CHAPTER-I

GENERAL INTRODUCTION

1.1 ENZYMES

Enzymes are biocatalysts in living cells to accelerate and coordinate the multitude of chemical reactions necessary to develop and sustain life processes. More than 3000 enzymes, which catalyze a wide variety of chemical reactions, are known. Among six classes of enzymes, hydrolases (proteases, amylases, cellulases and xylanases) have a wide range of biotechnological applications.

The activity of an enzyme is due to its catalytic nature. The reaction is at much higher rate when enzyme is present. Enzymes are highly specific and act only on certain types of substrates. Enzymes are non-toxic and biodegradable and can be produced from microorganism in large amounts without the need for special chemical resistant equipment.

Commercial exploitation of microbial enzymes began much before their nature and properties were worked out. For centuries, extracts of plants had been used to bring about hydrolysis of polymeric material, use of malted barley extracts in brewing of beer, application of animal dung in bating of hides in leather manufacturing are some examples of the uses of the enzymes in earlier days. However, these sources of enzymes were unreliable and expensive, hence search for alternative sources began. Largely these were found in the microbial cultures.

1.2 PRODUCTION, MARKETING AND APPLICATIONS OF INDUSTRIAL ENZYMES

The current estimated value of the worldwide sales of industrial enzymes is $1 billion (Meenu et al., 2000). Among the industrial enzymes 75% are hydrolytic. Among the hydrolytic enzymes proteases represent one of the three largest groups of industrial enzymes accounting for 30% of the total world wide enzyme production (Horikoshi, 1996; Richard et al., 1996; Banerjee et al., 1999 and Adinarayana and Ellaiah, 2002) and
about 60% of the total worldwide sale of enzymes, the distribution of industrial enzymes is shown in Fig-1 (Rao et al., 1998).

Fig-1: Distribution of enzyme sales. The contribution of different enzymes to the total sale of enzyme is indicated. The shaded colored portion indicates the total sale of proteases.
1.1 PROTEASES

Proteases form a large group of enzymes, ubiquitous in nature and found in a wide, variety of microorganisms. They are molecules of relatively small size and are compact, spherical structures that catalyze the peptide bond cleavage in proteins (Polgar, 1989). These enzymes are important in a number of diverse and crucial biological processes; for example, they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogenicity, and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao et al., 1998).

1.3.1 Source of proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. Among the various protease, bacterial proteases are the most significant compared with animal and fungal protease (Ward, 1985 and Adinarayana et al., 2003). The first report on the production of alkaline protease from Bacillus sp.No.221 was from Horikoshi (1971).

1.3.1.1 Plant proteases

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

1.3.1.2 Animal Proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins (Boyer, 1971; Hoffman, 1974 and Rao et al., 1998). These are prepared in pure form in bulk quantities. However, their production depends on the availability of livestock for slaughter, which in turn governed by political and agricultural policies.
1.3.1.3 Microbial proteases

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

1.3.2 Classification of proteases

Proteases are difficult to characterize because of their diversity of action and structure. Originally proteases were classified based on molecular size, charge or substrate specificity. However, with the advent of molecular biology, proteases are now grouped into families based on the chemical nature of the catalytic or active sites, mechanism(s) of action, and the evolutionary relationship of their three-dimensional structure (Beynon and Bond, 1989 and Rao et al., 1998).

Proteases are broadly divided into either exopeptidases or endopeptidases depending on their site of action. If the enzyme cleaves the peptide bond proximal to the amino or carboxy terminus of the substrate, they are classified as exopeptidases. If the enzyme cleaves peptide bonds distant from the termini of a substrate, they are classified as endopeptidases. Based on the functional group present at the active site and their
catalytic mechanism, proteases are then categorized into four groups; serine proteases, aspartic proteases, cysteine/thiol proteases, or metalloproteases. Four classes of endoproteases have been identified in living organisms and three of them have been isolated and purified in bacteria are serine, cysteine, and metalloproteases.

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (International Union of Biochemistry. 1992). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Barett, 1994). Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960).

Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I and (iv) Myxobacter II. The neutral proteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity. 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 also referred to as acidic, neutral, or alkaline proteases.

A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce alkaline proteases (Taguchi et al., 1983; Kim et al., 1993; Ogrydziak, 1993; Abraham and Breuil, 1996 and Meenu et al., 2000). 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 (Han and Damodaran, 1997). Alkaline proteases were in fact the first enzyme to be produced in bulk.

There are a few miscellaneous proteases, which do not precisely fit into the standard classification, e.g., ATP-dependent proteases which require ATP for activity (Menon and Goldberg, 1987). Based on their amino acid sequences, proteases are classified into different families (Argos, 1987) and further subdivided into “clans” to accommodate sets of peptidases that have diverged from a common ancestor (Rawlings and Barrett, 1993). Each family of peptidases has been assigned a code letter denoting the type of catalysis, viz., S, C, A, M, or U for serine, cysteine, aspartic, metallo-, or unknown type, respectively.
1.3.3 Genetic engineering of microbial proteases

Gene cloning is a rapidly progressing technology that has been instrumental in improving our understanding of the structure-function relationship of genetic systems. It provides an excellent method for the manipulation and control of genes. More than 50% of the industrially important enzymes are now produced from genetically engineered microorganisms (Hodgson, 1994).

1.3.4 Applications of proteases in various sectors

Proteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins; hence proteases are degradative enzymes, which catalyze the total hydrolysis of proteins.

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmental friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. The worldwide requirement of enzymes for individual applications varies considerably. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc.,
Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used.

1.3.4.1 Detergent industry

Microbes are the good sources of proteases, however only proteases from *Bacillus* Spp. have been found satisfactory in detergent applications (Sinha and Satyanarayana, 1991 and Nehra et al., 2002). Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures (Adil and Saleemuddin, 2000). The use of proteases in laundry detergents as an active ingredient in laundry detergents is the single largest application (Sinha and Saryanarayana, 1991) accounts for approximately 25% of the total worldwide sales of enzymes (Rao et al., 1998; Banerjee et al., 1999 and Adil and Anwar, 2000). The preparation of the first enzymatic detergent, “Burnus,” dates back to 1913; it consisted of sodium carbonate and a crude pancreatic extract. The first detergent containing the bacterial enzyme was introduced in 1956 under the
trade name BIO-40. In 1960, Novo Industry A/S introduced alcalase, produced by *Bacillus licheniformis*; its commercial name was BIOTEX. This was followed by Maxatase, a detergent made by Gist-Brocades. The biggest market for detergents is in the laundry industry, amounting to a worldwide production of 13 billion tons per year.

The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood, and other body secretions. Activity and stability at high pH, temperature and compatibility with other chelating and oxidizing agents added to the detergents are among the major prerequisites for the use of proteases in detergents (Richard et al., 1996 and Sandeep et al., 2001). The key parameter for the best performance of protease in a detergent is its pl. It is known that a protease is most suitable for this application if its pl coincides with the pH of the detergent solution. Esperase and Savinase T (Novo Industry), produced by Alkalophilic *Bacillus* spp. are two commercial preparations with very high isoelectric points (pl 11); hence, they can withstand higher pH ranges (Rani et al., 1999). Due to the present energy crisis and the awareness for energy conservation, it is desirable to use proteases that are active at lower temperatures. A
combination of lipase, amylase, and cellulase is expected to enhance the performance of protease in laundry detergents. All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. An alkaline protease from *Conidiobolus coronatus* was found to be compatible with commercial detergents used in India (Phadatare *et al.*, 1993) and retained 43% of its activity at 50°C for 50 min in the presence of Ca$^{2+}$ (25 mM) and glycine (1 M) (Bhosale *et al.*, 1995). The alkaline protease produced from *Bacillus* sp. JB-99 retained 90% of activity at 50°C at pH 11.0 in presence of 10 mM Ca$^{2+}$ (Johnvesly and Naik, 2001).
1.3.4.2 Leather industry

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 (Malathi and Chakraborty, 1991 and Berla et al., 2001). The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Poldermas, 1990 and Fox et al., 1991). At the end of 1998 there were 18 major companies manufacturing leather products (12 footwear and 6 leather works) primarily for the export markets (FITIB, 2001).

