein synthesis. The observation made with *E. album* in the present study with half of the amino acids are in agreement with the observation made for the above mentioned fungi, but the rest of the amino acids favored enhanced level of protease production.

Among the inorganic nitrogen sources tested, only ammonium hydrogen carbonate, ammonium nitrate and ammonium hydrogen phosphate have a positive effect on enzyme production. When compared to control, 94% increase in activity was observed with the addition of ammonium hydrogen carbonate, which yielded a maximal enzyme activity of 15,186.8 U/gIDS and specific activity of 380.4 U/mg protein. It was also noted that ammonium oxalate and ammonium iron sulphate as additional nitrogen source, totally inhibited enzyme production. In fact, the protein content in the enzyme extract did not show any correlation with protease activity. However, specific activity showed a similar trend very much like that of enzyme activity.

Among the different additional nitrogen sources tested, including organic, inorganic and amino acids, inorganic nitrogen source, ammonium hydrogen carbonate caused a 94% increase in enzyme activity whereas leucine and malt extract, as amino acids and organic nitrogen sources caused only 44.9 and 49 % increase in enzyme activity compared to the control respectively. Thus ammonium hydrogen carbonate can be considered as the effective nitrogen source for the production of protease by *E. album*.

Number of spores used for inoculation had a linear effect on the enzyme production. Data presented suggest that 4×10^6 cfu/ml was found to be optimal for maximal enzyme production. Apparently a minimum of 2×10^5 spores were

observed to be required for enzyme production. Even though the extracellular protein content increased for the inoculum with spore count of 6×10^8 spores, there was decline in enzyme production. Results also indicated that though, protein could be recorded in crude extract for all the levels of inoculum tested, protease activity could not be detected. There was no direct relation between the protein content of the enzyme extract and protease activity. These observations suggest that the other soluble proteins and other enzymes secreted by the fungus might have contributed to the protein level.

Sodium chloride was observed to significantly affect the protease production by *E. album* under SSF. Protease production in distilled water based medium was almost double fold compared to the sea water based medium. Sodium chloride showed an inverse effect on enzyme production both in seawater and distilled water based media. In the presence of seawater, addition of even 1% sodium chloride caused a 13% decrease in enzyme production. Further, addition of sodium chloride above 1% to sea water based medium and above 5% in distilled water based medium totally inhibited protease production. Observations made with *E. album* in response to NaCl is intriguing since the organism was isolated from a marine sediment where the NaCl concentration in sea water is usually high. However, it may be assumed that protease secretion in wheat bran medium is influenced by NaCl.

Studies conducted using *E. album* under submerged fermentation indicated that enzyme production in seawater based medium starts at an early stage than in distilled water based medium. It was also noticed that during purification and characterisation of the enzyme, the pure enzyme became inactive in the total absence of NaCl and the enzyme undergoes autolysis. It was reported that the tightly folded enzyme tend to be resistant to proteolysis whereas unfolded or partially unfolded proteins are generally susceptible to proteolytic degradation (Akasako et al., 1995; Daniel et al., 1982; Markert et al., 2001). Additionally, loss

of structure (unfolding) generally implies loss of enzymatic activity. Thus in *E. album*, the absence of NaCl may affect the stability and activity of the enzyme. Further studies on molecular mechanism of NaCl inhibition of protease synthesis is warranted.

Results obtained for the study conducted using different dilutions of sea water with distilled water on protease production by *E. album* suggest that this fungus can produce double fold protease under SSF using WB in distilled water than that in sea water. Addition of 25% distilled water to seawater (75% seawater) could induce significant levels of protease (11,470 U/gIDS). The protease activity increased along with increase in dilution of seawater with distilled water. Specific activity also showed a similar trend. In fact, a maximal specific activity for protease could be observed with 25% seawater + 75% distilled water. Nevertheless, the protein content in the enzyme extract recorded almost identical level and was independent of enzyme activity observed.

The use of sea water in the fermentation medium was reported to considerably influence the production of proteolytic activity in halotolerant strain of *Bacillus licheniformis* under submerged fermentation and the addition of NaCl also enhanced enzyme production (Manachini and Fortina, 1998). Perhaps the microbes differentially responded to NaCl levels in their cultivation medium and a detailed study may throw insight into the exact molecular mechanism in *E. album*.

Data obtained for the time course experiment conducted over a period of 216 days under optimised condition clearly evidence that the protease production commenced on second day (i.e., 48 hrs) and reached a peak after 120 hrs. Further incubation beyond 120 hrs did not favour enhanced enzyme activity and instead resulted in a decline. Rapid decline in enzyme activity occurred after 168 hrs. May be the protease, which accumulated in the fermented moldy wheat bran, got degraded on extended incubation and hence enzyme yield declined beyond 5 days.

