2.0 LITERATURE SURVEY

2.1 ENZYMES

Enzymes are protein molecules and are central to every biochemical process. They are the biological catalysts having high degree of specificity for their substrate and can accelerate the reaction tremendously as compared to synthetic or inorganic catalysts. Acting in organized sequence, they catalyse hundreds of reactions stepwise and can convert a simple precursor to industrially important biomolecule.

Except for some catalytic RNA molecules, most enzymes are proteins folded in their tertiary or quaternary structure. The region of the enzyme where the substrate molecule binds is called the active site and is specific to the substrate. Some enzymes require no chemical group for their action other than their amino acid residues, whereas others may require inorganic ions called cofactors such as Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, etc. or complex organic or metalloorganic molecules called as coenzymes. Some enzymes may require both cofactor as well as coenzyme. The whole enzyme along with its coenzyme/cofactor is called holoenzyme. The protein part of the enzyme is called apoenzyme or apoprotein, whereas the coenzyme/cofactor is called prosthetic group (Nelson and Cox, 2004).

2.2 PROTEASES

Proteases are the enzymes which conduct hydrolysis of peptide bonds via the process called as proteolysis. These are also known as peptidyl – peptide hydrolases (EC 3.4.21-24 and 99) and are industrially important enzymes.

Proteases represent one of the three largest groups of industrial enzymes and account for 60 % of total worldwide sales of enzymes (Figure. 2.1). The application of proteases in food and detergent industry is known for long time. Their growing applications in leather industry, peptide synthesis, silk degumming has conferred added biotechnological importance. The variety of proteases, in contrast to the specificity of their biocatalysis, has attracted global scientific interest in attempts to develop their physiological and biotechnological applications (Rao et.al, 1998).
Figure 2.1: Contribution of different enzymes to total sales of enzymes.


### 2.2.1 SOURCES OF PROTEASES

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

**Plant Proteases:** Plant proteases are obtained by extraction of the plant material, which is a very time consuming process and the amount of enzyme available is also very less. Moreover availability of plant material depends on several factors such as land for cultivation and suitability of cultivation conditions for growth.

E.g. of plant proteases: papain, keratinases, bromelain.

**Animal Proteases:** Animal Proteases are obtained by extraction of tissues and are prepared in pure and bulk quantities. However, their production is dependent on availability of livestock for slaughter, which in turn is controlled by socio-political scenario and agricultural policies.

E.g. of animal proteases: Renin, pepsin, trypsin, chymotrypsin
Microbial proteases: Because of the inability of plant proteases and animal proteases to meet the current market demand of enzymes due to the reasons explained above, microbial proteases have gained popularity in the recent years. Micro organisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods producing an abundant, regular supply of the desired product. Besides they can be genetically manipulated to produce new enzymes with altered properties that are advantageous for their various applications (Rao et al., 1998, Gupta et al., 2002). Although microbial proteases can be sourced from bacteria, fungi and viruses, but bacterial proteases are more extensively studied as they are easy to handle and consumes less time for production. E.g. of bacterial proteases: thrombin, plasmin, streptokinase, nattokinase

2.3 THERMOPHILIC PROTEASES
Proteases are physiologically essential molecules and their production is well-known in plants, animals and diverse strains of bacteria, fungi and yeasts. An excellent source of proteases are microorganisms, and among them great consideration is received by thermophilic bacteria and archaea. Thermophilic microorganisms are found in different biotopes, such as hot springs, geothermal sediments, marine solfatares or fermenting compost as well as in industrial environments, e.g., hot water pipelines (Rothschild and Manicineli, 2001). Thermophilic and hyperthermophilic microorganisms studied till date fulfill demand on different proteolytic enzymes that have optimum pH up to 12.0 and temperatures ranged from 45 - 110°C (Antranikian et al., 1995). Industrially important thermostable proteases are usually produced using thermophilic strains belonging to the genus Bacillus (Haaki and Rakshit, 2003). An example of such enzyme is thermolysin, a neutral metalloprotease isolated from Bacillus stearothermophilus with half-life of 1 h at 80°C (Rahman et al., 1994; Rao et al., 1998). The pyrolysins, serine protease which achieve maximal activity at 100°C was isolated from hyperthermophilic archaeon Pyrococcus furiosus (Antranikian et al., 1995). Other examples of well characterized heat resistant proteases are subtilisins of Bacillus sp. They display wide substrate specificity and have comparable properties such as optimal temperature of 60°C and an optimal pH of 10 (Klingberg et al., 1991; Rao et al., 1998). An important source of thermostable proteases seems also to be gram-negative, aerobic bacteria of the genus Thermus which have been isolated from numerous natural and artificial thermal environments. The strains belonging to the
genus *Thermus* use carbohydrates, amino acids, carboxylic acids and peptides and their optimal growth temperatures range from 55-85°C. High culture yields make this microorganism important source of different thermozyymes. Some *Thermus* strains, e.g., *Thermus aquaticus* and *Thermus thermophilus* can be used as a source of aqualyasin which is subtilisin type heat-stable serine protease. This enzyme is extracellularly secreted as zymogen, activated in the growth medium through autolysis (Pantazaki et al., 2002). Other proteolytic enzyme sourced from *Thermus* strains is ATP dependent zinc protease which catalyzes release of small peptides (Pantazaki et al., 2002). A number of proteases have been isolated from archaeons (Klingberg et al., 1991; Hanazawa et al., 1996). Among them *Desulfurococcus kamchatkensis* is able to grow at temperatures between 65 and 87°C and can hydrolyze wide range of substrates, including a-keratin, albumin or gelatin (Kublanov et al., 2009). Thermophilic microorganisms exhibiting keratinolytic properties have a great significance as they can be used for decomposition of waste by-products from poultry industry which are produce in amount about 10,000 tons per annum (Suzuki et al., 2006). Unfortunately, the properties of thermostable keratinases have been accounted only in a few cases. Among them keratinase isolated from *Fervidobacterium islandicum* AW-1 was characterized (Nam et al., 2002). In some cases thermostable keratinases active at temperatures about 70°C are produced by mesophiles (Dozie et al., 1994; Takami et al., 1992). Enzymes active towards collagen are handy for utilization of bones and some other wastes from meat industry. However, most mesophiles producing collagenases are capable to attack human tissues by damaging extracellular matrix and these confines the use of such pathogens (Watanabe, 2004). On the other hand, there are no pathogenic strains of thermophiles, even amongst those that degrade collagen. Regrettably, collagenases are rarely known in thermophilic sources. Collagenolytic activity of *Geobacillus collagenovorans*, growing at temperatures 50 - 70°C in a neutral pH range was reported (Miytake et al., 2005). Another example of thermophile active against collagen (reported in the article of Okamoto et al., 2001) is *Alicyclobacillus sendainensis* NTAP-1 which preferentially grows in acidic surroundings.

