1. INTRODUCTION

1.1 Enzymes General

Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. More than 3000 different enzymes described to date the majority have been isolated from mesophilic organisms. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known enzymes unrec...
Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta et al., 2002b). Proteases represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market.

Protease accounting for about 60% of total worldwide sale of enzymes (Rao et al., 1998; Cowan, 1994). This dominance of proteases in the industrial market is expected to increase further by the year 2005 (Gupta et al., 2002b). The estimated value of the worldwide sales of industrial enzymes was $1 billion including Rennins 10%, Trypsin 3%, alkaline proteases 25%, Amylases 18%, Other carbohydrases 10%, Analytical and pharmaceutical enzymes 10%, Lipases 3%, Other proteases 21% (Rao et al., 1998).

Proteases the most important group of enzymes produced commercially are used in detergent, protein, brewing, meat, photographic, leather and dairy industries (Anwar and Saleemuddin, 1998; Disney Ribeiro Dias et al., 2008). Proteases have a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals, is a relatively new development and has conferred added biotechnological importance (Rao et al., 1998). These enzymes have also become widely used in the detergent industry, since their introduction in 1914 as detergent additives (Gupta et al., 2002b).

Proteases of commercial importance are produced from microbial, animal and plant sources. They constitute a very large and complex group of enzymes with different properties of substrate specificity, active site and catalytic mechanism, pH
and temperature activity and stability profiles. Industrial proteases have application in a range of process taking advantage of the unique physical and catalytic properties of individual proteolytic enzyme types (Ward, 1991). This 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 (Rao et al., 1998).

1.3 Classification of Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). Proteases can be classified according to 3 major criteria. Such as; i) the reaction catalysed, ii) the chemical nature of the catalytic site, iii) the evolutionary relationship, as revealed by the structure.

Proteases are broadly classified as endo- or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine proteases, aspartic proteases, cysteine proteases, or metallo proteases depending on their catalytic mechanism. They are also classified into different families and clans depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimal activity, they are referred to as acidic, neutral, or alkaline proteases (Rao et al., 1998).

1.3.1 Types of Proteases

Proteases serine protease, cysteine protease, aspartic proteases and metallo protease constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market (Nunes and Martins 2001; Singh et al., 2001; Zeikus et al., 1998). Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases and among bacteria, Bacillus sp are specific producers of protease (Ward, 1985).
Microbes have both intra cellular and extra cellular proteases, the intracellular proteases are responsible for the maintenance of amino acid pool inside the cell by degrading the unwanted proteins and the extra cellular proteases hydrolyze proteins outside the cells into peptides and amino acid required by the cells for their growth. Proteases are classified into two major groups: the exopeptidases (peptidases) and the endopeptidases (proteinases). The peptidases hydrolyze the protein from C- or N-terminus releasing single amino acid and the endopeptidases as the name suggests hydrolyses the peptide bond in the middle of the amino acid chain. Further the proteases are also classified into alkaline, acid and neutral proteases based on their pH optima of activity (Banerjee et al., 1993).

1.3.2 Exoproteases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively (Rao et al., 1998).

1.3.3 Aminopeptidases

Amino peptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. They are known to remove the N-terminal Met that may be found in heterologously expressed proteins but not in many naturally occurring mature proteins. Amino peptidases occur in a wide variety of microbial species including bacteria and fungi. In general amino peptidases are intracellular enzymes, but there has been a single report on an extracellular peptidase produced by A. oryzae (Rao et al., 1998).

1.3.4 Carboxypeptidases

The carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallo carboxypeptidases, and
cysteinecarboxypeptidases, based on the nature of the amino acid residue at the active site of the enzymes (Rao et al., 1998).

1.3.5 Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine and (iv) metalloproteases (Rao et al., 1998).

1.3.6 Serine Proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms (Rao et al., 1998). Serine proteases are recognized by their irreversible inhibition by 3,4- dichloroisocoumarin (3,4-DCl), L-3 carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E-64), diisopropylfluorophosphate (DFP), phenyl methyl sulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity. Their molecular masses range between 18 and 35 kDa. The isoelectric points of serine proteases are generally between pH 4 and 6. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases (Rao et al., 1998).