The production of protease in complex growth media often promotes exuberant growth and high enzyme yields (Johnvesly and Naik, 2001; Joo et al., 2002). Their expensive cost makes them unsuitable for a large-scale production. Earlier studies conducted using different solid supports as substrates for SSF clearly indicates that among the various agrobyproducts used in different growth systems (SSF, SmF and two phase system), wheat bran was most effective in terms of protease production (Kaur et al., 2001; Malathi and Chakraborty, 1991). Earlier reports on the production of protease by solid state fermentation are limited to the genus *Bacillus* and some fungi of the *Penicillium* and *Aspergillus* sp. (Germano et al., 2003; Johnvesly and Naik, 2001; Kumar et al., 1999). The present study adds evidence to the fact that even marine fungus could perform well in protease synthesis in WB as SSF medium.

5.2 ENZYME PURIFICATION AND CHARACTERISATION

Protease enzyme purified by ammonium sulphate fractionation and ion exchange chromatography yielded 16 fold of purification with 8% of recovery and in preparative PAGE, a 45 fold of purification with 1.3% yield was obtained. It seems that a combination of several techniques and repetitive purification may be required to enhance the fold of purification.

Purified fraction subjected to Native and SDS-PAGE analysis yielded a single band, which confirms the homogeneity and purity of the enzyme. SDS-PAGE performed under reducing conditions also yielded a single band, evidencing the single polypeptide nature of the enzyme. The proteolytic activity of the purified enzyme protein, confirmed by zymogram analysis on X-ray film indicated a single type of extracellular protease.

The molecular mass of protease calculated by different methods like SDS-PAGE, Gel filtration chromatography, amino acid analysis and MALDI- MS are

listed in the Table 5.1. Molecular weight estimated for the *E. album* protease by comparing the electrophoretic mobility of marker protein in SDS-PAGE yielded a value of 38kDa. Whereas, gel filtration chromatography and amino acid analysis suggested a size of 30kDa and 24.8kDa respectively. On the other hand, MALDI-MS analysis indicated a size of 28.6kDa. MALDI-MS analysis is a more precise method with a mass accuracy of 0.05-0.1% (Jensen et al., 1997) and thus the molecular mass of *E. album* protease can be considered as 28.6kDa. Protease enzyme isolated from the most related genus of *E. album* like *Beauveria* (Chrzanowska et al., 2001) and *Tritirachium* (Jany et al., 1986) have a molecular weight of 32kDa and 28.5kDa respectively.

The molecular mass of most of the reported serine proteases is in the range between 15 and 30kDa (Gupta et al., 2002a; Huang et al., 2003; Kumar et al., 1999; Urtz and Rice, 2000). Of course, there are few exceptions like serine protease from *Blakeslea trispora*, which has a molecular mass of 126kDa (Govind et al., 1981) and enzyme from *Kurthia spiroforme* with an extremely low molecular weight of 8kDa .

Table 5.1 Physicochemical properties of purified protease from *E. album*

Property of Protease	Value obtained
Molecular weight by	
Gel filtration on Sephadex G 75	30kDa
SDS-PAGE	38kDa
Amino acid composition	24.8kDa
MALDI analysis	28.6kDa
pI	3 - 4
Km	4.727×10^{-1} mg/ml
pH optimum	10.0-11.0
pH stability	5.0-12.0
Temperature optimum	60°C
Temperature stability	Up to 60°C

Protease of *E. album* after purification has a pI value between 3-4. In related species of *E. album*, i.e., *Beauveria* and *Tritirachium*, the pI value was reported to be 7.5 and 4.5 respectively (Jany et al., 1986; Urtz and Rice, 2000). In some cases, the pI value coincides or are nearly in the range of the optimal pH of the enzyme (Huang et al., 2003).

The N-terminal sequencing of the *E. album* protease reported a probability of N-terminal blocking since sequence analysis got terminated after 3 cycles. Hence, MALDI analysis was done to get a peptide fingerprint.

Peptide fingerprint profile using MALDI analysis for the purified protease enzyme of *E. album* identified three internal peptide sequences which showed homology to *Tritirachium album* precursor Protease T and of these, one showed homology to the conserved stretch assigned to Subtilisin class Serine protease. Since, peptide fingerprint profile resulted only in a single hit for protease enzyme and biochemical studies conducted revealed several unique characteristics of *E. album* protease, the enzyme may be a novel one, which may have distinct structure and sequence homology compared to other reported proteases.

Purified protease has an optimum pH between 10.0 and 11.0 for maximal activity. In general, the protease was active over a pH range of 6.0-12.0 and increase in pH from 6.0 to 11.0 recorded proportionate increase in activity. The decrease in enzyme activity in the neutral pH and in the acidic range is more significant which confirms the enzyme as an alkaline protease.

Extreme pH conditions alter the structure of the surface of the enzymes, modifying the interaction between active site and substrate. Because of that, under strong acidic and alkaline conditions, enzymes are denatured, and as a consequence, their activity is totally or partially lost.