Thermophilic proteases like other enzymes from thermophilic and hyperthermophilic microorganisms (often called thermozyymes) are of particular importance in some applications because they withstand and are active at temperatures above 60-70°C. In
addition, they are more resistant than their mesophilic counterparts to organic solvents, detergents, low and high pH and other denaturing agents (Cowan et al., 1985; Cowan, 1997; Gupta and Khare, 2006). The use of proteases at higher temperatures is advantageous, because unfolded form of proteinaceous substrate is better susceptible for the still active thermostable protease. It results in higher specific activities for proteases sourced from thermophiles and optimization of some industrial processes, particularly with the enzymes that are active at temperatures near 100°C. Stability in organic solvents makes the thermostable proteases useful for synthesis of high molecular weight peptides, carried out in reaction media with low water content (Bruins et al., 2001; Sellek and Chaudhuri, 1998; Synowiecki, 2008). Performing enzyme reactions at elevated temperatures permit for higher substrate concentrations, lower viscosity, reduction of microbial contamination risk and high reaction rates (Bruins et al., 2001; Eichler, 2001). With the industrially desirable attributes of very high stability at elevated temperature, in wide range of pH and resistance to detergents, chelators, organic solvents along with increased susceptibility of protein substrates at high temperature, proteases from thermophilic organism are of considerable biotechnological interest and may open unexplored avenues of biocatalysis which were otherwise limited due to use of mesophilic proteases.

Given the potential uses of the thermostable proteases and their high demand, there exists a need for the screening strains of thermophilic bacteria that produce proteases with industrially desirable characteristics (as mentioned above) and the development of industrial fermentation processes for the same.

**GENERAL PROPERTIES**

The enzymatic and physico-chemical properties of thermophilic proteases have been studied extensively and are given below:

**Optimum temperature and stability:**

There are mainly two factors which affects the heat stability of enzymes alone or in combination. First is primary structure of enzyme. More the number of hydrophobic groups, more condensed will be the structure and the enzyme will not denature easily. Disulphide bridges impart stability against heat inactivation and chemical denaturation. Secondly, specific molecules such as polysaccharides and divalent ions confer stability to enzyme (Öztürk, 2001).
Generally thermophilic proteases are identified to be active over a wide range of temperature. The optimum temperatures of thermophilic proteases range from 60°C to 100°C. Some exceptions like the protease from an extreme thermophile *Pyrococcus furiosus* showed a very high optimum temperature of 115°C, this protease demonstrated good thermostability at high temperatures, with half life of 0.33 h at 105°C. The optimum temperature and thermal stability of some thermophilic proteases from various thermophiles is summarized in Table 2.1.

**Optimum pH:**
Enzymes are protein molecules containing side chains of weak basic and acidic amino acids i.e. they are amphoteric in nature and hence the surface charge of enzyme molecules changes with change in the pH of the environment. These effects are especially important in the zone of the active sites, which affects the activity, structural stability and solubility of the enzyme (Chaplin, 1990). Thermophilic proteases are generally alkaliphilic in nature active in the range of pH 9 and 11, with a few exceptions like *pyrococcus furiosus* active at pH 6 and *sulfolobus acidocaldarius* active at pH 2 (Table 2.1).

**Molecular weight:**
The proteases produced by thermophiles have molecular weight in range of 30kDa to 60kDa; however there are exceptions like *Pyrobaculum aerophilum* with very high molecular weight of 401kDa others like *Thermoactinomyces vulgaris* with molecular weight of 279kDa and *Pyrococcus furiosus* with 150 kDa (Table 2.1).
Table 2.1 Properties of thermophilic proteases

<table>
<thead>
<tr>
<th>Thermophilic Strain</th>
<th>Mol Wt (kDa)</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>Half-life</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeropyrum pernix</em></td>
<td>34</td>
<td>90</td>
<td>8</td>
<td>1h/100°C</td>
<td>Catara et al., 2003</td>
</tr>
<tr>
<td><em>Alicyclobacillus sendaiensis</em></td>
<td>37</td>
<td>3.9</td>
<td></td>
<td></td>
<td>Tsuruoka et al., 2003</td>
</tr>
<tr>
<td><em>Aquifex aeolicus</em></td>
<td>54</td>
<td>80</td>
<td>8.5</td>
<td>&gt;0.5/110°C</td>
<td>Khan et al., 2000</td>
</tr>
<tr>
<td><em>Aquifex pyrophilus</em></td>
<td>43</td>
<td>85</td>
<td>9</td>
<td>6h/105°C</td>
<td>Ward et al., 2002</td>
</tr>
<tr>
<td><em>Desulfurococcus mucosus</em></td>
<td>43–54</td>
<td>95</td>
<td>7.5</td>
<td>4.3h/95°C</td>
<td>Ward et al., 2002</td>
</tr>
<tr>
<td><em>Fervidobacterium islandicum</em></td>
<td>80</td>
<td>8.0</td>
<td></td>
<td></td>
<td>Godde et al., 2005</td>
</tr>
<tr>
<td><em>Fervidobacterium islandicum AW-1</em></td>
<td>&gt;200</td>
<td>100</td>
<td>9.0</td>
<td>1.5h/100°C</td>
<td>Nam et al., 2002</td>
</tr>
<tr>
<td><em>Fervidobacterium pennivorans</em></td>
<td>58</td>
<td>80</td>
<td>10.0</td>
<td></td>
<td>Kluskens et al., 2002</td>
</tr>
<tr>
<td><em>Geobacillus caldoproteolyticus</em></td>
<td>70–80</td>
<td>9.0</td>
<td></td>
<td>1h/80°C</td>
<td>Chen 2004</td>
</tr>
<tr>
<td><em>Pyrobaculum aerophilum</em></td>
<td>401</td>
<td>&gt;100</td>
<td>9</td>
<td></td>
<td>Ward et al., 2002</td>
</tr>
<tr>
<td><em>Pyrococcus abyssi</em></td>
<td>60</td>
<td>95</td>
<td>9.0</td>
<td></td>
<td>Ward et al., 2002</td>
</tr>
<tr>
<td><em>Pyrococcus furiosus</em></td>
<td>150</td>
<td>115</td>
<td>6–9</td>
<td>0.33h/105°C</td>
<td>Antranikian et al., 2005</td>
</tr>
<tr>
<td><em>Staphylothermus marinus</em></td>
<td>150</td>
<td>90</td>
<td>9.0</td>
<td></td>
<td>Antranikian et al., 2005</td>
</tr>
<tr>
<td><em>Sulfolobus acidocaldarius</em></td>
<td>46–51</td>
<td>90</td>
<td>2.0</td>
<td></td>
<td>Ward et al., 2002</td>
</tr>
<tr>
<td><em>Sulfolobus solfataricus</em></td>
<td>118</td>
<td>&gt;90</td>
<td>8</td>
<td></td>
<td>Ward et al., 2002</td>
</tr>
<tr>
<td><em>Thermoactinomyces sp.</em></td>
<td>31</td>
<td>85</td>
<td>11.0</td>
<td></td>
<td>Gupta et al., 2006</td>
</tr>
<tr>
<td><em>Thermoactinomyces vulgaris</em></td>
<td>279</td>
<td>60–65</td>
<td>7.5–9</td>
<td></td>
<td>Ward et al., 2002</td>
</tr>
</tbody>
</table>
Stability conferred by bonds and ions:

