Chapter 1

Introduction
The role of enzymes in biological processes has been known for a long time. Their existence was associated with the history of ancient Greece where they were using enzymes from microorganisms in baking, brewing, alcohol production, cheese making etc. With better knowledge and purification of enzymes the number of applications has increased many fold, and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged. Thermostable enzymes, isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Haki and Rakshit, 2003). Enzymes have been produced commercially from plant, animal and microbial sources. However microbial enzymes have an enormous advantage over the other sources because of the possibility of their production in large quantities by fermentation process. A large number of microorganisms including bacteria, yeast and fungi produce different groups of enzymes like amylases, β-galactosidases, inulinas, proteases, tannases, lipases, phytases, esterases, xylanases, cellulases, polygalacturonases, xylosidases, laccases, pectinases, hemicellulases, lignases, polymerases etc (Jonathan and Frank, 2006).

Proteases are one of the most important classes of enzymes and produced throughout the animal and plant kingdoms as well as in viruses and bacteria. The protease family has drawn special attention for drug target to cure several diseases such as osteoporosis, arthritis and cancer (Dubey et al., 2007). The current estimated value of the worldwide sales of industrial enzymes is US $1 billion (Godfrey and West, 1996). Among the specific types of industrial enzymes, proteases and amylases
lead the market with a current share of 25% and 20%, respectively. Both markets are expected to grow at approximately 2.8% average annual growth rate (AAGR). Geographically, North America market is currently leading with 36% of total market share and will continue to do so through the forecast period (upto 2009). Volume growth of industrial enzymes is between 4% and 5% AAGR, which is accompanied by decreasing prices, due to the increase in number of small players competing the market. As a result, the market is expected to rise at an AAGR of a little over 3% over the next two years, and the total industrial enzyme market in 2009 is expected to reach nearly US $ 2.4billion (Rajan, 2004). Of the industrial enzymes, 75% are hydrolytic. The protease family has drawn special attention as this constitutes one of the most important groups of industrial enzymes accounting for about 65% of the total world wide enzyme sales (Rao et al., 1998; Beg and Gupta, 2003; Ellaiah et al., 2003, Nascimento and Martins, 2004) (Fig. 1).

Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as haemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Their involvement in the life cycle of disease causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. Protease production is an inherent capacity of all microorganisms. A large number of bacterial species are known to produce alkaline proteases of serine type although very few are recognized as commercial producers. Only those microorganisms that produce substantial amounts of extracellular enzymes are of commercial importance. Several products based on alkaline proteases have
been launched successfully in the market in the past few years (Table 1). The vast
diversity of proteases, in contrast to the specificity of their action, has attracted
worldwide attention in attempts to exploit their physiological and biotechnological
applications (Poldermans, 1990; Fox et al., 1991).

1.1 SOURCES OF PROTEASES

Since proteases are physiologically necessary for living organisms,
they are ubiquitous, being found in a wide diversity of sources such as plants,
animals, and microorganisms.

1.1.1 Plant Proteases

The use of plants as a source of proteases is governed by several factors such
as the availability of land for cultivation and the suitability of climatic conditions for
growth. Moreover, production of proteases from plants is a time-consuming process.
Papain, bromelain, keratinases, and ficin represent some of the well-known proteases
of plant origin.

Papain is a traditional plant protease and has a long history of use (Schechler
et al., 1967). It is extracted from the latex of Carica papaya fruits, which are grown
in subtropical areas of west and central Africa and India. The crude preparation of the
enzyme has a broader specificity due to the presence of several proteinase and
peptidase isozymes. The performance of the enzyme depends on the plant source, the
climatic conditions for growth, and the methods used for its extraction and
purification. Three dimensional structure has been elucidated for papain, a
representative member of papain like cysteine proteases (Dubey et al., 2007).
<table>
<thead>
<tr>
<th>Supplier</th>
<th>Product trade name</th>
<th>Microbial source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novo Nordisk, Denmark</td>
<td>Alcalase</td>
<td><em>Bacillus licheniformis</em></td>
<td>Detergent, silk degumming</td>
</tr>
<tr>
<td></td>
<td>Savinase</td>
<td><em>Bacillus</em> sp.</td>
<td>Detergent, textile</td>
</tr>
<tr>
<td></td>
<td>Esperase</td>
<td><em>B. lentus</em></td>
<td>Detergent, food, silk degumming</td>
</tr>
<tr>
<td></td>
<td>Biofeed pro</td>
<td><em>B. licheniformis</em></td>
<td>Feed</td>
</tr>
<tr>
<td></td>
<td>Durazym</td>
<td><em>Bacillus</em> sp.</td>
<td>Detergent</td>
</tr>
<tr>
<td></td>
<td>Novozyme 471MP</td>
<td>n.s.</td>
<td>Photographic gelatin hydrolysis</td>
</tr>
<tr>
<td>Genencor International, USA</td>
<td>Purafact</td>
<td><em>B. lentus</em></td>
<td>Detergent</td>
</tr>
<tr>
<td></td>
<td>Primatan</td>
<td>Bacterial source</td>
<td>Leather</td>
</tr>
<tr>
<td>Gist-Brocades, The Netherlands</td>
<td>Subtilisin</td>
<td><em>B. alcalophilus</em></td>
<td>Detergent</td>
</tr>
<tr>
<td></td>
<td>Maxacal</td>
<td><em>Bacillus</em> sp.</td>
<td>Detergent</td>
</tr>
<tr>
<td></td>
<td>Maxatase</td>
<td><em>Bacillus</em> sp.</td>
<td>Detergent</td>
</tr>
<tr>
<td>Solvay Enzymes, Germany</td>
<td>Opticlean</td>
<td><em>B. alcalophilus</em></td>
<td>Detergent</td>
</tr>
<tr>
<td></td>
<td>Optimase</td>
<td><em>B. licheniformis</em></td>
<td>Detergent</td>
</tr>
<tr>
<td></td>
<td>Maxapem</td>
<td>Protein engineered variant</td>
<td>Detergent of <em>Bacillus</em> sp.</td>
</tr>
<tr>
<td></td>
<td>HT-proteolytic</td>
<td><em>B. subtilis</em></td>
<td>Alcohol, baking, brewing, feed, food, leather, photographic waste.</td>
</tr>
<tr>
<td>Company</td>
<td>Enzyme Type</td>
<td>Source</td>
<td>Industry</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------------</td>
<td>----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Amano Pharmaceuticals, Japan</td>
<td>Protease</td>
<td><em>B. licheniformis</em></td>
<td>Food, waste</td>
</tr>
<tr>
<td></td>
<td>Proleather</td>
<td><em>Bacillus sp.</em></td>
<td>Food</td>
</tr>
<tr>
<td></td>
<td>Collagenase</td>
<td><em>Clostridium sp.</em></td>
<td>Technical</td>
</tr>
<tr>
<td></td>
<td>Amano protease S</td>
<td><em>Bacillus sp.</em></td>
<td>Food</td>
</tr>
<tr>
<td>Enzyme Development, USA</td>
<td>Enzeco alkaline</td>
<td><em>B. licheniformis</em></td>
<td>Industrial</td>
</tr>
<tr>
<td></td>
<td>Enzeco alkaline</td>
<td><em>B. licheniformis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enzeco protease-L FG</td>
<td><em>B. licheniformis</em></td>
<td>Food</td>
</tr>
<tr>
<td></td>
<td>Enzeco high alkaline protease</td>
<td><em>Bacillus sp.</em></td>
<td>Industrial</td>
</tr>
<tr>
<td>Nagase Biochemicals, Japan</td>
<td>Bioprase concentrate</td>
<td><em>B. subtilis</em></td>
<td>Cosmetic, pharmaceuticals</td>
</tr>
<tr>
<td></td>
<td>Ps. protease</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Research</td>
</tr>
<tr>
<td></td>
<td>Ps. elastase</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Research</td>
</tr>
<tr>
<td></td>
<td>Cryst. protease</td>
<td><em>B. subtilis (K2)</em></td>
<td>Research</td>
</tr>
<tr>
<td></td>
<td>Cryst. protease</td>
<td><em>B. subtilis (bioteus)</em></td>
<td>Research</td>
</tr>
<tr>
<td></td>
<td>Bioprase</td>
<td><em>B. subtilis</em></td>
<td>Detergent, cleaning</td>
</tr>
<tr>
<td></td>
<td>Bioprase SP-10</td>
<td><em>B. subtilis</em></td>
<td>Food</td>
</tr>
<tr>
<td>Godo Shusei, Japan</td>
<td>Godo-Bap</td>
<td><em>B. licheniformis</em></td>
<td>Detergent, food</td>
</tr>
<tr>
<td>Rohm, Germany</td>
<td>Corolase 7089</td>
<td><em>B. subtilis</em></td>
<td>Food</td>
</tr>
<tr>
<td>Wuxi Synder</td>
<td>Wuxi</td>
<td><em>Bacillus sp.</em></td>
<td>Detergent</td>
</tr>
<tr>
<td>Advance Biochemicals, India</td>
<td>Protosol</td>
<td><em>Bacillus sp.</em></td>
<td>Detergent</td>
</tr>
<tr>
<td>n.s. Not specified.</td>
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</tr>
</tbody>
</table>
Cysteine proteases of the papain super family are widely distributed in nature. They are found in both prokaryotes and eukaryotes e.g. bacteria, parasites, plant invertebrates and vertebrates (Berti and Storer, 1995). The enzyme is active between pH 5 and 9 and is stable up to 80 - 90°C in the presence of substrates. It is extensively used for the preparation of highly soluble and flavored protein hydrolysates. Besides some of these proteases have also been used as model systems for studies on structure-function relationships and protein folding problems (Dubey and Jagannadham, 2003).

Bromelain is prepared from the stem and juice of pineapples. The major supplier of the enzyme is Great Food Biochem., Bangkok, Thailand. The enzyme is characterized as a cysteine protease and is active in the range of pH 5-9. Its inactivation temperature is 70°C, which is lower than that of papain (Rowan et al., 1990).

Some plants produce proteases which degrade hair. Digestion of hair and wool is important for the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems.

1.1.2 Animal Proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins (Boyer, 1971). These are prepared in pure form in bulk quantities. However, their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies.
Trypsin (Mr 23,300) is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues (Table 2). Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, this enzyme has received attention as a target for biocontrol of insect pests.

1.1.3 Microbial Proteases

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey, 1996). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.
Table 2. Specificity of proteases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Peptide bond cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>Lys (or Arg)</td>
</tr>
<tr>
<td>Chymotrypsin, Subtilisin</td>
<td>Trp (or Tyr, Phe, Leu)</td>
</tr>
<tr>
<td><em>Staphylococcus</em> V8 Proteases</td>
<td>Asp (or Glu)</td>
</tr>
<tr>
<td>Papain</td>
<td>Phe (or Val, Leu) Xaa</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>...... Leu (or Phe)</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Phe (or Tyr, Leu) Trp(or Phe,Tyr)</td>
</tr>
</tbody>
</table>

1.1.3.1 Bacteria

Commercial proteases are mostly from various sources and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria (Ferrero et al., 1996). The alkaline proteases from these species represent the subtilisins. Members of subtilisin super family of proteases have now been identified with different functions in all living organisms (Siezen and Leunissen, 1997). Thermophiles such as *Thermus aquaticus* (Gabriela et al., 2003), *Bacillus licheniformis* (Ferrero et al., 1996), *Bacillus stearothermophilus* (Boonayanan et al., 2000), *Bacillus pumilus* (Kumar, 2002), *Thermoanerobacter yonseensis* (Hyenung et al., 2002), *Bacillus thermoruber* (Manachini et al., 1988) and *Bacillus brevis* (Banerjee et al., 1999) have been studied for their capability to produce thermostable proteases. Thermostable proteases are advantageous as they are highly stable at elevated temperatures, reduce risk of contamination by other organisms, ideal models for studying thermal stability of protein, elucidation of mechanisms involved in thermostability of enzymes (Helmann, 1995). Most enzymes used are mainly derived from mesophilic sources that work in narrow ranges of pH and moderate temperatures. However, alkaline proteases exhibit optimum activity and stability at high pH, temperatures and in the presence of surfactants and chaotropic agents, which make them suitable candidates for industrial applications (Kumar, 1999). Bacterial alkaline proteases are characterized by their high activity at alkaline pH, (pH 10), 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.
1.1.3.2 Fungi

Fungi elaborate a wider variety of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. However, they have a lower reaction rate and low heat tolerance than the bacterial enzymes. Fungal enzymes can be conveniently produced in a Solid state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheese making industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases, active at pH 7.0 and inhibited by chelating agents. In view of the accompanying peptidase activity and their specific function in hydrolyzing bonds between hydrophobic amino acids, fungal neutral proteases supplement the action of plant, animal and bacterial proteases in reducing the bitterness of food protein hydrolysates. Fungal alkaline proteases are also used in food protein modification.

1.1.3.3. Viruses

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 *et al.*, 1993). All of the virus-encoded peptidases are endopeptidases and there are no metallopeptidases. Retroviral aspartyl proteases 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 (Kubo et al., 1989). Extensive research was focused on the three-dimensional structure of viral proteases and their interaction with synthetic inhibitors with a view to design potent inhibitors that can combat the relentlessly spreading and devastating epidemics of AIDS. Thus, although proteases are widespread in nature, microorganisms serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications.

1.2. CLASSIFICATION OF PROTEASES

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). 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 (Barret, 1994). Proteases are grossly subdivided into two major groups based on the type of reaction they catalyse 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 six prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, metalloproteases and unknown type (Hartley, 1960). The threonine protease is the recently discovered (Seemuller et al., 1995). There are a few miscellaneous proteases, which do not precisely fit into the standard
classification, e.g., ATP-dependent proteases which require ATP for activity (Menon et al., 1987). Based on their amino acid sequences, proteases are classified into different families (Argos, 1987) and further subdivided into “clans” to accommodate sets of peptidases that have diverged from a common ancestor (Rawlings et al., 1993). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, and U for serine, cysteine, aspartic, metallo and unknown type, respectively.

1.2.1. 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.

1.2.1.1. 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 *A. 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 profiles of the products of hydrolysis (Cerny, 1978). Aminopeptidase I from *Escherichia coli* is a large protease (400,000 Da) and has a broad pH optimum of 7.5 to 10.5 and requires Mg$^{2+}$
or Mn\(^{2+}\) for optimal activity (De Marco et al., 1978). *Bacillus licheniformis* aminopeptidase has a molecular weight of 34 kDa. It contains 1g-atom of Zn\(^{2+}\) per mol and its activity is enhanced by Co\(^{2+}\) ions. On the other hand, aminopeptidase II from *B. stearothermophilus* is a dimer with a molecular weight of 80 to 100 kDa and is activated by Zn\(^{2+}\), Mn\(^{2+}\) and Co\(^{2+}\) ions (Stoll et al., 1976).

1.2.1.2. Carboxypeptidases

The carboxypeptidases act at C terminus of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallo carboxypeptidases, 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* sp. *Saccharomyces* sp. and *Aspergillus* sp. 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* sp. (Felix et al., 1966) and *Pseudomonas* sp. (Lu et al., 1969; Gupta et al., 2005) require Zn\(^{2+}\) or Co\(^{2+}\) for their activity. The enzymes can also hydrolyze the peptides in which the peptidyl group is replaced by a pteroyl moiety or by acyl groups.

1.2.2. 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 endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases. To facilitate quick and unambiguous reference to a particular
family of peptidases, Rawlings and Barrett (1993) assigned a code letter denoting the catalytic type, i.e., S, C, A, M and U (serine, cysteine, aspartic, mettalo and unknown type respectively) followed by an assigned number. Hartley (1960) classified endoproteases into four groups on the basis of their active site and sensitivity to various inhibitors whose properties are briefed in Table 3.

Serine proteases

Serine proteases are characterized by the presence of a serine residue 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, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further, subdivided into about six clans with common ancestors (Barret, 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 (repressor LexA) 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.
Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as p-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity. Their molecular masses range between 18 and 35 kDa. The serine protease from *Blakeslea trispora*, which has a molecular mass of 126 kDa, was also reported (Govind *et al.*, 1981). The isoelectric point of serine proteases are generally between pH 4 and 6. The serine proteases are further classified on the basis of their side-chain specificity against the oxidized insulin β-chain (Morihara, 1974) and structural homology to well established proteases. A comprehensive account of subclasses of serine proteases is as follows.
<table>
<thead>
<tr>
<th>Properties</th>
<th>ECNo.</th>
<th>Molar mass range/ kDa</th>
<th>pH Optimum</th>
<th>Temperature optimum/°C</th>
<th>Metal ion requirement(s)</th>
<th>Active site amino acid(s)</th>
<th>Major inhibitor(s)</th>
<th>Major source(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic or carboxyl proteases</td>
<td>3.4.23</td>
<td>30–45</td>
<td>3–5</td>
<td>40–55</td>
<td>Ca²⁺</td>
<td>Aspartate or cysteine</td>
<td>Peptatin</td>
<td>Aspergillus, Mucor, Endothia, Rhizopus, Penicillium Neurospora, animal tissue (stomach)</td>
</tr>
<tr>
<td>Cysteine or thiol proteases</td>
<td>3.4.22</td>
<td>34–35</td>
<td>2–3</td>
<td>40–55</td>
<td>Aspartate or cysteine</td>
<td>Indacacetamide, p-CMB</td>
<td>Aspergillus stem of pineapple (Ananas comorus), latex of fig tree (Ficus sp.), papaya (Carica papaya), Streptococcus, Clostridium</td>
<td></td>
</tr>
<tr>
<td>Metallo proteases</td>
<td>3.4.24</td>
<td>19–37</td>
<td>5–7</td>
<td>65–85</td>
<td>Zn²⁺ Ca²⁺</td>
<td>Phenylalanine or leucine</td>
<td>Chelating agents such as EDTA, EGTA</td>
<td>Bacillus, Aspergillus, Penicillium, Pseudomonas, Streptomyces</td>
</tr>
<tr>
<td>Serine proteases</td>
<td>3.4.21</td>
<td>18–35</td>
<td>6–11</td>
<td>50–70</td>
<td>Ca²⁺</td>
<td>Serine, histidine and aspartate</td>
<td>PMSF, DIFP, EDTA, soybean trypsin inhibitor, phosphate buffers, indole, phenol, triamino acetic acid</td>
<td>Bacillus, Aspergillus, animal tissue (gut), Tritirachium album (thermostal-e)</td>
</tr>
</tbody>
</table>
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). 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, and their isoelectric point is around pH 9. Their molecular masses are in the range of 15 to 30 kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter*, and *Flavobacterium* sp. (Boguslowski *et al.*, 1983), subtilisins produced by *Bacillus* sp. are the best known (Maurer, 2004).

Alkaline proteases are also produced by *S. cerevisiae* (Mizuno *et al.*, 1984) and filamentous fungi such as *Conidiobolus* sp. (Phadatare *et al.*, 1993) *Aspergillus* sp. (Anandan *et al.*, 2007) and *Neurospora* sp. (Lindberg *et al.*, 1981).

Chymotrypsin-like proteases

Chymotrypsin, a mammalian digestive protease, has structural homology with trypsin, elastase and thrombin (Graycar, 1999). It is specific for basic amino acids and is most active at pH 8. The most important inhibitors of this class are *N*-tosyl-L-lysine chloromethyl ketone, *L*-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), DFP and soybean trypsin inhibitor. The molecular weight is generally around 20 kDa. This group is well represented by proteases of animal origin and those belonging to various species of *Streptomyces* like *S. erythreus*, *S. fradiae* and *S. griseus* (Ward, 1985; Kalisz, 1988).
Subtilisin-like proteases or subtilases

Subtilisin-like serine proteases are generally bacterial in origin, although they are reported in other organisms (Siezen and Leunissen, 1997). They are generally secreted extracellularly for the purpose of scavenging nutrients (Graycar, 1999). This class of proteases is specific for aromatic or hydrophobic residues (at position P1), such as tyrosine, phenylalanine and leucine. They are highly sensitive towards phenyl methyl sulphonyl fluoride (PMSF), DFP and potato inhibitor. They are most active around pH 10, with a molecular weight range of 15–30 kDa and an isoelectric point near 9. 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 Nagase (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 laundry detergents and in automatic dishwashing detergents. Their function is to degrade proteinaceous stains; typical stains include blood, milk, egg, grass and sauces (Aehle, 2004). 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 27.5 kDa 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. Both enzymes exhibit broad substrate specificity and have an active-site triad made up of Ser221, His64 and Asp32. The Carlsberg enzyme has a 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. This class is well represented by various species.
of *Bacillus* like *B. amyloliquifaciens, B. licheniformis* and *B. subtilis* (Rao et al., 1998). It is also produced by *Flavobacterium* (Morita et al., 1998). 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).

**Wheat serine carboxypeptidase II-like proteases**

These have a different catalytic triad residue in their active site and their tertiary folds are also completely different for each structural family. They are most active in the pH range of 4.5–5.5 suggesting an unusual low pKa value for its catalytic histidine residue (Liao and Remington, 1990).

**Prolyl oligopeptidase-like serine proteases**

Not much is known about this class of proteases but this group was defined based on the results obtained using sequence homology techniques (Rawlings et al., 1991).

**Myxobacter α-lytic proteases**

These exhibit strong bacteriolytic activity towards soil bacteria and are represented by various species of *Sorangium*. They are specific towards the carboxyl group of neutral and aliphatic amino acids. They show maximum activity around pH 9 and are sensitive towards DFP (Morihara, 1974).
Staphylococcal protease

*Staphylococcus aureus* produces a DFP sensitive protease of 12 kDa with maximum activity in the pH range of 4.0–7.8. The enzyme is specific towards peptide bonds with acidic amino acid residues at the carboxylic group (Morihara, 1974).

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. 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 (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. The members of the pepsin family have a bilobal structure with the active site cleft located between the lobes (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 diazoacetyl-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* sp.
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 differ among the families (Barret, 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 sp. 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 50 kDa, whereas the isoelectric point and molecular mass of streptopain are pH 8.4 and 32 kDa, respectively.

Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Barret, 1995). They are characterized by the requirement of 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 (Weaver et al., 1977; Hibbs et al., 1985; Okada et al., 1986; Wilhelm et al., 1987; Shannon et al., 1989). About 30 families of metalloproteases have been recognized, of which 17 contain endopeptidases, 12 contain only exopeptidases, and 1 (M3) only contains both endo- and exopeptidases. Families of metalloproteases have been grouped into different clans based on the nature of the amino acid that competes for 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 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 34 kDa. 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 broth of the anaerobic bacterium
**1.3 MECHANISM OF ACTION OF PROTEASES**

The mechanism of action of proteases has been a subject of great interest to researchers. 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, and gel filtration
techniques, have been well documented (Gupta et al., 2002). Preparative polyacrylamide gel electrophoresis has been used for the purification of proteases from *Conidiobolus coronatus* (Phadatare et al., 1993). Purification of staphylocoagulase to homogeneity was carried out from culture filtrates of *Staphylococcus aureus* by affinity chromatography with a bovine prothrombin-Sepharose 4B column (Igarashi et al., 1979) and gel filtration (Zen-Yogi et al., 1961).

A number of peptide hydrolases have been isolated and purified from *E. coli* by DEAE-cellulose chromatography (Perlmann et al., 1970). The catalytic site of proteases is flanked on one or both sides by specificity subsites, each able to accommodate the side chain of a single amino acid residue from the substrate. These sites are numbered from the catalytic site S1 through Sn toward the N terminus of the structure and S1 through Sn toward the C terminus. The residues which they accommodate from the substrate are numbered P1 through Pn and P1 through Pn, respectively (Fig. 2).
Fig. 2. Active sites of proteases. The catalytic site of proteases is indicated by * and the scissible bond is indicated by .......; S1 through Sn and S1' through Sn' are the specificity subsites on the enzyme, while P1 through Pn and P1' through Pn' are the residues on the substrate accommodated by the subsites on the enzyme.
1.3.1. 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 et al., 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. Serine endopeptidases can be classified into three groups based mainly on their primary substrate preference: (i) trypsin-like, which cleave after positively charged residues; (ii) chymotrypsin-like, which cleave after large hydrophobic residues; and (iii) elastase-like, which cleave after small hydrophobic residues. The Pl residue exclusively dictates the site of peptide bond cleavage. The primary specificity is affected only by the Pl residues; the residues at other positions affect the rate of cleavage. The subsite interactions are localized to specific amino acids around the Pl residue to a unique set of sequences on the enzyme. Some of the serine peptidases from Achromobacter sp. are lysine-specific enzymes (Masaki et al., 1978), whereas those from Clostridium sp. are arginine specific (clostripain) (Gilles et al., 1979) and those from Flavobacterium sp. are 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 S. 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 (Barret, 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* sp. 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 yeasts *Tritirachium* and *Metarhizium* sp. 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 *et al.*, 1976).

### 1.3.2. 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; Sielecky *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 Asp-Thr-Gly-Xaa motif in both the N- and C-terminal lobes of the enzyme, where Xaa is Ser or Thr, whose side chains can hydrogen bond to Asp. The catalytic site of proteases is indicated by p and the scissile bond is indicated by Å; S1 through Sn and S19 through Sn9 are the specificity subsites on the enzyme, while P1 through Pn and P19 through Pn9 are the residues on the substrate accommodated by the subsites on the enzyme. 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 (Lindberg et al., 1981). 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. 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).

1.3.3. Metalloproteases

The mechanism of action of metalloproteases 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 (Holmes et al., 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.

1.3.4. Cysteine Proteases

Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of serine proteases. A striking similarity is also observed in the reaction mechanism of several peptidases of different evolutionary origins. 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).

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 acid-base catalysis as their mechanisms of action. The activity of metalloproteases depends on the binding of a divalent metal ion to a His-Glu-Xaa-Xaa-His motif. Cysteine proteases adopt a hydrolysis mechanism involving a general acid-base formation followed by hydrolysis of an acyl-thiol intermediate.
1.4 PHYSIOLOGICAL FUNCTIONS OF PROTEASES

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. Proteases play a critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones, pharmacologically active peptides from precursor proteins and transport of secretory proteins across membranes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism. In contrast to the multitude of the roles contemplated for proteases, our knowledge about the mechanisms by which they perform these functions is very limited. Extensive research is being carried out to unravel the metabolic pathways in which proteases play an integral role, this research will continue to contribute significantly to our present state of information. Some of the major activities in which the proteases participate are described below.

1.4.1. Protein Turnover

All living cells maintain a particular rate of protein turnover by continuous, albeit balanced, degradation and synthesis of proteins. Catabolism of proteins provides a ready pool of amino acids as precursors for the synthesis of proteins. Intracellular proteases are known to participate in executing the proper protein turnover for the cell. In *E. coli*, ATP-dependent protease La, the *lon* gene product, is responsible for hydrolysis of abnormal proteins (Chung *et al.*, 1981). The turnover of intracellular proteins in eukaryotes is also affected by a pathway involving ATP-
dependent proteases (Hershko et al., 1984). Evidence for the participation of proteolytic activity in controlling the protein turnover was demonstrated by the lack of proper turnover in protease-deficient mutants.

1.4.2 Sporulation and Conidial Discharge

The formation of spores in bacteria (Kornberg et al., 1968), ascospores in yeasts (Esposito et al., 1981), fruiting bodies in slime molds (North, 1982) and conidial discharge in fungi (Phadatare et al., 1989) involve intensive protein turnover. The requirement of a protease for sporulation has been demonstrated by the use of protease inhibitors (Dancer et al., 1975). Ascospore formation in yeast diploids was shown to be related to the increase in protease activity (Esposito et al., 1981). Extensive protein degradation accompanied the formation of a fruiting body and its differentiation to a stalk in slime molds. The alkaline serine protease of *Conidiobolus coronatus* was shown to be involved in forcible conidial discharge by isolation of a mutant with less conidial formation (Phadatare et al., 1989). Formation of the less active protease by autoproteolysis represents a novel means of physiological regulation of protease activity in *C. coronatus* (Phadatare et al., 1993).

1.4.3. Germination

The dormant spores lack the amino acids required for germination. Degradation of proteins in dormant spores by serine endoproteinases makes amino acids and nitrogen available for the biosynthesis of new proteins and nucleotides. These proteases are specific only for storage proteins and do not affect other spore proteins. Their activity is rapidly lost on germination of the spores (Postemsky et al., 1978). Conidal germination and hyphal fusion also involve the participation of a specific alkaline serine protease (Leighton et al., 1970). Extracellular acid proteases
are believed to be involved in the breakage of cell wall polypeptide linkages during germination of *Dictyostelium discoideum* spores (Jackson *et al.*, 1984) and *Polysphondylium pallidum* microcysts (O'Day, 1976).

### 1.4.4. Enzyme Modification

Activation of the zymogenic precursor forms of enzymes and proteins by specific proteases represents an important step in the physiological regulation of many rate-controlling processes such as generation of protein hormones, assembly of fibrils and viruses, blood coagulation and fertilization of ova by sperm. Activation of zymogenic forms of chitin synthase by limited proteolysis has been observed in *Candida albicans*, *Mucor rouxii* and *Aspergillus nidulans*. Kex-2 protease (Kexin; EC 3.4.21.61), originally discovered in yeast, has emerged as a prototype of a family of eukaryotic precursor processing enzymes (Barret, 1994). It catalyzes the hydrolysis of prohormones and of integral membrane proteins of the secretory pathway by specific cleavage at the carboxyl side of pairs of basic residues such as Lys-Arg or Arg-Arg. Furin (EC 3.4.21.5) is a mammalian homologue of the Kex-2 protease that was discovered serendipitously and has been shown to catalyze the hydrolysis of a wide variety of precursor proteins at Arg-X-Lys or Arg-Arg sites within the constitutive secretory pathway (Smeekens, 1993). Pepsin, trypsin and chymotrypsin occur as their inactive zymogenic forms which are activated by the action of proteases. Proteolytic inactivation of enzymes, leading to irreversible loss of *in vivo* catalytic activity, is also a physiologically significant event. Several enzymes are known to be inactivated in response to physiological or developmental changes or after a metabolic shift. Proteinases A and B from yeast inactivate several enzymes in a two-step process involving covalent modification of proteins as a marking mechanism for proteolysis. Proteolytic modification of enzyme is known to result in a protein with altered
physiological function, e.g., leucyl-L-RNA synthetase from *E. coli* is converted into an enzyme which catalyzes leucine-dependent pyrophosphate exchange by removal of a small peptide from the native enzyme.

1.4.5. Nutrition

Proteases assist the hydrolysis of large polypeptides into smaller peptides and amino acids thus facilitating their absorption by the cell. The extracellular enzymes play a major role in nutrition due to their depolymerizing activity. The microbial enzymes and the mammalian extracellular enzymes such as those secreted by pancreas are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition.

1.4.6. Regulation of Gene Expression

Modulation of gene expression mediated by proteases has been demonstrated (Robert *et al.*, 1977). Proteolysis of a repressor by an ATP requiring protease resulted in a derepression of the gene. A change in the transcriptional specificity of the B subunit of *Bacillus thuringiensis* RNA polymerase was correlated with its proteolytic modification (Lecadet *et al.*, 1977). Modification of ribosomal proteins by proteases has been suggested to be responsible for the regulation of translation (Kalisz, 1988). Besides the general functions described so far, the proteases also mediate the degradation of a variety of regulatory proteins that control the heat shock response, the SOS response to DNA damage, the life cycle of bacteriophage (Gottesman *et al.*, 1992), and programmed bacterial cell death (Van Melderen *et al.*, 1996). Recently, a new physiological function has been attributed to the ATP-dependent proteases conserved between bacteria and eukaryotes. It is believed that they act as chaperones and mediate not only proteolysis but also the insertion of proteins into membranes and
the disassembly or oligomerization of protein complexes (Suzuki et al., 1997). In addition to the multitude of activities that are already assigned to proteases, many more new functions are likely to emerge in the near future.

1.5 APPLICATIONS OF PROTEASES

Proteases have a large variety of applications mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and 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 such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those used in medicine are produced in small amounts but require extensive purification.

Detergent industry

The history of detergent enzymes dates back to 1914, when two German scientists, Rohm and Haas used pancreatic proteases and sodium carbonate in washing detergents. The product was named “Burnus”, after the white Arab cloak. The first detergent containing the bacterial enzyme was introduced into the market in 1956 under the trade name Bio-40. However, it was only in 1963 an alkaline protease, “Alcalase” was effectively incorporated in the detergent powder and was marketed by Novo Industry, Denmark under the trade name Biotex. 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 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 Netherlands, Genencor International in the United States, Solvay in Belgium and Showa-Kenko in Japan. These suppliers marketed a full range of enzymes for liquid and powder detergents. In the beginning of the year 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. Today, Novo Nordisk and Genencor International are the major suppliers of detergent enzymes supplying up to 95% of the global market of proteases.

The biggest market for detergents is in the laundry industry amounting to a worldwide production of 13 billion tons per year. The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood, and other body secretions. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to the detergents are among the major prerequisites for the use of proteases in detergents.

The key parameter for the best performance of a protease in a detergent is its pi. It is known that a protease is most suitable for this application if its pi coincides with the pH of the detergent solution. Esperase and Savinase T (Novo Industry), produced by alkalophilic *Bacillus* sp. are two commercial preparations with very high isoelectric points (pi 11). Hence, they can withstand higher pH ranges. Due to the present energy crisis and the awareness for energy conservation, it is desirable to use proteases that
are active at lower temperatures. A combination of lipase, amylase, and cellulase is expected to enhance the performance of protease in laundry detergents. All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. An alkaline protease from *Conidiobolus coronatus* was found to be compatible with commercial detergents used in India (Phadatare *et al*., 1993) and retained 43% of its activity at 50°C for 50 min in the presence of CaCl₂ (25 mM) and glycine (1 M) (Bhosale *et al*., 1995).

**Leather Industry**

Leather processing involves several steps such as soaking, dehairing, bating, and tanning. The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards. Thus, for environmental reasons, the treatment of leather using an enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction, thus being ecofriendly (Andersen, 1998). Alkaline proteases with elastolytic and keratinolytic activity can be used in leather-processing industries. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular (Varela *et al*., 1997). Alkaline proteases speed up the process of 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. The use of *B. subtilis* IIQDB32 alkaline protease (Varela *et al*., 1997) for dehairing sheep skin, *B. amyloliquefaciens* alkaline protease for dehairing hides and
skins (George et al., 1995) and B. subtilis K2 alkaline protease in bating and leather processing (Hameed et al., 1999) is recently reported. Novo Nordisk reported three different proteases, Aquaderm, NUE and Pyrase, for use in soaking, dehairing, and bating, respectively.

**Food and Feed industry**

The use of proteases in the food industry dates back to antiquity. Traditionally, microbial proteases have been exploited in the food industries in many ways. Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks (Ward, 1985; Neklyudov et al., 2000). The basic function of proteases is to hydrolyze proteins and this property has been exploited for the preparation of protein hydrolysates of high nutritional value. The alkaline proteases are used in hydrolysate production from various natural protein substrates. The commercial protein hydrolysates are derived from casein (Miprodan, MD Foods), whey (Lacprodan, MD Foods) and soy protein (Proup, Novo Nordisk, Bagsvaerd, Denmark). B. subtilis proteases are used in the production of fish hydrolysates of high nutritional value (Rebeca et al., 1991). Matsui et al. (1993) reported on protease hydrolysates having angiotensin-I-converting enzyme-inhibitory activity from sardine muscle obtained after treatment with B. licheniformis alkaline protease. Fujimaki et al. (1970) used alkaline protease for the production of soy protein hydrolysates. Cheese whey is an abundant liquid by-product of the dairy industry with an estimated world production of 145X10^6 ton/year. Perea et al. (1993) used alkaline protease for the production of whey protein hydrolysate using cheese whey in an industrial whey bioconversion process. O’Meara and Munro (1984)
reported the up-grading of lean meat waste to edible products by alkaline protease hydrolysis of the meat waste using commercial alkaline proteases. Ohmiya et al. (1979) reported the use of immobilized alkaline protease (on Dowex MWA-1 by gluteraldehyde) in cheese-making. Tanimoto et al. (1991) reported the use of alkaline protease in the enzymatic modification of zein to produce a non-bitter peptide fraction with high Fischer ratio for patients with hepatic encephalopathy. Keratinolytic activity of alkaline protease has also been exploited in the production of proteinaceous fodder from waste feathers or keratin-containing materials. The use of alkaline proteases (B72 from B. subtilis and B. licheniformis PWD-1) for the hydrolysis of feather keratin to obtain a protein concentrate for fodder production is also reported (Dalev, 1994; Cheng et al., 1995).

**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 in the range 30 to 40 kDa. 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. The microbial enzymes exhibited two major drawbacks, i.e., (i) the presence of high levels of non-specific and heat-stable proteases which led to the development of bitterness in cheese after storage and (ii) a poor yield. 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 cheesemaking, the primary function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-k-casein and macropeptides. Chymosin is preferred due to its high specificity for casein which is responsible for its excellent performance in cheesemaking. The proteases produced by microbes such as *Mucor miehei*, *Bacillus subtilis*, and *Endothia parasitica* are gradually replacing chymosin in cheesemaking. In 1988, chymosin produced through recombinant DNA technology was first introduced to cheese makers for evaluation. Genencor International increased the production of chymosin in *Aspergillus niger* var. *awamori* to commercial levels. At present, their three recombinant chymosin products are available and are awaiting legislative approval for their use in cheesemaking (Godfrey *et al.*, 1996). Whey is a by-product of cheese making. It contains lactose, proteins, minerals, and lactic acid. The insoluble heat denatured whey protein is solubilized by treatment with immobilized trypsin.

**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. Bacterial proteases are used to improve the extensibility and strength of the dough.

**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. Proteolytic modification of soy proteins helps to improve their functional properties. Treatment of soy proteins with alcalase at pH 8 results in soluble hydrolysates with high solubility, good protein yield, and low bitterness. The hydrolysate is used in protein-fortified soft drinks and in the formulation of dietetic feeds.

**Debittering of protein hydrolysates**

Protein hydrolysates have several applications, e.g., as constituents of dietetic and health products, in infant formulae and clinical nutrition supplements 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.

**Synthesis of aspartame**

The Food and Drug Administration have approved the use of aspartame as a noncalorific artificial sweetener. Aspartame is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. The L configuration of the two amino
acids is responsible for the sweet taste of aspartame. Maintenance of the stereospecificity is crucial, but it adds to the cost of production by chemical methods. Enzymatic synthesis of aspartame is therefore preferred. Although proteases are generally regarded as hydrolytic enzymes, they catalyze the reverse reaction under certain kinetically controlled conditions. An immobilized preparation of thermolysin from *Bacillus thermoproteolyticus* is used for the enzymatic synthesis of aspartame. Toya Soda (Japan) and DSM (Netherlands) are the major industrial producers of aspartame.

**Pharmaceutical Industry**

The wide diversity and specificity of proteases are used to a 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 aspartagine from the bloodstream in the various forms of lymphocytic leukemia. Alkaline protease from *Conidiobolus coronatus* was found to be able to replace trypsin use in removal of monolayer animal cell cultures from flask (Chiplonkar *et al.*, 1985).

**Photographic industry**

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 polyester film base can be recycled. Alkaline protease from *B. subtilis* decomposed the gelatin layer within 30 min at 50–60 °C and released the silver (Fujiwara et al., 1989). Ishikawa et al. (1993) reported the use of alkaline protease of *Bacillus* sp. B21-2 for the enzymatic hydrolysis of gelatin layers of X-ray films to release silver particles. The alkaline proteases of *Bacillus* sp. B18 (Fujiwara et al., 1991) and *B. coagulans* PB-77 (Gajju et al., 1996) were also efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered.

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, as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.

1.5 **GENETIC ENGINEERING OF MICROBIAL PROTEASES**

Gene cloning is a rapidly progressing technology that has been instrumental in improving our understanding of the structure function relationship of genetic systems. It provides an excellent method for the manipulation and control of genes. More than 50% of the industrially important enzymes are now produced from genetically engineered microorganisms (Hodgson, 1994). Several reports have been published in the past decade on the isolation and manipulation of microbial protease genes with the
aim of (i) enzyme overproduction by the gene dosage effect, (ii) studying the primary structure of the protein and its role in the pathogenicity of the secreting microorganism, and (iii) protein engineering to locate the active-site residues and/or to alter the enzyme properties to suit its commercial applications. Protease genes from bacteria, fungi, and viruses have been cloned and sequenced.

1.6.1. *B. subtilis* as a host for cloning of protease genes from *Bacillus* sp.

The ability of *B. subtilis* to secrete various proteins into the culture medium and its lack of pathogenicity make it a potential host for the production of foreign polypeptides by recombinant DNA technology. Several *Bacillus* sp. secrete two major types of protease, a subtilisin or alkaline protease and a metalloprotease or neutral protease, which are of industrial importance. Studies of these extracellular proteases are significant not only from the point of view of overproduction but also for understanding their mechanism of secretion. *B. subtilis* 168 secretes at least six extracellular proteases into the culture medium at the end of the exponential phase. The structural genes encoding the alkaline protease (*apr*) or subtilisin (Stahl *et al.*, 1984), neutral protease A and B (*nprA* and *nprB*) (Yang *et al.*, 1984; Henner *et al.*, 1985; Tran *et al.*, 1991), minor extracellular protease (*epr*) (Brueckner *et al.*, 1990; Sloma *et al.*, 1988), bacillopeptidase F (*bpr*) (Sloma *et al.*, 1990), and metalloproteases (*mpr*) (Sloma *et al.*, 1990) have been cloned and characterized. These proteases are synthesized in the form of a "prepro" enzyme. To increase the expression of subtilisin and neutral proteases, Henner *et al.* (1985), replaced the natural promoters of *apr* and *npr* genes with the amylase promoter from *B. amyloliquefaciens* and the neutral protease promoter from *B. subtilis*, respectively. To understand the regulation of *npr A* gene expression, Toma *et al.* (1986) cloned the genes from *B. subtilis* 168 (normal producer) and Base 1A341 (overproducer). The
two genes were found to be highly homologous except for a stretch of 66 bp close to the promoter region which is absent in the Bac 1A341 gene. The epr gene shows partial homology to the apr gene and to the major intracellular serine protease (Isp-1) gene of B. subtilis (Koide et al., 1986). The epr gene was mapped at a locus different from the apr and npr loci on the B. subtilis chromosome and was shown not to be required for growth or sporulation, similar to apr or npr genes. Deletion of 240 amino acids from the C-terminal region of the epr gene product did not abolish the enzyme activity (Brueckner et al., 1990). The deduced amino acid sequence of the mature bpr gene product is similar to those of other serine proteases of B. subtilis, i.e., subtilisin, Isp-1, and Epr. B. subtilis strains containing mutations in five extracellular protease genes (apr, npr, epr, mpr, and bpr) have been constructed (Sloma et al., 1990) with the aim of expressing heterologous gene products in B. subtilis. The total amino acid sequence of B. subtilis Isp-1 deduced from the nucleotide sequence showed considerable homology (45%) to subtilisin. Highly conserved sequences are present around the essential amino acids, Ser, His, and Asp indicating that the genes for both the intra and extracellular serine proteases have a common ancestor. Yamagata et al. (1995) cloned and sequenced a 90-kDa serine protease gene (hspK) from B. subtilis (Natto) 16. The large size of the enzyme may represent an ancient form of bacterial serine protease. Analysis of DNA sequences of subtilisin BPN9 from B. amyloliquefaciens (Wells et al., 1983; Vasantha et al., 1984) and subtilisin Carlsberg from B. licheniformis (Jacobs et al., 1985) revealed that the two sequences are highly conserved in the coding region for the mature protein and must therefore have a common ancestral precursor. Yoshimoto et al. (1988, 1990) characterized the gene encoding subtilisin amylosacchariticus from B. subtilis sub sp. amylosacchariticus. The sequence was highly homologous to that of subtilisin E from
B. subtilis 168 (Stabile et al., 1996). The gene was expressed in B. subtilis ISW 1214 by using the vector pHY300PLK.

AIM & SCOPE

Enzymes from different sources (plants, animals and microorganisms) have wide application in different industries such as food, pharmaceuticals, leather, detergents, textiles, paper and pulp, wastemanagement, etc. While it is expected that some 25,000 enzymes exist, only 2100 have been identified and listed so far. Food industry is the largest consumer of enzymes and approximately 45% of bulk share goes to it. Detergents are second stake holder in enzyme consumption. Among all the industrial enzymes, hydrolytic enzymes account for 85%. The market size was approximately US $ 1.6 billions in 2002 and has witnessed ~12% annual growth over a period of last one decade. It is expected that the market will continue to grow fast and reach US $ 3 billions by 2008.

Recently there has been increasing demand for proteases because of their suitability for versatile applications, mainly as a detergent additive. The wide specificity of hydrolytic action of proteases finds extensive applications in different industries such as food, textile, pharmaceutical, silk and recovery of used X-ray films. Proteolytic enzymes are available from plant, animal and microbial sources. Microorganisms represent an alternative 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. Furthermore, microbial proteins have longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation
broth by producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals. However, microbes are considered for commercial exploitation due to their broad biochemical diversity and susceptibility to genetic manipulation.

In view of industrial importance of microbial proteases the present work was designed to study the production of protease by bacteria isolated from the soil polluted with detergent industry effluents.

Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that the enzymes are better suited for harsh industrial processes. Valuable advantages of conducting biotechnological processes at elevated temperatures include avoidance of the risk of contamination, bioavailability and solubility of organic compounds, higher reaction rates due to decrease in viscosity and increased solubility of substrates. Thermostable enzymes can also be used as models for the understanding of thermostability and thermoactivity which is useful in protein engineering. Because of these advantages it is proposed to isolate thermostable bacteria which produces protease enzyme.

The cost of enzyme production is one of the major obstacles in the successful production of proteases in industry. Major area of focus in the future concerning the production of protease is the optimization of media. Synthetic media have a great advantage over complex media in consistency of processes and production. Protease yields have been improved by optimization of the fermentation medium, use of immobilized cells and screening for hyper producing microbial strains. The use of agroindustrial waste such as bran will significantly reduce the cost of production.
Hence it is proposed to carry out production of protease by optimizing various conditions using rice bran as sole carbon source.

Immobilized enzymes or whole cells is advantageous because such biocatalysts display better operational stability, higher efficiency of catalysis and also reusable. Because of these advantages of immobilized cells it is proposed to carry out immobilization of protease producing bacteria in different matrices and protease production by using immobilized cells.

The present topic has been proposed for investigation with the following objectives:

- Isolation and screening of thermostable alkaline protease producing bacterial strains.
- Optimization of culture conditions for protease production using agricultural wastes.
- Purification of alkaline protease.
- Partial characterization of alkaline protease.
- Stability studies using different detergents.
- Immobilization studies using different matrices.