Serine alkaline proteases are produced by several bacteria, molds, yeast, and fungi. They hydrolyse a peptide bond, which has tyrosine, phenylalanine, or leucine at the carboxyl site of the splitting bond. The optimal pH of alkaline proteases is around pH 10, and their isoelectric point is around pH 9. Their molecular weights
are in the range at 15 and 30 kDa. Although serine alkaline proteases are produced by several bacteria such as *Arthrobacter, treptomyces, and Flavobacterium* spp., subtilisins produced by *Bacillus* spp. are the best known ones (Rao *et al*., 1998).

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg, and subtilisin Novo or bacterial proteases Nagase (BPN’), have been identified. Subtilisin Carlsberg produced by *Bacillus licheniformis* was discovered in 1947 by Linderstrom, Lang and Ottesen at the Carlsberg laboratory and is widely used in detergents. Less commercially important Subtilisin Novo or BPN’ is produced by *B. amyloliquefaciens*. Both subtilisins have a molecular weight of 27.5 kDa but differ from each other by 58 amino acids. They have similar properties such as an optimal temperature of 60°C and an optimal pH of 10 (Rao *et al*., 1998).

**1.3.7 Aspartic Proteases**

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin, retropepsin and enzymes from pararetroviruses. Most aspartic proteases show maximal activity at low pH and have isoelectric points in the range of pH 3 to 4.5. Their molecular weights are in the range of 30 to 45 kDa. The aspartic proteases are inhibited by pepstatin (Rao *et al*., 1998).

**1.3.8 Cysteine / Thiol Proteases**

Cysteine proteases occur in both prokaryotes and eukaryotes. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. In catalytic mechanism of cysteine peptidases the thiol group of a single cysteine residue plays an essential role. This group is susceptible to oxidation and can react with a variety of reagents; heavy metals, iodoacetate, N-ethyl-maleimide etc. (Kenny, 1999). Based on their side-chain specificity, they are broadly divided
into four groups: (i) papain-like, (ii) trypsin-like, (iii) specific to glutamic acid and (iv) others. Papain is the best-known cysteine proteases. Cysteine proteases have neutral pH optima.

1.3.9 Metalloproteases

Metallo proteases are characterized by the requirements for a divalent metal ion for their activity. Based on the specificity of their action, metalloproteases can be divided into 4 groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I and (iv) Myxobacter II. They are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP.

1.4 Sources of Proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, found in a wide diversity of sources such as plants, animals and microorganisms (Rao et al., 1998). Fortunately, enzymes can be separated from living cells and perform catalysis independent of their physiological environment. Commercial proteases are derived from animal tissues, plant cells and microbial cells via fermentation.

1.4.1 Plant Proteases

The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin (Rao et al., 1998). Papain and ficin are prepared by water extraction of crude material from Carica papaya and Ficus carica respectively. Bromelain is usually obtained from the stems of the pineapple plant by extraction and fractional solvent precipitation (Ward, 1985). Bromelain and papain are plant-derived proteases with a longstanding history of use in a diverse range of food applications. As plant-derived products, they are perceived as safe and “natural”
ingredients for use in the food applications and may offer unique benefits and functionality. However in applications where increasing the concentration of soluble solids or simple viscosity reduction are the primary objectives, bromelain and papain are usually not cost effective.

1.4.2. Animal Proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennin. These are prepared in pure form in bulk quantities. However their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies (Rao et al., 1998). Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins (Rao et al., 1998). Chymotrypsin is found in animal pancreatic extract. Pure chymotrypsin is an expensive enzyme and is used only for diagnostic and analytical applications (Rao et al., 1998). Pepsin is an acidic protease that is found in the stomachs of almost all vertebrates (Rao et al., 1998). Pepsin is prepared from the fundus portion of hog stomachs, by acid extraction and filtration (Ward, 1985). Pepsin was used in laundry detergents as early as 1913, but is now being replaced by a mixture of serine and metal microbial proteases that appear to be less degradable by soaps, alkaline conditions and high temperatures (Adinarayana and Ellaiah, 2002). Rennet is a pepsin-like protease that is produced as an inactive precursor in the stomachs of all nursing mammals. It is converted to active rennin by the action of pepsin. It is used extensively in the dairy industry to produce a stable curd with good flavour (Rao et al., 1998).