Thermal resistance of enzymes is determined by free energy consumption essential for transformation of molecules from folded to unfolded state (Shiraki et al., 2001). Slight changes of amino acids distribution and sequences increase the number of stabilizing interactions in the folded protein, such as: additional ion-pairs, disulphide bridges, hydrogen bonds and hydrophobic interactions (Farias and Bonato, 2003; Kumar and Nussinov, 2001; Mozhaev, 1993; Grütter et al., 1979; Hartley, Payton 1983). Alteration in amino acid sequence can affect stability, without any obvious structural alteration, by giving rise to a relatively small number of additional intramolecular interactions. In addition to side-chain interactions, thermophilic and hyperthermophilic microorganisms adopt other strategies for stabilizing proteins. This may be achieved by filling cavities in the molecular structure of the proteins, shortening of the loops and reduction of accessible hydrophilic surface area (Fukuchi and Nishikawa, 2001; Shiraki et al., 2001; Stetter, 1999; Thompson and Eisenberg, 1999; Voght et al., 1997). Other modifications include: metal ion binding and diminished amount of residues susceptible to deamidation or oxidation. Moreover, some thermozymes contain thermolabile residues in location in which they are not susceptible to degradation (Zeikus et al., 1998). Thermostability of some enzymes can be assured by environmental factors, e.g., increased intracellular salts and protein concentrations and synthesis of different stabilizers (Gupta, 1991; Vieille and Zeikus, 2001). However, there are no universal factors or their combinations that may be responsible for thermal stability of proteins. Furthermore, many features involved in thermostabilization of soluble proteins do not appear in case of membrane proteins (Schneider et al., 2002; Trivedi et al., 2006). Although, adaptation of thermozymes to act at elevated temperatures is mainly achieved by exchange of few amino acid residues and/or their different localization in molecule, the homologous thermostable and thermolabile enzymes are similar and have the same catalytic mechanisms (Vieille and Zeikus, 2001). Higher resistance of thermozymes as compared with their mesophilic counterparts is the result of increased rigidity, which preserves their catalytically active structure, but leads to reduced activity at lower temperatures (Shiraki et al., 2001). However, in some cases, thermozymes are more active than their mesophilic counterparts even at low temperatures. Such phenomenon suggests that their molecules exhibit local flexibility in the area of catalytic site with overall rigidity of the rest of the protein (Vieille and Zeikus, 2001). In proteases, specific
binding of metal ions (particularly of calcium) further improves molecular stability. For example, the increased stability of caldolysin over thermolysin can be attributed almost entirely to the binding of six calcium ions to the caldolysin (Khoo et al., 1984), as opposed to four calcium ions to the thermolysin (Roche and Voordouw, 1978). The abnormally high frequency of tyrosine in thermolysin (Ohta, 1967) has also been implicated in its thermostability, although this proposed mechanism seems to be unique. The stability of thermophilic proteases is not restricted to temperature but also includes resistance to denaturing agents, detergents and organic solvents (Cowan and Daniel, 1982; Taguchi et al., 1983; Ohta, 1967). Little evidence is available to suggest that their stability at extremes of pH is any greater than that of their mesophilic counterparts. The thermophilic alkaline serine proteases are quite stable in mildly alkaline conditions (<pH 11.5) but lose activity rapidly below pH 4 (Cowan and Daniel, 1982). In practical terms, hydrolyses can be performed satisfactorily at 75°C over extended periods at pH levels between 5 and 11. It is interesting to note that most of the heat resistant enzymes indicate maximal activity above the optimal growth temperature of the microorganisms from which they are isolated (Fujiwara, 2002; Niehaus et al., 1999).

**Production of thermophilic protease:**

Protease production in a number of strains of *Thermus* spp. has been investigated (Matsuzawa et al. 1983; Jones et al. 1988; Kanasawud et al. 1989) but most studies have concentrated on the properties of the enzymes (Cowan and Daniel 1982; Taguchi et al., 1983; Cowan et al. 1987; Saravani et al. 1989). Thermophilic bacteria investigated to date produce proteases at levels lower than do most mesophiles, and an increase in the level of production is necessary before thermophilic proteases can become competitive as industrial enzymes rather than specialty enzymes only (Cowan et al. 1985). While a few investigations have been made on the apparently inducible or constitutive nature of *Thermus* proteinases (Jones et al., 1988; Cowan et al., 1985), medium optimization studies have been done only in a preliminary manner (e.g., Kanasawud et al., 1989).

Protease-producing thermophiles are routinely grown on undefined complex media (O’brien & Campbell, 1957; Sidler & Zuber, 1980; Mizusawa & Yoshida, 1973; Heinen & Heinen, 1972; Cowan & Daniel, 1982). In the laboratory, yeast extract and
commercially available protein digests are normally used, while larger scale commercial producers substitute cheaper nutrient sources such as corn-steep liquor, fish meal and soybean extract (Keay L, et al., 1972) which usually have high C: N ratios. While the thermophilic bacilli are rather nonspecific in their nutrient requirements, *Thermus* species grow poorly in media containing high concentrations of carbohydrate (Zeikus, 1979). Proteases are excreted at relatively low levels by most thermophilic bacteria. Strict comparisons between mesophilic and thermophilic protease activities are complicated by different assay temperatures and procedures. However, it is apparent that substantial increases in protease production, whether brought about by genetic manipulation or by nutrient optimization, would be necessary before these thermophilic proteases could become competitive as industrial enzymes. Production expenses and productivity would be less critical factors if thermophilic proteases were to be considered only as specialty enzymes. Little information is available on the mechanisms controlling protease production in extreme thermophiles. A study of the effects of amino acids, ammonium salts and sugars on the production of extracellular protease by *Thermus aquaticus* strain T351 showed that no significant induction or repression occurred (Cowan, et al., 1985). Study by Altintas & Ulgen, 1999 showed that replacement of yeast extract and tryptone with organic nitrogen [NaNO₃ or (NH₄)₂SO₄] did not support growth of thermus strain also the strain did not respond to addition of biotin. However, a strong association was noted between growth rate and extracellular proteolytic activity. Although there are numerous examples of the induction and repression of the synthesis of proteases by mesophilic microorganisms (Ward, 1983), Limited data on similar effects in extremely thermophilic bacteria are currently available. Study by Colin et al., 1988 showed that the regulation of protease synthesis occurs in a manner which is dependent on repression/derepression rather than induction. Derepression of protease synthesis occurs under conditions of carbon/energy starvation.

**Nitrogen source:**

In most micro organisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components. The protease comprises 15.6% nitrogen (Kole et.al, 1988) and its production is reliant on the availability of both carbon and nitrogen sources in the medium (Kole et.al, 1988). Although complex nitrogen sources are usually used for
protease production, the requirement for a specific nitrogen supplement differs from organism to organism.

Protease production is also subject to repression by various nitrogen sources, the order in which they are effective (urea > ammonium chloride > amino acid) probably reflecting their ability to increase the steady-state concentration of ammonia within the cell. The role of the extracellular protease is to act as a scavenger of carbon/energy or nitrogen, i.e. to hydrolyse extracellular protein into a mixture of amino acids and small peptides which can subsequently be transported into the cell to alleviate starvation. Protease synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium in bacillus species (Frankena et al., 1986; Giesecke et al., 1991; Nehete et al., 1986)

Several reports have demonstrated the use of organic nitrogen sources leading to higher enzyme production than the inorganic nitrogen sources. Fujiwara and Yamamoto, 1987 recorded maximum enzyme yields using a combination of 3% soybean meal and 1.5% bonito extract. Soybean meal was also reported to be a suitable nitrogen source for protease production (Sen & Satyanarayana, 1993; Chandrasekaran & Dhar, 1983; Tsai et al., 1988; Cheng et al., 1995). In addition, by using an acid hydrolysate of soybean in place of conventional soymeal, a threefold increase in total enzyme activity was noted (Takagi et al., 1995).

Addition of certain amino compounds has been reported to effect the production of extracellular enzymes by alkaliphilic Bacillus sp. (Ikura & Horikoshi, 1987). Glycine had inhibitory effects on protease production. Casamino acids were also found to inhibit protease production (Ong & Gaucher, 1976). In some studies, use of oil cakes as a nitrogen source did not favour enzyme production (Sen & Satyanarayana, 1993; Sinha & Satyanarayana, 1991).

**Carbon source:**

Studies have also indicated a reduction in protease production due to catabolite repression by glucose (Kole et al., 1988; Frankena et al., 1986; Hanlon et al., 1982; Frankena et al., 1985). On the other hand, Zamost et al., 1990, correlated the low yields of protease production with the lowering of pH brought about by the rapid
growth of the organism. In commercial practice, high carbohydrate concentrations repressed enzyme production. Therefore, carbohydrate was added either continuously or in aliquots throughout the fermentation to replenish the exhausted component and keep the volume limited and thereby reduce the power requirements (Aunstrup, 1980). Increased yields of alkaline proteases were reported by researchers who used different sugars such as lactose (Malathi & Chakraborty, 1991), maltose (Tsuchiya et al., 1991), sucrose (Phadatare et al., 1993) and fructose (Sen & Satyanarayana, 1993). However, a repression in enzyme synthesis was observed with these sugars at high concentrations.

Whey, a waste by-product of the dairy industry containing mainly lactose and salts, has been demonstrated as a potential substrate for alkaline protease production (Donaghy & McKay, 1993). Similarly, maximum alkaline protease secretion was observed in Thermomonospora fusca YX, which used pure cellulose (Solka-floc) as the principal carbon source (Gusek et al., 1988).

Various organic acids, such as acetic acid (Ikeda et al., 1974), methyl acetate (Kita & Horikoshi, 1976) and citric acid or sodium citrate (Takii et al., 1990; Kumar et al., 1997) have been demonstrated to increase production of proteases at alkaline pH. The use of these organic acids was interesting in view of their economy as well as their ability to control pH variations. n-paraffins were also found to be used by Fusarium sp. for the production of increased amounts of alkaline proteases (Nakao et al., 1973).

**Metal ion requirement for protease production:**

Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese, and molybdenum are required in the fermentation medium for optimum production of alkaline proteases. However, the requirement for specific metal ions depends on the source of enzyme.

Earlier investigations by Peek et al (Peek et al., 1990) had shown that Ca$^{2+}$ had a significant stabilizing effect on the purified protease. Janssen et al., 1991 showed that removal of chelating agents, lowering the inorganic phosphate concentration and increasing the calcium levels all had positive effects on the half-life of protease activity in batch culture. The final medium composition improved the protease yield by 45% and greatly increased the protease half-life, from 5.9 h to 90 h. The use of
AgNO$_3$ at a concentration of 0.05 mg/100 ml or ZnSO$_4$ at a concentration of 125 mg/100 ml resulted in an increase in protease activity in *Rhizopus oryzae* (Banerjee & Bhattacharya, 1992a; Banerjee & Bhattacharya, 1992b). Potassium phosphate has been used as a source of phosphate in most studies (Mao et al., 1992; Moon & Parulker, 1991; Hubner et al., 1993) and as a buffer in the medium. Phosphate at the concentration of 2 g/l was found optimal for protease production. However, amounts in excess of this concentration showed an inhibition in cell growth and repression in protease production (Moon & Parulker, 1991). When the phosphate concentration was more than 4 g/l, precipitation of the medium on autoclaving was observed (Moon & Parulker, 1993). This problem, however, could be overcome by the supplementation of the disodium salt of EDTA in the medium (Heineken & O'Connor, 1972). In at least one case, the salts did not have any effect on the protease yields (Phadatare et al., 1993).

**Temperature and pH of protease production:**

There is a good correlation between the growth temperature of a source organism and the stability of its extracellular proteases (Table 2.2). It follows, therefore, that a search for bacteria capable of existing at even higher temperatures should yield proteases of even greater thermostability.

Most of the thermophilic protease producer’s reported are alkaliphilic in nature. The important trait of most alkaliphilic microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production. For increased protease yields from these alkaliphiles, the pH of the medium must be maintained above 7.5 throughout the fermentation period (Aunstrup, 1980). The advantage in the use of carbonate in the medium for an alkaline protease has been well demonstrated (Horikoshi & Akiba, 1982).
Table 1.2 Thermostability of mesophilic and thermophilic proteases

<table>
<thead>
<tr>
<th>Source organism</th>
<th>Growth temp (°C)</th>
<th>Half-life (min)</th>
<th>Incubation temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>37</td>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>37</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td><em>B. subtilis N'</em></td>
<td>37</td>
<td>12-18</td>
<td>50</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em> NCIB 8924</td>
<td>55</td>
<td>15</td>
<td>74</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em> NRRL 3880</td>
<td>55</td>
<td>15</td>
<td>87</td>
</tr>
<tr>
<td><em>Malbranchea pulchella</em> var. sulfurea</td>
<td>55</td>
<td>120</td>
<td>73</td>
</tr>
<tr>
<td><em>Streptomyces rectus</em> var Proteolyticus</td>
<td>50</td>
<td>30</td>
<td>82</td>
</tr>
<tr>
<td><em>B. thermoproteolyticus</em></td>
<td>55</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td><em>Thermus aquaticus</em> T351</td>
<td>75</td>
<td>1800</td>
<td>80</td>
</tr>
<tr>
<td><em>T. aquaticus</em> YT1</td>
<td>75</td>
<td>180</td>
<td>80</td>
</tr>
<tr>
<td><em>B. caldolyticus</em></td>
<td>72</td>
<td>&gt;480</td>
<td>80</td>
</tr>
<tr>
<td><em>T. caldophilus</em></td>
<td>70</td>
<td>120</td>
<td>80</td>
</tr>
</tbody>
</table>


The culture pH also strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon & Parulker, 1991). When ammonium ions were used, the medium turned acidic, while it turned alkaline when organic nitrogen, such as amino acids or peptides were metabolized (Moon & Parulker, 1993). The decline in the pH may also be due to production of acidic products (Moon & Parulker, 1991). In view of a close relationship between protease synthesis and the utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the protease production, such as the start and end of the protease production period.
**Aeration and agitation:**
During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be obtained by: (i) variations in the aeration rate; (ii) variations in the agitation speed of the bioreactor; or (iii) use of oxygen-rich or oxygen-deficient gas phase (appropriate air-oxygen or air-nitrogen mixtures) as the oxygen source (Moon & Parulker, 1991; Michalik et al., 1995).