Leather processing involves several steps such as soaking, dehairing, bating, and tanning (Sinha and Satyanarayana, 1991 and Sandeep et al., 2001). The major building blocks of skin and hair are proteinaceous. The conventional methods of leather processing involve hazardous chemicals such as sodium sulfide, which create problems of pollution and effluent disposal (Malthi and Chakraborty, 1991). The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution. Proteases are used for selective hydrolysis of noncollagenous constituents of the skin and for removal of nonfibrillar proteins such as albumins and globulins. The purpose of soaking is to swell the hide. Traditionally, this step was performed with alkali. Currently, microbial alkaline proteases are used to ensure faster absorption of
water and to reduce the time required for soaking. The use of nonionic and, to some extent, anionic surfactants are compatible with the use of enzymes. Microbial proteases and animal proteases are also used in dewooling of animal skin and recovery of soluble proteins and amino acids from chrome leather wastes (Taylor et al., 1993 and Berla and Susheela, 2002). The conventional method of dehairing and dewooling consists of development of an extremely alkaline condition followed by treatment with sulfide to solubilize the proteins of the hair root. At present, alkaline proteases with hydrated lime and sodium chloride are used for dehairing, resulting in a significant reduction in the amount of wastewater generated. Earlier methods of bating were based on the use of animal feces as the source of proteases; these methods were unpleasant and unreliable and were replaced by methods involving pancreatic trypsin. Currently, trypsin is used in combination with other Bacillus and Aspergillus proteases for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin, and the amount of enzyme needed depends on the type of leather (soft or hard) to be produced. Increased usage of enzymes for dehairing and bating not only prevents pollution problems but also is effective in saving energy.
Novo Nordisk manufactures three different proteases, Aquaderm, NUE, and Pyrase, for use in soaking, dehairing, and bating, respectively.

1.3.4.3 Food industry

The use of proteases in the food industry dates back to antiquity (Rani et al., 1999). They have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates, and meat tenderization (Rao et al., 1998 and Sandeep et al., 2001).

1.3.4.4 Dairy industry

The major application of proteases in the dairy industry is in the manufacture of cheese (Fairbairn and Law, 1986 and Koka, Weimer, 2000 and Nehra et al., 2002). The milk-coagulating enzymes fall into three main categories, (i) animal rennets, (ii) microbial milk coagulants and (iii) genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have molecular weights between 30,000 to 40,000 daltons. Rennet extracted from the fourth stomach of unweaned calves contains the highest ratio of chymosin.
(EC 3.4.23.4) to pepsin activity. A world shortage of calf rennet due to the increased demand for cheese production has intensified the search for alternative microbial milk coagulants. The microbial enzymes exhibited two major drawbacks, i.e., the presence of high levels of nonspecific and heat-stable proteases, which led to the development of bitterness in cheese after storage; and (ii) a poor yield. Extensive research in this area has resulted in the production of enzymes that are completely inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases. In cheese making, the primary function of proteases is to hydrolyze the specific peptide bond (Phe105-Met106) to generate para-k-casein and macro peptides. Chymosin is preferred due to its high specificity for casein, which is responsible for its excellent performance in cheese making. The proteases produced by GRAS (genetically regarded as safe)-cleared microbes such as Mucor michei, Bacillus subtilis and Endothia parasitica are gradually replacing chymosin in cheese making. In 1988, chymosin produced through recombinant DNA technology was first introduced to cheese makers for evaluation. Genencor International increased the production of chymosin in Aspergillus niger var. awamori to commercial levels. The three recombinant chymosin products are
available and are awaiting legislative approval for their use in cheese making (Godfrey and West, 1996 and Koka and Weimer, 2000). Whey is a by-product of cheese manufacture. It contains lactose, proteins, minerals, and lactic acid. The insoluble heat denatured whey protein is solubilized by treatment with immobilized trypsin.

1.3.4.5 Baking industry

Proteases are also commonly used in baking industries (Gennari et al., 1998 and Sandeep et al., 2001). Wheat flour is a major component of baking processes. It contains an insoluble protein called gluten, which determines the properties of the bakery doughs. Endo and exoproteinases from Aspergillus oryzae have been used to modify wheat gluten by limited proteolysis. Enzymatic treatment of the dough facilitates its handling and machining permits the production of a wider range of products. The addition of proteases reduces the mixing time and results in increased loaf volumes. Bacterial proteases are used to improve the extensibility and strength of the dough.
1.3.4.6 Manufacture of soy products

Soybeans serve as a rich source of food, due to their high content of good-quality protein. Proteases have been used from ancient times to prepare soy sauce and other soy products. The alkaline and neutral proteases of fungal origin play an important role in the processing of soy sauce (Rao et al., 1998). Proteolytic modification of soy proteins helps to improve their functional properties. Treatment of soy proteins with alcalase at pH-8 results in soluble hydrolysates with high solubility, good protein yield, and low bitterness. The hydrolysate is used in protein-fortified soft drinks and in the formulation of dietetic feeds.

1.3.4.7 Debittering of protein hydrolysates

Protein hydrolysates have several applications, e.g., as constituents of dietetic and health products, in infant formulae and clinical nutrition supplements, and as flavoring agents. The bitter taste of protein hydrolysates is a major barrier to their use in food and health care products. The intensity of the bitterness is proportional to the number of hydrophobic amino acids in the hydrolysate. The presence of a proline residue in the center of the
peptide also contributes to the bitterness. The peptidases that can cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolysates. Amino peptidases from lactic acid bacteria are available under the trade name Debitrase. Carboxypeptidase A has a high specificity for hydrophobic amino acids and hence has a great potential for debittering. A careful combination of an endoprotease for the primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness (Rao et al., 1998).

1.3.4.8 Synthesis of aspartame

The use of aspartame as a noncalorific artificial sweetener has been approved by the Food and Drug Administration. Aspartame is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. The L configuration of the two amino acids is responsible for the sweet taste of aspartame. Maintenance of the stereospecificity is crucial, but it adds to the cost of production by chemical methods. Enzymatic synthesis of aspartame is therefore preferred. Although proteases are generally regarded as hydrolytic enzymes, they catalyze the reverse reaction under certain kinetically
controlled conditions. An immobilized preparation of thermolysin from *Bacillus thermoproteolyticus* is used for the enzymatic synthesis of aspartame. Toya Soda (Japan) and DSM (The Netherlands) are the major industrial producers of aspartame (Rao et al., 1998).

**1.3.4.9 Pharmaceutical industry**

The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Oral administration of proteases from *Aspergillus oryzae* (Luizym and Nortase) has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. *Clostridial collagenase* or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. An asparginase isolated from *Escherichia coli* is used to eliminate asparagines from the bloodstream in the various forms of lymphocytic leukemia. Alkaline protease from *Conidiobolus coronatus* was found to be able to replace trypsin in animal cell cultures (Chiplonkar et al., 1985 and Sandeep et al., 2001). Nehra et al., (2002) reported the use of proteases in pharmaceutical industries.

**1.3.4.10 Proteases and health**

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms (Wandersman, 1989 and Son and Kim, 2002). Proteases play a critical role in many physiological processes including protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens (Delepelaire and Wandersman, 1989 and Son and Kim, 2002), release of hormones and pharmacologically active peptides from precursor proteins, and transport
of secretory proteins across membranes (Rawlings and Barett, 1993 and Son and Kim, 2002).

Proteases are degradation enzymes, which catalyze the total hydrolysis of proteins. In general, extracellular proteases catalyse the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell, whereas intracellular proteases play a critical role in the regulation of metabolism. Bacterial proteases are also important virulence factors in many diseases (Farrell and Crosa, 1991).

The wide diversity and specificity of proteases is used to great advantages in developing effective therapeutic agents. The enzyme has been used as digestive aids in gastrointestinal disorders such as dyspepsia. Streptokinase from Streptococcus pyogenes is used for treatment of thrombosis. Alkaline protease from Bacillus licheniformis has been used in denture cleanser tablet. Proteases from Serratia marcescens are used for debridement of dermal ulcers and burns. Clostridial collagenases or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds.