It could be inferred that the way in which the activity of the enzyme is altered (when they are exposed to different pH for a period of time) may be attributed to their primary and tertiary structure rather than been an adaptation to the pH of the habitat where the producing strains strive.

Highest optimal pH for activity of alkaline protease reported earlier was between 10.0-10.5 (Beg and Gupta, 2003; Huang et al., 2003; Rashbehari et al., 2003). Of course, few reports are available where the optimal pH of activity was at 11.0 or above for some enzymes from some *Bacillus* sp. (Horikoshi, 1990; Kumar et al., 1999). *Bacillus* sp. KSM-K16 exhibited a higher pH optima of 12.3 (Kobayashi et al., 1995). Data obtained from the pH stability studies of the protease evidence that the enzyme is stable over a wide range of pH from 5.0-12.0 although, maximal residual enzyme activity was recorded with pH 9.0. Protease retained more than 85% of residual activity between pH 5.0 and pH 12.0, but lost their activity at pH 13.0. The pH stability profile qualifies this protease for their application in industrial processes that are carried out at a pH range in the alkaline or extreme alkaline conditions. This stability at high pH compromises their likely use as additive in washing detergents, leather processing or other applications at pH higher than 9.0.

Protease from *E. album* was active over a broad range of incubation temperature with maximal activity at 60°C. Temperature above 60°C led to a sharp decline in enzyme activity and was totally nil above 85°C. Interestingly, the enzyme showed activity even at 5°C (67.9U/ml), though it is negligible considering the activity recorded at 60°C (4,658 U/ml). In fact, the protease activity showed a linear increase along with increase in temperature and particularly, the increase was rapid during 40°C-60°C. Relative activity of protease observed at different temperatures indicated clearly the preference for a higher temperature of 50°C-60°C for maximal activity

Temperature stability studies conducted using protease testify that the enzyme could retain 100% of activity even after 24 hrs at 30°C- 40°C and at both 50°C (retained 95% of residual activity) and at 4°C (control) there was only marginal decrease in residual activity. Results also suggest that at high temperatures above 60°C the enzyme had denatured and lost activity within 1 hour. Velocity of enzymatic reaction is enhanced by a raise in temperature, but at temperatures close to that supporting maximal activity, the enzyme suffers denaturation and thus inactivation (Dixon, 1979).

From the results, it is inferred that the protease is thermostable and could be harnessed for application that demands activity at 60°C. The higher temperature optima up to 75°C have been reported for alkaline protease from *Bacillus stearothermophilus* F1 (Rahman et al., 2005). A number of alkaline proteases isolated from *Bacillus* sp. have high optimal temperatures of about 55-70°C (Adinarayana et al., 2003; Banerjee et al., 1999; Kumar et al., 1999) whereas, there are only few reports on the fungal protease with high temperature optima (Li et al., 1997). This is an important characteristic required for use of these enzymes as detergent additives. Temperature and pH optima of *E. album* protease observed in the present study are very similar to those alkaline proteases produced commercially by *Bacillus* sp., which is currently used in detergent industry under the trade name Savinase and Esperase (http://www.novozymesbiotech.com).

The current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the use of low washing temperatures. This has pushed enzyme manufacturers to look for novel enzyme that can act under low temperatures. Novo Nordisk Bioindustry in Japan has developed a detergent protease called Kannase, which keeps its high efficiency, even at very low temperatures (10–20°C). There are only a few reports on the proteases that are active over a wide range of temperatures. Protease isolated from *Penicillium chrysogenum* Pg 222 active against meat proteins showed activity

in a wide range of temperature from 10-60°C (Benito et al., 2002). In their natural habitats, marine microbes never have to cope up with a temperature higher than 30°C. So their enzymes can shift their activity temperatures for activity towards lower value (Feller, 1995). The protease enzyme isolated from *E. album* is also active at lower temperatures, which shows its potential to be used in cold washing conditions.

Effect of stabilizers on thermal stability was studied by the addition of different reported stabilizers and incubating at 65°C and 70°C. Among the different stabilizers tested, starch (1%) could play the role of stabilizer promoting thermal stability at both the temperatures, whereas, $CaCl_2$ at a concentration of 1mM showed a maximal residual activity at 65°C.