The variation in the agitation speed influences the extent of mixing in the shake flasks or the bioreactor and will also affect the nutrient availability. Optimum yields of alkaline protease are produced at 200 rpm for *B. subtilis* ATCC 14416 (Chu et al., 1992) and *B. licheniformis* (Sen & Satyanarayana, 1993). In one study, *Bacillus* sp. B21-2 produced increased enzyme titres when agitated at 600 rpm and aerated at 0.5 volume per volume per min (Fujiwara & Yamamoto, 1987). Similarly, *Bacillus firmus* exhibited maximum enzyme yields at an aeration rate of 7.0 l min$^{-1}$ and an agitation rate of 360 rpm. However, lowering the aeration rate to 0.1 l min$^{-1}$ caused a drastic reduction in the protease yields (Moon & Parulker, 1991). This indicates that a reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis.

Foam built up on the surface of the culture had a dramatic reduction of protease activity. This could be attributed to denaturation at air-surface interfaces and subsequent increased susceptibility to autolysis (Janssen et al., 1991).

**Growth and enzyme production:**
The production of an enzyme exhibits a characteristic relationship with regard to the growth phase of that organism. *Thermus* sp. Rt41A protease production is concomitant with growth and substrate utilization (Peek et al. 1990), suggesting that protease production occurs only while the organism is growing. Preliminary investigations indicate some form of control may be present (Jones et al., 1988; Janssen et al., 1991), but detailed investigation is necessary to elucidate the mechanism of control. *Thermus* sp. Rt41A grown in pH-controlled batch culture on medium 162 with 2 g/l Na L-glutamate as the carbon
source produced protease concomitant with growth and substrate utilization. When the substrate exhausted, growth stopped, the dissolved oxygen concentration rapidly increased to 100% saturation at 70°C, and the protease level began to fall. This implied that protease production was directly linked to the culture being metabolically active and growing. There was no further protease production once growth ceased (Janssen et al., 1991). Since protease production occurs in parallel with culture growth. Increasing the levels of limiting nutrients like Fe and/or S in addition to anabolic phosphate addition results in a marked increase in protease yield (Janssen et al., 1991)

2.4 STASTICAL TOOL

Optimization of media compounds by the traditional “one-variable at a-time” strategy is the most frequently used operation in biotechnology (Haaland, 1989). This strategy is extremely time consuming and expensive when a large number of variables need to be considered. Additionally, this method is unable to detect true optimal conditions including the interactions among different production factors.

In recent years, the use of statistical approaches involving Plackett–Burman designs and response surface methodology (RSM) has gained momentum in medium optimization. These methods are also useful for understanding interactions among various physicochemical parameters using a minimal number of experiments. The Plackett–Burman design allows for the screening of the main factors from a large number of variables, and this information can be retained in further optimization. RSM is a collection of statistical techniques that is useful for designing experiments, building models, evaluating the effects of different factors and searching for optimal conditions of studied factors for desirable responses (Coninck et al., 2000). RSM has been successfully applied in many areas of biotechnology, such as α-amylase production (Kunamneni et al., 2005) and protease production (Dutta et al., 2004).

**Box behnken design:**

Box–Behnken designs (BB designs) are 3-level second-order designs (SODs) introduced by Box and Behnken (1958, 1960), for fitting the second-order response surface model \( y = X\beta + \varepsilon \) (1) for \( m \) factors \( x_1, \ldots, x_m \) in \( n \) runs where \( y \) is the \( n \times 1 \) response vector, \( X \) is a \( n \times p \) model matrix with \( n \times 1 \) row vectors \( x = (x_1, x_1, \ldots, x_m) \)
\( x_1, x_2, \ldots, x_{m-1}, x_m \), \( \beta \) is a \( p \times 1 \) vector of parameters to be estimated and \( \varepsilon \) is a \( n \times 1 \) vector of errors with zero mean and covariance matrix \( I_n \sigma^2 \). BB designs are available for 3–12 and 16 factors. BB designs are spherical designs because all design points are either on a sphere or at the centre of a sphere. These designs are used when there is little or no interest in predicting responses at the extremes, i.e. the corners of the cube BB designs are either rotatable (for those with 4 and 7 factors) or near-rotatable. All BB designs except the ones with 3 and 11 factors can be orthogonally blocked. For an orthogonally blocked design, the inclusion of blocks does not affect the estimated regression coefficients for the second-order model in (1), and as such the primary effect of blocking is to potentially reduce the magnitude of the experimental error. Blocking, however, also reduces the number of degrees of freedom for estimating this potentially reduced variance (Nguyen & Borkowski, 2008).

BB designs and central-composite designs of Box and Wilson (1951) (subject to the appropriate choice of factor levels) are either rotatable or near-rotatable and can be orthogonally blocked. In addition, they satisfy several goodness criteria judged as essential for a response surface design and are very popular second order designs.

2.5 PURIFICATION

The development of techniques for protein purification has been an essential prerequisite for many of the advancements made in biotechnology. Protein purification varies from simple one-step precipitation procedures to large scale validated production processes. The key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required.

Occasionally when a sample is readily available, purity can be achieved by simply adding or repeating steps. However, experience shows that even for the most challenging applications, high purity and yield can be achieved efficiently in fewer than four well-chosen and optimized purification steps. Techniques should be organized in a logical sequence to avoid the need for conditioning steps. The need to obtain a protein efficiently and economically in sufficient purity and quantity applies
to every purification process. All the information concerning properties of the target protein and contaminants helps during purification development. Factors that must be considered in designing a downstream processing scheme include the nature, concentration and stability of the product, the desired purity and end use. As far as possible, the requisite purification and concentration should be achieved with the few processing steps; generally no more than six to seven steps are used, a situation quite different from that in chemistry and biochemistry laboratories, where the number of individual steps is often not a major consideration and purity of the product is usually more important than overall yield or costs. Separation schemes which utilize different physicochemical interactions as the basis of separation are likely to achieve the greatest performance for a given number of steps. Speed of processing is another factor that significantly affects the design of a recovery scheme.

To minimize reduction of the overall yield, high-resolution separations such as chromatography should be utilized as early as possible. Most purification schemes involve some form of chromatography. Different chromatographic techniques with different selectivity can form powerful combinations for the purification of any biomolecule.

For thermophilic enzymes, purification processes involving one step or combination of the following can be developed:

a) Cell Separation
b) Concentration
c) Precipitation
d) Chromatography

a) Cell separation:
Cell separation is the first step after harvesting the broth and before employing any purification technique. It refers to removal of cells, solids and colloids from the fermentation broth and is done generally using filters or centrifuge.

b) Concentration:
Since the amount of enzyme is very low in cell free broth, it is usually concentrated before further purification. The concentration is done usually by ultrafiltration. It is a pressure driven inexpensive technique which results in both purification as well as
concentration with little loss of enzyme activity. The main drawback of the process is
clogging of filters due to precipitates formed in the end of filtration. This clogging can
usually be alleviated or overcome by treatment with detergents, proteases, or acids
and alkalies.

Han and associates used a temperature-sensitive hydrogel ultrafiltration for
concentrating an alkaline protease. This hydrogel comprised poly (N-isopropyl-
acrylamide), which changed its volume reversibly by the changes in temperature. The
separation efficiency of the enzyme was dependent on the temperature and was 84%
at temperatures of 15°C and 20°C. However, at temperatures above 25°C, a decrease
in the separation efficiency was observed.

c) Precipitation:
Protein precipitation achieves separation by the conversion of soluble proteins to an
insoluble state, which subsequently can be removed by various means. Precipitation
can be used to remove components in cell culture media that may interfere with
downstream purification methods. Precipitation also results in both concentration and
purification. Thus, precipitation is often used early in the sequence of downstream
purification, reducing the volume and increasing the purity of the protein prior to any
chromatography steps.

Methods of Precipitation
There are several methods to reduce the solubility of proteins, some of which are:
ionic precipitation (e.g. ammonium sulfate, sodium chloride), temperature, pH, metal
ions (e.g. Cu²⁺, Zn²⁺ and Fe²⁺), nonionic polymers [e.g. polyethylene glycol (PEG)],
organic solvents (e.g. ethanol, acetone), tannic acids, heparin, dextran sulfates,
cationic polyelectrolytes (e.g. protamines), short chain fatty acids (e.g. caprylic acid), trichloracetic acid (TCA), lectins (e.g. concanavalin A), group-specific dyes (e.g. Procion Blue) and ligand-antibody interaction. Ionic precipitation, utilizing inorganic salts, is the most common precipitation method. Precipitation by various salts or non-ionic polymers is the preferred method to utilize whenever possible. These precipitations typically yield stable non-denatured products. The use of temperature, pH or organic solvents can lead to denaturation and should be performed with care to minimize any decrease in yield or activity (Protein Purification, Vol 1).

**Ammonium Sulphate Precipitation**

Inorganic salts can be utilized for the precipitation of proteins, with ammonium sulphate being the most common. The concentration of ammonium sulphate required for precipitation varies from protein to protein and should be determined empirically. Typically, ammonium sulphate is used in a series of steps performed involving increasing the ammonium sulphate concentration gradually at 2°C to 8°C. The temperature is kept lower to avoid the denaturation of enzyme. The collected precipitates are resuspended in the minimal volume of buffer suitable for the next step in the purification process and the salt is removed typically via dialysis (Protein Purification, Vol 1).

**d) Chromatography:**

Chromatography refers to a technique used to separate the molecule of interest from mixture of substances. The mixture is dissolved in a fluid called as mobile phase and is loaded on a solid matrix called as stationary phase. The various constituents of the mixture travel at different speed, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases.

The most common chromatographic techniques used for the separation of alkaline protease are:

- Gel Filtration chromatography
- Ion exchange chromatography
**Gel filtration:**
Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. To perform a separation, gel filtration medium is packed into a column to form a packed bed.

The medium is a porous matrix in the form of spherical particles that have been chosen from their chemical and physical stability and inertness. The packed bed is equilibrated with buffer which fills the pores of the matrix and the space in between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the liquids outside the particles, referred to as the mobile phase. The samples are eluted isocratically, i.e. there is no need to use different buffers during the separation. However, a wash step using the running buffer is usually included at the end of separation to facilitate the removal of any molecules that may have been retained on the column and to prepare the column for a new run.

**Ion exchange:**
Ion exchange chromatography separates molecules on the basis of differences in their net negative charge. In case of proteins, net surface charge changes gradually with change in pH of environment. Ion Exchange chromatography takes the advantage of this fact that the relationship between net surface charge and pH is unique for a specific protein. In IEX separation, reversible interactions between charged molecules and oppositely charged IEX media are controlled in order to favour binding or elution of specific molecules and the separation is achieved.

The solution to be injected is usually called a sample and the individually separated components are called analytes or fractions. A sample is introduced either manually or with an auto sampler. A buffered aqueous solution known as the mobile phase carries the sample from the loop onto a column that contains some form of stationary phase material. The stationary phase is typically a resin or gel matrix consisting of agarose or cellulose beads with covalently bonded charged functional groups. The molecule of interest i.e. analyte is retained on the stationary phase and is eluted on the later stage by increasing the concentration of a similarly charged species that will displace the analyte from the stationary phase.
A combination of Ammonium sulphate precipitation, DEAE cellulose and CM cellulose chromatography has been employed for purification of thermophilic protease from thermus strain (Matsuzawa et al., 1988). Gel permeation chromatography followed by ammonium sulphate precipitation and DEAE sephadex chromatography has been reported to purify thermophilic keratinase (Prakash et al., 2010). Similarly various purification schemes involving ammonium sulphate precipitation and ion exchange chromatography have been carried out at lab scale.

**Newer low cost purification techniques - three phase partitioning:**

The conventional methods used for protein purification like column chromatography are either very costly or other simpler methods like concentration, ultrafiltration results in loss of activity. Thus an alternative method is required to overcome these drawbacks. One of such method is three phase partitioning.

Three-phase partitioning (TPP) has been reported as an efficient alternative method for concentration and purification of various industrially important enzymes (Dennison & Lovrein, 1997). This technique uses a combination of ammonium sulfate and t-butanol to precipitate proteins from crude extracts. t-butanol binds to the precipitated proteins, thereby increasing their buoyancy and causing the precipitates to float above the denser aqueous salt layer. Optimum pH, temperature, ammonium sulfate and t-butanol concentrations can selectively precipitate proteins at the interface of the organic and aqueous phases. Kosmotropy, salting out, co-solvent precipitation, isoionic precipitation, osmolytic electrostatic forces, conformation tightening and protein hydration shifts all contribute to protein precipitation at the interface (Dennison & Lovrein. 1997; Lovrein et al., 1987). In many cases, TPP enhances the activity of various enzymes, resulting in apparent higher yields of 100–1000% (Dennison & Lovrein, 1997, Singh et al., 2001b). In addition to concentration, purification with TPP was found comparable to chromatographic techniques. For example, Saxena et al. obtained a 20.1-fold purification of a wheat germ protease/amylase bifunctional inhibitor using TPP (Saxena et al., 2007). In comparison, a combination of fractional ammonium sulfate, affinity, ion exchange and gel filtration yielded a maximum 22-fold purification (Saxena et al., 2010). Thus, the novelty of TPP lies in its ability to concentrate proteins from crude broths with higher purification than conventional concentration methods. Scalability, rapid
recovery and a requirement for only minimal pre-treatment are additional advantages of TPP (Rajeeva & Lele, 2011).

2.6 APPLICATIONS OF THERMOPHILIC PROTEASES

The ability of thermophilic proteases to preserve a significant level of activity after extended periods at high temperatures in aqueous solution (e.g. 4-5 h at 85 °C) must impart certain biotechnological advantages (Cowan & Daniel, 1982). The difficulty lies in categorizing which enzymatic processes could be acceptably performed at higher temperatures: most are carried out at less than 60°C (Ward, 1983). A multitude of industrial protein recovery or solubilization processes are likely candidates (Ward, 1983; Cowan, 1996). Advantages such as more efficient hydrolysis, reduced mesophilic contamination and reduced viscosity will be to a degree counterbalanced by the cost of maintaining the reactor temperature. In all these examples, the cost of the protease preparation is also likely to be an important consideration.

<p>| Table 2.3 Suitability of proteases for use in detergent preparations |</p>
<table>
<thead>
<tr>
<th>Mesophilic proteases</th>
<th>Thermophilic proteases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzyme</strong></td>
<td><strong>Papain</strong></td>
</tr>
<tr>
<td><strong>Properties</strong></td>
<td>Low</td>
</tr>
<tr>
<td><strong>Activity at (pH 9-10.5)</strong></td>
<td>Low</td>
</tr>
<tr>
<td><strong>Stability</strong></td>
<td>Low</td>
</tr>
<tr>
<td><strong>Effect of chelation</strong></td>
<td>None</td>
</tr>
<tr>
<td><strong>Effect of oxidation</strong></td>
<td>Very high</td>
</tr>
<tr>
<td><strong>Cost</strong></td>
<td>Low</td>
</tr>
</tbody>
</table>


Nevertheless, commercial processes usually have multiple requirements for specificity etc. as well as stability. In the detergent industry, proteases must also be
resistant to the effects of high pH, oxidizing agents and EDTA. Several thermophilic proteases can in fact fulfill these requirements (Table 2.3) but would need to be synthesized in much higher yields before they could become economically competitive. The same argument applies to many of the other existing commercial applications of proteases.

Table 2.4 Commercial and industrial applications of mesophilic bacterial proteases

<table>
<thead>
<tr>
<th>Industry</th>
<th>Example of application</th>
<th>Are existing thermophilic proteases appropriate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washing / cleaning</td>
<td>Detergent preparations</td>
<td>Yes</td>
</tr>
<tr>
<td>Tanning</td>
<td>Dehairing / bating</td>
<td>Probably not</td>
</tr>
<tr>
<td>Protein recovery / hydrolysis</td>
<td>Soy protein</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Meat and fish hydrolysates</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Gelatin hydrolysis</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Tenderization</td>
<td>Yes</td>
</tr>
<tr>
<td>Organic synthesis</td>
<td>Aspartame synthesis</td>
<td>Yes</td>
</tr>
<tr>
<td>Beverage</td>
<td>Clarifying wine and beer</td>
<td>No</td>
</tr>
<tr>
<td>Milling/baking</td>
<td>Gluten hydrolysis</td>
<td>Probably not</td>
</tr>
<tr>
<td>Medicine</td>
<td>Digestive aids, treatment of burns, ulcers, etc</td>
<td>No</td>
</tr>
<tr>
<td>Photography</td>
<td>Recovery of silver from emulsions</td>
<td>Yes</td>
</tr>
</tbody>
</table>


If the restraint on cost of enzyme because of low yield of thermophilic protease is mitigated the proteases from thermophiles can be applied for industrial application in detergent, protein hydrolysate, organic synthesis and recovery of silver from photographic/used X-ray films (Table 2.4). Thermophilic proteases may be of more immediate value in processes where the reaction environment precludes using cheaper mesophilic enzymes. One example could be the use of thermophilic proteases in organic synthesis; the ability of proteases to synthesize peptide bonds (reviewed by Glass JD, 1981) is enhanced in the presence of organic solvents but these solvents themselves may cause loss of enzyme activity. This loss can be minimized by using:
(1) immiscible solvents, (2) immobilized (stabilized) proteases or (3) proteases of greater intrinsic stability. Immobilized enzyme preparations are expensive to produce, inevitably lose activity during synthesis and often have lower Vmax values than the free enzyme. The stability of thermophilic proteases in organic solvents permits their use while minimizing activity losses. Indeed, several peptide syntheses in miscible and immiscible organic/aqueous solvents by thermophilic proteases have been reported (Jakubke, 1987; Konnecke et al., 1981).