1.4.3. Microbial Proteases

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases (Rao et al., 1998). Though several microorganisms such as bacteria, fungi, yeast, plant, and mammalian tissues are known to produce alkaline protease, with increasing
industrial demand for proteases it is expected that hyperactive strains will emerge and that the enzymes produced by new exotic microbial strains could be used as biocatalysts in the presently growing biotechnological era (Prakasham et al., 2005; Varun et al., 2008). Bacteria also secrete proteases to hydrolyze (digest) the peptide bonds in proteins and therefore break the proteins down into their constituent monomers. A secreted bacterial protease may also act as an exotoxin, and be an example of a virulence factor in bacterial pathogenesis. Bacterial exotoxic proteases destroy extracellular structures. Protease enzymes are also used extensively in the bread industry. *Bacillus sp*, as one of the best alkaline protease producers, has shown various physiological capabilities (Holt et al., 1994; Ram et al., 1994; Sneath et al., 1986). *Bacillus* sp isolated from alkaline soil was able to producing extra cellular alkaline protease (Muhammad Asif et al., 2012). The detection and isolation methods of isolate was based on resistance of their endospores to high temperatures ranging 70 – 80 °C (Emtiazi et al., 2005; Holt et al., 1994; Sneath et al., 1986; Vela, 1974). Proteases are the most useful industrial enzymes that are produced 500 tones annually. In industry, proteases are produced from bacteria and fungi.

Proteases of bacteria, fungi and viruses are increasingly studied due to its importance and subsequent applications in industry and biotechnology. Commercial application of microbial proteases is attractive due to the relative ease of large-scale production as compared to proteases from plant and animals. Microbial proteases account for approximately 40% of the total worldwide enzyme sales. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they posses almost all the characteristics desired for their biotechnological applications (Rao et al., 1998). Microorganisms 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 generate new enzymes with altered properties that are desirable for their various applications (Rao
et al., 1998, Gupta et al., 2002a). In general microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals (Gupta et al., 2002a). Microbial proteases, especially from Bacillus sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulations (Beg et al., 2003).

1.5 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 are of metallo-type (metalloprotease); and they are the most important group of enzymes exploited commercially (Gupta et al., 2002b). Alkaline proteases are most active at pH values of about pH 10. They are sensitive to DFP and a potato inhibitor but not to TLCK or tosyl-L-phenylalanine chloromethyl ketone (TPCK). 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 and Saleemuddin, 1998).

Alkaline proteases being a physiologically and commercially important group of enzymes are used primarily as detergent additives. These enzymes have broad substrate specificities and will function to some extent under the rather 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).
In recent years there has also been a phenomenal increase in the use of alkaline protease as industrial catalysts. In Japan, 1994 alkaline protease sales were estimated at $116 million. There is expected to be an upward trend in the use of alkaline proteases so that by the turn of the decade the total value for industrial enzymes is likely to reach $700 million or more (Kumar and Takagi, 1999).

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 (Agrawal et al., 2004, Gupta et al., 2002a).

Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues (Singh et al., 2001a). Despite this 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. 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 60ºC. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao et al., 1998). From all the alkaliphilic bacteria that have been screened for use in various industrial applications, members of the genus *Bacillus*, mainly strains *B. subtilis* and *B. licheniformis* were found to be predominant and a prolific source of alkaline proteases (Kumar and Takagi, 1999).

### 1.6 Alkaline Proteases of Alkaliphilic *Bacillus* Strains

Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent source. A myriad of *Bacillus* species from many different exotic environments have been explored and exploited for alkaline
protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, and *B. majovensis* (Gupta *et al.*, 2002a). Alkaline proteases produced by thermophilic and alkaliphilic bacilli can withstand high temperature, pH, chemical denaturing agents and in non-aqueous environments (Johnvesly and Naik, 2001; Wang Shuai *et al.*, 2012).

Bacteria belonging to *Bacillus* sp. are by far the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give rise to products that are in turn stable in a wide range of harsh environments. *Bacillus* is a rod-shaped, gram positive, spore forming, aerobic, usually catalase positive, chemooorganotropic bacterium. Alkaliphilic *Bacillus* can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources (Akbalık, 2003).

The first report concerning an alkaline enzyme was published by Horikoshi in 1971. Horikoshi reported the production of an extracellular alkaline serine protease from alkaliphilic *Bacillus* strain 221. This strain, isolated from soil, produced large amounts of alkaline protease that differed from the subtilisin group. The optimum pH of the purified enzyme was 11.5 and 75% of the activity was maintained at pH 13.0. The enzyme was completely inhibited by diisopropylfluorophosphate or 6 M urea but not by EDTA or *p*-chloromercuribenzoate. The molecular weight of the enzyme was 30,000 Da, which is slightly higher than those of other alkaline proteases. The addition of a 5mM solution of calcium ions increased the activity by 70% at the optimum temperature of 60°C (Horikoshi, 1999). Moreover, various types of alkaline protease have been characterized and their potential industrial applications have been explored. The
major applications of these enzymes are in detergent formulation, the food industry, leather processing, chemical synthesis and waste management (Gupta et al., 2002a).

1.7 Properties of Alkaline Proteases

1.7.1. Optimum Temperature and Thermostability of Alkaline Proteases

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 (Öztürk, 2001). 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 (Rahman et al., 1994).

Therefore, a wide range of microbial proteases from thermophilic species has been extensively purified and characterized. These include Thermus sp., Desulfuroccoccus strain Tok12S1 and Bacillus sp.. Among them alkaline proteases derived from alkaliphilic bacilli, are known to be active and stable in highly alkaline conditions (Rahman et al., 1994). The earliest thermophilic and alkaliphilic Bacillus sp. was B.stearothermophilus strain F1 isolated by Salleh and friends in 1977, which was stable at 60ºC (Haki and Rakshit, 2003). Further studies on microbial alkaline proteases have been done in view of their structural-function relationship and industrial applications, as they needed stable biocatalysts capable of withstanding harsh conditions of operation (Rahman et al., 1994).

Generally alkaline proteases produced from alkaliphilic Bacillus are known to be active over a wide range of temperature. The optimum temperatures of alkaline proteases range from 40 to 80ºC. In addition, the enzyme from an
obligatory alkaliphilic *Bacillus* P-2 showed an exceptionally high optimum temperature of 90°C. The protease has also good thermostability at high temperatures, being thermostable at 90°C for more than 1 h and retained 95% and 37% of its activity at 99°C (boiling) and 121°C (autoclaving temperature), respectively. *Bacillus* P-2 was the only mesophile reported until 2001, which produced a proteolytic enzyme that was stable for so long even at autoclaving (121 °C) and boiling temperatures (Kaur et al., 2001). In some studies it has also been observed that the addition of Ca2+ further enhanced enzyme thermostability (Takami et al., 1989, Gessesse, 1997, Rahman et al., 1994).

1.7.2. 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 will 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., 2002a). 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

### 1.7.3. 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 moves farther 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 (Ozturk, 2001).

### 1.7.4. The Molecular Weight of Alkaline Proteases

The molecular weights of alkaline proteases generally range from 15 to 30 kDa (Kumar and Takagi, 1999) with few reports of higher molecular weights of 32.0 kDa (Huang *et al.*, 2003), 33.5 kDa (Rahman *et al.*, 1994), 36.0 kDa (Durham *et al.*, 1987).

