Chapter 1

Introduction and Review of literature
1.1 Thraustochytrids

Originally thought to be primitive fungi, thraustochytrids have more recently been assigned to the subclass Thraustochytridae (Chromista, Heterokonta), aligning them more closely with the heterokont algae such as brown algae and diatoms (Cavalier-Smith et al., 1994). Following an early description of thraustochytrids (Sparrow, 1936), little research concerning this group of organisms occurred until the 1960s, when a number of descriptive and ecological studies were carried out (e.g., Goldstein, 1963; Gaertner, 1968; Bahnweg and Sparrow, 1974; Raghukumar, 1986, 1987, 1992).

The thraustochytrids are one group of organisms that has been identified as an extremely common component of the marine microbial consortia and often reaching biomass content comparable to that of bacteria (Naganuma et al., 1997; Kimura et al., 1999; Raghukumar et al., 2000, 2001). They are heterotrophic, obligate marine protists belonging to the kingdom Straminipila, which also includes several heterotrophic flagellates, oomycetous fungi, diatoms, and brown algae. Organisms belonging to this kingdom have tubular cristae in the mitochondria and produce heterokont zoospores with tripartite hairs (Porter, 1990; Raghukumar, 2002). They are widespread and have been isolated from numerous habitats in coastal and oceanic regions. Although usually saprophytic, some are parasitic, causing diseases in a few marine animals. They have been shown to be present in microbial films that form on submerged surfaces in the sea (Raghukumar et al., 2000; Raghukumar, 2002).

In view of their abundant presence in the sea, it is of intrinsic interest to examine the production of hydrolytic enzymes by thraustochytrids from the
perspective of their ecological role as well as their biotechnological importance. This study addresses the production of two such hydrolytic enzymes, viz., proteases and lipases, by thraustochytrids.

1.1.1 The microbial food chain and thraustochytrids

Marine pelagic ecosystems are sustained by two types of food chains, namely the classical grazing food chain and the microbial food chain (Valiela, 1995). The microbial food chain serves to salvage organic materials such as phytoplankton exudates and phyto-/zoo-detritus from the grazing food chain (Pomeroy and Wiebe, 1993). At the base of pelagic microbial food chains is a large pool of dissolved organic matter (DOM) primarily utilized by bacterioplankton. Production of bacterioplankton often accounts for a considerable part of pelagic secondary production (Naganuma, 1997). Another agent of DOM utilization is mycoplankton, which have drawn less attention from ecologists due to their relatively low abundance (Naganuma et al., 1998; Kimura et al., 1999) compared to that of bacterioplankton. Marine mycoplankton are largely comprised of fungoid protists, the thraustochytrids. Thraustochytrids are a forgotten agent of the marine microbial food chain but the importance of thraustochytrids in pelagic secondary production has been increasingly noticed. Production of thraustochytrids is likely associated with the degradation of refractory DOM that is not readily utilized by bacterioplankton (Bremer, 1995). It can thus be simplistically formulated that while mycoplankton serve as scavengers of refractory DOM which is often allochthonous (riverine) in nature, bacterioplankton salvage the autochthonous (marine) DOM exuded from phytoplankton.
1.1.2 Taxonomy of thraustochytrids

Modern taxonomy classifies thraustochytrids in the phylum Heterokonta, which is in turn placed in the kingdom Chromista, based on 18S rDNA sequencing (Cavalier-Smith et al., 1994). The phylum Heterokonta includes chromophytes such as diatoms and kelps as well as oomycetes such as thraustochytrids. The thraustochytrids are thus no longer classified in a fungal group and are not necessarily to be regarded as mycoplankton. Nevertheless, in as much as diatoms and kelps are traditionally called phytoplankton and algae although they are no longer so, thraustochytrids may still be called mycoplankton (Kimura and Naganuma, 2001).

Thraustochytrids have been described as a group of non-photosynthetic, heterotrophic, marine fungoid protoctists (Moss, 1986; Porter, 1989). This group has a typical structure consisting of an ectoplasmic net and a non-cellulosic, sulfurylated cell wall (Darley et al., 1973). The ectoplasmic net is thought to be the site of organic degradation using excreted enzymes. Recent years have seen several publications on the prevalence in the sea of this group of osmoheterotrophic fungoid protists. The presence (and often dense populations) of these single-celled microorganisms has been reported from numerous habitats, including including the water column, sediments, algae, particulate detritus and invertebrates (Raghukumar, 1990). The mode of reproduction in these protists is by means of motile zoospores which settle on suitable substrata that offer organic nutrients. Vegetative cells developing from encysted zoospores derive their nutrition by producing extracellular hydrolytic enzymes (Coleman and Vestal 1987).
The taxonomic position of thraustochytrids is indicated below:

(Dick, 2001) (Cavalier-Smith, 1994)

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Straminipila</th>
<th>Kingdom</th>
<th>Chromista</th>
</tr>
</thead>
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<tr>
<td>Phylum</td>
<td>Heteroknota</td>
<td>Phylum</td>
<td>Heteroknota</td>
</tr>
<tr>
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<td>Sub-Phylum</td>
<td>Labyrinthista</td>
</tr>
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<td>Labyrinthulea</td>
</tr>
<tr>
<td>Family</td>
<td>Thraustochytriaceae</td>
<td>Family</td>
<td>Thraustochytridae</td>
</tr>
</tbody>
</table>

*(from Raghukumar, 2002)*

1.1.3 Characterization and recognition

The phylum Labyrinthulomycota consists of two groups of primarily marine organisms, the labyrinthulids and the thraustochytrids. The clubbing together of labyrinthulids and thraustochytrids in this phylum is based primarily on their membrane-bounded ectoplasmic networks devoid of cytoplasmic constituents and which are produced by the cells from specialized organelles at the cell surface called sagenogens or bothrosomes. In addition, both groups produce thin wall scales in the golgi apparatus and when present, have heteroknot, biflagellate zoospores. The membranes of ectoplasmic networks are continuous with the plasma membrane at the sagenogens. Nuclei of these organisms have a prominent central nucleolus. Mitochondria have tubular cristae. In the thraustochytrids, unlike in the labyrinthulids, the network does not surround the developing sori but emanates from the basal side where it arises from a single or cluster of sagenogens.
The life cycle of *Thraustochytrium* and its ultra structural features are shown in Fig 1.1a and b (*Porter, 1990*).

**Fig 1.1a** Life cycle of *Thraustochytrium*  
**Fig 1.1 b** Ultra structural features of *Thraustochytrium*

**Fig 1 a:**
A young thallus (a) grows by enlargement and nuclear division (b) Progressive cleavage of the protoplast c) occurs in the sorous d) which by dissolution of the wall e) releases zoospores f) thay settle down and grow to form a new thallus.

*Thraustochytrium*

**Fig 1 b:**

a) A young thallus- Ectoplasmic net formed from bothrosomes; trophic cells have a single layer of golgi-derived scales  
b) Zoospores lack an eyespot and are surrounded by a single layer of scales
Seven genera have been described in the family *Thraustichytriidae*: *Thraustochytrium*, *Schizochytrium*, *Ulkenia*, *Labyrinthuloides*, *Japonochytrium*, *Aplanochytrium* and *Althornia*. They are distinguished by the development and form of the sorous and by the spore type. According to Gaertner (1968), these organisms can be identified only if observed under standardized conditions such as when pollen-baited sea water is used. Identification on agar plates may not be possible because abnormal development is frequently observed.

Typically, thraustochytrids are not colonial but grow by enlargement of cells which develop into sori and then release spores. This process is the result of mitotic divisions. No sexual stages are known for any thraustochytrids. Spore formation occurs in most genera by cleavage of the multi-nucleate sorous protoplast (as in *Thraustochytrium*). Spores may also be produced by successive bipartition of the developing sorous (as in *Labyrinthuloides*). In *Schizochytrium*, mitotic division of a single cell produces a cluster of cells, each of which may develop into a sorous. Most species of thraustochytrids produce heteroknot zoospores that have a thin layer of wall scales. They lack sagenogens and do not have the pigmented eyespot found in *Labyrinthula*. The genus *Labyrinthuloides* is distinct in that it forms ovoid spores that move by a gliding motility on the ectoplasmic net elements associated with each spore.

### 1.1.4 Ecology of thraustochytrids

Previous studies have determined the abundance of thraustochytrids over a wide range of habitats, including the water column, algae, particulate detritus and invertebrates (Raghukumar, 1990; Riemann and Schaumann, 1993;
Naganuma et al., 1998). The thraustochytrids are sized largely from 5-20 \( \mu m \). They are thus thought to serve as important food sources for picoplankton-feeders and contribute to the enhancement of pelagic secondary production (Naganuma et al., 1998). Thraustochytrids uniquely produce polyunsaturated fatty acids such as docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Nakahara et al., 1996), which are essential nutrients for many animals and for the growth of fish larvae.

Fresh water thraustochytrids are not known although many have been isolated from habitats reflecting a wide range of salinities from weakly brackish waters to briny salt evaporation ponds. Thraustochytrids are thus considered euryhaline, however, many species and isolates, found in habitats of limited salinities, probably stenohalic. At the high salinities of salt ponds, most of the thraustochytrids are likely to be found in some kind of resting state (Porter, 1990).

1.1.5 Ecological importance

The microbial food chain is a pathway of particle size increments: from \( \mu m \)-sized bacteria to flagellates, to ciliates, and to mm-sized zooplankton, involving four trophic levels. When the chain starts from 10 \( \mu m \)-sized thraustochytrids, only three trophic levels are involved and thus the whole transfer efficiency would be ten times higher than the chain starting from bacterioplankton. Planktonic thraustochytrids are probably a suitable food item in terms of size for 100 \( \mu m \)-sized protozoans (Rassoulzadegan et al., 1988), heterotrophic dinoflagellates (Hansen, 1992; Hansen et al., 1996) and filter-feeding bivalves
(Jørgensen, 1996). This is another aspect of thraustochytrid contribution to enhancing the efficiency of the marine microbial food chain. A different aspect of thraustochytrid importance is the nutritional value to predators. Thraustochytrids are known to contain high cellular contents of the long chain polyunsaturated fatty acids DHA and DPA. Both DHA and DPA are essential fatty acids for many marine animals and must thus be obtained through the diet. Thraustochytrids provide not only material high in carbon but also diets high in DHA/DPA (Fell and Newell, 1998).

Another and more important ecological role of thraustochytrids may be in the decomposition of refractory organic substrates in marine ecosystems (Heald and Odum, 1970). Thraustochytrids often occur in association with decaying plant material such as algal tissue (Sathe-Pathak et al., 1995). It is likely that thraustochytrids also grow on refractory substrates of terrestrial origin, such as cellulose and lignin contained in river water (Bremer, 1995; Bremer and Talbot, 1995). In addition to the capability of recycling of refractory organic matter, cells of thraustochytrids may have higher carbon contents, giving larger impact on C-cycling than a bacterioplankton cell would probably do.

Fungi play a key role in the decomposition and biochemical transformation of macrophytic detritus. Production of degradative enzymes is the key to biochemical transformation. Microorganisms that break down these recalcitrant compounds will have a major role in detrital dynamics (Raghukumar et al., 1994).
As a matter of fact, marine detrital fungi producing lignocellulolytic enzymes appear to have much biotechnological potential in bioremediation and in the paper and pulp industry (Raghukumar, 2002). In addition, the role of thraustochytrids in possible lignocellulose degradation has not been addressed. Thraustochytrids do elaborate cellulases (Raghukumar et al., 1994; Bremer, 1995) and it is worth examining their lignin-degrading enzymes. Fungal degradation of lignocellulose may result in dissolved organic carbon, which might be utilized by bacteria or contribute to the humic material of the surrounding environment. Thraustochytrids present in detritus of the macroalga Sargassum cinereum were seen to produce proteases but not alginases which degrade the structural polysaccharides of the brown algal cell wall.