Only few thermostable proteases are commercially available. One of them is alcalase isolated from *Bacillus licheniformis* a subtilisin, which is an endoprotease of serine type, exhibiting highest activity at 60°C and pH of 8.3. Alcalase found many applications in the food industry, e.g., by reason of their low specificity towards different proteins from plant and animal sources. For example, this enzyme is important in the processing of soy meal which results in soluble, non-bitter hydrolysate, used as component of protein-fortified soft drinks and dietetic food (Synowiecki, 2008). Alcalase is also useful for recovery of proteins from by-products of the meat and fish industry and from crustacean shell waste during chitin production. In addition, thermostable proteases that are resistant to anionic or non-ionic surfactants and are active at temperatures above 60°C found application as component of dishwashing detergents (Banerjee et al., 1999; Niehaus et al., 1999). Such enzymes can be also used for cleaning ultrafiltration membranes at high temperatures, increasing the efficiency of this process (Bruins et al., 2001). The other prospective application of heat-resistant proteases is meat tenderizing. It is due to the great difference of enzymatic activity at moderate and high temperatures. The mesophilic proteases injected into the tissue show a residual activity during the whole period of post-slaughter storage of the meat cuts leading to an excessive fragmentation of the protein molecules. The use of enzymes with an essential activity only during cooking allows the stoppage of proteolysis just by cooling. The stability of heat-resistant proteases in aqueous/organic and non-aqueous media leads to the modification of the reaction equilibria, creating new peptide bonds. Such reverse reactions may be used to improve nutritional quality and functionality of protein hydrolyzates and to decrease their bitterness. It is achieved by increased molecular weight of peptides as well as by introduction of desirable amino acid residues. Among the thermostable proteases used on an industrial scale, immobilized thermolysin from
*Bacillus thermoproteolyticus* is involved in the synthesis of aspartylphenylalanine-1-methyl ester, known as aspartame (De Martin et al., 2001). This product is commonly used as sweetener in many low-caloric food and beverages. Enzymatic synthesis eliminates contamination of the product by non-sweet and bitter isomers. Poultry industry generates a large amount of feathers. Traditionally, this by-product is degraded by alkali hydrolysis and steam pressure cooking. Such way of processing destroy some essential amino acids and lead to formation of non-nutritive lysinoalanine and lantionine. By this reason, a great significance has non-polluting, biotechnological utilization of keratin-containing wastes (Gousterova et al., 2005; Grazziotin et al., 2006). However, the commonly produced proteases could not degrade keratin. It is caused by tightly packed filament structure of keratin, stabilized by a large number of disulfide and hydrogen bonds as well as by hydrophobic interactions (Parry and North, 1998). This insoluble protein can be broken down by some microorganisms secreting keratinases (EC 3.4.99.11) and obtained hydrolyzates have been used as fertilizers and dietary protein supplement for animal feed (Grazziotin et al., 2006). In addition, keratinases have potential use as de-hairing agent in leather and cosmetic industry, and as constituent of detergents and edible films (Cortezi et al., 2008). There have been many reports on purification of thermophilic keratinases from microorganisms (Ferrero et al., 1996; Riffel et al., 2007; Riessen and Antranikian, 2001; Zhang et al., 2009). Depending on protease source, keratinolytic enzymes show different activity and substrate specificity. For instance, keratinase from *B. licheniformis* is capable of hydrolyzing bovine serum albumin, collagen and elastin. Conversely, a thermostable protease synthesized by *Cryzosphorium keratinophilum* is active only towards keratin (Dozie et al., 1994). The use of keratinolytic enzymes from thermophiles leads to increased rate of keratin degradation. Moreover, such keratinases usually have low collagenolytic activity, which are the requirements of enzymatic de-hairing in the leather industry. The conventional method of de-hairing in alkaline condition by treatment with sodium sulfide poses problems of environmental pollution (Gupta and Ramnani, 2006; Macedo et al., 2005). Properties of keratinases are dependent on microorganism and synthesis of these enzymes can be induced by keratin added to the growth media. Essential for keratinases production are cultivation conditions, such as pH, temperature and media composition. The majority of known keratinases are extracellular or outer-membrane bound endopeptidases belonging to the serine
protease family, but aspartic, cysteine and metallo-proteases are also found (Suzuki et al., 2006). Keratinases produced by mesophilic bacteria and saprophytic fungi are mostly active at temperatures up to 50°C. However, in a few cases, thermoactive proteases which maintain keratinolytic activity at elevated temperatures are produced by some mesophiles (Dozie et al., 1994). Such phenomenon was observed in the case of *B. licheniformis* K-19 production, during cultivation at 37°C; keratinolytic protease performed enzymatic activity at temperatures from 30 to 90°C. This protease has highest activity at 60°C and pH 7.5 - 8.0 (Xu et al., 2009). In contrast, protease from thermophilic *Fervidobacterium islandicum* AW-1 shows optimal keratinolytic activity at 100°C and pH 9.0, and has half-life of 90 min at 100°C (Nam et al., 2002). The biodegradation employing purified keratinase could be replaced by the action of thermophilic microorganisms growing in reaction media or the use of culture filtrates containing the keratinase alone without microorganism cells (Balint et al., 2005; Fredrich and Antranikian, 1996; Gousterova et al., 2005; Wang and Yeh, 2006). This process is efficient enough and usually requires mild conditions and smaller energy input, because microbial decomposition generates heat during cultivation. It reduces the costs of processing and no pathogenic bacteria (mostly mesophiles) can grow at elevated temperature. Recently, the bacterium *Meiothermus ruber* H-328 was used for utilization of chicken feathers. Aerobic cultivation of this moderate thermophile at 55°C for 6 days causes almost complete degradation of the feathers into amino acids and oligopeptides (Matsui et al., 2009). The novel possible application of keratinases and some other microbial proteases is their use for degradation of infectious form of prion proteins formed through aggregation to abnormal amyloid structure designated as PrPSc (Johnson, 2005; Priola, 2001). Generation of this form causes bovine spongiform encephalopathy and Creutzfeldt-Jacob disease in human. Aggregated prion molecules are strongly resistant to conventional proteases and different methods used for pathogen inactivation, including autoclaving at 121°C (Langeveld et al., 2003). Investigations have shown that amyloid form of prion can be degraded by some microbial proteases through the use of denaturing pretreatments, such as pre-heating and treatment with detergents (Tsiroulnikow et al., 2004). Promising source of enzymes degrading PrPSc without detergents or under non-alkaline conditions are some thermophiles and microorganisms belonging to the genus of *Streptomyces* (Hui et al., 2004). Furthermore, thermophiles belonging to *Thermoanaerobacter* and *Thermococcus* were found to hydrolyze the thermally denatured amyloid form of
prion and could be used for decontamination of animal wastes (Suzuki et al., 2006). The activity of the membrane-bound proteases from thermophilic *Geobacillus colagenovorans* MO-1 towards collagen shows that their substrate binding-domains could be useful in enhancing drug delivery in some tissues. For instance, a fusion protein carrying the epidermal growth factor at the collagen binding-domain, when injected into nude mice, remained around sites of injection up to 10 days, whereas not fused substance was not detectable 24 h after injection (Nishi et al., 1998). Thermostable protease from *Thermus* sp. is used for cleanup of DNA before polymerase chain reaction (PCR). The application of thermostable enzyme for this purpose is dependent on compatibility with an existing high-temperature process (Bruins et al., 2001).

### 2.7 IMMOBILIZATION

Protein stability can be increased by various chemical modification procedures. Reports of increasing the thermostability of thermophilic proteases by immobilization (Cowan and Daniel, 1982; Kumakura et al., 1984) and metal ion substitution (Khoo et al., 1984) have been published. Other methods of protein stabilization such as by covalent cross linking are well established (Mozhaev & Martinek, 1984; Maneepun & Klibanov, 1982). Immobilization of enzymes on magnetic micro and nano particles has been done for obvious advantages of ease of separation, increased half life and reusability (Huang et al., 2010; Yiu & Keane 2012; Rossi et al., 2004).

### 2.8 CLONING OF THERMOPHILIC PROTEASE GENE IN MESOPHILE

Fujii and coworkers (Fujii et al., 1983) have cloned the structural gene for a *B. stearothermophilus* neutral protease into a plasmid. When the recombinant plasmid was expressed in *B. stearothermophilus*, protease production increased 15-fold. There have been no reports of attempts to increase protease production in cultures of extreme thermophiles by genetic manipulation. Attempts to select a highly productive, UV-induced mutant of *T. aquaticus* strain T351 have yielded a variant producing significantly more extracellular protease than the wild type (Cowan et al., 1985). It is quite possible that selection, mutation and genetic manipulation techniques will allow the isolation of thermophilic protease producers comparable to mesophilic organisms in their enzyme production.