In liquid formulations, physical isolation of enzymes is more difficult and the presence of solvent (water) amplifies the detrimental effect of surfactants and enhances the rate of undesirable reactions like autolytic degradation, oxidation, denaturation etc. of the enzyme (Lalonde et al., 1995). This has pursued to the development of various enzyme stabilization strategies based on chemical additives. There are reports in literature regarding the stabilisation of enzyme using carboxylic acid salts, calcium chloride (Crossin, 1989), boron compounds (boric acid, borate salt) especially in conjunction with pyrrols and polyols like propyline glycol, glycerol, mannitol, surbitol etc. (Asther and Meunier, 1990; Boskamp, 1984; Gonzalez et al., 1992; Severson, 1984). The protective effect of these could be explained by the strengthening of the hydrophobic interactions inside the protein molecule and by the indirect action of these compounds on water structure. Sucrose has been used to protect the proteins against oxidation, aggregation and damage during lyophilization. The stabilizing effect of sucrose is due to a preferential exclusion mechanism (Timasheff, 1998). Calcium ions have been shown to contribute approximately 3 kCal/mol to the enzyme's total kinetic thermal stability in Subtilisin (Voordouw et al., 1976).

Calcium ions stabilize the protein through specific and nonspecific binding sites, and may also allow for additional bonding within the enzyme molecule preventing unfolding at higher temperatures, as has been demonstrated for protease from thermophilic bacteria, particularly thermolysin (James et al., 1991). The improvement in protease thermal stability against thermal inactivation in the presence of Ca^{2+} may be explained by strengthening of interaction inside protein molecules and by the binding of Ca^{2+} ions to autolysis sites (Ghorbel et al., 2003).

Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements and the nature of the active center (Sigma and Mooser, 1975). Among all the inhibitors tested, PMSF, an irreversible inhibitor of serine protease, significantly inhibited protease activity at all the concentrations tried and at 50mM concentration the residual activity was almost nil. A reduced level of inhibition was detected in the presence of aprotinin, a reversible serine protease inhibitor. The activity was not inhibited by 1,10-Phenanthroline (metallo-protease inhibitor) or Pepstatin (Aspartic protease inhibitors), whereas, slightly by EDTA (metallo-protease inhibitors); Iodo acetamide, another cysteine protease inhibitor at its higher concentration caused a slight inhibition on enzyme activity. Since PMSF inhibited 98% of protease activity and aprotinin, a reversible inhibitor of the enzyme caused 10% inhibition, it is concluded that the enzyme belongs to the serine protease group where PMSF sulfonates the essential serine residue in the active site of the protease which result in the complete loss of enzyme activity (Gold and Fahrney, 1964).

Generally serine proteases are not inhibited by metal chelating agents. However, there are examples of serine proteases that are affected by EDTA (Gnosspelius, 1978; Izotova et al., 1983; Kato et al., 1974). Studies investigating the conformational integrity of subtilisin have shown that calcium chelating agents lead to autolytic digestion (Wells and Estell, 1988). Removal of calcium cause

flexibility of the protein and there by its rate of autolysis. Thus protein may need to unfold to become a substrate for autolysis (Siezen et al., 1991).

The stability of the enzyme in the presence of EDTA is advantageous for use of enzymes as detergent additive. This is because detergents contain high amount of chelating agents, which function as water softeners and also assist in stain removal. These agents specifically bind to and chelate metal ions in the enzyme making them unavailable in the detergent solution (Beg and Gupta, 2003).

Substrate specificity of protease studied indicates that the enzyme has highest affinity for casein followed by haemoglobin while Gelatin and BSA were not much preferred by the enzyme. The ability to hydrolyse several protein substrates is a criterion of protease potency (Grebeshova et al., 1999). The highest affinity for casein as a substrate was also reported for the protease of the fungus *Conidiobolus coronatus* (Phadatare et al., 1993). The substrate specificity towards haemoglobin and casein of *E. album* protease may be advantageous for its use in detergent against blood and milk protein stains. Most of the previous studies have revealed that alkaline proteases show highest activity towards casein relative to other native and modified proteins (Gupta et al., 2002a; Kumar et al., 1999; Phadatare et al., 1993).

Protease kinetic studies conducted using casein as the substrate revealed that K_m and V_{max} of protease of *E. album* were 4.727 x 10^{-3} and 394.7 U respectively. K_{cat} of the enzyme was estimated to be 4.2175 x 10^{7} s^{-1}.

In the case of bacteria, the K_m of protease from *Pseudomonas flourescens* AR 11 was 3mg/ml (Alichanidis and Andrews, 1977) and *Pseudomonas* sp. B-25 was 4mg/ml (Malik and Mathur, 1984). There are no similar reports available on other fungal protease for a direct comparison.

Commercial detergent formulas often includes water softening "builders" (Sachdev and Krishnan, 1997) Hence, it is worthwhile to evaluate the protease for their response to varying concentration of different metal ions towards evaluating the potential of protease for application in detergent industry. Most of the metal ions studied for their effect on enzyme activity showed either no effect or exhibited a slight stimulatory effect at lower concentrations. While even at lower concentrations, Hg^{2+}, Cu^{2+} and Fe^{3+} had very adverse effect on enzyme activity, Zn^{2+}, Cd^{2+} and Al^{3+} inhibited enzyme activity at higher concentration, whereas, interestingly 145% of residual activity was obtained for 1mM concentration of Co^{2+} compared to control. However, activity was reduced to 34% at higher concentration of 20mM

Studies have indicated that metal ions impart thermodynamic stabilization to the native state of the protein by binding to the enzyme active site (Wyman and Gill, 1990) According to some authors, Ca^{2+} ions are important for catalysis. It is presumed, that they stabilize the protein through specific and nonspecific binding sites, and may also allow for additional bonding within the enzyme molecule preventing unfolding at higher temperatures, as has been demonstrated for protease from thermophilic bacteria, particularly thermolysin (James et al., 1991). Ca^{2+} has been reported to bind to the inner surface and autolytic sites of protein molecule thereby strengthening the interaction inside the molecule (Ghorbel et al., 2003). The same may be the reason behind the enhanced activity of protease in presence of some of the metal ions, especially as in the case of Co^{2+} in *E. album* protease.

Copper ions may cause the denaturing of protease (Demina and Lysenko, 1995) whereas, Hg^{2+} and organo mercurials interact with -SH and S-S groups of proteins in a multitude of systems thereby causing conformational changes in proteins which have been reviewed thoroughly by Vallee and Ulmer (1972). The reduction in enzyme activity in presence of some of the metal ions studied may be attributed to the above said reason

A reduction in protease activity with Cu^{2+} ions and a two fold increase in activity with Ca^{2+} and Ba^{2+} ions was observed in *Streptomyces* sp. (Azeredo et al., 2004). Ca^{2+}, Mg^{2+}, and Mn^{2+} at 5mM concentrations strongly activated serine protease of *Bacillus subtilis* PE 11 (Adinarayana et al., 2003). Protease activity in *Bacillus mojavensis* was enhanced by Fe^{2+} and Mn^{2+} (Beg and Gupta, 2003). In *Aspergillus parasiticus*, strong stability was observed in the presence of metal ions such as Hg^{2+}, Co^{2+} and Sn^{2+} (Rashbehari et al., 2003). In the above case, 100% activity was retained in the presence of Co^{2+}, whereas *E. album* protease showed an enhancement in enzyme activity to 45% when compared to the control.

Results obtained for the effect of various detergents on protease activity shows that in the case of Triton X-100, after an initial decrease, enzyme activity increased later along with increase in concentration and at 0.6%, 114% of residual activity was obtained. Tween 80 also showed a similar pattern. In the case of Tween 20, more than 80% of activity was conserved even at 5% concentration. In the presence of SDS, at lower concentration the enzyme was inactive. However, at 0.6% concentration it regained more than 85% relative activity although at higher concentration enzyme lost its activity. In the case of Brij 35, all the concentrations retained more than 70% of relative activity.

The inhibition of enzyme at higher concentration of SDS and other detergents may be the result of combined effect of factors such as reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure and the direct interactions with the protein molecule. The inhibitory effect of SDS is well documented by Creighton (1989). The increase in activity in the presence of detergents may be attributed to the increased substrate accessibility of the enzyme (Bressollier et al., 1999).

The property of stability towards oxidizing agents and SDS is important because oxidation and SDS stable enzymes from wild-type microorganisms are not

generally known except for few alkaliphiles, such as *Bacillus* sp. KSM-KP 43 (Saeki et al., 2002) and extremophiles, such as *Pyrococcus furiosus* (Blumentals et al., 1990) and *Thermococcus stetteri* (Klingeberg et al., 1995). The latest trend in enzyme based detergents is use of rDNA and protein engineering technologies to produce bio-engineered enzymes to improve catalytic efficiency and better stability towards temperature, oxidizing agents and changing washing conditions (Gupta et al., 2002b; Rao et al., 1998).

In the present study, on evaluation it was observed that H_2O_2 has a drastic inhibitory effect on protease even at the lowest concentration tried. Whereas, the reducing agents have a positive effect on protease activity except at highest concentration tried. Most of the proteases are inactive in the presence of oxidizing and reducing agents with a few exceptions like *Bacillus mojavensis* thiol dependent alkaline serine protease which was strongly stimulated up to a level of 54 and 12% in the presence of the oxidizing agents H_2O_2 and sodium hypochlorite respectively (Beg and Gupta, 2003).

A 'Met' residue located next to the catalytic serine residue is responsible for oxidative instability of subtilisin (Stezen and Leunissen, 1997) since they are readily activated by oxidants (Stauffer and Etson, 1969). To improve the oxidative stability in bleach based detergent formulations, the susceptible 'Met' residue is replaced with non-oxidisable amino acids (Estell et al., 1985).

The effect of ionic strength on protease activity suggests that increase in the ionic strength of NaCl led to decrease in enzyme activity. Thus, at 1M concentration, the enzyme retains 78% of activity and on increase in NaCl concentration, activity decreases and reaches to 7% at 4.5M concentration. In the present study, it was observed that the presence of NaCl in the fermentation medium or in the assay mixture led to a decrease in enzyme activity. Whereas, during purification and characterisation of the enzyme, it was also noticed that the

pure enzyme became inactive in the total absence of NaCl and the enzyme undergoes autolytic digestion. This suggests that even if NaCl causes decrease in enzyme activity, it has some effect on the stability of protease. Observations made in the present study indicate the scope for further research on the effect of NaCl on molecular mechanism of enzyme stabilization and activity in marine fungus.

Similar reports are available in literature were 1M NaCl caused retention of more than 80% of residual activity (Johnvesly and Naik, 2001). Activity of the protease enzyme in the presence of sodium chloride is of interest in industry for processing of dry cured meat products since, these products usually contain 1-2 M NaCl which act as a powerful inhibitor of the endogenous proteolytic enzymes (Ordonez et al., 1999).

Application of proteases for the production of certain oligopeptides has received great attention as a viable alternative to chemical approach (Fruton, 1982; Lee et al., 1993). However, the use of proteases for peptide synthesis is limited by the specificity and the instability of the enzyme in the presence of organic solvents since the reaction occurs in organic media. Proteases have been used successfully for the synthesis of dipeptide (Barros et al., 1999) and tripeptide (So et al., 2000), regioselective sugar esterification (Riva et al., 1988) and dia-stereoselective hydrolysis of peptide esters (Chen et al., 1991b). A number of reports are available on the use of alkaline protease in peptide synthesis and the resolution of racemates of amino acids. The nature and type of organic solvent have a strong effect on protease activity in organic solvents (Kawashiro et al., 1997). In the present study, *E. album* protease retained considerable amount of activity in the presence of most of the organic solvents tested. This shows its probable application in peptide synthesis and other esterification reactions; however, further investigations are required to arrive at a conclusive assessment.

Purified *E. album* protease was relatively stable for the observed period of one year. Protease stability is often measured in terms of inactivation over time. However, inactivation can occur via a number of different pathways such as autolysis, aggregation, oxidation and unfolding/denaturation (Bryan, 2000). The tightly folded enzyme tends to be resistant to proteolysis whereas unfolded or partially unfolded proteins are generally susceptible to proteolytic degradation. Additionally, loss of structure (unfolding) generally implies loss of enzymatic activity (Akasako et al., 1995; Daniel et al., 1982; Markert et al., 2001).

Effect of hydrocarbons including petrol, kerosene, used engine oil, used lubricant oil, diesel and grease on protease activity was evaluated and the results obtained indicated that there was no marked inhibition of enzyme activity. In general, the *E. album* protease retained more than 80% of its initial activity even at the highest concentration tested.

In all the oils tested, the enzyme retained more than 95% of its initial activity even at the highest concentration. It may be said that all the natural oils did not have any drastic effect on protease activity and the enzyme seems to compromise with its microenvironment demonstrating its activity. Reports on similar studies are not available for any other fungi for a direct comparison and discussion.

5.3 APPLICATION STUDIES

Commercial detergent compatibility of the enzyme tested indicated that in all the detergents, except Harpic toilet cleaner more than 90% of activity was retained even after 3hrs of incubation. In fact, Surf Excel, Surf Excel Automatic, Ariel, Rin, Wheel, Speed and Godrej Dish Wash were observed to be 100% compatible to the protease since there was no decrease in enzyme activity. This observation is complimented by the pH value of each solution where all the detergents, except Harpic, have an alkaline or near neutral pH. Harpic have a pH of

2.13 and may contain certain strong bleaches. Interestingly, the enzyme incubated in Godrej dish wash, having a pH of 6.72 also retained 100% of its activity even after 3hrs of incubation suggesting a novel property of fungal protease for industrial application. Further, visual examination of the stained cloth pieces subjected to wash treatment exhibited the effectiveness of *E. album* protease in removal of stains.

Incorporating enzymes into detergent formulations possess numerous practical problems. Proteases are susceptible to autolytic degradation, oxidation and denaturation, processes that are often enhanced by surfactants, bleaches and water softening builders that must be included in laundry detergent products. Additionally, protease catalyse the lytic degradation of other detergent enzymes that may be also present in the formulations (Crutzen and Douglass, 1999). In general, all currently used detergent compatible enzymes are alkaline and thermostable in nature with a high pH and temperature optima of 9.0-12.0 and 50 to 70°C respectively since the pH and the washing conditions are in that range. In this context, the observations made in the present study with *E. album* protease add strength to the faith that marine microbes could return many industrial enzymes of potential application.

Stability of *E. album* protease and other proteases in the presence of commercial detergents at higher temperature was studied by incubating various proteases in Surf Excel Automatic (7mg/ml) for 3hrs at 60°C and residual activity was calculated at every 30 minutes. Specific activity determined after 30 minutes of incubation showed that *E. album* protease retained the maximal specific activity. Well known protease-Esperase, retained less specific activity compared to *E. album* protease and the protease subtilisin Calsberg was totally inactive after 30 minutes of incubation at 60°C. However, in the presence of commercial detergent, except Esperase, Pronase E and *B. licheniformis* protease, all the others were totally inactive after 30 minutes of incubation. Adinarayana et al (2003) reported

that the supplementation of calcium chloride and glycine to alkaline protease from *Bacillus subtilis* PE 11 caused retention of more than 50% of activity with detergents even after 3 hrs of incubation at 60°C. This may also be applicable to the *E. album* protease and the addition of stabilizers may enhance the stability of the enzyme in the presence of detergents.

Results indicated that the enzyme has a higher affinity towards the short chain fatty acid derivatives like p-nitrophenyl butyrate (four carbon containing fatty acid) followed by p-nitrophenyl caprylate (eight carbon containing fatty acid). The enzyme is not able to cleave p-nitrophenyl laurate and p-nitrophenyl palmitate, which are the ideal substrates for lipase. This clearly indicates the esterase activity of the *E. album* protease. It was also confirmed by activity staining, which released the fluorescent 4-methylumbelliferone (MUF) that appeared as a blue band in the gel.

Hydrolytic activity of protease on the gelatin layer of the X-ray film was evaluated by incubating X-ray film in enzyme solution. Protein content of the supernatant estimated after incubation clearly evidence the ability of the enzyme to degrade the protein layer of the X- ray film even in distilled water. Visual observation of the film conclusively marks the ability of enzyme to decompose the gelatin layer of the X-ray film.

Chapter 6

SUMMARY AND CONCLUSION

Protease production by the fungus *Engiodontium album* BTMF S10, isolated from marine sediments under solid state fermentation was evaluated. The fungus was identified at MTCC, IMTECH, Chandigarh. Various process parameters affecting protease production under SSF were optimised towards maximal enzyme production. Strategy adopted for the optimisation was to evaluate the effect of various parameters individually on protease production under SSF and conduct finally a time course experiment under optimised condition.

Maximum enzyme activity of 4,351 U/gIDS was recorded for 60% moisture content at 120hrs of incubation with a specific activity of 172 U/mg proteins. Considerable level of enzyme production (3,754 U/gIDS) was also recorded for 70% moisture content at 120 hrs.

Wheat bran with particle size of <425µ supported maximum enzyme activity, protein content and specific activity (12,089 U/gIDS, 47 mg/gIDS and 254 U/mg protein respectively). As the particle size increased, there was a considerable decrease in enzyme activity. *E. album* preferred an ambient temperature of 25°C and alkaline pH of 10.0 for maximal enzyme production.

None of the additional proteinaceous substrates tested had a profound effect on enzyme production, and among the additional carbon sources tested, sucrose favoured highest enzyme yield (15,912 U/gIDS) and specific activity (341 U/mg protein). Arabinose, ribose and xylose totally inhibited enzyme production.

All the organic nitrogen sources tested, except urea showed a positive effect on enzyme production. Malt extract, soyabean meal and tryptone have almost similar effect, which caused a 47-49% increase in enzyme activity. Among the inorganic nitrogen sources tested, addition of ammonium hydrogen carbonate caused 94% increase when compared to control. Among the 19 different amino acids tested as nitrogen source, 50% had a positive effect on enzyme production. Of these, leucine caused a 44.9% increase in enzyme activity. Methionine and tryptophan caused a 92.2% and 89.6% reduction in enzyme activity when compared to the control.

Inoculum concentration of 4×10^6 cfu/ml was found to be optimal for maximal enzyme production (enzyme activity of 5,830 U/gIDS and specific activity of 199 U/mg protein).

Addition of sodium chloride to the seawater based and distilled water based media had an inverse effect on enzyme production. In the presence of seawater and distilled water based media, addition of NaCl above 1% and 5% totally inhibited protease production respectively. Similar pattern was also clearly displayed in seawater: distilled water combination.

From the time course experiment conducted under optimised condition, it was inferred that *E. albus* produced maximal protease (11,540 U/gIDS) at 120hrs.

Protease was purified employing standard protein purification procedures, which included ammonium sulphate fractionation followed by dialysis, ion-exchange chromatography and electrophoresis. It was observed that 40-90% saturation of ammonium sulphate was optimal for the complete precipitation of *E. albus* protease.

Ion exchange chromatography followed by preparative polyacrylamide gel electrophoresis furnished a single peak having protease activity with 16 and 45 fold of purification respectively.

Enzyme protein, which was eluted with 0.2 M NaCl, gave a single band in Native-PAGE. SDS-PAGE under non-reducing and reducing conditions also yielded a single band, which endorse the single polypeptide nature of the enzyme. The molecular mass of protease estimated by electrophoretic mobility showed that the *E. album* protease has an apparent molecular mass of 38kDa.The proteolytic activity of the enzyme protein band was confirmed by zymogram analysis on X-ray film. In Isoelectric focusing, the enzyme appeared as a single band testifying the purity of protease band with a pI value between 3 and 4.

Molecular weight calculated by Gel filtration chromatography yielded a value of 30kDa. Antibodies produced gave a single band in the Immunodiffusion experiment, which testified the purity of the protease.

Based on the amino acid composition, it was inferred that the enzyme protein contain approximately 255 amino acids and the molecular weight calculated for this protein gave a value of 24.8 kDa and MALDI-MS spectrum of the purified protease shows that the enzyme is pure with a single peak having the molecular weight of 28.6 kDa. MALDI-MS analysis is a more precise method with a mass accuracy of 0.05-0.1% and thus the molecular mass of *E. album* protease was taken as 28.6kDa.

Peptide finger print analysis identified three internal peptide sequences, which showed homology to *Tritirachium album* precursor Protease T. Of these, one showed homology to the conserved stretch assigned to Subtilisin class of Serine protease.

E. album protease has an optimum pH between 10.0 and 11.0 with stability over a wide range of pH from 5.0-12.0. Enzyme was active over a broad range of temperature with maximal activity at temperature 60°C and the enzyme retained 66% of activity after 12hrs of incubation at this temperature. 1mM $CaCl_2$, starch and sucrose were found to be thermal stabilizers of the enzyme at 65°C, and at 70°C starch was found to be a better thermal stabilizer of the enzyme followed by sucrose and sorbitol.

Enzyme inhibition by the serine protease inhibitors PMSF and aprotinin suggests that the protease belongs to the serine protease family. This enzyme has highest affinity for the substrate casein followed by Hemoglobin. K_m and V_{max} of the reaction was calculated as 4.727×10^{-2} and 394.68 U respectively. K_{cat} of the enzyme was estimated to be $4.2175 \times 10^{-2}\ s^{-1}$

Even lower concentrations of Hg^{2+}, Cu^{2+} and Fe^{3+} and higher concentrations of Zn^{2+}, Cd^{2+} and Al^{3+} inhibited enzyme activity. However, 1mM concentration of Co^{2+} caused an increase in enzyme activity to 45% when compared to control

In the presence of 1% H_2O_2 as oxidizing agent 57% of activity was retained by the enzyme. Reducing agents, except at its highest concentration, caused increase in activity and enzyme showed considerable level of activity even in the presence of detergents. In the presence of 1M concentration of NaCl, the enzyme retained 78% of activity. However, further increase in NaCl concentration caused inactivation of the enzyme

The enzyme showed considerable amount of activity in the presence of most of the organic solvents tested. Enzyme stability studies conducted for a period of one year testify that the enzyme is relatively stable for the observed period.

The enzyme retained more than 80% of its initial activity in all the hydrocarbons tested and more than 95% activity in the presence of natural oils even at the highest concentration tested

Commercial detergent compatibility of the enzyme tested indicates that in all the detergents, enzyme retained more than 90% of activity even after 3hrs of incubation Comparison of stability of *E. album* protease and other proteases in the presence of commercial detergent (Surf Excel Automatic) at higher temperature (60°C) showed that *E. album* protease retained maximal specific activity compared to others after 30 minutes of incubation. Further, wash performance analysis confirmed the effectiveness of *E. album* protease in stain removal.

Esterase activity of the protease was indicated by the cleavage of p-nitrophenyl derivatives like p-nitrophenyl butyrate and p-nitrophenyl caprylate and was confirmed by activity staining using methylumbelliferyl butyrate

The ability of protease for stripping the gelatin layer of the X-ray film for the recovery of silver from the used X-ray film was also demonstrated.

Conclusion

The results obtained during the course of this study indicate the scope for the utilization of this marine fungus *E. album* for extracellular protease production employing solid state fermentation. High productivity in solid state fermentation, activity and stability in alkaline pH and high temperatures, detergents, reducing and oxidizing agents, metal ions and storage stability of the enzyme advocates the potential of this enzyme for various industrial applications. Further activity in the presence of standard commercial detergents, wash performance studies, esterase activity and the ability to hydrolyse the gelatin layer on X-ray film for the recovery of silver adds evidence to its potential industrial applications. To the best of our knowledge, this is the first report on alkaline protease production by any marine

fungus especially *Engyodontium* sp that has probable industrial application. It is concluded that a scale up study on protease production in pilot scale SSF bioreactor, would contribute an ideal strain as well as bioprocess for indigenous industrial production of this enzyme and application in detergent industry. There is ample scope for further research investigation on the biochemistry of the enzyme, structure elucidation and enzyme engineering towards a wide range of further applications, besides enriching scientific knowledge on marine enzyme.

Chapter 7

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