Proteases of the subtilisin group are used in the treatment of burns and wounds. Oral administration of protease produces an anti-inflammatory response in burn patients and speeds up the process of healing (Bogner and Snyder, 1962; Shaw, 1969; Tsomides and Foldberg, 1969 and Thangam and Rajkumar, 2002). Latha et al., (1998) reported that trypsin and chymotrypsin preparation acted as anti-inflammatory and antioxidant agents in burn wounds in humans.

Advances in analytical techniques have demonstrated that proteases conduct highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes.

Proteases execute a large variety of functions, extending from the cellular level to the organ and organism level, to produce cascade systems such as hemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Their
involvement in the life cycle of disease-causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS.

Rice chaff was used as the substrate for the production of fibrinolytic enzyme by *Fusarium oxysporium* in solid state fermentation (Sun *et al.*, 1997). The occlusion of a cerebral or coronary artery by a blood clot accounts for the majority of death and it is responsible for significant in capacitation and morbidity (Haber *et al.*, 1989). Despite their widespread use, all currently available fibrinolytic agents suffer from a number of significant limitation (Lijnen and Collen, 1991) fibrinolytic enzymes occur in bacteria, earthworms and snake toxin, but up to now, there have been few production of fibrinolytic enzymes from fungi (Sun *et al.*, 1997).

### 1.3.4.11 Proteases and environment

*Bacillus* species have been successfully used in degradation of proteinaceous wastes into useful biomass by many investigators. Most of the proteases used in waste bioconversion are alkaline proteases (Venugopal *et al.*, 1989; Dalev and Simeonova, 1992; Atalo and Gashe, 1993; Dalev, 1994 and Yang *et al.*, 2000).

Proteases are by far the most important group of enzyme produced commercially and are used in many areas of applications, such as detergent, brewing, meat, photographic, leather and dairy industries (Kalisz, 1988). In recent years, ample successes in degradation of proteinaceous waste into useful biomass by proteases have also been demonstrated (Venugopal *et al.*, 1989; Dalev and Simeonova, 1992; Atalo and Gashe, 1993 and Dalev, 1994). There were few reports comparing the deproteinisation effects between microbes and enzymes. Bustos and Michael (1994) have compared the effects of microbial and enzymatic deproteinisation.

A protease producing microorganism *Bacillus subtilis, Bacillus subtilis* Y-108 has been used for deproteinisation of crustacean wastes in the preparation of chitin and for
deproteinisation tests, liquid phase fermentation untreated shrimp shell, crab shell, and lobster shell wastes (Yang et al., 2000).

Chitin a homopolymer of N-acetyl-d-glucosamine (GlcNAc) residues linked by $\beta$-1-4 bonds, is the most abundant renewable natural resources after cellulose (Deshapande, 1986). Chitin and its derivatives hold great economic value because of their versatile biological activities and agrochemical applications (Cosio et al., 1982; Flach et al., 1992; Wang et al., 1995 and Hirano, 1996). Marine crustaceans wastes are very rich in chitin. It is estimated that the world wide annual recovery of chitin from the processing of marine crustaceans wastes is 37,300 metric ions, Shaikh and Deshapande (1993) have reported that shrimp crab shell (SCS) contains chitin, protein and inorganic compounds such as calcium carbonate. Conveniently, preparation of chitin from marine waste material involves demineralization and deproteinisation with the use of strong acids or bases (Gagne and Simpson, 1993 and Wang et al., 1997), however the use of these chemicals may cause a partial deacetylation of chitin and hydrolysis of the polymer, resulting in a final inconsistent physiological properties (Brine and Austin, 1981; Shimahara and Takaguchi, 1988; Gagne and Simpson, 1993 and Wang et al., 1997). These chemical treatments also create waste disposal problems, because neutralization and detoxification of the discharged wastewater may be necessary. Furthermore the value of the deproteinisation liquid is diminished due to the presence of sodium hydroxide (Gagne and Simpson, 1993). To overcome the shortage of the chemical treatments studies have been conducted using microorganisms (Shimahara and Takaguchi, 1988 and Bustos and Michael, 1994) or proteolytic enzymes (Takeda and Abe, 1962; Takeda and Katsura, 1964; Broussignac, 1968; Gagne and Simpson, 1993 and Bustos and Michael, 1994) for deproteinisation of marine crustacean wastes.

