Proteases and Lipases from the Marine Protists, Thraustochytrids

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for the degree of

Doctor of Philosophy in Biotechnology

by

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M.Sc (Biochemistry)

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CERTIFICATE

This is to certify that the thesis entitled ***"Proteases and Lipases from the Marine Protists, Thraustochytrids"*** *submitted by Mrs.R.Kanchana for the award of the Degree of Doctor of Philosophy in Biotechnology is based on original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any university or institution.*

Place : Goa University
Date :22.12.2007

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Certified that the necessary corrections have been incorporated and the thesis is recommended for the award of Ph.D degree

30/9/'08

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STATEMENT

As required under the University ordinance OB- 9.9 (ii), I state that the present thesis entitled ***"Proteases and Lipases from the Marine Protists, Thraustochytrids"*** *is my original contribution and that the same has not been submitted on any previous occasion for any degree. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.*

Place: Goa, India

Date: 22.12. 2007

R.Kanchana

R.Kanchana

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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)	
Kingdom	Straminipila	**Kingdom**	Chromista
Phylum	Heteroknota	**Phylum**	Heteroknota
Class	Labyrinthista	**Sub-Phylum**	Labyrinthista
Order	Thraustochytriales	**Class**	Labyrinthulea
Family	Thraustochytriaceae	**Family**	Thraustochytridae

* (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 1.1 a and b.(Porter, 1990).

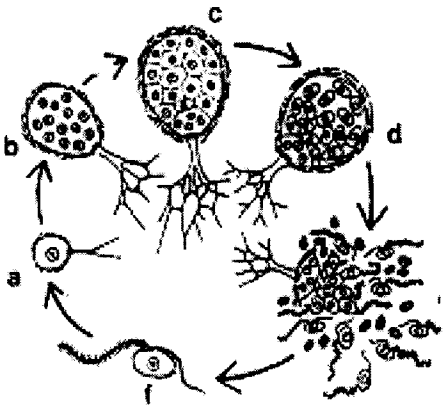


Fig 1.1a Life cycle of *Thraustochytrium*

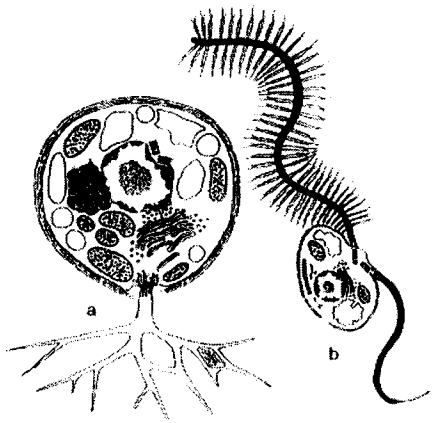


Fig 1.1 b Ultra structural features of *Thraustochytrium*

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 μm. 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 μm-sized bacteria to flagellates, to ciliates, and to mm-sized zooplankton, involving four trophic levels. When the chain starts from 10 μm-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 μm-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. Thraustochytids 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.

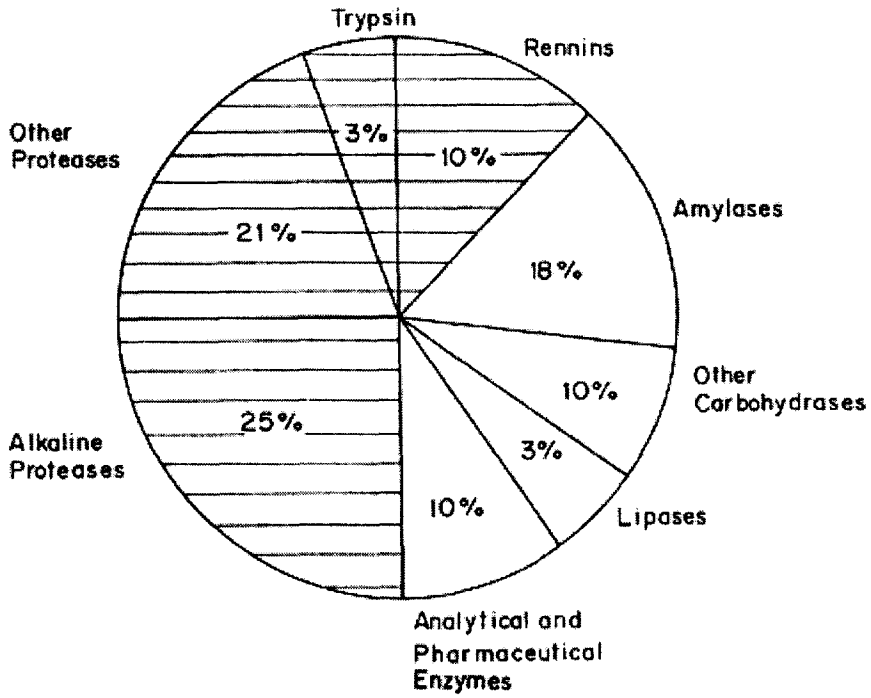


FIG. 1.2 Distribution of enzyme sales(Rao *et al.*,1998)

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 (M_r 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 (M_r 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 (M_r 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 (M_r 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, metallocarboxypeptidases and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes. Metallocarboxypeptidases 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.

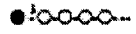
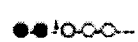
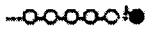
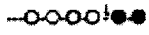
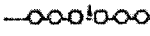
Other exopeptidases include dipeptidases, which cleave a dipeptide and omega peptidases which release modified residues from N- or C- termini.

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)

Protease	Mode of action	EC no:
Exopeptidase		
Aminopeptidases		3.4.11
Dipeptidyl peptidase		3.4.14
Tripeptidyl peptidase		3.4.14
Carboxypeptidase		3.4.16–3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl Dipeptidases		3.4.15
Dipeptidases		3.4.13
Omega peptidases		3.4.19
		3.4.19
Endopeptidases		3 .4.21–3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
		3.4.24
Metalloprotease		3.4.99
Endopeptidases of unknown catalytic mechanism		

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 diffesr 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.

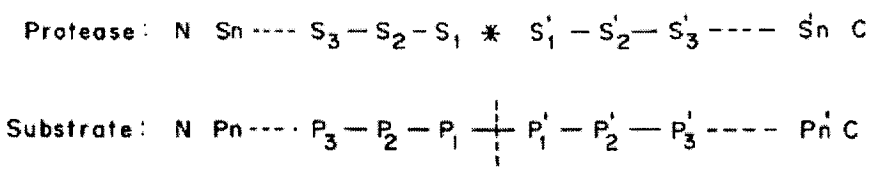
iv) Metalloproteases - Metalloproteases are the most diverse types of proteases (Barett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of

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 PI through Pn and P1' through Pn', respectively.



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)

Micro organism	pH	Temperature (°C)	Agitation (rpm)	Incubation period (h)	Preferred/optimized nitrogen sources	Preferred/optimized carbon sources
Alcaligenes faecalis	8	30	200	48	Soybean meal	None[a]
Bacillus sp. IS-3	10.5	37	200	72	Soybean meal	Glucose
Bacillus sp. JB99	10	55	180	24	KNO_3	Citric acid
Bacillus sp. K2	7	37	300-500	60-72	Casein hydrolyzate, gelatine	Glycerol
Bacillus sp. P-2	9.5	30	-	24	Peptone, yeast extract	Glucose
Bacillus sp. RGR-14	7	37	200	72-96	Soybean meal; peptone	Starch
Bacillus sp. SSR-1	10	40	150	18	Biopeptone, yeast extract	Beef extract, lactose
B. brevis MTCC B0016	10.5	37	200	96	Soybean meal	Lactose
B. licheniformis ATCC 21415	7	30	250-400	48	Soybean, $(NH_4)_2PO_3$	Lactose, glucose
B. mojavensis	7	50	200-250	24	Casein or casamino acids	Glucose
B. pumilis MK6-5	9.6	35	250	60	Cornsteep liquor, tryptone	Glucose, sodium citrate
B. sphaericus	n.s.[b]	30	300	n.s.	Biopeptone, yeast extract	Glucose
B. subtilis 168	n.s.	36	250	6-8	Nutrient broth; yeast extract	Glucose; yeast extract

Contd.....

Flavobacterium balustinum	7.4	10	150	72	Polypeptone, yeast extract, casein	None[a]
Serratia marcescens ATCC 25419	n.s.	30; 36	250	24; 16-18	Yeast extract, tryptone, asparagine, NH_4Cl	Whey; sucrose
Conidiobolus coronatus (NCL 86.8.20)	7-7.5	28	220	48	Ammonium nitrate, tryptone, casein	Sucrose
Ophiostoma piceae	n.s.	23	250	9 days	Soydrink from soybean meal	Starch
Tritirachium album	5.9	28	200	24-120	Peptone, yeast extract, $NaNO_3$, $(NH_4)_2SO_4$	Glucose
Streptomyces sp. NCIB 10070	7	n.s.	n.s.	24	Rapemeal	None[a]
Thermoactinomyces sp. E79	7.2	50	250	16	Soytone	Starch

[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

Substrate	Wavelength (nm)
Casein	700
	670
	500
	660
	750
Hammerstein casein	660
	275
DNHB casein	366
Immobilized ostazin blue S-2G dyed-casein	620
Thermally modified casein complexed with black drawing ink	400
Azocasein	440
	340
	420
	480
Thermally modified azocasein	366-400
Azoalbumin	440
$_{l}$-Crystalline aggregate	405
Thermally modified gelatin complexed with congo red or nigrosin	490, 570
Chemically modified (formaldehyde/gluteraldehyde mediated) gelatin complexed with black drawing ink	800-900

Tripeptide substrate	400

Fluorescent oligopeptide energy transfer assay

Dansylated hexapeptide	310-410

ELISA-based protease assay

Biotinylated BSA	405

Magnet-based protease assay

Magnet dye stained gelatin	605

Fluorescence-based protease assay

FITC casein, FTC hemoglobin	575 nm with excitation at 490 nm

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 pI. It is known that a protease is most suitable for this application if its pI 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 (pI 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, bating 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_{max}, K_m, K_{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.

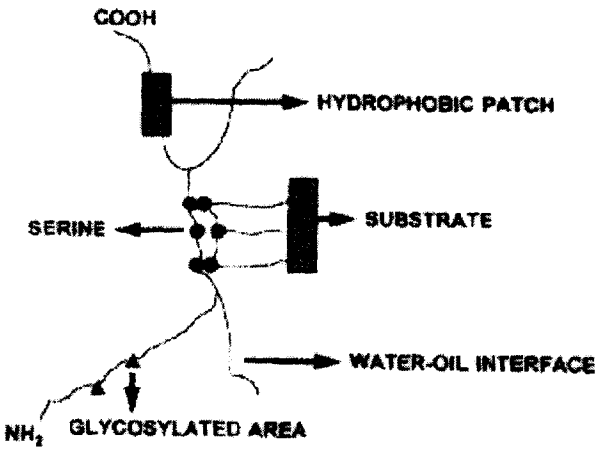


Figure 1.3 Diagrammatic representation of a lipase molecule showing its main features. Substrate can be any triglyceride (Saxena *et al.*, 1999)

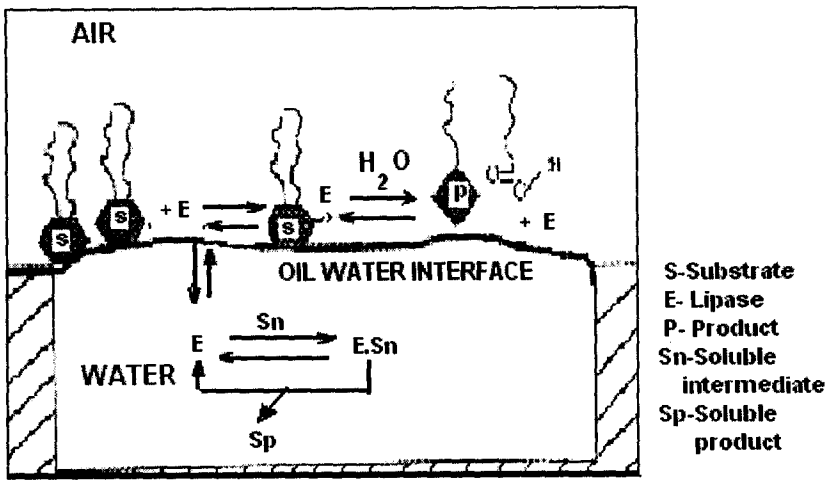


Figure 1.4 Lipolytic reaction at the oil–water interface (Saxena *et al.*, 1999)

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 CO_2 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)

Organism	C- source	N-source	Lipase U/ml	Assay method	Reference
Acinetobacter radioresistens	Olive oil and n-hexadecane	Tryptone, yeast extract, NH_4Cl	2	Titrimetry using olive oil	Chen *et al.* 1998
Aeromonas sobria	Whey, Soybean meal	Soybean meal, yeast extract	40	Spectrophoto-metry using *p*NPP	Lotrakul and Dharmsthiti 1997a
Bacillus circulans	Sucrose	$NaNO_3$, leucine	3890 mg/ml	-	Elwan *et al.* 1985
Bacillus circulans	Soluble starch	Soybean meal, peptone	2.4	Titrimetry using tributyrin	Sztajer and Maliszewska 1988a
Bacillus thermoleovorans ID-1	Olive oil	Tryptone, yeast extract	0.7	Spectrophoto metry using *p*NP-butyrate	Lee *et al.* 1999
Bacillus sp.	Sucrose	Soybean meal,	2.7	Titrimetry using	Sztajer and Maliszewska

Contd....

Bacillus sp.	Tween 80	NH_4NO_3	4	Titrimetry using olive oil
Bacillus sp. THL027	Rice bran oil	Yeast extract, peptone	8.3	Titrimetry using olive oil
Bacillus licheniformis	Maltose	Soybean meal	2.8	Titrimetry using tributyrin
Flavobacterium	Tween 80	Yeast extract, peptone	0.2	Spectrophoto-metry using *p*NPP
Pseudomonas fluorescens	Starch	Soybean meal	1.4	Titrimetry using tributyrin
Pseudomonas aeruginosa EF2	Tween 80	KNO_3	8.3	Titrimetry using olive oil
Pseudomonas sp.	Ground soybean, Soluble starch	Corn steep liquor	87.5	Titrimetry using olive oil
Pseudomonas nitroreduscens	Soybean meal, soluble starch	$(NH_4)_2SO_4$, Urea	500	Titrimetry using olive oil
Pseudomonas fragi	Soybean meal, soluble starch	$(NH_4)_2SO_4$, Urea	30	Titrimetry using olive oil
Pseudomonas aeruginosa YS-7	Soybean oil	-	85	Spectrophoto metry using *p*NP laurate
Pseudomonas aeruginosa MB 5001	Peptonized milk	Beef extract, peptone	100	Titrymetry using olive oil
Pseudomonas pseudoalcaligenes	Soymeal	Peptone, yeast extract	100	Spectrophoto metry using *p*NP laurate
Pseudomonas sp.	Castor oil	Polypep-tone	105	Titrimetry using olive oil
Streptomyces sp.	Soluble starch	Peptone	1.4	Titrimetry using tributyrin

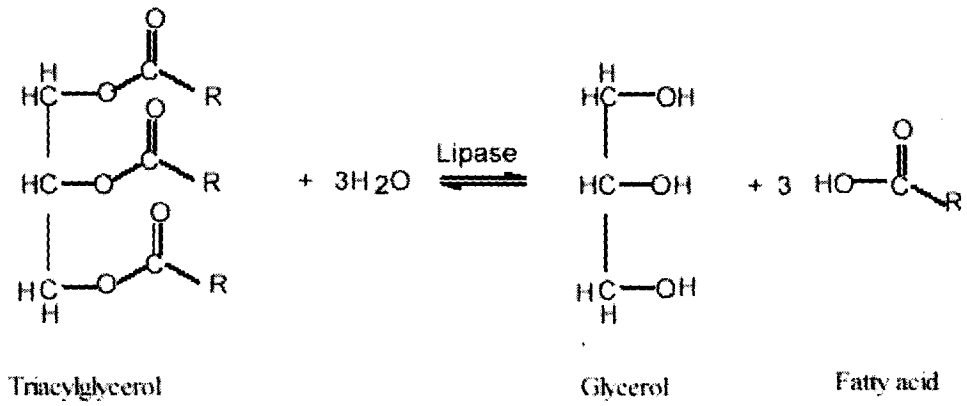


Figure 1.5 Hydrolysis of triacylglycerol by lipase

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 1μM 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.

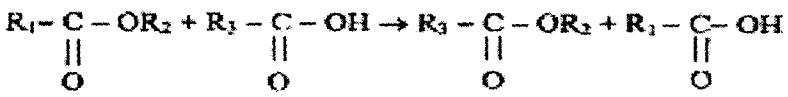
The transesterification can be further divided into glycerolysis and alcoholysis, involving transfer of acyl group from triglyceride to either glycerol or an alcohol. In interesterification, the acyl group is exchanged between a glyceride and either a fatty acid (acidolysis) or a fatty acid ester. Interesterification 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

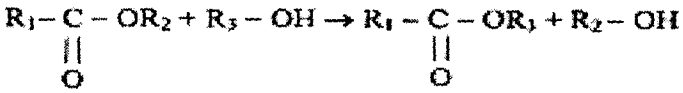
There are numerous methods available for lipase activity estimation and they have been well reviewed in literature (Jensen, 1983; Jaeger *et al.*, 1994; Beisson *et al.*, 2000). Most of these methods are designed to estimate the products of hydrolytic reactions. These assay methods may be classified 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

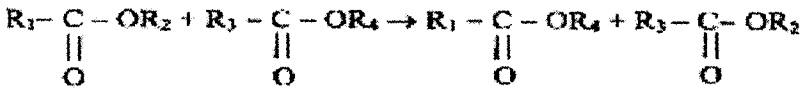
(a) Acidolysis



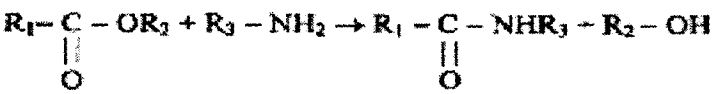
(b) Alcoholysis



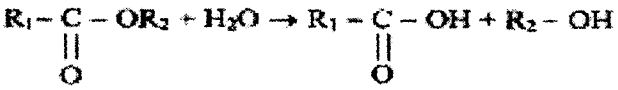
(c) Ester Exchange



(d) Aminolysis



Hydrolysis



Ester Synthesis

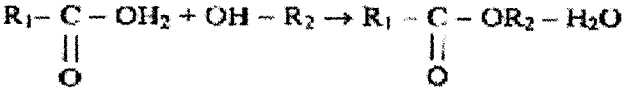


Figure 1.6 Various lipase mediated reactions

Table 1.5 An overview of Lipase assays (Kulkarni, 2002)

Plate assays		
Substrate	Reaction product	Method
Triacylglycerols	Free fatty acids	Coloured indicators (Victoria blue, methyl red, phenol red, rhodamine B)
Titrimetric assay		
Triacylglycerols	Free fatty acids	pH determination
Interfacial tensiometry		
Dicaprin	Free fatty acids	Measurement of barrier movement
Triacylglycerols	Free fatty acids	Measurement of drop volume or decrease in surface tension

Contd ...

Spectrophotometry				
Substrate	Product	Method	Final product	Wavelength nm
2,3-dimercaptopropan-1-ol Tributyrate	Glycerol analogue (2 over 3 positions)	Reaction with DTNB	TNB	412
p- Nitro phenyl esters	p-nitro-phenol	Product is colored		410
Glycerides (triolein)	Free fatty acid	Enzymatic conversion	NAD	340
Glycerides (triolein)	Free fatty acid	Negative charge	Safranine	520/560
Aryleethene derivatives	Hydrolysis products are colored			Variable
Glycerides	Free fatty acid	Complex formation	Rhodamine 6G	513
Glycerides	Free fatty acid	Complex formation	Cu (II) salt	715
1-2- diglycerides	Glycerol	Enzymatic conversion	quinone	550
Fluorescence				
Substrate	Reaction product	Method	Final product	Wavelength nm
Glycerides containing pyrene ring	Free acid analogues or aggregated substrate	Fluorescence shift	Free acid analogues or glyceride analogues	Ex. 340 em. 400 450
Glycerides	Free fatty acid	Complex formation	11-(dansylamino)undecanoic acid	Ex. 350 em 500

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 epidermidi* 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

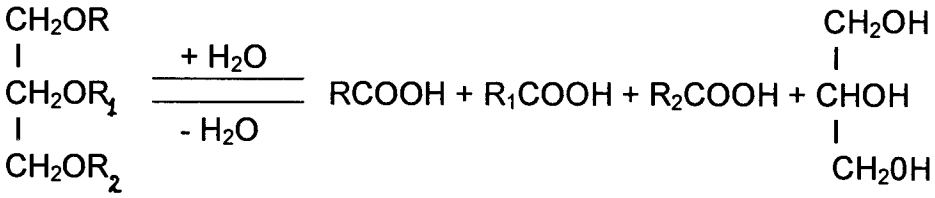
lipopolysaccharide aggregates (Steur *et al.*, 1986). During purification of lipases, such aggregates were dissociated by treatment with detergents such as Triton X-100 or CHAPS (Chartrain *et al.*, 1993; Steur *et al.*, 1986) or solvents like isopropanol (Dünhaupt *et al.*, 1992).

1.3.6 Types of lipases and the reactions catalyzed

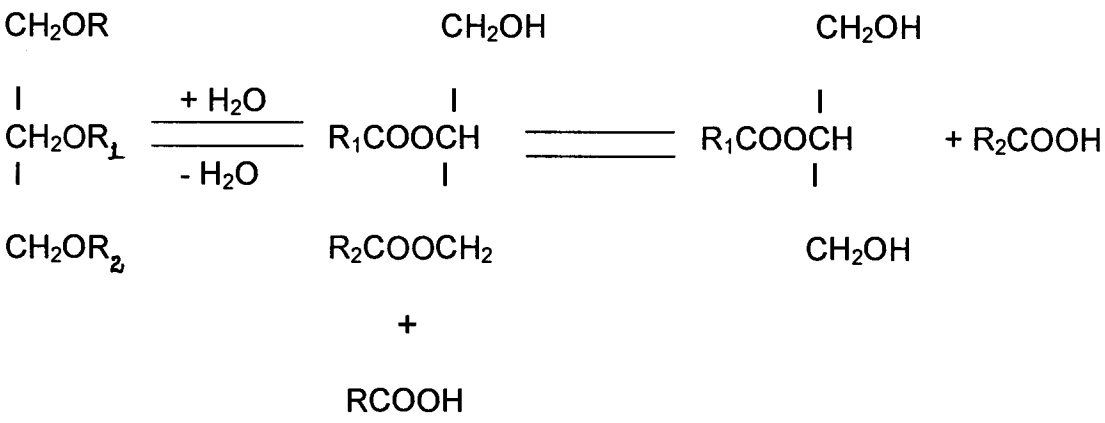
a) According to their substrate specificity microbial lipases can be classified into three groups – non-specific, regio-specific and fatty acid specific (Fig1.7). Non-specific lipases show neither positional nor fatty acid specificity and thus result in complete breakdown of triacylglyceride to fatty 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 trasesterfication or interesterification. Transesterification involves an exchange of acyl radicals between a triacylglyceride and a fatty acid (acidolysis), an alcohol (alcoholysis),

Nonspecific Lipases



1, 3 Specific Lipases



Fatty acid specific lipases

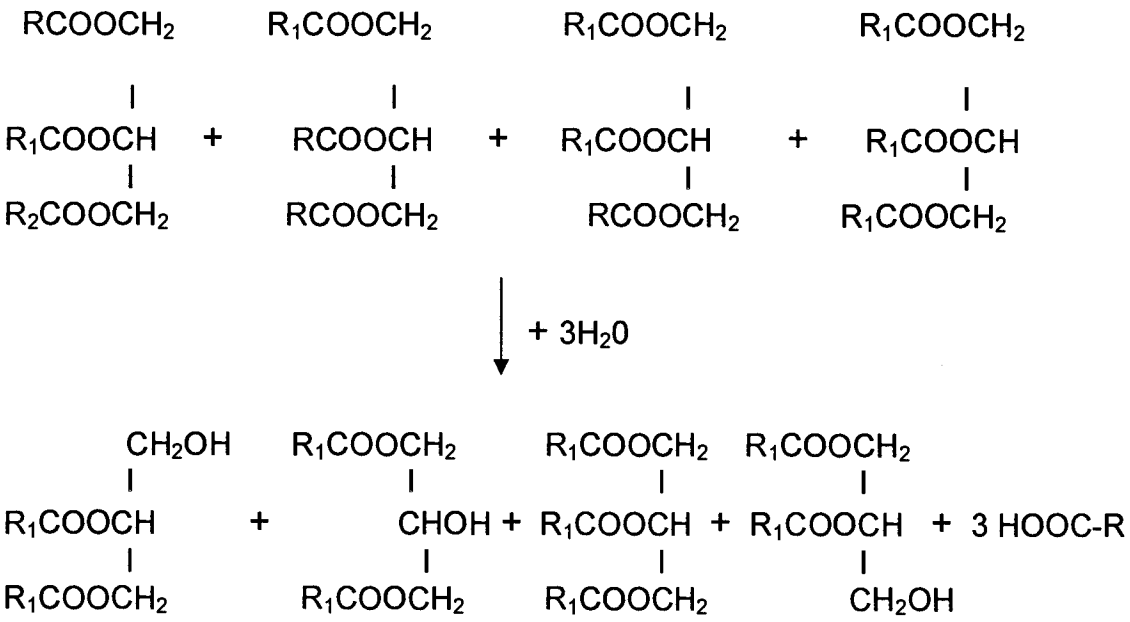


Fig 1.7 Types of lipases and the reactions catalyzed

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 (*Pseudomonas oleovorans*, *Haemophilus influenzae*, *Acetobacter pasteurianus*) as well as from cold-adapted (*Moraxella* sp., *Psychrobacter immobilis*) or heat-adapted (*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 *Arthrobacter globiformis* *Streptomyces chrysomallus* and *Pseudomonas fluorescens*.

1.3.7 Applications of lipases

Lipases possess the unique feature of acting at an interface between the aqueous and nonaqueous (*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 thermophilc 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)

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and di-glycerides
Pharmaceuticals	Transesterification, hydrolysis	Specially lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

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 moacylglycerol 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)

Chapter 2

Screening of Thraustochytrids for alkaline protease production

Microorganisms are the most suitable resources for industrial production of protease. As protease-producing microorganisms are easily cultivated on a large scale, protease yields from them are very high. Different proteases produced by microorganisms have different biochemical and physical characteristics and physiological functions (Kumar and Tagaki, 1999).

Thraustochytrid protists appear to be an untouched bioresource for enzyme production. Screening and isolation of thraustochytrids with high protease activities and optimization of medium and cultivation conditions for alkaline protease production by them thus became a primary objective of the present study.

Proteolytic activity can be detected by either qualitative or quantitative methods. The basic principle underlying both types of methods involves measurement of either the products of protein hydrolysis or of the residual protein itself. The methods available for detection and assay of proteolytic activity vary in their simplicity, rapidity, range of detection and sensitivity. Qualitative assays usually rely on the formation of a clear zone of proteolysis on agar plates, as a result of protease production.

The protein agar plate assay is commonly used for the initial screening of proteolytic activity and depending upon the needs and strategy of screening, different protein substrates are selected. The most commonly used protein substrates for selective screening are skim milk (Rajamani and Hilda, 1987), casein, gelatin, bovine serum albumin (BSA) (Vermelho *et al.*, 1996) and

keratin (Friedrich *et al.*, 1999). The plate assay can also be used to distinguish the type of protease as neutral / acid / alkaline by manipulating the buffer system (Rajamani and Hilda, 1987).

Quantification of protease activity would measure the extent of proteolytic potential of the enzyme. The commonly used methods employ natural or synthetic substrates using techniques such as spectrophotometry, fluorimetry, radiometry or enzyme-linked immunosorbent assay-based (ELISA) assays. Spectrophotometric methods are the most generally preferred of these. A widely 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 such as BSA, casein, hammerstein casein or hemoglobin. These peptide residues are estimated either by absorption at 280 nm (direct estimation method) or by using the conventional Folin's reagent (colorimetric method).

This Chapter discusses the isolation of thraustochytrids from different ecosystems of Goa and the screening of these and other coastal isolates for alkaline protease production.

2.1 Materials and Methods

All chemicals were of analytical grade and glass double distilled water was used throughout. In all experiments, the measurements were carried out with duplicated parallel cultures.

2.1.1 Sample collection

Samples of mangrove leaf detritus and sediments from the Zuari River and the Chorao Island of Goa, India (Fig 2.1) were collected aseptically during the post monsoon season (Oct-Nov, 2004) and immediately transferred to the laboratory (Fig.2.1). The mangrove forests of Goa are distributed along the estuaries of the Mandovi and Zuari rivers. Chorao Island (15°31'N; 73°52'E), which is the major mangrove and spread over 160 ha, is surrounded by the river Mandovi on three sides. It harbours a good mangrove flora comprising about 12 mangrove species.

The city if Panaji (Goa) is flanked by two rivers, the Mandovi and the Zuari which flow into the Arabian Sea.

2.1.2 Isolation of thraustochytrids

With minimum delay after collection, the samples were subjected to repeated wash with sterile distilled water. Mangrove leaf detritus were cut into small pieces, inoculated on Modified Vishniacs (MV) agar plates (0.8% agar) with 1% antibiotics (Streptomycin and Penicillin, to prevent bacterial contamination) by spread plating and spot inoculation methods and incubated at room temperature (30$\pm$2°C). At 24 h intervals the plates were checked for growth of thraustochytrids. On the third day, colonies started appearing, which were isolated and purified by repeated streaking on MV agar plates. Micrographs of the vegetative cells of one of these isolates are shown in Fig 2.2 a, b. Coastal isolates used in this research were very kindly provided by Dr. S. Raghukumar, National Institute of Oceanography, Goa.

Mangroves of Chorao island, Goa

Mangroves along the banks of Zuari, Goa

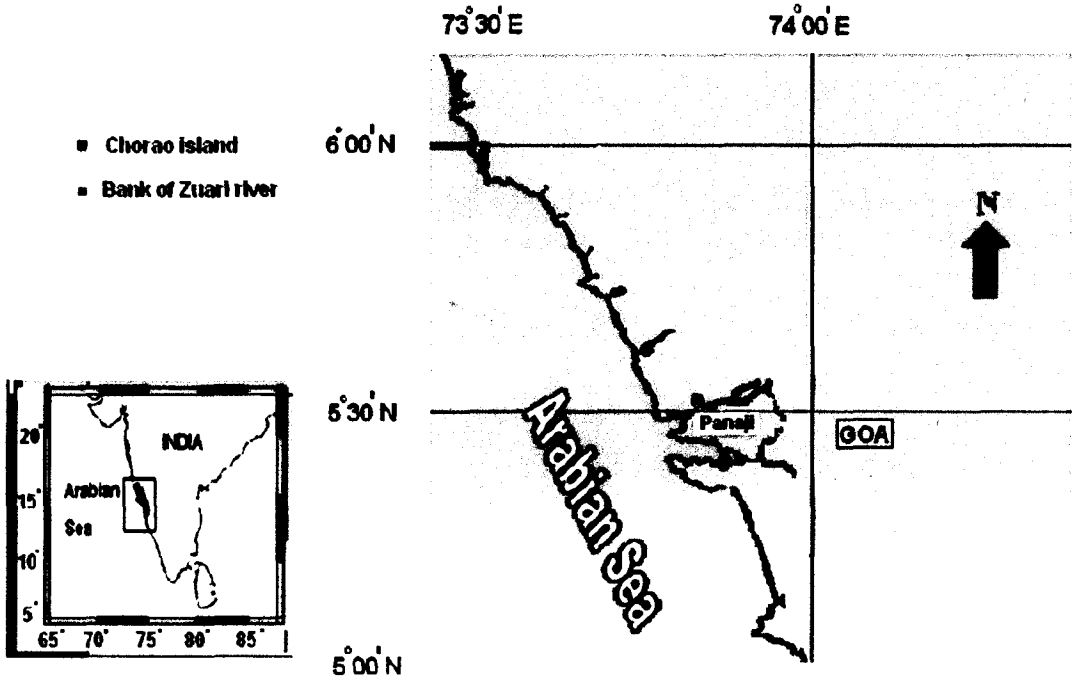


Fig 2.1 Sampling site

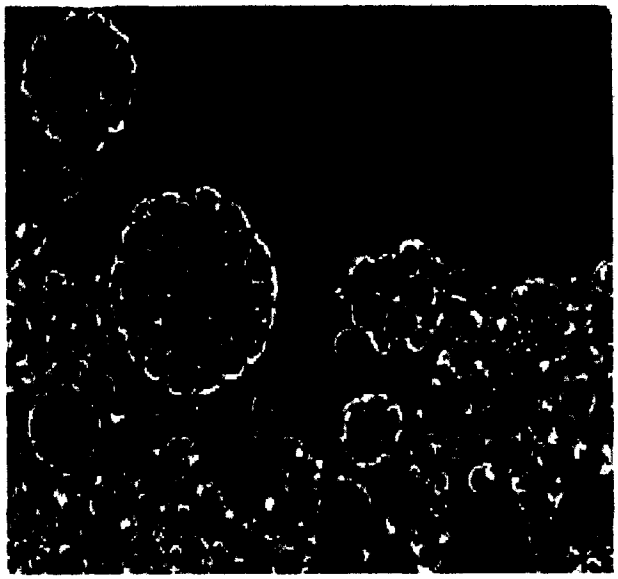


Fig 2 .2a Micrographs of vegetative cells

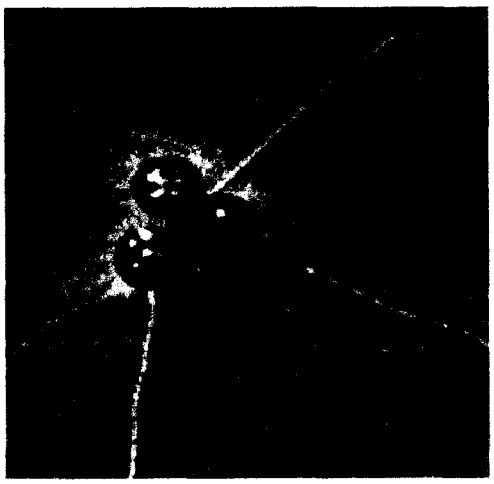


Fig 2.2b Micrographs of vegetative cells with ectoplasmic net elements (Scale bar 20 μm)

2.1.3 Maintenance of thraustochytrids

Purified cultures were maintained on MV agar stabs (0.6% agar) at 4^0C and periodic transfers were done every month. Between the transfers stocks could be maintained successfully at 15-20^0 C on slants or parafilm sealed petri dishes for two months. On dry agar plates, most of the thraustochytrids would not survive more than 1-2 weeks. Pollen-baited sea water flasks could also be used to maintain thraustochytrids.These typically survived several months between transfers.

2.1.4 Qualitative screening for protease production

All the isolates were checked qualitatively for proteolytic activity by spotting on MV agar medium containing 1% skimmed milk powder. The production of protease was confirmed by the appearance of clearance zones on the plates. The ratio of the diameter of the clearance zone to that of the colony was calculated, which served as the index for preliminary selection of protease high-yielding strains.

2.1.5 Preparation of crude culture supernatant

Isolates wherein protease producing property was detected qualitatively were raised in sterile M. V. broth containing 0%, 0.5% or 1.0% skimmed milk powder. This helped in deducing the constitutive or inducible nature of the enzyme production by the thraustochytrids.The flasks were kept on an orbital shaker (120 rpm) for 72 h at room temperature (30 ± 2^o C).

Protease production could be observed by the clearance of the broth which was then centrifuged at 5°C (10,000 rpm, 15 min). The culture filtrate thus obtained was stored in aliquots at -20° C until further use.

2.1.6 Alkaline protease assay

Quantitative estimation of alkaline proteolytic activity was carried out at 37°C for 30 min using appropriate volume of crude enzyme incubated with casein (0.5%) in borate buffer (50mM, pH 9.0) to a final volume of 2 ml.The reaction was stopped by adding 2 ml of 20% TCA and the tubes were kept at 0°C for 15 min. The precipitated protein was pelleted down by centrifugation (10000 rpm, 15 min) and the supernatant assayed for liberated tyrosine by the method of Lowry *et al.* (1951). Appropriate enzyme blanks and substrate blanks were also included in the reaction. One unit of enzyme activity (1U) is defined as that which liberates 1μg of tyrosine per min under the above standardized assay conditions. Protein was measured by the method of Lowry *et al.* (1951) using BSA as the standard.

For purposes of rapid comparision, the results of enzyme activity measurements have been presented in this Chapter as U/ml.

2.2 Results and Discussion

Clearance zones on agar plates were visualized on the third day after inoculation. Plates showing clearance zone on MV agar medium with 1% skimmed milk powder, before and after staining with Coomassie Brilliant Blue, are shown in Fig 2.3a and b. The results of this first round of qualitative screening for proteases in general is summarized in Table 2.1.

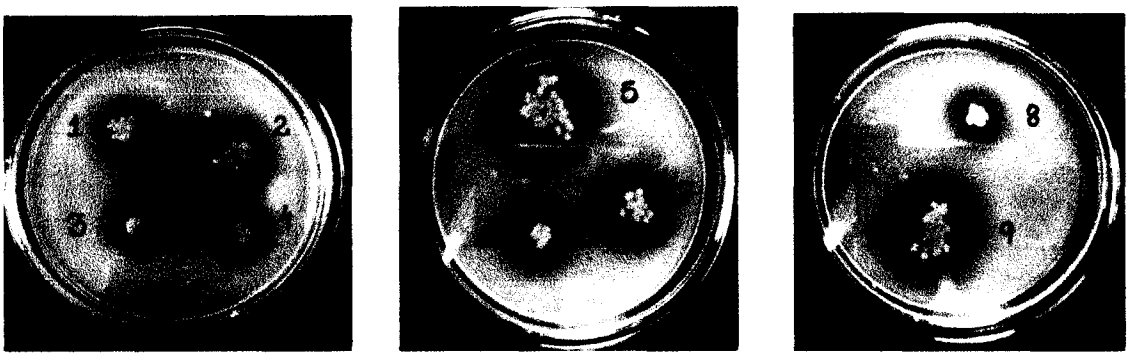


Fig 2.3 a Clearance zone on M V agar medium

Isolated cultures of thraustochytrids spotted on MV agar plate supplemented with 1.0% skimmed milk powder showing clearance zones. (Composition of MV medium is given in the Appendix I).

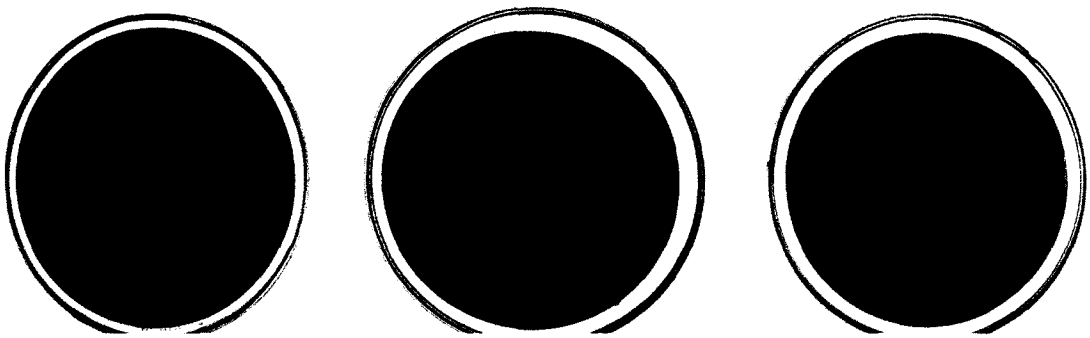


Fig 2.3 b Plate stained with Coomassie Blue

The plate shown in Fig 2.3 a after staining with Coomassie Blue.

1. Isolate TC-1
2. Isolate A6T
3. Isolate AH-2
4. Isolate MH1
5. Isolate TZ
6. Isolate TC-2
7. Isolate HOF1
8. Isolate Mar3
9. Isolate 7091

Table 2.1. Qualitative screening for proteases

Habitat	Isolate	Ratio of clearance zone to colony size
Coastal	HOF1	5.0
	A6T	4.0
	AH-2	2.2
	7091	5.0
	Mar3	3.0
	MH1	2.5
	SL	0
	CW	0
Mangrove Detritus	TC-1	3.0
	TC-2	3.2
	TZ	5.5

All but two of the tested isolates produced proteases (Table2.1).These results are not surprising and are reminiscent of those of Bahnweg (1979) who while investigating 21 isolates of marine thraustochytrids found all of them to be proteolytic. Raghukumar *et al.* (1994) had reported production of cellulases, amylases, xylanases, proteases and pectinases by *Schizochytrium mangrovei* from mangrove leaf detritus. Sharma *et al* (1994) found only proteases while Investigating the enzymatic activities by thraustochytrid *Ulkenia visurgensis* from brown algal detritus,

The importance of the mangrove ecosystem in terms of export of plant detritus and faunal biomass supporting offshore biological production is well documented (Lee and Rhee, 1995). Microbes are responsible for the transformation of the polymeric compounds into dissolved or particulate organic matter utilizable by other consumers in the food web. These microbes include the bacteria, the eumycotic fungi including the ascomycetes, the mitosporic fungi, the chytrids and the chromistan group which were formerly known as the oomycetes, the labyrinthulids and the thraustochytrids. There are numerous reports describing the association of these various groups with decaying plant litter in mangroves and their presence in sediments (Fell and Master, 1973). Comparatively little information is however, available on the enzyme activity of these microbes and therefore their role in the recycling of nutrients remains unclear. Such investigations were essentially hampered by the slow development of methods for the ecological study of the aquatic mycelial eukaryotic decomposers compared with the well established (published and field-tested) methods used for the measurement of prokaryotic productivity

(Newell, 1992). The recently published or refined methods for measuring mycelial mass and productivity have enabled a better understanding of the role of the mycelial decomposers in different marine ecosytems.

Thraustochytrids generally represent a negligible fraction of microbial abundance and a minor fraction of the total benthic microbial mass (Bongiorni and Dini, 2002, Bongiorni *et al.*, 2005). As at present, the only available information on their enzymatic activities deals with a few species from the water column, mangrove leaves, brown algae and faecal pellets and that too for a limited number of enzymes (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 and do produce extracellular enzymes (Raghukumar, 1999). While a large array of assay protocols is available for protease estimation, in view of their reliability, ease of performance and cost effectiveness, the skimmed milk agar assay and the casein-based spectrophotometric assays remain the methods of choice for routine laboratory analysis.

Culture filtrates from 9 of the 11 isolates that showed indications of protease production (Table 2.1) were assayed for alkaline protease activity. From Table 2.2 it can be observed that two strains, namely, TZ and AH-2 showed higher activities and these were therefore selected for subsequent studies. The enzyme production by the isolate A6T, although showing positive results during

qualitative screening, could not be detectable by the quantitative assay. The reason could be due to the fact that the quantitative estimation was done at alkaline pH to specifically screen for the alkaline proteases whereas preliminary qualitative screening was done at near neutral pH. The alkaline protease production by the isolates HOF1, 7091, MH1, TC-1 and TC-2 were inducible with 0.5% or 1.0% skimmed milk powder in the culture medium. The enzyme production by the isolates AH2, