1.1 General Introduction

Proteases, proteinases, or peptidases are enzymes that are essential for all forms of life. They are biology's version of Swiss army knives, cutting long sequences of amino acids (peptides) into fragments that fold into proteins (Seife, 1997). Proteases are essential for the synthesis of all proteins, controlling protein composition, size, shape, turnover and ultimate destruction. Their actions are exquisitely selective, each protease being responsible for splitting very specific sequences of amino acids under a preferred set of environmental conditions. According to International Protease Network, there are over 500 human proteases, accounting for 2% of human genes and similar numbers of proteases occur in every plant, insect, marine organism and in all infectious organisms that cause disease. Consistent with the important and widespread role of proteases in human biology and pathology, human proteases have been validated as therapeutic targets both for inhibitors and agonists and have also been used as therapeutic agents (Ramachandran Rithwik. et al., 2012).

Modern industries have begun to explore the advantages of enzymes. Microorganisms used in the past have now been replaced by purified enzymes. This has led to the growing market for industrial enzymes. The global market for industrial enzymes is estimated at $3.3 billion in 2010. This market is expected to reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5-year forecast period (PR Newswire, 2011). Research and development (R&D) spending, along with increasing competition, patent expiries, and new technologies are taking the global market for industrial enzymes in a new direction. Proteases are the most studied microbial enzymes at commercial, industrial, pharmaceutical, analytical, diagnostic, effluent abatement, etc sectors. These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons; for example they exhibit high catalytic activity, a high degree of substrate specificity can be produced in large amounts and are economically viable. Microbial alkaline proteases dominate the worldwide
enzyme market, accounting for two-third of the share of the detergent industry. Although production is inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. Their application web is increasing day by day as the newer functional roles of these enzymes are discovered. 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 (Fox et al., 1991; Poldermans, 1990).

1.2 Review of Literature

1.2.1 Proteases

Proteases are the single class of enzymes, which occupy a key position with respect to their applications in both physiological and commercial fields. Although they catalyze a single reaction the hydrolysis of peptide bond, the various ways they achieve this. Proteases play pivotal regulatory roles in conception, birth, digestion, growth, maturation, ageing, and death of all living cells. Proteases regulate most physiological processes by controlling the activation, synthesis and turnover of proteins. Proteases are also essential in viruses, bacteria and parasites for their replication and the spread of infectious diseases, in all insects, living cells and animals for effective transmission of disease, and in human and animal hosts for the mediation and sustenance of diseases. Proteases execute a large variety of complex physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis and development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. Besides the general functions that are described so far, the proteases also mediate the degradation of a variety of
regulatory proteins that control the heat shock response, the SOS response to DNA damage, the life cycle of bacteriophage and programmed bacterial cell death. (Aizenman et al., 1996; Van Melderen et al., 1994) Proteolysis of a repressor by an ATP-requiring protease resulted in a derepression of the gene. Change in the transcriptional specificity of the β-subunit of RNA polymerase in *Bacillus thuringiensis* was correlated to its proteolytic modification (Henning stahlberg et al., 1999). A new physiological function has been attributed to the ATP-dependent proteases conserved between bacteria and eukaryotes. Modification of ribosomal proteins by proteases has been suggested to be responsible for the regulation of translation (Suzuki et al., 1997). 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. Their ubiquitous distribution among all life forms, their multiplicity of locations inside, outside and at the surface of cells and above all their enormous diversity of function makes them one of the most fascinating groups of enzymes. They are presumed to have arisen in the earliest phases of biological evolution, some billion years ago since the present digestive proteases can be shown to have a common ancestry with those of the microbial origin. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. 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.

The extracellular proteases are having commercial value and find multiple applications in various industrial sectors. The production of extracellular protease is governed, at least in part, of available individual nutrient (Periasamy Anbu et al., 2008). Since microorganisms can be made to propagate rapidly and profusely, they are an ideal source of enzymes (Rehm, 1980). Among these, strains of *Bacillus* sp. dominate the industrial sector (Gupta et al., 2002).
With increasing industrial demands for the biocatalysts that can cope with industrial processes at harsh conditions, the isolation and characterization of new promising strains is a recent approach to increase the yield of such enzymes with defined biological properties.

1.2.2 Protease Classification

Proteases belong to the hydrolase class of enzymes, although they catalyze the typical hydrolytic reaction of peptide bonds, there are various ways of achieving this goal. The widely used term “protease” is synonymous with peptidase. The International Union of Biochemistry and Molecular Biology has recommended using the term peptidase for the subset of peptide bond hydrolases (Subclass E.C 3.4.). Seventy five percent of the industrial enzymes are hydrolytic. 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 (Barett, 1994):

(I) Type of reaction catalyzed,
(II) Chemical nature of catalytic site, and
(III) Evolutionary relationship with reference to structure

Depending on their site of action, proteases are grossly subdivided into two major groups, i.e. exopeptidases and endopeptidases (table 1.1).
Table 1.1 Classification of Protease

### PROTEASES

**EXOPEPTIDASES**

- Aminopeptidases
  - (3.4.11) \( \lambda \rightarrow \text{O-O-O-O-O-O} \)
- Dipeptidyl peptidase
  - (3.4.14) \( \lambda \rightarrow \text{O-O-O-O-O-O} \)
- Tripeptidyl peptidase
  - (3.4.14) \( \lambda \rightarrow \lambda \rightarrow \text{O-O-O-O-O-O} \)

**CARBOXYPEPTIDASES**

- Carboxypeptidase (serine) (3.4.16)
- Carboxypeptidase (metallo) (3.4.17)
- Carboxypeptidase (cysteine) (3.4.18)

**PEPTIDYL Dipeptidases**

- (3.4.15) \( \text{O-O-O-O-O-O} \rightarrow \lambda \)

**Dipeptidases**

- (3.4.19) \( \lambda \rightarrow \lambda \)

**Omegepeptidases**

- (3.4.19) \( \text{O-O-O-O-O} \rightarrow \lambda \rightarrow \lambda \rightarrow \text{O-O-O-O-O} \)

**ENDOPEPTIDASES**

- (Based on Active sites)
  - Serine endopeptidases (3.4.21)
  - Cysteine endopeptidases (3.4.22)
  - Aspartic endopeptidases (3.4.23)
  - Metallo endopeptidases (3.4.24)
  - Endopeptidases of unknown catalytic mechanism (3.4.99)

Outer circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and the triangles signify the blocked termini. Arrows show the sites of action of the enzyme. (Rao Mala B et al., 1998)

*Exopeptidases* act only near the ends of polypeptide chains, further classified as aminoo-carboxyopeptidases based on their site of action at the N or C terminus respectively. Amino peptidases liberate a single amino acid residue, a dipeptide (dipeptidyl peptidase) or a tripeptide (tripeptidyl peptidase). Aminopeptidases
can also be classified as aminopeptidase N or aminopeptidase A, depending on their preference for neutral (uncharged) or acidic side chains respectively. Most of the aminopeptidases are metalloenzymes. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes. The enzymes can also hydrolyze the peptides in which the peptidyl group is replaced by a pteroyl moiety or by acyl groups. Other exopeptidases include dipeptidases, which cleave a dipeptide and omega peptidases which release modified residues from N- or C- termini.

**Endopeptidases** are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N or C termini. They are divided into five subgroups on the basis of their catalytic mechanism as Serine proteases, Cysteine proteases, Aspartic proteases, Metalloproteases, Threonine proteases and/or unknown type. The classification of peptidases based on the evolutionary relationships has been given in Barett et al., (1998), and MEROPS – the peptidase database (Richard, 2003).

1.2.2.1 **Serine proteases**

Serine proteases are characterized by the presence of a serine group in their active site. Based on their structural similarities, they have been grouped into 20 families, which have been further subdivided into about six clans with common ancestors (Barett, 1994). Another interesting feature of the serine proteases is the conservation of glycine residues near the catalytic serine residue to form the motif Gly-Xaa-Ser-Yaa-Gly (Brenner, 1988). They are recognized by their irreversible inhibition by 3, 4- DCI (3, 4-dichloroisocoumarin), E.64 (L-3carboxytrans 2, 3-epoxypropyl-leucylamido (4- guanidine) butane, DFP (Di-isopropyl fluorophosphate), PMSF (Phenyl methyl sulfonyl fluoride) and TLCK (Tosyl-L-lysine chloromethyl ketone). Some are inhibited by thiol reagents such as pCMB (p-chloromercuribenzoate) due to the presence of a cysteine residue.
near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity. Their molecular masses range between 18 and 35 kDa. Their isoelectric points are generally between pH 4 and 6. Trypsin and chymotrypsin are the well studied proteases of this subgroup. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases.

Subtilisins of Bacillus origin represent the second largest family of serine proteases. Two different types of alkaline proteases, Subtilisin Carlsberg produced by Bacillus licheniformis and Subtilisin Novo or BPN’ produced by B. amyloliquefaciens have been identified. Subtilisin Carlsberg is widely used in detergents. 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).

1.2.2.2 Aspartic proteases

Commonly known as acid proteases. These proteases depend on aspartic acid residues for their catalytic activity. They have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (Barett, 1995) and have been placed in the clan AA. Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. The members of the pepsin family have a bilobal structure with the active-site cleft located between the lobes (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 acid proteases are inhibited by pepstatin and diazocompounds such as DAN (Diazacetyl-DL-norleucine
methyl ester) and EPNP [1, 2-Epoxy-3-(p-nitrophenoxy) propane] in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin. They can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus* and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* sp.

1.2.2.3  **Cysteine/thiol proteases**

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

1.2.2.4  **Metalloproteases**

These are the most diverse of the catalytic types of proteases (Page, 1996) characterized by the requirement of a divalent metal ion for their activity. Out of 30 families of metalloproteases, 17 contain only endopeptidases, 12 contain only exopeptidases and 1 (M3) contains both endo- and exopeptidases. Families of metalloproteases have been grouped into different clans based on the nature of the amino acid that completes the metal-binding site; e.g., clan MA has the sequence HEXXH-E and clan MB corresponds to the motif HEXXH-H. Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I, and (iv) Myxobacter II. The neutral proteases show specificity for hydrophobic amino acids, while alkaline proteases
possess a very broad specificity. Myxobacter I is specific for small amino acid residues on either side of the cleavage bond, whereas Myxobacter II is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP. Thermolysin, collagenase and elastase are the well studied metalloproteases. Matrix metalloproteases play a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing, and may be useful in the treatment of diseases such as cancer and arthritis (Browner, et al., 1995).

1.2.2.5 Threonine proteases

Threonine proteases are a family of proteolytic enzymes harboring a threonine (Thr) residue within the active site. It is responsible for functioning proteasome, the large protein-degrading apparatus. Threonine proteases have a conserved N-terminal threonine at each active site. Pre-proteins, which are catalytic beta subunits, are activated when the N-terminus is cleaved off. This makes threonine the N-terminal residue. Threonine proteases are activated by primary amines. The mechanism for the threonine protease was described first in 1995. The mechanism showed the cleaving of a peptide bond which made an amino acid residue (usually serine, threonine, or cysteine) or a water molecule become a good nucleophile which could perform a nucleophilic attack on the carboxyl group of the peptide. The amino acid residue is usually activated by a histidine residue.

1.2.3 Mechanism of action

The mechanism of action of proteases have been a subject of great interest to researchers as it forms a basis for exploring various ways of modifying its activity to make it suitable for its biotechnological application. Studies on the mechanism of proteases have revealed that they exhibit different types of mechanism based on their active-site configuration. The catalytic site of proteases is flanked on one or both sides by specificity 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 (figure 1.1).

![Diagram of protease and substrate](image)

**Protease:** N Sn ----- S3 – S2 – S1  κ  S1’ – S2’ – S3’ ----- Sn’ C

**Substrate:** N Pn ----- P3 – P2 – P1 –|– P1’ – P2’ – P3’ ----- Pn’ C

Figure 1.1 Active sites of proteases

The mechanism of action of metalloprotease is slightly different from that of the serine and other 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.
Figure 1.2 Catalytic mechanism of metalloprotease (Raih et al., 2005)
Figure 1.3 Mechanism of the active site of thermolysin (Ref: PDB, Inf)

Crystal structure of Thermolysin in complex with S-1,2-Propanediol from Bacillus thermoproteolyticus (Dr. Jürgen Behnen et al., 2012)

Figure 1.4 3D structure of protease
1.2.4 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.

1.2.4.1 Plant Proteases

Papain, bromelain, and ficin represent some of the well-known proteases of plant origin. Papain is extracted from the latex of *Carica papaya* fruits and is extensively used in industry for the preparation of protein hydrolysates (Schechler, *et al.*, 1967). Bromelain and ficin are derived from papaya fruit and the pineapple plant sources. 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 (Boyer, 1971). 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.

1.2.4.2 Animal Proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins (Boyer, 1971; Hoffman, 1974), which are usually transformed from their zymogens either by auto catalysis or by the hydrolytic action of other enzymes. These are prepared in pure form in bulk quantities. Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. Chymotrypsin is found in animal pancreatic extract and is used for diagnostic and analytical purposes. Pepsin is an acidic protease found in the stomachs of all vertebrates while rennet is a pepsin-like protease produced in the stomachs of all nursing mammals and is used extensively in the dairy industry to produce a stable curd with good flavor. However, their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies.

1.2.4.3 Microbial Proteases
Chapter 1: Introduction

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

**Bacteria:** Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) 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. 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, 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.

**Fungi:** The fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity, for example *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. However, they have a lower reaction rate and not as good as heat tolerance than do the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid-state fermentation process. Fungal acid proteases are particularly useful in the cheese making industry due to their narrow pH and temperature specificities.
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). 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.

Although proteases are widespread in nature, microbes are considered to be the gold mines of proteases and represent the preferred source of enzymes in view of their rapid growth, less space required for cultivation and accessibility to genetic manipulation to generate new enzymes with altered properties that are desirable for their various applications.

1.2.5 Alkaline Proteases and its Properties

1.2.5.1 Alkaline Proteases

Alkaline proteases (EC.3.4.21-24, 99) are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre (serine protease) or metallo-type (metalloprotease); and they are the most important group of enzymes exploited commercially (Gupta et al., 2002). They are all specific against aromatic or hydrophobic amino acid residues at the carboxyl side of the splitting point (Ward, 1985). These enzymes also offer advantages over the use of conventional chemical catalysts for numerous reasons. For example they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts and are economically viable (Anwar, 1998). Especially, alkaline proteases of microbial origin, which dominate the worldwide enzyme market, possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agarwal et al., 2004; Gupta et al., 2002). Alkaline proteases are produced by a wide range of
microorganisms including bacteria, molds, yeasts and also mammalian tissues (Anvari Masumeh and Khayati Gholam, 2011). Despite the interest in other microbial sources, survey of the literature conclusively shows that bacteria are by far the most popular source of commercial alkaline proteases to date.

These enzymes have broad substrate specificities and will function to some extent under the extreme conditions encountered in domestic washing temperatures of 20 to 70°C, a pH up to 11 and at high concentrations of detergents, polyphosphates, chelating agents such as EDTA and oxidizing agents such as sodium perborate (Cowan, 1994). These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao, 1998). 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. amyloliquefaciens*, and *B. majovensis*. The different alkaline protease-producing *Bacillus* species and strains are summarized in table-1.2. Some of the Gram-negative bacteria producing alkaline proteases were identified as *Pseudomonas aeruginosa*; *Pseudomonas maltophilia* (Kobayashi et al., 1985); *Pseudomonas* sp. strain B45 (Chakraborty and Srinivasan, 1993); *Xanthomonas maltophilia* (Debette, 1991); *Vibrio alginolyticus*; and *Vibrio metschnikovii* strain RH530 (Kwon et al., 1994).
**Table 1.2: The different alkaline protease producing *Bacillus* species and strains**

<table>
<thead>
<tr>
<th>Alkaline protease producing <em>Bacillus</em> species*</th>
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<tr>
<td><em>B. alkalophilus</em> ATCC 21522 (<em>Bacillus</em> sp. no.221)</td>
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<td><em>B. alkalophilus</em></td>
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<tr>
<td><em>B. alkalophilus</em> subsp. <em>halodurans</em> KP1239</td>
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<td><em>B.amyloliquefaciens</em></td>
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<td><em>B.circulans</em></td>
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<td><em>B.coagulans</em> PB-77</td>
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<td><em>B.firmus</em></td>
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<td><em>B.proteolyticus</em></td>
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<td><em>B.pumilus</em></td>
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<td><em>B.sphaericus</em></td>
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<td><em>B.subtilis</em></td>
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<td><em>B.subtilis</em> var. <em>amylosacchariticus</em></td>
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<td><em>B.thuringiensis</em></td>
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<td><em>Bacillus</em> sp. Ya-B</td>
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<td><em>Bacillus</em> sp. NKS-21</td>
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<td><em>Bacillus</em> sp. B21-2</td>
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<td><em>Bacillus</em> sp. Y</td>
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<td><em>Bacillus</em> sp. KSM-K16</td>
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<td><em>Bacillus</em> sp. MK5-6</td>
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* Ganesh Kumar and Takagi, 1999
1.2.5.2 Properties of Alkaline Proteases

1.2.5.2.1 Optimum Temperature and Thermostability

Generally alkaline proteases produced from alkaliophilic Bacillus are known to be active over a wide range of temperature. The heat stability of enzymes is affected by at least two factors alone or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondly, specific components such as polysaccharides and divalent cations, if any, can stabilize the molecule (Hande, 2004). Even though there is no firm evidence to suggest that thermostable enzymes are necessarily derived from thermophilic organisms, nevertheless there is a greater chance of finding thermostable proteins from thermophilic bacteria (Raja Noor Zaliha Abd. Rahman et al., 1994). Therefore, a wide range of microbial proteases from thermophilic species has been extensively purified and characterized. These include Thermus sp., Desulfurococcus strain Tok12S1 and Bacillus sp. Among them alkaline proteases derived from alkaliophilic bacilli, are known to be active and stable in highly alkaline conditions (Raja Noor Zaliha Abd. Rahman et al., 1994). The earliest thermophilic and alkaliophilic Bacillus sp. was B. stearothermophilus strain F1 isolated by Salleh and friends in 1977, which was stable at 60°C (Haki and Rakshit, 2003). Further studies on microbial alkaline proteases have been done in view of their structure-function relationship and industrial applications, as they needed stable biocatalysts capable of withstanding harsh conditions of operation. In some studies it has also been observed that the addition of Ca$^{2+}$ further enhanced enzyme thermostability (Takami et al., 1989; Gessesse, 1997).
1.2.5.2.2 **Optimum pH of Alkaline Proteases**

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly located on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This affect the total net charge of the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990). In general, all currently used detergent-compatible proteases are alkaline in nature with a high pH optimum; therefore they fit the pH of laundry detergents, which is generally in the range of 8 to 12. Therefore, most of the commercially available subtilisin-type proteases are also active in the pH range of 8-12 (Gupta et al., 2002). A good example for this is the well-known detergent enzymes, subtilisin Carlsberg and subtilisin Novo or BPN’ which show maximum activity at pH 10.5 (Banerjee et al., 1999). Alkaline proteases of the genus *Bacillus* show an optimal activity and a good stability at high alkaline pH values (Margesin et al., 1992). The optimum pH range of *Bacillus* alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima of 11.5 (Fujiwara and Yamamoto, 1987), 11-12 (Kumar and Takagi, 1999), 12-13 (Takami et al., 1989; Ferrero et al., 1996; Kumar and Takagi, 1999).

1.2.5.2.3 **The Isoelectric Point**

The pH referred as isoelectric point (pI) at which the net charge on the molecule is zero, is a characteristic of each enzyme, where solubility in aqueous solutions is generally minimum. In aqueous solution, charged groups interact with polar water molecules and stabilize the protein, which is intrinsically hydrophobic. A low number of charged groups and a high number of aliphatic or aromatic side chains characterize a protein that is less soluble in water. As one move further from pI, the number of ionized groups increases therefore the
solubility tends to increase. Hence the isoelectric point is important as it affects the solubility of proteins as well as interaction between them (Öztürk, 2001).

1.2.5.2.4 The Molecular Weight

The molecular weights of alkaline proteases have been reported of 32.0 kDa (Huang et al., 2003), 33.5 kDa (Rahman et al., 1994), 36.0 kDa (Durham et al., 1987). Extracellular alkaline proteases with very low molecular masses, 8, 11 and 12.5 KDa have also been isolated from Kurthia spirofome sp. nov. (Steele et al., 1992), Streptomyces diasticus (Chaphalkar and Deys, 1998) and Staphylococcus aureus V8 (Anridson et al., 1973) respectively.

1.2.5.2.5 Metal Ion Requirement and Inhibitors of Alkaline Proteases

Alkaline proteases require a divalent cation like Ca\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) or a combination of these cations, for maximum activity. These cations were also found to enhance the thermal stability of a Bacillus alkaline protease. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar et al., 1999). Inhibition studies give insight into the nature of the enzyme, its cofactor requirements, and the nature of the active site. Alkaline proteases are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP). This inhibition profile classifies these proteases as serine hydrolases. In addition, some of the alkaline proteases were found to be metal ion dependent in view of their sensitivity to metal chelating agents, such as EDTA. Thiol inhibitors have little effect on alkaline proteases of Bacillus spp., although they do affect the alkaline enzymes produced by Streptomyces sp. (Kumar et al., 1999; Hande, 2004).

1.2.6 Genetic engineering of alkaline proteases

A century after the pioneering work of Louis Pasteur, the science of microbiology has reached its pinnacle. In a relatively short time, modern biotechnology has grown dramatically from a laboratory curiosity to a commercial activity. Advances in microbiology and biotechnology have created a
favorable niche for the development of proteases and will continue to facilitate their applications to provide a sustainable environment for mankind and to improve the quality of human life. Despite the systematic application of recombinant technology and protein engineering to alter the properties of proteases, it has now been possible to obtain microbial proteases that are ideal for their biotechnological applications. Industrial applications of proteases have posed several problems and challenges for their further improvements. The biodiversity represents an invaluable resource for biotechnological innovations and plays an important role in the search for improved strains of microorganisms used in the industry. There is a renewed interest in proteases as targets for developing therapeutic agents against relentlessly spreading fatal diseases such as cancer, malaria, and AIDS. The advent of techniques for rapid sequencing of cloned DNA has yielded an explosive increase in protease sequence information. Analysis of sequences for acidic, alkaline, and neutral proteases has provided new insights into the evolutionary relationships of proteases. The existing knowledge about the structure-function relationship of proteases, coupled with genetic engineering, promises a fair chance of success, in the near future, in evolving proteases that were never made in nature and that would meet the requirements of the multitude of protease applications. Advance in genetic manipulation of microorganisms opens new possibilities for the introduction of predesigned changes resulting in the production of tailor-made proteases with novel and desirable properties.

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 means for the manipulation and control of genes. More than 50% of the industrially important enzymes are now produced from genetically engineered microorganisms (Hodgson, 1994) with the aim of (i) enzyme overproduction by gene dosage effect (ii) studying the primary structure of the protease and its role in
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 several bacteria, fungi and viruses have been cloned and sequenced. Several genes encoding proteolytic enzymes have been cloned with the purpose of enhancing protease production as well as to gain a better understanding of gene expression and mechanism of protein secretion.

1.2.7 Computation Techniques

1.2.7.1 Sequence Homology

Studies on DNA and protein sequence homology are important for a variety of purposes and have, therefore, become a routine task in computational molecular biology. They serve as a prelude to phylogenetic analysis of proteins and assist in predicting the secondary structure of DNA and proteins. Proteases are a complex group of enzymes, which vary enormously in their physicochemical and catalytic properties. The nucleotide and amino acid sequences of a number of proteases have been determined and their comparison is useful for elucidating the structure-function relationship (Mark, 1987). Homology of proteases with respect to the nature of the catalytic site has been studied (Katsuhiko et al., 1998; BO LIN et al., 1996). It has been shown that the residues involved in the substrate and metal ion binding, catalysis, disulfide bond formation and those forming the active site are conserved. Accordingly, the enzymes have been allocated to evolutionary families and clans. It has been suggested that there may be as many as 60 evolutionary lines of peptidases with separate origins. Some of these contain members with quite diverse peptidase activities and yet there are some striking examples of convergence (Rawlings et al., 1993). A number of reports are available regarding the homology of proteases. Takagi et al. found that the thermostable proteases of *B. stearothermophilus* and *B. thermoproteolyticus* are 85% homologous and the thermolabile proteases of *B. subtilis* and *B. amyloliquefaciens* are 82% homologous.
whereas the thermostable protease of *B. stearothermophilus* shares only 30% homology with the thermolaile protease of *B. subtilis* (Takagi *et al.*, 1985). The amino acid sequences of intracellular serine proteases from *B. subtilis* was compared with that of subtilisin Carlsberg and subtilisin BPN' and showed them to be 45% homologous (Koide *et al.*, 1986). The sequences around the catalytic triad of serine, aspartate and histidine are highly conserved suggesting that the genes for both the intracellular and extracellular proteases have evolved from a common ancestor by divergent evolution (Neurath, 1984). Proteases are present in all living organisms and are considered to have arisen in the earliest phases of biological evolution, some billion years ago. Comparisons of amino acid sequences, three dimensional structures and mechanism of action of proteases assist in deciphering of their course of evolution. Changes in molecular structure have accompanied the demands for altered functions of proteases during evolution.

1.2.7.2  *Bioinformatics*

Bioinformatics, a new interdisciplinary science, is essential for managing, understanding, and harnessing clinical benefit from new genetic data. Bioinformatics, a term coined for the applications of computer science in biology is now emerging as a major element in contemporary biology and biomedical research. Bioinformatics deals with the exponential growth in biological data which has led to the development of primary and secondary databases of nucleic acid sequences, protein sequences and structures. Some of the well known databases include GenBank, SWISS-PROT, PDB, CATH etc. These databases are available as public domain information and hosted on various Internet servers across the world. Basic research and modeling is done using these databases with the help of sequence analysis tools like BLAST, FASTA, ClustalW, etc., and the model structures are visualized using visualization tools such as WebLab, MOLMOL, Rasmol, etc. Apart from analysis of genome sequence data, bioinformatics is now being used for a vast array of other important tasks,
including analysis of gene variation and expression, analysis and prediction of gene and protein structure and function, prediction and detection of gene regulation networks, simulate environments for whole cell modeling, complex modeling of gene regulatory dynamics and networks, and presentation and analysis of molecular pathways in order to understand gene-disease interactions. On a smaller scale, simpler bioinformatics tasks valuable to the clinical researcher can vary from designing primers to predicting the function of gene products.

1.2.8 Industrial applications

Enzymes have long been used as alternatives to chemicals to improve the efficiency and cost-effectiveness of a wide range of industrial systems and processes. They are currently used in basic and applied arenas of research as well as in a wide range of product design and manufacturing processes, such as those pertaining to the food, beverage, pharmaceutical, detergent, leather processing, and peptide synthesis (Gupta et al., 2002). According to estimates, these enzymes account for nearly 65% of total worldwide enzyme sales (Anonyme, 2007; Rao et al., 1998). They are widely distributed in nature and play a vital role in life processes. They are particularly known for their capacity to hydrolyze peptide bonds in aqueous environments and to synthesize peptide bonds in non-aqueous biocatalysis.

1.2.8.1 Immobilization

Enzymes accelerate different chemical reactions with high specificity and are not permanently modified by their participation in reactions. But enzymes are costlier than chemical catalysts, in general, and cost effectiveness of enzyme-based processes could be reached by the repeated use of enzymes. Since enzymes remain in solution with products, it is not possible to recover them easily from the reaction mixture. Repeated use of an enzyme becomes possible, if they are made insoluble or stationary in active forms. Immobilization is the process by which an enzyme is made insoluble or stationary with the retention of full or
substantial activity. Immobilization is also localization or confinement of enzymes during a process, which permits separation of the enzyme from substrate and product for its repeated use. Enzyme immobilization offers advantages over free enzymes in terms of multiple or repetitive use of a single batch of enzyme, rapid termination of reactions, controlled product formation, increased stability of the enzyme, predictable decay rates and adaptability to various engineering designs. The interest in immobilized enzymes and their application to bioprocessing, analytical system and enzymatic therapy has grown in past decade. However, it is understood that some changes of physical and chemical properties of immobilized enzymes may take place because of the development of new microenvironment around enzyme by supporting matrix. The changes are usually expressed to various extents by altered stability and kinetic parameters of the enzymes. Stability of the enzyme either increases or decreases on immobilization, depending on the effect of the microenvironment and denaturation of the enzyme. Specific activity of an enzyme usually decreases upon immobilization, possibly due to partial denaturation of protein depending on the process of coupling between enzyme and matrix. The application of proteases for peptide synthesis has been stimulated to a considerable degree by the availability of immobilized enzymes. Covalently bound proteases catalyze peptide bond formation as effectively as the native enzymes since substrate specificity and stereo specificity remain unchanged. The peptides synthesized are free of contamination by proteolytic activities and denatured protein. A novel enzyme biosensor for the detection of protein was made using a protease immobilized on an immunodyne membrane and by placing it on an electrode. The method was very effective for detecting a very low level of protein (Klibanov, 1983). The effects of site-specific immobilization on the thermal stability of mutants of the thermolysin-like protease from *Bacillus stearothermophilus* showed that the protein stabilization by immobilization is most effective if the protein is attached to the carrier at that region where unfolding is
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initiated (Channe et al., 1998). There is no best-known method for the immobilization of any specific enzyme. The support, the enzyme, the substrate and technique, are all involved in the development of an effective process (Subhabrata Sengupta et al., 2006).

1.2.8.2 Commercial applications of microbial proteases

Detergent industry

The detergent industry is one of the major users of proteases. These days, the use of alkaline protease-based detergents is preferred over the conventional synthetic ones. This is partly because of their better cleaning properties, higher performance efficiency at lower washing temperature, and safer dirt removal conditions (Gupta et al., 2002). Typically, a detergent protease needs to be active, stable, and compatible with the alkaline environment encountered under harsh washing conditions: pH 9 - 11, temperature of 20 - 60°C, as well as high concentrations of salt, bleach, and surfactant. Presently, the alkaline serine protease from *B. licheniformis* is the commercial detergent protease of choice. Some of the alkaline proteases that are particularly preferred in contemporary detergent formulations include Savinase™ (Subtilisin 309), Subtilisin Novo (BPN’), Alcalase™ (Subtilisin Carlsberg; SC), Maxacal™ (Novozymes A/S, Denmark), BLAP Sb (Henkel, Germany) and Properase™ (Genecor Int. USA). They are often reported to be stable at conditions of elevated temperatures and pH. Most of them have, however, been criticized for their limited efficiency in the presence of liquid or solid laundry detergents wherein their stability decreases (Beg and Gupta, 2003; Maurer, 2004). Therefore, the search for and screening of alternative microorganisms that produce detergent-stable enzymes and preserve their high activity and stability at extreme conditions would be highly desired, with powerful, safe and healthy cleansing abilities (Bassem Jaouadi et al., 2011).
Leather industry

Proteases are used for selective hydrolysis of non-collagenous constituents of the skin and to remove nonfibrillar proteins such as albumins and globulins. Currently, microbial alkaline proteases are used to ensure faster absorption of water and to reduce time required for soaking. The use of proteases as alternatives to hazardous chemicals such as sodium sulphide has proved successful in improving leather quality and in reducing environmental pollution. Alkaline proteases with hydrated lime and sodium chloride are used for dehairing resulting in significant reduction in wastewater. Trypsin in combination with other proteases of *Bacillus* and *Aspergillus* origin is used for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin and the amount of enzyme depends on the type of leather (soft or hard) desired to be produced. Increased usage of enzymes for dehairing and bating not only prevents pollution problems but is also effective in saving energy. Novo Nordisk manufactures three different proteases viz. Aquaderm TM, NUE, and Pyrase for use in soaking, dehairing and bating respectively.

Food processing industry

The enzymes have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates and meat tenderization. The use of proteases in food industry dates back to antiquity. In cheese making, the primary function of proteases is to hydrolyse the specific peptide bond viz. Phe105-Met106 bond to generate parakappa-casein and macropeptides. Chymosin is preferred due to its highest 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 miehei*, *Bacillus subtilis*, *Endothia parasitica* are gradually replacing chymosin in cheese making. Genencor International increased its production in *Aspergillus niger* var. *awamori* to commercial levels. Whey is a byproduct of
cheese manufacture. The insoluble heat denatured whey protein is solubilised by treatment with immobilized trypsin. Endo and exoproteases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. 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. Proteases have been used from ancient times to prepare soya sauce and other soya products. Proteolytic modification of soya proteins helps to improve their functional properties. Treatment of soya proteins with alcalase at pH 8 results in the 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. The peptidases that can cleave hydrophobic amino acids and proline are valuable in debittering of protein hydrolysates. Aminopeptidases from lactic acid bacteria are available under the trade name Debitrase. Carboxy peptidase-A has a high specificity for hydrophobic amino acids and hence has a great potential for debittering. A careful combination of endoprotease for the primary hydrolysis and the aminopeptidase in the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness. Immobilized preparation of thermolysin from *Bacillus thermoproteolyticus* is used for the enzymatic synthesis of aspartame, which is used as a non-calorific artificial sweetener.

**Pharmaceutical Industry**

The wide diversity and specificity of proteases are of great advantage in developing effective therapeutic agents. Oral administration of proteases from *Aspergillus oryzae* (Chiplonkar *et al.*, 1985; Rao *et al.*, 1998) 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 asparaginase isolated from *E. coli* is used to eliminate aspargine from the bloodstream in the various forms of lymphocytic leukemia.
**Peptide mapping and sequencing**

Limited proteolysis is necessary to study initial cleavage products, to monitor the time course of a reaction or to generate large peptide fragments. Examples of limited proteolysis in vivo are zymogen activation, prohormone processing or cleaving out peptides of a polypeptide chain. In most cases both peptide mapping and sequencing of proteins require a complete fragmentation of the protein, resulting in a strongly defined and reproducible peptide pattern. Proteases of lower specificity like chymotrypsin, thermolysin, subtilisin or pepsin cleave the protein adjacent to several amino acid residues, thereby yielding more and shorter peptide fragments. Coagulation factor Xa, thrombin and enteropeptidase are especially used during the isolation of recombinant proteins (Tanksale Aparna, 2001) and their sites are cloned into positions where they allow cleaving fragments or proteins of interest out of a construct or fusion protein. Exopeptidases are valuable tools for cleaving off N-terminal blocking groups, like N-acetyl or formyl groups or a pyroglutamate residue. Carboxypeptidases are used to get sequence information from the C-terminal end of the protein.

**Study of protein conformation**

Limited proteolysis can be used as a classical biochemical method to probe structure and dynamics of proteins in solution, providing experimental results which are easy to obtain and well complement as compared to those derived from the use of other classical physicochemical methods and approaches. The most suitable proteases for such a study are that displaying broad substrate specificity, such as subtilisin, thermolysin, proteinase K and pepsin. The 17 protein fragment mixture generated by limited proteolysis can be analyzed by electrophoretic or chromatographic methods. Limited proteolysis can be used for monitoring the overall unfolding of a globular protein to the random-coil polypeptide chain or local unfoldling of a protein molecule when exposed to a denaturing environment. It can be used to dissect multidomain
proteins into fragments capable of an independent folding since the peptides between the domains are usually flexible and serve as sites of preferential proteolysis compared to the individual domains of tight and rigid conformation. The most classical cases being the dissection of immunoglobulin molecule into Fab and Fc pieces and of calmodulin. Limited proteolysis can be used to remove loose, flexible parts of a protein e.g. Hirudin wherein a disordered C terminal tail was separated from the well-structured, rigid N-terminal core domain using a variety of proteases (subtilisin, thermolysin, trypsin, and V8 protease). Recently, using the same rationale, the minimum size of a folded fragment of thermolysin was determined. Recently, limited proteolysis has been used to probe the structural and dynamic differences between the holo and apo form of horse myoglobin (Mb). A variety of proteases (subtilisin, thermolysin, chymotrypsin and trypsin) cleave apoMb at the level of chain segment 89-96, whereas holo Mb is fully resistant to proteolysis, thus showing that only the F-helix in apo Mb is largely disrupted which was earlier inferred from spectroscopic measurements. Proteolytic enzymes can also be used as probes of structural features of partly folded states or molten globule states of proteins (Tanksale Aparna, 2001).

**Study of membrane proteins**

Association of the glycoproteins with the cell surface and the types of these associations can be determined by treatment of intact cells or vesicular membranes with limited dilutions of proteolytic enzymes such as trypsin. When the inside-out membrane vesicles are treated with limited amounts of trypsin, a reduction in the apparent molecular weight on SDS-gels of angiotensin converting enzyme and the Lyt-2/3 (CD-8) antigen of cytotoxic T-lymphocytes indicated the presence of cytoplasmic tails and hence of a transmembrane orientation of these proteins. The exposure of proteolytic sites is dependent on the specific conformation of the protein. Misfolded proteins are more susceptible to proteolytic digestion and in contrast to correctly folded proteins, can be easily degraded by proteolytic enzymes (Lüscher et al., 1985; Naim et al., 1988).
Tissue culture

Since the discovery of Rous and Johns (1916), proteases have been used extensively for the primary dissociation of tissues and for detaching cells in monolayers for subsequent replating. Proteases with broad substrate specificity such as pronase, trypsin, collagenase, dispase, are used in tissue culture for various purposes. Large numbers of viable cells from several human tissues can be isolated by combining mechanical disintegration with 0.1mg/ml trypsin or 0.5mg/ml collagenase and 0.1mmol EGTA (Ethylene glycol tetraacetic acid). Proteolytic enzymes have been shown to adsorb to cell surfaces and persist in an active form as long as 24 h thereafter. They were found to prevent the formation of glycoprotein cell coat material at the surface and to interfere with the attachment, spreading and growth of cells on glass. Ficin was found to be the most suitable enzyme compared to trypsin, papain and bromelain for isolation of bovine pulp cells due to its even rate of cell removal, good initial viability, subsequent growth of the separated cells in monolayer culture. Overgrowth of cultured keratinocyte preparations by fibroblasts could be significantly reduced by utilizing thermolysin since this enzyme selectively digests the dermal-epidermal junctions. Alkaline protease from Conidiobolus coronatus was able to replace trypsin in animal cell cultures (Chiplonkar et al., 1985).

Protease-catalyzed peptide synthesis

The use of proteases to perform selective transformations in peptide synthesis is advantageous since chemical ligation methods are prone to racemization and suffer from time-consuming side-chain protection/deprotection necessities. Important approaches towards suppressing competitive reaction in the reversal of proteolysis are: leaving group manipulations of the acyl donor ester in kinetically-controlled synthesis; peptide synthesis in frozen aqueous systems and zymogen-catalyzed peptide synthesis. In addition, in both equilibrium-controlled synthesis and in the kinetic approach, kinetically controlled syntheses promise favorable results for use with
immobilized proteases as only low concentrations of enzymes and organic solvents are needed to dissolve the reactants. The examples of immobilized proteases used for peptide synthesis are: α-chymotrypsin attached to macroporous silica, thermolysin immobilized on Enzacryl AH. Cryoenzymatic synthesis is the upcoming branch of peptide synthesis. Freezing decreases the rate of proteolysis and enhances hydroxylaminolysis. α-Chymotrypsin is able to act as a reverse carboxypeptidase catalyzing coupling of free amino acids as amino components in frozen 19 aqueous systems and was used for the synthesis of the luteinizing hormone releasing hormone (LHRH). Frozen state enzymology opens completely new possibilities in enzymatic peptide synthesis. The simplest strategy of peptide bond formation, which cannot be performed by chemical methods, is using N-terminal free amino acid or peptide esters as acyl donors. Water-based high density media can also lead to a high peptide yields e.g., the artificial low-calorie sweetener precursor Z-aspartame is synthesized in semi-preparative scale by thermolysin-catalyzed coupling of Z-Asp-OH and H-Phe-OMe (Ganesh Kumar et al., 1999).

**Management of Industrial and Household waste**

Proteases solubilize proteinaceous waste and thus help to lower the biological oxygen demand of aquatic systems. Recently the use alkaline protease in the management of wastes from various food processing industries and household activities opened up a new era in the use proteases in waste management. Dalev (1994) used alkaline protease from *Bacillus subtilis* for the management of waste feathers from poultry slaughterhouses.

**Silk Degumming**

One of the least explored areas for the use of proteases is the silk industry and only a few patents have been filed describing the use of proteases for the degumming of silk (Kanehisa et al., 2000).
Medical Usage

Alkaline proteases are also used for developing products of medical importance. Kudrya and Simonenka (1994) exploited the elastolytic activity of \textit{B.\textit{subtilis}} 316M for the preparation of elastoerase which was applied for the treatment of burns, purulent wounds, carbuncles furuncles and deep abscesses. Kim et al. (1996) reported the use of alkaline protease from \textit{Bacillus sp}. Strain CK-114 as a thrombolytic agent having fibrinolytic activity.

Photographic Industry

Alkaline protease plays a crucial role in the bio-processing 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. Alkaline protease of \textit{Bacillus sp}. B18’ (fujiwara et. al., 1991) and \textit{B. coagulans} PB-77 (Gajju et al., 1996) were efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered.

Other applications of proteases

Several other applications of proteases have been reported, includes; its incorporation into animal feed to improve the nutritional quality, in the production of single cell proteins, in extraction of flavour and colour compounds from plants, in preparation of culture media especially for microbes, in equipment clearing procedure, in the preparation of digestive aids in gastrointestinal disorders such as dyspepsia, in disease treatment of clotting disorders and for patients on chronic haemodialysis, gelatin hydrolysates are prepared utilizing alkaline proteases which in turn used in cosmetic industry as additives to shampoos and ointments.

1.3 Aim of study

The aim of present work was to get a better strain as alkaline protease source and characterization of alkaline proteases for various industrial
applications. Proteases are the most studied microbial enzymes at commercial, industrial, pharmaceutical, analytical, diagnostic, effluent abatement, etc. Their application web is increasing day by day as the newer functional roles of these enzymes are discovered. The study was undertaken to screen the microorganisms from the soil and water samples from various industrial area and alkaline rich soil region. The optimization of growth conditions and other parameters which have been predicted to play a significant role in enhancing the production of alkaline proteases, therefore, objective of this study was manual and statistical optimization of process parameters and to determine the selection of medium components for enhancing the protease yield. It was considered of significance to purify and characterize the enzyme, this work was undertaken to purify alkaline protease from Bacillus thuringiensis strain cc7 and to elucidate some basic enzymatic properties and study the factors affecting the activity to present potential and possible application for industrial purposes. However, there are few reports dealing with the cloning and expression of alkaline proteases from Bacillus thuringiensis strains. The obtained enzyme was hence taken into consideration for the identification of gene encoding alkaline protease, cloning & expression. The present investigation has also attempted to study the molecular and structural properties alkaline protease enzyme using bioinformatics tools.