Seasonal studies indicate poor correlation with phytoplankton blooms but often reveal thraustochytrid biomass equivalent to that of bacteria during times of phytoplankton decay. It is concluded that they may play an important role in mineralisation of phyto- and zoo- plankton detritus in the sea. Their high content of omega-3 polyunsaturated fatty acids suggests that they may form an important link in the foodweb. More knowledge about their ability to degrade various forms and concentration levels of organic carbon in the sea, as well as possible phagotrophy will help to resolve their exact ecological niche vis-a-vis the bacteria in the marine ecosystem (Raghukumar, 2002).

Thraustochytrids generally represent a negligible fraction of microbial abundance and a minor fraction of the total benthic microbial mass (Bongiorni & Dini 2002, Bongiorni et al.2005). Nevertheless, their role in the degradation of
different substrates and mineralization of organic matter has been hypothesized as significant, with regard to their ability to pervade various solid substrates (Raghukumar, 2002). At present, the only available information on their enzymatic activities deals with a few species from water column, mangrove leaves, brown algae and faecal pellets of marine invertebrates for a limited number of enzymes (Raghukumar et al., 1994; Sharma et al., 1994; Raghukumar and Raghukumar, 1999). Enzymatic activity is a key step in the degradation of high molecular weight organic matter. In the marine environment, it is assumed that most of these activities are almost exclusively carried out by bacteria but it is known that protists can produce extracellular enzymes (Mohapatra and Fukami, 2004).

These thraustochytrids are typically encountered in association with refractory substrates but the extent of their role in organic matter decomposition is still unknown. The investigated strains by Bongiorni et al. (2005) exhibited a wide spectrum of enzymes involved in the hydrolysis of all classes of organic compounds, suggesting that thraustochytrids are capable of degrading a large variety of substrates. The enzymatic pools were similar among all strains tested and exhibited a good production of lipase, a selection of protease and a poor pool of carbohydrate degradation enzymes. However, different isolates displayed different spectra and intensities of enzymatic activities. In the present study, the ability of thraustochytrids to produce a variety of enzymes has been examined for the first time.
1.2 Proteases

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications both physiologically and in the commercial field. Proteolytic enzymes are degradative enzymes that catalyze the cleavage of peptide bonds in other proteins. 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.

The current estimated value of the worldwide sales of industrial enzymes is $1 billion, of which 75% are hydrolytic, and proteases account for about 60% of this (Fig.1.2). 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. The major producers of proteases distributed worldwide are Novo Industries (Denmark), Gist-Brocades (Netherlands), Genencor International and Miles Laboratories (United States) (Rao et al., 1998).

1.2.1 Sources of Proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms.
FIG. 1.2 Distribution of enzyme sales (Rao et al., 1998)

The contribution of different enzymes to the total sale of enzymes is indicated. The shaded portion indicates the total sale of proteases.
a) 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.

i) Papain - Papain is a traditional plant protease and is extracted from the latex of Carica papaya fruits, which are grown in subtropical areas of west and central Africa and India. The crude preparation of the enzyme has a broad specificity due to the presence of several proteinase and peptidase isozymes. The enzyme is active between pH 5 and 9 and is stable up to 80 or 90°C in the presence of substrates. It is extensively used in industry for the preparation of highly soluble and flavored protein hydrolysates.

ii) Bromelain - Bromelain is prepared from the stem and juice of pineapples. The major supplier of the enzyme is Great Food Biochem, Bangkok, Thailand. The enzyme has been characterized as a cysteine protease and is active from pH 5 to 9. Its inactivation temperature is 70°C, which is lower than that of papain (Friedrich, 1999).

iii) Keratinases – These are the proteases, which degrade hair and is important for prevention of clogging of wastewater systems.

b) Animal Proteases The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins (Boyer, 1971).
i) Trypsin - Trypsin (Mr 23,300) is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by lysine and arginine residues. Trypsin is used in the preparation of bacterial media and in some specialized medical applications.

ii) Chymotrypsin - Chymotrypsin (Mr 23,800), found in animal pancreatic extracts, is stored in the pancreas in the form of a precursor and is activated by trypsin in a multistep process. Pure chymotrypsin is an expensive enzyme and is used only for diagnostic and analytical applications. It is used extensively in the deallergenizing of milk protein hydrolysates.

iii) Pepsin - Pepsin (Mr 34,500) is an acidic protease that is found in the stomachs of almost all vertebrates. The active enzyme is released from its zymogen (pepsinogen) by autocatalysis in the presence of hydrochloric acid.

iv) Rennin - Rennet is a pepsin-like protease (rennin, chymosin; EC 3.4.23.4) that is produced as an inactive precursor, prorennin, in the stomachs of all nursing mammals. It is converted to active rennin (Mr 30,700) by the action of pepsin or by its autocatalysis. It is used extensively in the dairy industry to produce a stable curd with good flavor.

c) Microbial Proteases
The inability of 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 and microbial proteases are
therefore preferred to those from plant and animal sources. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Rao et al., 1998)

i) **Bacterial** - Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in the pH range of pH 5 to 8 and have relatively low thermotolerance. Neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant proteinase inhibitors and are therefore useful in the brewing industry. Bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates which require a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity while others are serine proteinases.

Bacterial alkaline proteases are characterized by their high activity at alkaline pH and their broad substrate specificity. Their optimal temperature is usually around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

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

**iii) Viral** - Viral proteases have gained importance due to their functional involvement in the processing of proteins of viruses that cause certain fatal diseases such as AIDS and cancer. Serine, aspartic and cysteine peptidases are found in various viruses (Rawlings and Barrett, 1993). All of the virus-encoded peptidases are endopeptidases; there are no metallopeptidases. Extensive research has focussed on the three-dimensional structure of viral proteases and their interaction with synthetic inhibitors with a view to designing potent inhibitors that can combat the relentlessly spreading and devastating epidemic of AIDS.

Although proteases are widespread in nature, microbes thus serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be...
genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications.

1.2.2 Classification of Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed (ii) chemical nature of the catalytic site and (iii) evolutionary relationship with reference to structure. Proteases are grossly subdivided into two major groups, viz, exopeptidases and endopeptidases, depending on the site of their action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, viz, serine proteases, aspartic proteases, cysteine proteases and metalloproteases. There are a few miscellaneous proteases which do not precisely fit into the standard classification, e.g., the ATP-dependent proteases which require ATP for activity (Menon and Goldberg, 1987). Based on their amino acid sequences, proteases are classified into different families 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, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo or
unknown type, respectively. The classification of proteases is shown in Table 1.1

a) Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N- or C-terminus, they are classified as amino- and carboxy-peptidases, respectively.

i) Aminopeptidases - Aminopeptidases act at a free N-terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide or a tripeptide. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi. In general, aminopeptidases are intracellular enzymes but there has been a lone report on an extracellular aminopeptidase produced by *A. oryzae* (Cerny, 1978).

ii) Carboxypeptidases - The carboxypeptidases act at C- terminal of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallo-carboxypeptidases and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes. Metallo-carboxypeptidases from *Saccharomyces* spp. and *Pseudomonas* spp require Zn$^{2+}$ or Co$^{2+}$ for their activity (Rao *et al.*, 1998). The enzymes can also hydrolyze the peptides in which the peptidyl group is replaced by a pteroyl moiety or by acyl groups.

Other exopeptidases include dipeptidases, which cleave a dipeptide and omega peptidases which release modified residues from N- or C- termini.
b) 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. Endopeptidases are divided into four subgroups based on their catalytic mechanism: (i) serine proteases (ii) aspartic proteases (iii) cysteine proteases and (iv) metalloproteases.

i) Serine proteases - Serine proteases are characterized by the presence of a serine group at their active site. They are numerous and widespread among viruses, bacteria and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further, subdivided into about six clans with common ancestors (Barett, 1995)

Serine proteases are recognized by their irreversible inhibition by 3, 4-dichloroisocoumarin (3,4-DCI), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as p-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They are known to 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.
Table 1.1 Classification of proteases (Rao et al., 1998)

<table>
<thead>
<tr>
<th>Protease</th>
<th>Mode of action</th>
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<td>Endopeptidases of unknown catalytic mechanism</td>
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</table>

a Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and stars signify the blocked termini. Arrows show the sites of action of the enzyme.
**Serine alkaline proteases** - Serine alkaline proteases are produced by several bacteria, molds, yeasts and fungi. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or TLCK. Their substrate specificity is similar to but less stringent than that of chymotrypsin. They hydrolyze a peptide bond which has tyrosine, phenylalanine or leucine at the carboxyl side of the splitting bond. The optimal pH of alkaline serine proteases is around pH 10 and their isoelectric point is around pH 9. Their molecular masses are in the range of 15 to 30 kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces* and *Flavobacterium* spp. (Boguslawski et al., 1983), subtilisins produced by *Bacillus* spp. are the best known to date. Alkaline proteases are also produced by *S. cerevisiae* and filamentous fungi such as *Conidiobolus* spp. (Phadatare et al., 1993) as well as *Aspergillus* and *Neurospora* spp.

**Subtilisins** - Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protease Nagase (BPN'), have been identified. Subtilisin Carlsberg is widely used in detergents. The active-site conformation of subtilisins is similar to that of trypsin and chymotrypsin despite the dissimilarity in their overall molecular arrangements. The serine alkaline protease from the fungus *Conidiobolus coronatus* was shown to possess a distinctly different structure from Subtilisin Carlsberg in spite of their functional similarities (Phadatare et al., 1992).
ii) 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, viz, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (Barett, 1995) and have been placed in clan AA. Most aspartic proteases show maximal activity at low pH (3 to 4) and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa. Microbial aspartic proteases can be broadly divided into two groups (i) pepsin-like enzymes produced by Aspergillus, Penicillium, Rhizopus and Neurospora and (ii) rennin-like enzymes produced by Endothia and Mucor spp.

iii) Cysteine/thiol proteases - Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differs among the families (Barett, 1994). Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin-like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid and (iv) others. Papain is the best-known cysteine protease. Cysteine are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents.

iv) Metalloproteases - Metalloproteases are the most diverse types of proteases (Barett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of
origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms and thermolysin from bacteria (Rao et al., 1998).

Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral (ii) alkaline (iii) Myxobacter I and (iv) Myxobacter II. The neutral proteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity. Myxobacter protease I is specific for small amino acid residues on either side of the cleavage bond, whereas protease II is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP.