### 1.7.5. Metal Ion Requirement and Inhibitors of Alkaline Proteases

Alkaline proteases require a divalent cation like Ca²⁺, Mg²⁺ and Mn²⁺ 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 and Takagi, 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). In this regard, PMSF sulfonates the essential serine residue in the active site, results in the complete loss of activity. 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 and Takagi, 1999).

1.8 Enzyme Production by Solid State Fermentation / Submerged Fermentation

Enzyme production by fermentation can be carried out by both submerged fermentation (SmF) and solid-state fermentation (SSF). SSF has been established as a superior technique (as compared to submerged fermentation) for the production of enzymes (Pandey *et al.*, 2001; Sandhya *et al.*, 2005). Solid-state fermentations involve microbial modification of a solid, undissolved substrate in which microbial cultures are grown on a moist solid with little or no free water, although capillary water may be present (Mudgett, 1986). The production can be recovered in highly concentrated form as compared to those obtained by submerged fermentations. Traditionally, synthetic substrates were used for fermentations, which are now being largely replaced by agro and agro-industrial by-products. These are not only providing a natural substrate for fungal growth and fermentation, they also result in improved value of these agro-industrial residues (Pandey *et al.*, 2001).

In order to overcome the high prices of the industrial proteases specially those used in the food and pharmaceutical industries several works are going on the fungal SSF and the feasibility of the process and its positive implications on the
protease production have been showed, however studies on the photolytic specificity and selected applications are need to be done (Barthomeuf et al., 1992, Banerjee et al., 1993; Fernandez Lahore, 1997). Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites (Adrio et al., 2003). Proteases represent an important group of enzymes produced industrially and account for 60% of the worldwide sales value of the total industrial enzymes (Godfrey, 1996). Proteases are capable of cleaving proteins into peptides and amino acids, they are characterized by their optimal pH (acid, neutral or alkaline), their temperature, their ability to hydrolyze specific proteins (collagenase, keratinase etc), their homology to well characterized enzymes as chymosine, chymotrypsin, pepsin, trypsin (trypsin-like, pepsin-like etc.), and their stability.

1.9 Substrate involved in Protease Enzyme Production

Sugarcane crop is mainly directed for production of ethyl alcohol, sugar and spirits. Sugarcane bagasse is a by-product resulting from juice extraction. This waste basically consists of 50% of cellulose, 30% sugar and 2.4% of ashes (Pandey et al., 2000). Agro-based industrial waste such as groundnut cake, coconut cake, soy cake and wheat bran were also used as substrate for protease enzyme production.

1.10 Applications of Proteases

The major uses of proteases are in the biotechnological production of detergents (pepsin) (Bailey et al., 1977), in dairy industries as milk-clotting agents (calf rennet composed mainly of chymosine and pepsin) (Fox, 1982) and as an agent for meat tenderization. Proteases have also clinical and medical application (reduction of tissue inflammation) (Bailey et al., 1977; Nout et al., 1990). Proteases of microbial origin have long been used in industry; they are replaced by fungal proteases (Ogrydziak, 1993; Pavlukova et al., 1998), easily extracted and separated from mycelium (Phadatare et al., 1993). Acid proteases are synthesised by Mucor miehei (Brown, 1991; Escobar et al., 1993; Fernandez- Lahore et al., 1999; Rickert,
1970), *Mucor hiemalis*, *Mucor racemosus* and *Mucor bacilliformis* (Fernandez-Lahore et al., 1997; Fernandez-Lahore et al., 1998). The *Mucor* rennin acid protease production by isolated sp of *Penicillium*. Proteases are derived from *Mucor pusillus* (Arima et al., 1968; Iwasaki et al., 1967) and other pepsin like acid proteases are synthesized by *Aspergillus* sp (Tremacoldi et al., 2004) and *Rhizopus* sp.

Biotechnological importance of protease enzymes has been realized by the leather industries for the purpose of dehairing and bating hides as a substitute toxic chemical (Bhosale et al., 1995; Zambare et al., 2007; Mahjabeen Saleem et al., 2012). In pharmaceutical industry, they are used as ingredients of ointments for debridement of wards and in medicine preparation (Jany et al., 1986).