A few studies on use of proteolytic enzyme for the deproteinisation of crustacean wastes have been reported. Broussignac (1968) demonstrated that use of papain, trypsin or pepsin produced chitin with as little deacetylation as possible. Tuna proteinase, papain, and a bacterial proteinase have also been used for the deproteinisation step. The residual protein associate the chitin after enzyme treatments was about 5% (Takeda and Abe,
1962 and Takeda and Katsura, 1964). Recently, Gagne and Simpson also investigated the utilization of chymotrypsin and papain on deproteinisation of shrimp wastes.

Feathers consisting primarily of keratin (Fraser 1969), are generated in a huge quantities as a waste by product in commercial poultry processing plants. In its native state, keratin is not degradable by common proteolytic enzymes; its degradation by microorganisms is performed by specific proteases (keratinases). The properties secretion and use of microbial keratinases have recently been reviewed (Onifade et al., 1998). Being a substrate for microbial degradation, keratin does not accumulate in substantial quantity in nature. Keratinolytic activity has been reported in many fungi (El-Naghy et al., 1998 and Gradisar et al., 2000) and bacteria eg., streptomycyes (Garcia-Kirchner et al., 1998). Thermoactinomycyes (Ignatova et al., 1999) and vibrio (Sangali and Brandelli, 2000). The secretion of alkaline serine protease is a frequent phenomenon amongst the different isolates of Bacillus licheniformis (Manachini and Fortina, 1988, Han et al., 1995 and Joo et al., 2003). The most effective keratin-degrading strains in the Bacillus genus belong to B. licheniformis. The keratinases of Bacillus licheniformis PWD-1 is a serine protease that effectively degrades leather keratin. When PWD-1 is grown on feathers, subtilisin-like enzymes are also induced (Williams et al., 1990 and Evans et al., 2000). Until recently, feather waste was utilized mainly as a dietary protein supplement for animal feedstuff. Biodegradation by microorganisms possessing keratinolytic activity is an alternative method for improvement of the nutritional value of feather waste and for avoidance of the destruction of certain amino acids (Steiner et al., 1983 and Papadopoulos, 1985). A keratin degrading strain of Bacillus licheniformis (K-508) was isolated from partially degraded feathers and characterized. It has high chicken feather-degrading activity when cultured in feather-containing broth, with a growth optimum at pH 7 and 47°C (Rozs et al., 2001). Broth filtrates were active towards N-Bz-Phe-Val-Arg-p-nitroanilide and N-Suc—Ala-Ala-Pro-Phe-p-nitroanilide, as chromogenic protease substrates at pH 8.0. Strain k-508 displays keratinolytic activity against native feather keratin (without any pretreatment) in the presence of SH-reducing compounds. It constitutively secreted both trypsin-like and chymotrypsin-like proteases.
Compost material has recently been used for the isolation of feather-degrading bacteria (Lin et al., 1999). Their keratinolytic activities were confirmed by assets in which azokeratin was applied as substrate. Five isolates that hydrolyzed feathers were from *Bacillus licheniformis*. Protease inhibition studies indicated that the predominant proteolytic enzymes of these feather-degrading isolates are serine proteases.

### 1.3.4.12 Other applications

Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure function relationship, in the synthesis of peptides, and in the sequencing of proteins. In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in the food, detergent, leather, and pharmaceutical industries, as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.

### 1.3.5 Protein engineering

Many industrial applications of proteases require enzymes with properties that are non physiological. Protein engineering allows the introduction of pre-designed changes into the gene for the synthesis of a protein with an altered function that is desired for the application. Recent advances in recombinant DNA technology and the
ability to selectively exchange amino acids by site-directed mutagenesis (SDM) have been responsible for the rapid progress of protein engineering. Identification of the gene and knowledge of the three-dimensional structure of the protein in question are the two main prerequisites for protein engineering. The X-ray crystallographic structures of several proteases have been determined (Cooper et al., 1990; Sobek et al., 1990; Pickersgill et al., 1990 and Koszelak et al., 1997). Proteases from bacteria, fungi, and viruses have been engineered to improve their properties to suit their particular applications.