1.2.3 Mechanism of Action of Proteases

The mechanism of action of proteases has been a subject of great interest to researchers. Purification of proteases to homogeneity is a prerequisite for studying their mechanism of action. The catalytic site of proteases is flanked on one or both sides by specificity subsites, each able to accommodate the side chain of a single amino acid residue from the substrate. These sites are numbered from the catalytic site $S_1$ through $S_n$ toward the N terminus of the structure and $S_1'$ through $S_n'$ toward the C terminus. The residues which they accommodate from the substrate are numbered $P_1$ through $P_n$ and $P_1'$ through $P_n'$, respectively.

\[
\text{Protease: } N \overset{S_n \cdots S_3 \cdots S_2 \cdots S_1}{\cdots} \overset{S_{i} \cdots S'_{1} \cdots S'_3 \cdots}{\cdots} \overset{i}{\cdots} \overset{S_n}{\cdots} \overset{C}{\cdots}
\]

\[
\text{Substrate: } N \overset{P_n \cdots P_3 \cdots P_2 \cdots P_1}{\cdots} \overset{P_{i} \cdots P'_{1} \cdots P'_3 \cdots}{\cdots} \overset{i}{\cdots} \overset{P_n}{\cdots} \overset{C}{\cdots}
\]
Studies on the mechanism of action of proteases have revealed that they exhibit different types of mechanism based on their active-site configuration. The serine proteases contain a Ser-His-Asp catalytic triad and the hydrolysis of the peptide bond involves an acylation step followed by a deacylation step. The carboxypeptidases are unusual among the serine-dependent enzymes in that they are maximally active at acidic pH. These enzymes are known to possess a Glu residue preceding the catalytic Ser, which is believed to be responsible for their acidic pH optimum. Although the majority of the serine proteases contain the catalytic triad Ser-His-Asp, a few use the Ser-base catalytic dyad. The Glu-specific proteases display a pronounced preference for Glu-Xaa bonds over Asp-Xaa bonds. Aspartic proteases are characterized by an Asp-Thr-Gly motif in their active site and by acid-base catalysis as their mechanisms of action. The mechanism of action of metalloproteases is slightly different from that of the above-described proteases. These enzymes depend on the presence of bound divalent cations and can be inactivated by dialysis or by the addition of chelating agents. The activity of metalloproteases depends on the binding of a divalent metal ion to a His-Glu-Xaa-Xaa-His motif. Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of serine proteases.

1.2.4 Physiological Functions of Proteases

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

**a) Protein Turnover**

All living cells maintain a particular rate of protein turnover by continuous, albeit balanced, degradation and synthesis of proteins. Catabolism of proteins provides a ready pool of amino acids as precursors of the synthesis of proteins. Proteases assist the hydrolysis of large polypeptides into smaller peptides and amino acids, thus facilitating their absorption by the cell. The extracellular enzymes play a major role in nutrition due to their depolymerizing activity. The microbial enzymes and the mammalian extracellular enzymes such as those secreted by the pancreas are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition. Intracellular proteases are known to participate in executing the proper protein turnover for
the cell. For instance, in *E. coli* an ATP-dependent protease is responsible for hydrolysis of abnormal proteins (Rao et al., 1998).

**b) Sporulation and Germination**

The formation of spores in bacteria, ascospores in yeasts, fruiting bodies in slime molds and conidial discharge in fungi all involve intensive protein turnover. The requirement of a protease for sporulation has been demonstrated by the use of protease inhibitors (Dancer and Mandelstam, 1975.). The alkaline serine protease of *Conidiobolus coronatus* was shown to be involved in forcible conidial discharge by isolation of a mutant with less conidial formation. Formation of the less active protease by autoproteolysis thus represents a novel means of physiological regulation of protease activity in *C. coronatus* (Phadatare et al., 1993).

The dormant spores lack the amino acids required for germination. Degradation of proteins in dormant spores by serine endoproteinases makes amino acids and nitrogen available for the biosynthesis of new proteins and nucleotides. These proteases are specific only for storage proteins and do not affect other spore proteins. Their activity is rapidly lost on germination of the spores. Extracellular acid proteases are believed to be involved in the breakage of cell wall polypeptide linkages during germination of *Dictyostelium discoideum* spores and *Polysphondylium pallidum* microcysts (O'Day, 1976).

**c) Enzyme Modification**

Activation of the zymogenic precursor forms of enzymes and proteins by specific proteases represents an important step in the physiological regulation
of many rate-controlling processes such as generation of protein hormones, assembly of fibrils and viruses, blood coagulation, and fertilization of ova by sperm. Activation of zymogenic forms of chitin synthase by limited proteolysis has been observed in *Candida albicans*, *Mucor rouxii*, and *Aspergillus nidulans*. Pepsin, trypsin, and chymotrypsin occur as their inactive zymogenic forms, which are activated by the action of proteases.

**d) Regulation of Gene Expression**

Modulation of gene expression mediated by protease has been demonstrated. Proteolysis of a repressor by an ATP-requiring protease resulted in a derepression of the gene. A change in the transcriptional specificity of the B subunit of *Bacillus thuringiensis* RNA polymerase was correlated with its proteolytic modification. Modification of ribosomal proteins by proteases has been suggested to be responsible for the regulation of translation.

Besides the general functions described so far, proteases also mediate the degradation of a variety of regulatory proteins that control the heat shock response, the SOS response to DNA damage, the life cycle of bacteriophage and programmed bacterial cell death (Van Melderen, 1996). Recently, a new physiological function has been attributed to the ATP-dependent proteases conserved between bacteria and eukaryotes. It is believed that they act as chaperones and mediate not only proteolysis but also the insertion of proteins into membranes and the disassembly or oligomerization of protein complexes. In addition to the multitude of activities that are already assigned to proteases, many more new functions are likely to emerge in the near future.
1.2.5 Regulation of protease biosynthesis

Although protease production is an inherent property of all organisms and these enzymes are usually constitutive, at times they are partially inducible (Kalisz, 1988). Proteases are largely produced during the stationary phase of microbial growth and are thus generally regulated by carbon and nitrogen stress. The onset of the stationary phase is marked by the transition from vegetative growth to sporulation stage in spore-formers. Therefore, protease production is often related to the sporulation stage in many bacilli, such as *B. subtilis* (O'Hara and Hageman, 1990) and *B. licheniformis* (Hanlon and Hodges, 1981). On the contrary, a few reports also suggest that sporulation and protease production - although co-occurring - are not related, as spore-deficient strains of *B. licheniformis* were not protease-deficient (Fleming *et al.*, 1995). Final protease yield during this phase is however, also determined by the biomass produced during exponential phase. Therefore, medium manipulation is needed to maximize growth and hence protease yields.

To regulate protease synthesis, different methods in submerged fermentations have been used with strategies combining fed-batch, continuous and chemostat cultures (Gupta *et al.*, 2002; Hameed *et al.*, 1999). Such strategies can achieve high yields of alkaline protease in the fermentation medium over a longer period of incubation during prolonged stationary state. Alkaline proteases are generally produced by submerged fermentation and on a commercial scale this is preferred over solid-state fermentation. Optimization of the medium is associated with a large number of physiological and nutritional parameters that effect protease production, viz. pH, temperature, incubation period and agitation, effect of carbon and nitrogen and divalent cations. A
comprehensive account of culture conditions for protease production from various microorganisms is given in Table 1.2.

1.2.6 Quantitation of proteolytic activity

The methods available for detection and assay of proteolytic activity vary in their simplicity, rapidity, range of detection and sensitivity. Qualitative assays rely on the formation of a clear zone of proteolysis on agar plates as a result of protease production. The most commonly used qualitative assays include protein agar plate assay, radial diffusion and thin-layer enzyme assay. Table 1.3 gives a comparative overview of the commonly followed assays for the measurement of proteolytic activity.

A commonly accepted procedure for estimating the activity of proteases is to determine the quantity of peptides in acid-soluble hydrolyzed product fractions after proteolytic action on a protein substrate (BSA, casein, Hammerstein casein, hemoglobin etc.). These peptide residues are estimated either by absorption at 280 nm (direct estimation method) or using the conventional Folin's reagent (colorimetric method).

Chromogenic or insoluble chromolytic substrates are also used for spectrophotometric determination of proteolytic activity in plants, animals and microbial samples. Both naturally occurring insoluble proteins, e.g., fibrin, elastin, gelatin, keratin, collagen or soluble proteins rendered insoluble either by cross-linking with bifunctional agents (Safarik, 1989) or entrapment into appropriate polymer matrix (Safarik, 1988) thermally modified substrates.
Table 1.2. Optimized production conditions for alkaline-protease-producing microorganisms (Gupta et al., 2002)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Agitation (rpm)</th>
<th>Incubation period (h)</th>
<th>Preferred/optimized nitrogen sources</th>
<th>Preferred/optimized carbon sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcaligene s faecalis</td>
<td>8</td>
<td>30</td>
<td>200</td>
<td>48</td>
<td>Soybean meal</td>
<td>None*</td>
</tr>
<tr>
<td>Bacillus sp. IS-3</td>
<td>10.5</td>
<td>37</td>
<td>200</td>
<td>72</td>
<td>Soybean meal</td>
<td>Glucose</td>
</tr>
<tr>
<td>Bacillus sp. JB99</td>
<td>10</td>
<td>55</td>
<td>180</td>
<td>24</td>
<td>KNO₃</td>
<td>Citric acid</td>
</tr>
<tr>
<td>Bacillus sp. K2</td>
<td>7</td>
<td>37</td>
<td>300-500</td>
<td>60-72</td>
<td>Casein hydrolyzate, gelatine</td>
<td>Glycerol</td>
</tr>
<tr>
<td>Bacillus sp. P-2</td>
<td>9.5</td>
<td>30</td>
<td>-</td>
<td>24</td>
<td>Peptone, yeast extract</td>
<td>Glucose</td>
</tr>
<tr>
<td>Bacillus sp. RGR-14</td>
<td>7</td>
<td>37</td>
<td>200</td>
<td>72-96</td>
<td>Soybean meal; peptone</td>
<td>Starch</td>
</tr>
<tr>
<td>Bacillus sp. SSR-1</td>
<td>10</td>
<td>40</td>
<td>150</td>
<td>18</td>
<td>Biopeptone, yeast extract</td>
<td>Beef extract, lactose</td>
</tr>
<tr>
<td>B. brevis MTCC B0016</td>
<td>10.5</td>
<td>37</td>
<td>200</td>
<td>96</td>
<td>Soybean meal</td>
<td>Lactose</td>
</tr>
<tr>
<td>B. licheniformis ATCC 21415</td>
<td>7</td>
<td>30</td>
<td>250-400</td>
<td>48</td>
<td>Soybean, (NH₄)₂PO₃</td>
<td>Lactose, glucose</td>
</tr>
<tr>
<td>B. mojavensis</td>
<td>7</td>
<td>50</td>
<td>200-250</td>
<td>24</td>
<td>Casein or casamino acids</td>
<td>Glucose</td>
</tr>
<tr>
<td>B. pumilis MK6-5</td>
<td>9.6</td>
<td>35</td>
<td>250</td>
<td>60</td>
<td>Cornsteep liquor, tryptone</td>
<td>Glucose, sodium citrate</td>
</tr>
<tr>
<td>B. sphaericus</td>
<td>n.s.</td>
<td>30</td>
<td>300</td>
<td>n.s.</td>
<td>Biopeptone, yeast extract</td>
<td>Glucose</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>n.s.</td>
<td>36</td>
<td>250</td>
<td>6-8</td>
<td>Nutrient broth; yeast extract</td>
<td>Glucose; yeast extract</td>
</tr>
</tbody>
</table>

Contd........
<table>
<thead>
<tr>
<th>Organism</th>
<th>pH</th>
<th>Triplicate 1</th>
<th>Triplicate 2</th>
<th>Triplicate 3</th>
<th>Carbon Source</th>
<th>Nitrogen Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Serratia marcescens</em> ATCC 25419</td>
<td>7.4</td>
<td>10</td>
<td>150</td>
<td>72</td>
<td>Polypeptone, yeast extract, casein</td>
<td>None(^a)</td>
</tr>
<tr>
<td><em>Flavobacterium balustinum</em></td>
<td>n.s.</td>
<td>30; 36</td>
<td>250</td>
<td>24; 16-18</td>
<td>Yeast extract, tryptone, asparagine, (\text{NH}_4\text{Cl})</td>
<td>Whey; sucrose</td>
</tr>
<tr>
<td><em>Conidiobolus coronatus</em> (NCL 86.8.20)</td>
<td>7-7.5</td>
<td>28</td>
<td>220</td>
<td>48</td>
<td>Ammonium nitrate, tryptone, casein</td>
<td>Sucrose</td>
</tr>
<tr>
<td><em>Ophiostoma piceae</em></td>
<td>n.s.</td>
<td>23</td>
<td>250</td>
<td>9 days</td>
<td>Soydrink from soybean meal</td>
<td>Starch</td>
</tr>
<tr>
<td><em>Tritirachium album</em></td>
<td>5.9</td>
<td>28</td>
<td>200</td>
<td>24-120</td>
<td>Peptone, yeast extract, (\text{NaNO}_3), ((\text{NH}_4\text{)}_2\text{SO}_4)</td>
<td>Glucose</td>
</tr>
<tr>
<td><em>Streptomyces sp.</em> NCIB 10070</td>
<td>7</td>
<td>n.s.</td>
<td>n.s.</td>
<td>24</td>
<td>Rapemeal</td>
<td>None(^a)</td>
</tr>
<tr>
<td><em>Thermoaactinomyces sp.</em> E79</td>
<td>7.2</td>
<td>50</td>
<td>250</td>
<td>16</td>
<td>Soytone</td>
<td>Starch</td>
</tr>
</tbody>
</table>

\(^a\)No carbon source was present in the medium and the major organic nitrogen source supplied the required carbon  
\(^b\)Not specified
Table 1.3 Protease assay methods: an overview

*(Gupta et al., 2002)*

**Spectrophotometric assays**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>670</td>
</tr>
<tr>
<td></td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>660</td>
</tr>
<tr>
<td></td>
<td>750</td>
</tr>
<tr>
<td>Hammerstein casein</td>
<td>660</td>
</tr>
<tr>
<td>DNHB casein</td>
<td>275</td>
</tr>
<tr>
<td>Immobilized ostazin blue S-2G dyed-casein</td>
<td>366</td>
</tr>
<tr>
<td>Thermally modified casein complexed with black</td>
<td>620</td>
</tr>
<tr>
<td>drawing ink</td>
<td></td>
</tr>
<tr>
<td>Thermally modified azocasein</td>
<td>400</td>
</tr>
<tr>
<td>Thermally modified azocasein</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>480</td>
</tr>
<tr>
<td>Thermally modified azocasein</td>
<td>366-400</td>
</tr>
<tr>
<td>Azoalbumin</td>
<td>440</td>
</tr>
<tr>
<td>r-Crystalline aggregate</td>
<td>405</td>
</tr>
<tr>
<td>Thermally modified gelatin complexed with congo</td>
<td>490, 570</td>
</tr>
<tr>
<td>red or nigrosin</td>
<td></td>
</tr>
<tr>
<td>Chemically modified (formaldehyde/glutaraldeh</td>
<td>800-900</td>
</tr>
<tr>
<td>y mediated) gelatin complexed with black drawing ink</td>
<td></td>
</tr>
<tr>
<td><strong>Tripeptide substrate</strong></td>
<td>400</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----</td>
</tr>
</tbody>
</table>

**Fluorescent oligopeptide energy transfer assay**

<table>
<thead>
<tr>
<th><strong>Dansylated hexapeptide</strong></th>
<th>310-410</th>
</tr>
</thead>
</table>

**ELISA-based protease assay**

<table>
<thead>
<tr>
<th><strong>Biotinylated BSA</strong></th>
<th>405</th>
</tr>
</thead>
</table>

**Magnet-based protease assay**

<table>
<thead>
<tr>
<th><strong>Magnet dye stained gelatin</strong></th>
<th>605</th>
</tr>
</thead>
</table>

**Fluorescence-based protease assay**

<table>
<thead>
<tr>
<th><strong>FITC casein, FTC hemoglobin</strong></th>
<th>575 nm with excitation at 490 nm</th>
</tr>
</thead>
</table>

*DNHB 3, 5 Dinitro-hydroxy benzene, FITC fluorescein isothiocyanate, FTC fluorescein thiocarbamoyl, ELISA enzyme-linked immunosorbent assay, BSA*
(Safarik, 1987) or synthesized chromogenic substrates using 3,5-dinitro-salicylic acid (Gallegos et al., 1996) can be used.

Hatakeyama et al. (1992) developed a photometric assay for proteases in which casein, with its amino groups chemically succinylated, was used as the substrate. The extent of hydrolysis of substrate was determined using trinitrobenzene sulfonate (TNBS). The increase in absorbance due to reaction between TNBS and the newly formed amino groups in the substrate was determined using a microtiter plate reader ($A_{405nm}$). Unlike casein, succinyl casein is easily dissolved at pH values greater than 4 and serves as the substrate of choice for acidic proteases. This colorimetric point assay is tedious and cannot be used with enzymes that require reducing agents such as dithiothreitol.

### 1.2.7 Applications of Proteases

Proteases have a large variety of applications, with a long history in the detergent and food industries. They are also 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.

#### a) Detergents

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. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood and other body secretions. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents routinely added to the detergents are among the major prerequisites for the use of proteases in detergents. The key parameter for the best performance of a protease in a detergent is its 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).

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.

Most of the 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.

b) Leather Industry

Leather processing involves several steps such as soaking, dehairing, bathing and tanning. 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. 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 is compatible with the use of enzymes. 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 is also effective in saving energy. Novo Nordisk manufactures three different proteases, Aquaderm, NUE, and Pyrase, for use in soaking, dehairing and bating respectively.
c) **Food Industry**

The use of proteases in the food industry dates back to antiquity. They have been routinely used for various purposes such as cheesemaking, baking, preparation of soya hydrolysates and meat tenderization.

i) **Dairy industry**

The major application of proteases in the dairy industry is in the manufacture of cheese. The milk-coagulating enzymes fall into three main categories: (i) animal rennets, (ii) microbial milk coagulants and (iii) genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have molecular weights between 30,000 to 40,000 kDa. Rennet extracted from the fourth stomach of unweaned calves contains the highest ratio of chymosin 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) 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 cheesemaking, the primary function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate para-casein and macropeptides. Chymosin is preferred due to its high specificity for casein, which is responsible for its excellent performance in cheesemaking. The proteases produced by GRAS (generally regarded as
safe)-cleared microbes such as *Mucor miehei, Bacillus subtilis* and *Endothia parasitica* are gradually replacing chymosin in cheesemaking (Gupta et al., 1998). 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.

**ii) Baking industry**

Wheat flour is a major component of baking processes. It contains an insoluble protein called gluten, which determines the properties of the bakery doughs. Endo- and exo-proteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. Enzymatic treatment of the dough facilitates its handling and machining and permits the production of a wider range of products. The addition of proteases reduces the mixing time and results in increased loaf volumes. Bacterial proteases are used to improve the extensibility and strength of the dough.

**iii) Manufacture of soy products**

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

v) 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 thus crucial but it adds to the cost of production by chemical methods and enzymatic synthesis of aspartame is 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.
**d) 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 *E. coli* is used to eliminate aspargine 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, 1985).

**e) 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 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.

**1.2.8 Properties of alkaline proteases**

Alkaline proteases from several microorganisms have been studied extensively and based on their properties, used in various industries. A brief account of individual properties is presented below:
a) **pH and temperature kinetics**

In general, all currently used detergent-compatible proteases are alkaline and thermostable in nature with a high pH optimum - the pH of laundry detergents is generally in the range of 8 to 12 and have varying thermostabilities at laundry temperatures (50-70°C). Therefore, most of the commercially available subtilisin-type proteases are also active in the pH and temperature ranges 8-12 and 50-70°C, respectively. In addition, a recent trend in the detergent industry is a requirement for alkaline protease active at low washing temperatures; for example, Kannase - marketed by Novozymes - is active even at temperatures as low as 10-20°C.

b) **Effect of stabilizers/additives and metal ions**

Some of the major commercial uses of alkaline proteases necessitate high temperatures, thus improving the thermal stability of the enzyme is distinctly advantageous. Thermostability can be enhanced either by adding certain stabilizers (PEG, polyhydric alcohols, starch) to the reaction mixture or by manipulating the tertiary structure of enzyme by protein engineering. A thermostabilization effect of up to a 2-fold increase in the half-life of *Cucurbita ficifolia* protease at 65°C has been reported by using polyhydric alcohols, PEG and casein (Gonzalez *et al.*, 1992). The ion Ca\(^{2+}\) is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline protease at higher temperatures (Kumar, 2002; Lee *et al.*, 1996). Other metal ions such as Ba\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), Co\(^{2+}\), Fe\(^{3+}\) and Zn\(^{2+}\) are also used for stabilizing proteases (Johnvesly and Naik, 2001; Rattray *et al.*, 1995). These
metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at higher temperatures.

c) Substrate specificity
Alkaline proteases in general have broad substrate specificity and are active against a number of synthetic substrates and natural proteins. There are however, specific types of alkaline proteases, viz, collagenase, elastase, keratinase (Friedrich et al., 1999), which are active against specific protein substrates (collagen, elastin, keratin and cuticle, respectively). Alkaline proteases are also specific against aromatic or hydrophobic amino acid residues such as tyrosine and phenylalanine or leucine at the carboxylic side of the cleavage site.

d) Kinetic parameters
To develop an enzyme-based process, prior information about kinetic parameters of the enzyme in question is of utmost importance. To be precise, kinetic properties like $V_{\text{max}}$, $K_m$, $K_{\text{cat}}$ and $E_a$ are important, being not only enzyme-specific, but also substrate- and environment-specific and knowledge of these is essential for designing enzyme reactors or quantifying the applications of the enzyme under different conditions.

1.2.9 Current problems in protease research and potential Solutions (Gupta et al., 2002)
Proteases are a complex group of enzymes which differ in their properties such as substrate specificity, active site and catalytic mechanism. Their exquisite
specificities provide a basis for their numerous physiological and commercial applications. Despite the extensive research on several aspects of proteases from ancient times, there are several gaps in our knowledge of these enzymes and there is tremendous scope for improving their properties to suit projected applications. The future lines of development would include (i) genetic approaches to generate microbial strains for hyper-production of the enzymes (ii) application of site-directed mutagenesis (SDM) to design proteases with unique specificity and increased resistance to heat and alkaline pH (iii) synthesis of peptides (synzymes) to mimic proteases (iv) production of abzymes (catalytic antibodies) with specific protease activity and (v) understanding of the structure-function relationship of the enzymes. Some of the important problems faced in the desired usages of proteases and the possible solutions to overcome these hurdles are discussed below:

a) Enhancement of Thermostability

The industrial use of proteases in detergents or for leather processing requires that the enzyme be stable at higher temperatures. One of the common strategies to enhance the thermostability of the enzyme is to introduce disulfide bonds into the protease by SDM. Introduction of a disulfide bond into subtilisin E from *Bacillus subtilis* resulted in an increase of 4.5°C in the $T_m$ of the mutant enzyme without causing any change in its catalytic efficiency (Takami, 1990). But the properties of the mutant enzyme were found to revert to those of the wild-type enzyme. Enhanced stability of subtilisin was observed as a result of mutations of Asn109 and Asn218 to Ser. The analog containing both the mutations showed an additive effect on thermal stability. Thermostability of the
alkaline protease from Aspergillus oryzae is important because of its extensive use in the manufacture of soy sauce. The optimal temperature of the wild-type enzyme was enhanced from 51 to 56°C by the introduction of a disulfide (Cys 169-Cys 200) bond. Another strategy for improving the stability of the protease was by replacing the polar amino acid groups by hydrophobic groups. Further research involving cassette mutagenesis, etc., is necessary to yield an enzyme with substantially enhanced thermostability.

b) Prevention of Autoproteolytic Inactivation

Subtilisin, an extensively studied protease, is widely used in detergent formulations due to its stability at alkaline pH. Its autolytic digestion however, presents a major problem for its use in industry. It was deduced that there is a correlation between the autolytic and conformational stabilities. Introduction of a disulfide bond increased the stability of the mutant to a level less than or equal to that of the wild-type enzyme. It appears logical that mutations in the amino acids involved at the site of autoproteolysis may prevent the protease inactivation caused by self-digestion.

c) Alteration of pH Optimum

Different applications of proteases require specific optimal pHs for the best performance of the enzyme. Protein engineering enables us to tailor the pH dependence of the enzyme catalysis to optimize the industrial processes. Modifications in the overall surface charge of the proteins are known to alter the optimal pH of the enzyme. A change of Asp99 to Ser in subtilisin from Bacillus amyloliquefaciens has demonstrated the potential of altering the optimal pH of
the enzyme by systematic multiple mutations on the surface of the protein (Thomas, 1985).

d) Changing of Substrate Specificity

The properties needed for industrial applications of proteases differ from their physiological properties. The natural substrates of the enzyme are usually different from those desired for their industrial applications. Despite extensive research on proteases, relatively little is known about the factors that control their specificities toward nonphysiological substrates. Strategies involving SDM are being explored to tailor these specificities at will.

e) Improvement of Yield

The cost of enzyme production is a major obstacle in the successful application of proteases in industry. Protease yields have been improved by screening for hyperproducing strains and/or by optimization of the fermentation medium. Strain improvement by either conventional mutagenesis or recombinant-DNA technology has been useful in improving the production of proteases. There are many major problems in the commercialization of proteases. Although they are being addressed by both conventional and novel methods of genetic manipulation, there are no entirely satisfactory solutions and many of these problems remain unanswered.

1.2.10 Future Scope (Rao et al., 1998; Gupta et al., 2002)

Industrial applications of proteases have posed several problems and challenges for their further improvements. The biodiversity represents an
invaluable resource for biotechnological innovations and plays an important role in the search for improved strains of microorganisms used in the industry. A recent trend has involved conducting industrial reactions with enzymes reaped from exotic microorganisms that inhabit hot waters, freezing Arctic waters, saline waters or extremely acidic or alkaline habitats. The proteases isolated from extremophilic organisms are likely to mimic some of the unnatural properties of the enzymes that are desirable for their commercial applications. Exploitation of biodiversity to provide microorganisms that produce proteases well suited for their diverse applications is considered to be one of the most promising future alternatives. Introduction of extremophilic proteases into industrial processes is hampered by the difficulties encountered in growing the extremophiles as laboratory cultures. Revolutionary robotic approaches such as DNA shuffling are being developed to rationalize the use of enzymes from extremophiles. The existing knowledge about the structure-function relationship of proteases, coupled with gene-shuffling techniques, promises a fair chance of success in the near future, in evolving proteases that were never made in nature and that would meet the requirements of the multitude of protease applications.

A century after the pioneering work of Louis Pasteur, the science of microbiology has reached its pinnacle. In a relatively short time, modern biotechnology has grown dramatically from a laboratory curiosity to a commercial activity. Advances in microbiology and biotechnology have created a favorable niche for the development of proteases and will continue to facilitate their applications to provide a sustainable environment for mankind and to improve the quality of human life.
As the research work involves methodology covering aspects of the microbial (growth conditions) and biochemical (characteristics and purification of enzymes from two different enzyme subclasses) studies on thraustochytrids as well as the biotechnological applications of the enzymes produced by them, the thesis has been compiled into 11 small Chapters to favour clarity of presentation.
1.3 Lipases

It is in the last decade that lipases have gained importance to a certain extent over proteases and amylases, especially in the area of organic synthesis. The enantioselective and regioselective nature of lipases has been utilized for the resolution of chiral drugs, fat modification, synthesis of cocoa butter substituents, biofuels and for synthesis of personal care products and flavor enhancers. Lipases thus, appear to be the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists and biochemists (Saxena et al., 1999).

Lipases (triacylglycerol acylhydrolases) belong to the class of serine hydrolases and do not require any cofactor. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved (Fig. 1.3 and 1.4). Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The occurrence of the lipase reaction at an interface between the substrate and the aqueous phase causes difficulties in the assay and kinetic analysis of the reaction.
Figure 1.3 Diagrammatic representation of a lipase molecule showing its main features. Substrate can be any triglyceride (Saxena et al., 1999)

Figure 1.4 Lipolytic reaction at the oil–water interface (Saxena et al., 1999)
Lipases (E.C. 3.1.1.3) are known to be produced by several microorganisms such as bacteria, fungi, archaea and eucaryotes, as well as by animals and plants (Olson et al., 1994). The interest in lipases arises due to the ability of these enzymes to catalyze the hydrolysis as well as synthesis of fatty acid esters. They act on a variety of substrates including natural oils, synthetic triglycerides and esters of fatty acids. Many lipases are resistant to solvents and hence find use in the synthesis of chiral drugs.

1.3.1 Lipase producing microorganisms

Lipase production has been reported from a variety of bacteria, fungi and actinomycetes (Sztajer et al., 1988; Rapp and Backhaus, 1992). The presence of lipases in bacteria had been observed as early as 1901 for Bacillus prodigiosus, Bacillus pyocyneus and Bacillus fluorescens (Jaeger et al., 1994) which represent some of today's best studied lipase producers, now named, Serratia marcescens, Pseudomonas aeruginosa and Pseudomonas fluorescens, respectively. Lipase producers have been isolated mainly from soil or spoiled food material that contains vegetable oils. Lipases also represent an important virulence factor of many plant and animal pathogens. Lipases with novel properties have been discovered from microorganisms isolated from the Antarctic Ocean (Feller et al., 1990), hot springs (Gowland et al., 1987; Lee et al., 1999), compost heaps (Gowland et al., 1987; Rathi et al., 2000) and highly salty or sugary environments (Elwan et al., 1985; Ghanem et al., 2000). Lipase producers have been reported to grow at varied pH and temperatures. The fungi in general are reported to require acidic pH for growth and lipase production (Arima et al., 1972; Pokorny et al., 1994). Many bacteria are found to prefer neutral pH but there are reports of lipase production by alkalophic
bacteria also (Gao et al., 2000; Ghanem et al., 2000). Psychrophilic and thermophilic organisms as well as organisms having different oxygen demand (aerobic, microaerophilic and anaerobic) have been reported to produce lipases (Kulkarni and Gadre, 1999).

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acid, fatty acid alcohol or fatty acid ester for induction. There are, however, a few reports of constitutive lipase production by bacteria (Elwan et al., 1983; Gao et al., 2000). Lipases are usually secreted out in the culture medium although there are a few reports of the presence of intracellular lipases (Lee and Lee, 1989) as well as membrane bound lipases (Large et al., 1999).

The onset of lipase production is organism-specific but in general, the enzyme is released during late logarithmic or stationary phase (Matselis and Roussis, 1992; Makhzoum et al., 1995). Cultivation period from 3.5 to 168 h have been reported for the different lipase producing organism. Fast growing organisms were normally found to secrete the lipase within 12-24 h (Chartrain et al., 1993; Imamura and Kitaura, 2000). The production of lipases from bacteria is heavily patented although the published work is rather scanty (Inoue et al. 1987; Ishida et al. 1995). Most of the fermentations for lipase production have been performed in submerged, batch mode.

There are also a few reports of fed-batch or continuous culture studies. Suzuki et al. (1988) have reported mass production of lipase by Pseudomonas fluorescens in fed-batch culture. Lipase production as high as 2000 U/ml was
achieved by feeding olive oil, in which the feed rate of oil was controlled automatically based on CO₂ evolution rate. Wang and Chen (1998) were able to obtain increased yield of lipase by fill-and-draw culture of *Acinetobacter radioresistens*. Lechner *et al.* (1988) have used a dialysis fermenter for production of lipase from *Staphylococcus carnosus*. Gilbert *et al.* (1991) have optimized the lipase production by *Pseudomonas aeruginosa* EF2 by continuous culture using response surface analysis. Gerritse *et al.* (1998) have reported high production of a lipase from a recombinant *Pseudomonas pseudoalcaligenes*. Table 1.4 presents a brief overview of lipase production by several bacteria in batch mode, in shake flasks or in fermenters.

### 1.3.2 Mechanism of lipolysis

Lipases act on a variety of substrates such as triacylglycerols, cholesterol esters and wax esters which are insoluble in water. A typical reaction catalyzed by a lipase can be represented as shown in Fig 1.5.

Triacylglycerols are the main substrates of lipases. These uncharged lipids with long chain fatty acids esterified with glycerol are insoluble in water, although those with short-chain fatty acids are sparingly soluble in water. Triacylglycerols normally form emulsions in aqueous solutions at concentrations greater than their saturation value. Phospholipids are natural substrates for phospholipases. Phospholipids are also insoluble in water but they form micelles when exceeding the maximum concentration of dissolved
Table 1.4 Overview of lipase production in batch mode

(Kulkarni, 2002)

<table>
<thead>
<tr>
<th>Organism</th>
<th>C-source</th>
<th>N-source</th>
<th>Lipase U/ml</th>
<th>Assay method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter radioresistens</td>
<td>Olive oil and n-hexadecane</td>
<td>Tryptone, yeast extract, NH₄Cl</td>
<td>2</td>
<td>Titrimetry using olive oil</td>
<td>Chen et al. 1998</td>
</tr>
<tr>
<td>Aeromonas sobria</td>
<td>Whey, Soybean meal</td>
<td>Soybean meal, yeast extract</td>
<td>40</td>
<td>Spectrophotometry using pNPP</td>
<td>Lotrakul and Dharmisthiti 1997a</td>
</tr>
<tr>
<td>Bacillus circulans</td>
<td>Sucrose</td>
<td>NaNO₃, leucine</td>
<td>3890 mg/ml</td>
<td>-</td>
<td>Elwan et al. 1985</td>
</tr>
<tr>
<td>Bacillus circulans</td>
<td>Soluble starch</td>
<td>Soybean meal, peptone</td>
<td>2.1</td>
<td>Titrimetry using tributyrin</td>
<td>Sztajer and Maliszewska 1988a</td>
</tr>
<tr>
<td>Bacillus thermoleovorans ID-1</td>
<td>Olive oil</td>
<td>Tryptone, yeast extract</td>
<td>0.7</td>
<td>Spectrophotometry using pNP-butyrate</td>
<td>Lee et al 1999</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>Sucrose</td>
<td>Soybean meal</td>
<td>2.7</td>
<td>Titrimetry using</td>
<td>Sztajer and Maliszewska</td>
</tr>
</tbody>
</table>

Contd....
<table>
<thead>
<tr>
<th><strong>Bacillus sp.</strong></th>
<th><strong>Tween 80</strong></th>
<th><strong>NH₄NO₃</strong></th>
<th>4</th>
<th><strong>Titrimetry using olive oil</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus</strong></td>
<td>Rice bran oil</td>
<td>Yeast extract, peptone</td>
<td>8.3</td>
<td>Titrimetry using olive oil</td>
</tr>
<tr>
<td><strong>Bacillus</strong></td>
<td>Maltose</td>
<td>Soybean meal</td>
<td>2.8</td>
<td>Titrimetry using tributyrin</td>
</tr>
<tr>
<td><strong>Bacillus</strong></td>
<td>Tween 80</td>
<td>Yeast extract, peptone</td>
<td>0.2</td>
<td>Spectrophotometry using pNPP</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Starch</td>
<td>Soybean meal</td>
<td>1.4</td>
<td>Titrimetry using tributyrin</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Tween 80</td>
<td>KNO₃</td>
<td>8.3</td>
<td>Titrimetry using olive oil</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Ground soybean, Soluble starch</td>
<td>Corn steep liquor</td>
<td>87.5</td>
<td>Titrimetry using olive oil</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Soybean meal, soluble starch</td>
<td>(NH₄)₂SO₄</td>
<td>500</td>
<td>Titrimetry using olive oil</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Soybean meal, soluble starch</td>
<td>(NH₄)₂SO₄</td>
<td>30</td>
<td>Titrimetry using olive oil</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Soybean oil</td>
<td>-</td>
<td>85</td>
<td>Spectrophotometry using pNP laurate</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Peptonized milk</td>
<td>Beef extract, peptone</td>
<td>100</td>
<td>Titrimetry using olive oil</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Soy meal</td>
<td>Peptone, yeast extract</td>
<td>100</td>
<td>Spectrophotometry using pNP laurate</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td>Castor oil</td>
<td>Polypeptone</td>
<td>105</td>
<td>Titrimetry using olive oil</td>
</tr>
<tr>
<td><strong>Streptomyces sp.</strong></td>
<td>Soluble starch</td>
<td>Peptone</td>
<td>1.4</td>
<td>Titrimetry using tributyrin</td>
</tr>
</tbody>
</table>
Figure 1.5 Hydrolysis of triacylglycerol by lipase
monomer at a point called critical micelle concentration. While the maximum saturation value in water for triacylglycerols can be as high as 0.330 M in the case of triacetin, it can be less than 1 μM for long-chain triacylglycerols (Jaeger et al., 1994). Lipolysis by lipases occurs exclusively at the lipid-water interface, implying that the concentration of substrate molecules at this interface directly determines the rate of lipolysis.

Emulsions are characterized by a 'core' or a bulk lipid phase surrounded by surface monolayer of amphipathic molecules. The formation of emulsion requires some amount of energy input such as mechanical dispersion of bulk lipid in an aqueous phase. In the absence of other system components, the dispersed lipid droplets tend to coalesce in order to minimize the apolar surface exposed to water. Amphipathic molecules must be present to form a surface monolayer on the dispersed apolar lipid and thus to stabilize the emulsion. In the typical biological system, the surface components of oil emulsions can be lipids, denatured proteins or other type of amphipathic compounds. In synthetic emulsions, such as an emulsion of triolein in gum acacia solution, gum acacia acts as the amphipathic compound. Emulsion is the most popular lipid system used for estimation of lipase activity in laboratories.

1.3.2.1 Activation of lipolytic enzymes by interfaces

Verger (1980) demonstrated a fundamental difference between esterase and pancreatic lipase based upon ability or inability to be activated by interfaces. They observed that in contrast to esterases, which show normal Michaelis-Menten activity dependence on substrate concentration, the lipase displayed
almost no activity when the substrate was in the monomeric state. However, when the concentration of substrate (i.e. triacetin) exceeded its solubility limit resulting in formation of an emulsion, there was a sharp increase in the observed enzyme activity with the same substrate in emulsified state.

Kinetics of lipase action cannot be easily described with the Michaelis-Menten model because this model is valid only in the case of a single homogenous phase of soluble enzyme and substrate. A new model was proposed by Verger et al. (1980) to explain catalysis by lipolytic enzymes. The model consisted of two successive equilibria. It first described the penetration of a water-soluble enzyme into an interface (E-E*). This was followed by a second equilibrium, in which one molecule of penetrated enzyme got bound to one substrate molecule, giving the complex E*S. This was equivalent in two dimensions of classical Michaelis-Menten equilibrium. Once the complex E*S was formed, the catalytic reaction took place, regenerating the enzyme in the form E* along with the liberation of the products.

1.3.2.2 Hydrolysis versus synthesis

The hydrolysis of fats and oils is a reversible reaction and it is possible to change the direction of the reaction toward synthesis by modifying the reaction conditions. The water content of the reaction mixture controls the equilibrium between forward and reverse reactions. In non-aqueous environment, lipases catalyze ester synthesis reaction. The ester synthesis reaction can be classified into simple esterification, transesterification and interesterification, depending on the nature of reaction (Fig 1.6). The esterification reaction involves synthesis
of glyceryl esters from glycerol and fatty acid. In transesterification, in place of fatty acid the acyl donor is an ester.

The transesterification can be further divided into glycerolysis and alcoholysis, involving transfer of acyl group from triglyceride to either glycerol or an alcohol. In interesterification, the acyl group is exchanged between a glyceride and either a fatty acid (acidolysis) or a fatty acid ester. Interesterification requires a small amount of water, in addition to the amount needed for the enzyme to maintain active, hydrated state. Since the lipases act on a variety of substrates, have complex kinetics and varied applications, a variety of assay methods have been developed for their qualitative and quantitative estimation.

1.3.3 Lipase assay methods

There are numerous methods available for lipase activity estimation and they have been well reviewed in literature (Jensen, 1983; Jaeger et al., 1994; Beisson et al., 2000). Most of these methods are designed to estimate the products of hydrolytic reactions. These assay methods may be classified under (1) titrimetry (2) interfacial tensiometry, (3) spectroscopy (photometry, fluorimetry, Infrared and turbidimetry) (4) chromatography (5) immunochemistry or conductimetry. Tables 1.5 give an overview of the different lipase assay methods. Some of the assay procedures which have also been attempted in the present study are outlined below:
**Transesterification Reactions**

(a) **Acidolysis**

\[
R_1 - C - OR_2 + R_3 - C - OH \rightarrow R_3 - C - OR_2 + R_1 - C - OH
\]

(b) **Alcoholysis**

\[
R_1 - C - OR_2 + R_3 - OH \rightarrow R_1 - C - OR_3 + R_2 - OH
\]

(c) **Ester Exchange**

\[
R_1 - C - OR_2 + R_3 - C - OR_4 \rightarrow R_1 - C - OR_4 + R_3 - C - OR_2
\]

(d) **Aminolysis**

\[
R_1 - C - OR_2 + R_3 - NH_2 \rightarrow R_1 - C - NHR_3 + R_2 - OH
\]

**Hydrolysis**

\[
R_1 - C - OR_2 + H_2O \rightarrow R_1 - C - OH + R_2 - OH
\]

**Ester Synthesis**

\[
R_1 - C - OH_2 + OH - R_2 \rightarrow R_1 - C - OR_2 - H_2O
\]

*Figure 1.6 Various lipase mediated reactions*
Table 1.5 An overview of Lipase assays (Kulkarni, 2002)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction product</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerols</td>
<td>Free fatty acids</td>
<td>Coloured indicators (Victoria blue, methyl red, phenol red, rhodamine B)</td>
</tr>
</tbody>
</table>

**Titrimetric assay**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction product</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerols</td>
<td>Free fatty acids</td>
<td>pH determination</td>
</tr>
</tbody>
</table>

**Interfacial tensiometry**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction product</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicaprin</td>
<td>Free fatty acids</td>
<td>Measurement of barrier movement</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>Free fatty acids</td>
<td>Measurement of drop volume or decrease in surface tension</td>
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Contd ...
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Method</th>
<th>Final product</th>
<th>Wavelength 4nm</th>
</tr>
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<tbody>
<tr>
<td>2,3-dimercaptopropan-1-ol Tributyrin</td>
<td>Glycerol analogue (2 over 3</td>
<td>Reaction with DTNB</td>
<td>TNB</td>
<td>412</td>
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<tr>
<td></td>
<td>positions)</td>
<td></td>
<td></td>
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<tr>
<td>p-Nitrophenyl esters</td>
<td>p-nitro-phenol</td>
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<td>Product is colored</td>
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<tr>
<td>Glycerides (triolein)</td>
<td>Free fatty acid</td>
<td>Enzymatic conversion</td>
<td>NAD</td>
<td>340</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Free fatty acid</td>
<td>Negative charge</td>
<td>Safranine</td>
<td>520/560</td>
</tr>
<tr>
<td>Arylethene derivatives</td>
<td>Hydrolysis products are colored</td>
<td></td>
<td>Variable</td>
<td></td>
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</tr>
<tr>
<td>Glycerides</td>
<td>Free fatty acid</td>
<td>Complex formation</td>
<td>Rhodamine 6G</td>
<td>513</td>
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<tr>
<td>Glycerides</td>
<td>Free fatty acid</td>
<td>Complex formation</td>
<td>Cu (II) salt</td>
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<td>1-2-diglycerides</td>
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<td>quinone</td>
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<td>Fluorescence</td>
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<tr>
<td>Substrate</td>
<td>Reaction product</td>
<td>Method</td>
<td>Final product</td>
<td>Wavelength 4nm</td>
</tr>
<tr>
<td></td>
<td>Free acid analogues or aggregated</td>
<td>Fluorescence shift</td>
<td>Free acid analogues or</td>
<td>Ex. 340</td>
</tr>
<tr>
<td></td>
<td>substrate</td>
<td></td>
<td>glyceride analogues</td>
<td>em. 400-450</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerides containing pyrene ring</td>
<td></td>
<td>Complex formation</td>
<td>11-(dansylamino)undecanoic acid</td>
<td>Ex. 350</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>em 500</td>
</tr>
</tbody>
</table>
a) **Plate assays**

Lipases secreted by microbial cultures can be detected in agar media. Plate assays have been described for screening of lipase-producing microorganisms using coloured compounds such as Methyl Red, Phenol Red, Rhodamine B or Victoria Blue B as indicators (Kouker and Jaeger 1987; Samad et al. 1989). In these methods, emulsified oils were added to growth medium containing indicator dyes. The hydrolysis of oils caused the formation of coloured or fluorescent halos or zones around the microbial colonies.

b) **Titrimetry**

The lipolytic reaction liberates an acid which can be titrimetrically assayed. Rowe and Gilmour (1981) have described a method for the estimation of serum lipase using olive oil as the substrate. At the end of the assay period, the reaction mixture was titrated with an alkali solution and the fatty acids produced were quantified. In another method called “pH-stat”, the pH of reaction mixture was kept constant by continuous addition of NaOH solution and the volume of alkali added was monitored as a function of time (Gargouri et al., 1986).

c) **Spectroscopy**

Several assays for lipase activity estimation are based on spectroscopic measurements. Rollof et al. (1984) developed an assay which involved direct turbidometric estimation of residual lipids after reaction of the lipase with lipid emulsion. Yet another turbidometric assay was developed by Robinson et al. (1989) for estimation of lipase activity in serum. Triacylglycerols are natural substrates of lipases and many spectrophotometric methods use them as
substrates. A few spectrophotometric assays are based on methods which render colour to fatty acids released after hydrolysis of triacylglycerols. Rhodamine 6G was used for complexation with free fatty acids liberated during lipolysis. A pink colour appeared and the absorbance was monitored at 513 nm. Medcova et al. (1981) assayed the monoglyceride lipase activity using Tween 20 as substrate. The lauric acid released was converted to copper laureate and measured spectrophotometrically at 435 nm. Safarik (1991) developed a method using immobilized triacylglycerols. In this method, the fatty acids released after hydrolysis were extracted with benzene and converted to their corresponding Cu (II) salts, which were measured spectrophotometrically. A method that used metachromatic properties of the cationic dye, safranine, to detect a change in the net negative charge at the lipid-water interface, which was monitored by the change in the colour of safranine is very sensitive and very low quantities of lipolytic enzyme can be detected using this method (Gupta et al., 2003).

There are enzymatic assays based on estimation of either glycerol or fatty acids released after action of lipase on triacylglycerols. Fossati et al. (1992) described a kinetic colorimetric method for assaying lipase activity in serum by using a natural long-chain fatty acid 1,2-diglyceride. In the presence of co-lipase, deoxycholate and calcium ions, pancreatic lipase hydrolyzed the clear substrate solution to produce a 2-monoglyceride, which in turn, released glycerol by the action of a 2-monoglyceride lipase. Glycerol was then assayed by a sequence of enzymic reactions (glycerol kinase, glycerol phosphate oxidase, and peroxidase) that produced a violet quinone monoimine dye with
peak absorption at 550 nm. Woollett et al. (1984) described an enzymatic method for the determination of the amount of free fatty acids released from triglyceride by lipoprotein lipase. The quantity of free fatty acids present in the medium before and after incubation was measured spectrophotometrically by the oxidation of NADH in the final reaction of a series of coupled enzymic reactions.

In some of the spectroscopic assays, synthetic substrates are used. McKellar (1986) determined lipase activity in skimmed milk using β-naphthyl caprylate as substrate and the product (β-naphthol) formed was measured spectrophotometrically at 560 nm. Kurooka et al. (1977) described an assay using 2,3-dimercaptopropan-1-ol tributyrate as substrate and 5,5'dithiobis (2-nitro-benzoic acid) as chromogenic reagent. Richardson et al. (1989) used substituted arylethene derivatives as substrate. The hydrolysis products of these compounds are coloured and many of them are water-soluble, making them suitable precursors for chromogenic enzyme substrates. Besides measurements of the coloured product, spectroscopic assays are also based on precipitation of fatty acids with calcium or copper, the increase in absorbance being then measured at 500 nm. Para-nitrophenyl esters of various chain length fatty acids are also used as substrates (Winkler and Stuckmann, 1979).

Fluorescent compounds have also been used for the lipase assay. Some assays make use of the action of lipase on substrates derived from different fatty acyl ester derivatives of fluorophore 4-methylumbelliferon (Roy, 1980;
Cooper and Morgan, 1981) Wilton (1990) had designed a continuous assay procedure in which displacement of the fluorescent fatty acid probe 11-(dansylamino)undecanoic acid from a fatty acid binding protein was measured. The long-chain fatty acids released as a result of lipase activity displaced the fluorescent fatty acid. It was also possible to use triacylglycerols having one of the alkyl groups substituted with a fluorescent group e.g. pyrenyl (Negre-Salvayre et al. 1991). In an aggregated substrate, the pyrene groups are close to each other and fluoresce at 450 nm. When fatty acids were cleaved fluorescence shifted to 400 nm.

An infrared spectroscope method for measuring lipase-catalyzed hydrolysis of triglycerides in reverse micelles was devised by Walde and Luisi (1989). Using this method, lipolysis can be monitored by recording the Fourier Transformed Infrared spectrum of the entire reaction mixture. Fatty acid esters and the free fatty acids can be quantified based on their molar extinction coefficient and Beer's law.

Chromatographic procedures such as HPLC, GC and TLC have been used by various workers for assaying lipolytic activity (Veeraraghavan, 1990; Kashyap et al., 1980)

f) Other assays

It was also possible to use Nuclear magnetic resonance (NMR) for the quantification of lipase activity in biphasic macroemulsions (O'Connor et al. 1992). A conductometric method has been described using the short-chain
substrate triacetin. There are a few immunological assays developed particularly to detect lipase in milk and dairy products (Birkeland et al. 1984).

1.3.4 Lipase purification

The purification of lipase normally involves several steps depending on the extent of purity desired. In case of extracellular lipases, invariably the first step is the removal of cells by centrifugation or filtration. In case of intracellular lipases, an additional step of cell lysis is required. The crude lipase preparation can then be concentrated by ultrafiltration or can be subjected to optional solvent or salt precipitation. In most of the cases, either ion exchange chromatography or hydrophobic interaction chromatography has been effectively used for further purification of the concentrated enzyme. The final step of gel filtration normally yields a homogenous product (Sharma et al., 2001).

In most of the lipase purification procedures, the diethylaminoethyl (DEAE) anion exchanger is used. Lipases show natural affinity for hydrophobic substances, as their substrates are hydrophobic molecules. Hydrophobic interaction chromatography (HIC) is a very popular technique for purification of lipases. HIC is either used as the first or second step in the purification procedure. Bornscheuer et al. (1994) have reported single-step purification of lipase from a commercial preparation (Pseudomonas cepacia lipase) using phenyl Sepharose. Queiroz et al. (1999) and Diogo et al. (1999) have purified the Chromobacterium lipase using specially designed HIC matrices. The use of HIC was generally found to result in satisfactory enzyme recovery and fold-
purification. In some cases, lipases did bind very strongly to HIC matrices and could not be eluted even by the use of solvents (Ihara et al. 1991).

Affinity matrices with fatty acids as ligands have been used for purification of microbial lipases (Kamimura et al. 1999). *Staphylococcus epidermidis* lipase has been purified using metal-affinity chromatography (Simons et al. 1996). A one-step purification of cloned *Bacillus licheniformis* lipase was reported by Nthangeni et al. (2001).

Lipases have also been purified by aqueous-two-phase extraction systems. Aires-Barros and Cabral (1991) have reported separation of two lipases from *Chromobacterium viscosum* by aqueous two-phase extraction.

Aggregate formation has been reported in lipases of both Gram-positive and Gram-negative bacteria. Among Gram-positive bacteria, aggregation has been reported with crude as well as purified *Staphylococcus aureus* lipase (Kotting et al., 1983). Such aggregation was also observed with purified lipase produced by *Bacillus subtilis*, *Bacillus thermocatenulatus* and *Bacillus* sp. THL027 (Lesuisse et al., 1993; Dharmsthiti and Luchai, 1999).

In Gram-negative bacteria, it has been well documented for the members of genus *Pseudomonas*. Aggregates were reported to be formed of either pure *Pseudomonas* lipases (Fox and Stepaniack, 1983, Gilbert et al., 1991) or of the lipases associated with lipophilic molecules (Kordel et al., 1991). The *Pseudomonas aeruginosa* PAC 1R was shown to form lipase
lipopolysaccharide aggregates (Steur et al., 1986). During purification of lipases, such aggregates were dissociated by treatment with detergents such as Triton X-100 or CHAPS (Chartrain et al., 1993; Steur et al., 1986) or solvents like isopropanol (Dünhaupt et al., 1992).

1.3.6 Types of lipases and the reactions catalyzed

a) According to their substrate specificity microbial lipases can be classified into three groups — non-specific, regio-specific and fatty acid specific (Fig1.7). Non-specific lipases show neither positional nor fatty acid specificity and thus result in complete breakdown of triacylglyceride to fatty acid and glycerol. Regio-specific lipases hydrolyse only primary ester bonds at atoms C1 and C3 of glycerol and thus hydrolyse triacylglyceride to give free fatty acids, 1,2- (2,3) diglyceride and 2-monoglyceride. The third group exhibits a pronounced fatty acid preference. As per report to date, no bacterial lipase belongs to this group. Lipase B from Geotrichum candidum is specific for fatty acids with a double bond between C9 and C10 (Chartrain et al., 1993).

Regiospecific lipases catalyse synthetic reactions either by trasesterification or interesterification. Transesterification involves an exchange of acyl radicals between a triacylglyceride and a fatty acid (acidolysis), an alcohol (alcoholysis),
**Nonspecific Lipases**

\[
\begin{align*}
\text{CH}_2\text{OR}_1 & + \text{H}_2\text{O} \rightarrow \text{RCOOH} + \text{R}_1\text{COOH} + \text{R}_2\text{COOH} + \text{CHOH} \\
\text{CH}_2\text{OR}_2 & - \text{H}_2\text{O} \rightarrow \text{CH}_2\text{OH}
\end{align*}
\]

**1, 3 Specific Lipases**

\[
\begin{align*}
\text{CH}_2\text{OR}_1 & + \text{H}_2\text{O} \rightarrow \text{R}_1\text{COOCH} + \text{R}_1\text{COOCH} + \text{R}_2\text{COOH} \\
\text{CH}_2\text{OR}_2 & - \text{H}_2\text{O} \rightarrow \text{R}_2\text{COOCH}_2 + \text{CH}_2\text{OH} + \text{RCOOH}
\end{align*}
\]

**Fatty acid specific lipases**

\[
\begin{align*}
\text{RCOOCH}_2 & + \text{R}_1\text{COOCH}_2 + \text{R}_1\text{COOCH}_2 + \text{R}_1\text{COOCH}_2 \\
\text{R}_1\text{COOCH} & + \text{RCOOCH} + \text{R}_1\text{COOCH} + \text{R}_1\text{COOCH} \\
\text{R}_2\text{COOCH}_2 & + \text{RCOOCH}_2 + \text{RCOOCH}_2 + \text{R}_1\text{COOCH}_2 \\
\text{CH}_2\text{OH} & + 3\text{H}_2\text{O} \\
\text{R}_1\text{COOCH} & + \text{CHOH} + \text{R}_1\text{COOCH} + \text{R}_1\text{COOCH} + 3\text{HOOC-R} \\
\text{R}_1\text{COOCH}_2 & + \text{R}_1\text{COOCH}_2 + \text{R}_1\text{COOCH}_2 + \text{CH}_2\text{OH}
\end{align*}
\]

Fig 1.7 Types of lipases and the reactions catalyzed
or glycerol (glycerolysis) and interesterification involves exchange of acyl radicals between two triacylglycerides.

b) Biochemical Classification of the bacterial lipases

Arpigny and Jaeger (1999) have classified 47 different bacterial lipases into eight families on the basis of amino acid sequence homology.

Family I

Family I comprised of a total of 22 members sub grouped into six subfamilies. *Pseudomonas* lipases were classified to subfamilies I.1 and I.2 on the basis of amino acid sequence comparison. Ogierman et al. (1997) have shown that the gene coding for the 33 kDa *Vibrio cholerae* O1 lipase was highly homologous to the lipase gene of *Pseudomonas aeruginosa*.

Subfamily I.3 contained enzymes from at least two distinct species: *Pseudomonas fluorescens* and *Serratia marcescens*. These lipases had in common a higher molecular mass than lipases from subfamilies I.1 and I.2 (*Pseudomonas fluorescens*, 50 kDa; *S. marcescens*, 65 kDa) and the absence of an N-terminal signal peptide and of Cysteine residues.

The various lipases produced by *Bacillus* sp. were found to have an alanine residue replacing the first glycine in the conserved pentapeptide: Ala-Xaa-Ser-Xaa-Gly. The lipases from the two mesophilic strains *B. subtilis* and *B. pumilus* were different from those other from *Bacillus* lipases. They were the smallest true lipases known (approximate molecular mass 20 kDa) and shared very little
sequence similarity (approximately 15%) with the other lipases. They were included in family I.4.

The high molecular weight *Bacillus* lipases and *Staphylococcus* lipases were included in subfamily I.5. *B. thermocatenulatus* and *B. stearothermophilus* produced lipases with similar properties. Their molecular mass was approx. 45 kDa and they displayed maximal activity around pH 9.0 and 65 °C. Staphylococcal lipases were enzymes with larger molecular mass (approx. 75 kDa) that were secreted as precursors and cleaved in the extracellular medium by a specific protease, yielding a mature protein of approximately 400 residues.

*Family II (The GDSL family)*

The enzymes grouped in family II did not exhibit the conventional penta-peptide Gly- Xaa-Ser-Xaa-Gly but rather displayed a Gly-Asp-Ser-(Leu) [GDS(L)] motif containing the active-site serine residue. In these proteins, this important residue was found to lie much closer to the N-terminus than in other lipolytic enzymes.

*Family III*

This family of lipases was identified primarily by Cruz et al. (1994) who solved the 3D structure of the *Streptomyces exfoliatus* (M11) lipase. This enzyme displays the canonical fold of α/β-hydrolases and contains a typical catalytic triad.

*Family IV-The hormone-sensitive lipase (HSL) family*

A number of bacterial enzymes (family IV) displayed a striking amino acid sequence similarity to the mammalian HSL. They were included in family IV.
Family V
Like proteins in the HSL family, enzymes grouped in family V originated from mesophilic bacteria (\textit{Pseudomonas oleovorans}, \textit{Haemophilus influenzae}, \textit{Acetobacter pasteurianus}) as well as from cold-adapted (\textit{Moraxella} sp., \textit{Psychrobacter immobilis}) or heat-adapted (\textit{Sulfolobus acidocaldarius}) organisms.

Family VI
With a molecular mass in the range 23–26 kDa, the enzymes in this family were among the smallest esterases known.

Family VII
A number of rather large bacterial esterases (55 kDa) share significant amino acid sequence homology (30% identity, 40% similarity) with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases. They were classified in family VII.

Family VIII
Enzymes grouped in family VIII originated from \textit{Arthrobacter globiformis} \textit{Streptomyces chrysomallus} and \textit{Pseudomonas fluorescens}.

1.3.7 Applications of lipases
Lipases possess the unique feature of acting at an interface between the aqueous and nonaqueous (\textit{i.e.} organic) phase; this feature distinguishes them from esterases. Lipase activity generally depends on the available surface
area. They are the most versatile biocatalysts and they bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. Lipases act under extremely mild conditions (Sharon et al. 1998). They can be used in a variety of organic solvents and often show selectivity for a specific reaction type.

Alkaline thermophilic lipases find application in the detergent industry. Many fatty food stains and human sebum contain triglycerides which are hydrolyzed by lipases to produce fatty acids, monoglycerides and diglycerides, which are easier to remove than unhydrolyzed triglycerides (Fuji et al., 1986). Table 1.6 enumerates a few of the most significant industrial applications of microbial lipases.

a) Lipases in food industry

Lipases have become an integral part of the modern food industry. The use of enzymes to improve the traditional chemical processes of food manufacture has been developed in the past few years. Yoneda et al. (1996) have patented a process on *Pseudomonas* lipase, which was claimed to be useful in, for example, food processing and oil manufacture. Alcoholysis of cod liver oil for the production of omega-3 polyunsaturated fatty acids was investigated by using *Pseudomonas* lipase (Zuyi and Ward, 1993). A few bacteria produce flavour esters and find use in cheese industry. The production of flavour esters by lipases of *Staphylococcus warneri* and *Staphylococcus xylosus* has been described by Talon et al. (1996). Synthesis of fatty acid esters by a recombinant *Staphylococcus epidermidis* lipase has been described by Chang et al. (2001). *Chromobacterium viscosum* lipase was shown to have good
### Table 1.6 Industrial applications of microbial lipases (from Sharma et al., 2001)

<table>
<thead>
<tr>
<th>Industry</th>
<th>Action</th>
<th>Product or application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergents</td>
<td>Hydrolysis of fats</td>
<td>Removal of oil stains from fabrics</td>
</tr>
<tr>
<td>Dairy foods</td>
<td>Hydrolysis of milk fat, cheese ripening, modification of butter fat</td>
<td>Development of flavoring agents in milk, cheese, and butter</td>
</tr>
<tr>
<td>Bakery foods</td>
<td>Flavor improvement</td>
<td>Shelf-life prolongation</td>
</tr>
<tr>
<td>Beverages</td>
<td>Improved aroma</td>
<td>Beverages</td>
</tr>
<tr>
<td>Food dressings</td>
<td>Quality improvement</td>
<td>Mayonnaise, dressings, and whippings</td>
</tr>
<tr>
<td>Health foods</td>
<td>Transesterification</td>
<td>Health foods</td>
</tr>
<tr>
<td>Meat and fish</td>
<td>Flavor development</td>
<td>Meat and fish products; fat removal</td>
</tr>
<tr>
<td>Fats and oils</td>
<td>Transesterification; hydrolysis</td>
<td>Cocoa butter, margarine, fatty acids, glycerol, mono-, and di-glycerides</td>
</tr>
<tr>
<td>Pharmaceuticals</td>
<td>Transesterification, hydrolysis</td>
<td>Specially lipids, digestive aids</td>
</tr>
<tr>
<td>Cosmetics</td>
<td>Synthesis</td>
<td>Emulsifiers, moisturizers</td>
</tr>
<tr>
<td>Leather</td>
<td>Hydrolysis</td>
<td>Leather products</td>
</tr>
<tr>
<td>Paper</td>
<td>Hydrolysis</td>
<td>Paper with improved quality</td>
</tr>
<tr>
<td>Cleaning</td>
<td>Hydrolysis</td>
<td>Removal of fats</td>
</tr>
</tbody>
</table>
potential for the instant generation of aroma and flavour compounds and could be stored at least for one month. In this case, the lipase activity was immediately regenerated on dehydration (Carlile et al. 1996).

b) Lipases in biomedical application

Owing to their excellent capability for specific regioselective reactions in a variety of organic solvents with broad substrate recognition, lipases have emerged as an important biocatalyst in biomedical applications. Recently, Parmar et al. (1996) have reviewed a variety of substrates accepted by hydrolytic enzymes, including lipases, to produce compounds in high enantiomeric excess, which can be used as chiral building blocks for the synthesis of compounds of pharmaceutical interest.

Conventional chemical synthesis of drugs containing a chiral center generally yields equal mixtures of enantiomers. During the past decade, many studies have shown that racemic drugs usually have the desired therapeutic activity residing mainly in one of the enantiomers and the other enantiomer might interact with different receptor sites, which can cause unwanted side effects (Pandey et al., 1999).

Akita et al. (1997) have performed enzymic hydrolysis in organic solvents for the kinetic resolution of water-insoluble acetyloxy esters using immobilized Pseudomonas sp. lipase to produce chiral intermediates for the synthesis of the antibiotic (-)-indolmycin. A method was developed by Jimenez et al. (1997) to synthesize methyl (R)- and (S)-2-tetradecyloxiranecarboxylate through
sequential kinetic resolution catalysed by *Pseudomopnas* sp. lipase. Both the enantiomers are a potent anti-diabetic and antioxidant agent.

c) *Lipases in pesticides*

A variety of pesticides (insecticides, herbicides, fungicides or their precursors) incorporating the applications of lipases are currently in use (Pandey *et al.*, 1999). The most important application of lipases has been in the organic synthesis of pesticides for the production of optically active compound (Reddy 1992). Generally, these compounds were produced through the resolution of racemic mixtures of alcohol or carboxylic esters; stereospecific synthesis reactions were also employed. Akita *et al.* (1997) described a highly stereospecific synthesis of the versatile chiral synthon possessing two stereogenic centres, which was subsequently converted into a homochiral intermediate for the synthesis of the biologically active potent pesticide nikkomycin-B. Mitsuda *et al.* (1990) have reported use of *Achromobacter* lipase for enantioselective hydrolysis of the acetic acid ester of racemic cyano-3- phenoxybenzyl alcohol (CPBA) for the production of (S)-CPBA, an active insecticidal stereoisomer.

d) *Lipases in detergents*

Lipases have been generally added to the detergents primarily in combination with proteases and cellulases. In addition other enzymes such as amylases, peroxidases and oxidases are also reported to be added in detergent preparations (Kottwitz *et al.*, 1994).
Removal of oil/fatty deposits by lipase is attractive owing to its suitability under milder washing conditions. To be a suitable additive in detergents, lipases should be both thermostable as well as alkalophilic and capable of functioning in the presence of the various components of washing powder formulations (Jaeger et al., 1994).

*Pseudomonas* lipase preparations have been used for preparation of washing powder formulations. *Pseudomonas medocina* (Lumafast.) and *Pseudomonas alcaligenes* (Lipomax.) lipases have been manufactured by Genencor International USA as detergent additives (Jaeger et al., 1994; Reetz and Jaeger., 1998). The Novo group has reported a highly alkaline, positionally non-specific lipase from a strain of *Streptomyces* sp. that was useful in laundry and dish-washing detergents as well as industrial cleaners (Pandey et al., 1999). Several lipase-producing organisms and their manufacturing processes have been patented for preparation of detergent lipases (Holmes, 1993; Lawler and Smith, 2000).

e) Lipases in the leather industry

Leather processing involves the removal of subcutaneous fat, de-hairing and stuffing. Tanning processes are usually performed in an alkaline environment, so alkalophilic microbes ought to be better for exploration. Many *Bacillus* sp. strains which grew successfully under highly alkaline conditions were found to be useful in leather processing (Haalck et al., 1992).
f) Lipases in environmental management

Lipases have been used for the degradation of wastewater contaminants such from oil mills (Vitolo et al., 1998). The treatment process involved the cultivation of lipase-producing microbial strains in the effluents. Wakelin and Forster (1997) investigated the microbial treatment of waste from fast-food restaurants for the removal of fats, oils and greases. They cultivated pure and mixed microbial flora known to produce lipases and other enzymes. *Acinetobacter* sp. was the most effective of the pure cultures, typically degrading 60–65% of the fatty material.

g) Lipases in the cosmetics and perfume industry

Monoacylglycerols and diacylglycerols prepared by the lipase-catalysed esterification of glycerol are useful as surfactants in cosmetics (Pandey et al., 1999). The monoacylglycerol synthesis has been reported using *Pseudomonas* sp. LP7315 moiacylglycerol lipase. Izumi et al., (1997) performed the transesterification of 3, 7-dimethyl-4, 7-octadien-1-ol with lipases from various microbial sources to prepare rose oxide, which is an important fragrance ingredient in the perfume industry.

The ability of lipases to show high stability and selectivity in organic solvents has been exploited by various researchers as reviewed by Gupta et al. (2003). Biotransformations on polyacetoxy arylmethyl ketones, benzylphenylketone peracetates, esters of polyacetoxy aromatic acids and peracetylated benzopyranones using commercial lipases have been carried out by Parmar et al., (1996). The work being carried out in Indian laboratories has made considerable progress in recent years. Novel lipases with properties of chemo-,
regio- and enantio-selectivity have been isolated, which may be eligible for exploitation at commercial level for industrial applications in course of time. In fact, some of the indigenously developed technologies for the production of lipases are already in the commercial production stage. Furthermore, comparison of some of the lipases produced by microorganisms indigenously has shown that they are at par or even better than the well-known commercially available imported lipases. Utilizing these lipases should thus greatly boost many biotechnology-based industries in the 21st century (Saxena et al., 1999)