1.2 SOLID STATE FERMENTATION (SSF)

Solid state fermentation (SSF) has recently, come to attract a great deal of scientific attention as a untapped potential for industrial exploration. SSF involves the growth of microorganisms on moist solid substrate in the absence of free water and stimulates the fermentation reactions that occur in nature (Raghavarao et al., 1993). The growth environment of SSF which is similar to that existing in nature and therefore provides ideal conditions of growth for certain microorganisms for the production of certain metabolites. Although SSF has been practiced historically in the Eastern world including Japan, China, Korea etc., (where it is referred to as ‘Koji’ fermentation), for the production of enzymes and fermented foods like soya sauce, tempeh, it had been largely neglected in the western world. This was mainly due to the popularity of the industrial production through submerged fermentation wherein the engineering and scale up principles of chemical process engineering could be easily applied. It was also due to the difficulties associated with the measurement and control of SSF products formed, as well
as measurement of the physical properties of the system. For example, measurement of organism growth in solid culture is much more difficult than in liquid culture. Also, the poorly described nature and structure of natural solid substrates, such as unrefined starches and the poorly elucidated relationships between microbial and physical transport phenomena greatly complicate kinetics of SSF.

Two types of mass transfer take place in SSF, interparticle mass transfer and intraparticle mass transfer. The former refers to the transfer of oxygen to the growing microorganisms and the latter to the transfer of nutrients and enzymes within the substrate solid mass (Ramana murthy et al., 1993). In general the degradation of substrate in SSF is attributed to the release of extracellular enzymes or cell bound enzymes to the external environment (Knapp and Howell, 1980; Moo et al., 1983 and Nandakumar et al., 1994).

However, over the past two decade, there has been a resurgence in research on SSF technology due to the need to lower manufacturing costs by using crude agro resources as also the potential of producing new and high value biomolecules through this technique.

1.3 IMMobilization

Enzyme and microbial technology has influenced the process industry significantly in the recent years by improvement of existing processes as well as in the development of eco-friendly industrial bioprocesses. One of the techniques, which have played a significant role, is the immobilization of enzymes and cells.

Immobilization is the important technique, which has emerged in the past two decades to solve the problems of enzymes cost and product purity (D’Souza, 2002). Major steps involved in the development of an immobilization enzymes technology are enzyme production, enzyme purification, and immobilization of enzyme and design of an enzyme reactor. Applications of immobilized enzymes cover food and pharmaceutical industries, waste treatment, environmental pollution control, clinical medicine, immunodiagnostics, silver less photography and analytical instrumentation (Shewale and
Naik, 1991). Recently, Singh and Singh (2002), have reported the removal of phenols from industrial effluents by immobilization techniques.

The use of immobilized whole microbial cells eliminates, the often tedious, time consuming and expensive steps involved in isolation and purification of intracellular enzymes. It also tends to enhance the stability of the enzymes by retaining its natural surroundings during immobilization and subsequent continuous operation. The ease of conversion of batch processes into a continuous mode and maintenance of high cell activity has advantages of immobilized cell systems. The metabolically active cell immobilization is particularly preferred where cofactors are necessary for the catalytic reactions (Ramakrishna and Prakasham, 1999).

Microorganisms are the best sources for the production of useful enzymes. Cell immobilization technology is aptly suited to produce extracellular enzymes. There is growing interest in applying cell immobilization techniques for continuous production of enzymes. Among the microbial enzymes immobilized cells are used for production of amylases, protease, pectinase, invertase etc.

Currently whole cells are gaining importance as a source of immobilized enzymes. Whole cells can be immobilized either in a viable or non-viable form. By permeabilising cells using physical (freezing and thawing), or chemical (organic solvents/detergents), diffusion of substrates and products through the cell membrane can be controlled, in case of intracellular or membrane bound enzymes. (Patil and D’souza, 1997). For periplasmic enzymes whole cells can be used as a source of enzymes without permeabilisation (Svitel et al., 1998). Immobilization viable cell technology can eliminate most of the constraints faced with the freedom to determine the cell density prior to fermentation. It also facilitates operation of fermentation on a continuous mode without cell wash or even at high dilution rates. The immobilization cell technology process also decouples microbial growth from cellular synthesis of favored compound.

1.5.1 Techniques of whole cell immobilization
A large number of techniques are now available for immobilization of cells on different supports. In principle, four different types of immobilization methods can be distinguished. These include entrapment, covalent binding, cross-linking and adsorption. A combination of two or more of these methods has also been employed. No single system can be applicable to all enzymes or cells in view of the differences in their composition and overall charge distribution. The substrate characteristics may also influence the choice of the method of immobilization (Messing, 1975).

1.5.1.1 Entrapment of cells

Entrapment of cells represents a more definite means of immobilization that does not have a significant dependence on cellular properties. Entrapment is the most frequently used method for immobilizing whole cell systems. The principle behind the entrapment technique is to form a polymeric network around the material to be trapped. The resultant gel must have sufficient porosity to allow the transport of substrates in and products out while retaining the cells. Though the technique imposes minimal constraints on the cells, some localized charge effects are likely to be introduced by the matrix material (Mosbach, 1987).

Various natural polymers like alginate, carrageenan, cellulose, agar, agarose, hen egg white, gelatin, collagen and synthetic polymers like polyacrylamide and other acrylic polymers, photo cross-linkable resins have been used for preparation of immobilized viable cell systems (Tampion and Tampion, 1987).
1.5.1.2 Covalent binding

The technique of covalent coupling is the creation of permanent chemical bonds and has been extensively used in the immobilization of enzymes. Covalent binding of an enzyme or cell to a solid matrix has the advantages of an attachment, which is not reversed by the pH or ionic strength. One of the general problems with covalent binding is that the cells are exposed to potent reactive groups and other harsh reaction conditions thus affecting their viability. There may also be a loss during continuous use, leading to loss of intracellular enzymes. Covalent binding of cells for obtaining many useful products has been reported by D’souza and Marolina (1999) and Abelyan (2000).

Covalent binding technique, in principle, is applicable to the immobilization of any cell. The outer surface of cells contain a variety of reactive groups, such as hydroxyl, aldehyde, ketone, carboxyl, amino, sulphhydryl, imidazole and various substituted aromatic rings. There is a great potential therefore for the creation of covalent bonds with suitable activated carriers using essentially the techniques used for immobilizing enzymes (Tampion and Tampion, 1987).

1.5.1.3 Adsorption

Immobilization of enzymes and cells through adsorption perhaps is the simplest of all the techniques. Adsorption is the oldest method of immobilization cells. Techniques for the adhesion of whole cells on polymeric surface including glass, cotton fabric and synthetic polymeric membranes using polyethylene imine (PEI) (D’Souza and Melo, 2001).

1.5.1.4 Cross-linking

Enzymes and cells can be immobilized by cross-linking with bifunctional reagents such as glutaraledehyde, cyanuric chloride, imidiates etc. unlike enzymes, cells have been normally immobilized in the presence of an inert protein like hens egg white, gelatin or collagen using glutaraldehyde as cross-linker (Bajpal and Margaritis, 1985).
1.5.2 Applications of immobilization technology

Immobilized cells are used as an alternative technology for variety of environmental application in agriculture, biocontrol, pesticide application and pollutant degradation in contaminated soils. Microbial inoculants have been investigated for soil applications such as enhancement of symbiotic or associative nitrogen fixation, biological control of soil-borne plant pathogens, reduction of aflotoxins and biodegradation of xenobiotic compounds (Cassidy et al., 1996 and Daigle and Cotty, 1997).

1.5.2.1 Analytical and medical applications

Immobilized proteins, enzyme cells and cellular organelles have been widely used in the field of analysis and medicine. The use of the immobilization materials in these directions can be divided into two major categories, i.e., biosensors and artificial organs. Some of the current developments and future potentials in biosensor field have been recently reported by D'Souza (2001).

The application of immobilized whole cells for the production of metabolic products by microorganisms have been widely studied and several valuable products such as enzymes, organic acids, amino acids, steroids and antibiotic have been successfully obtained. The continuous production of citric acid from dairy wastewater was investigated using calcium-alginate immobilized Aspergillus niger ATCC 9142 (Kwon et al., 2002).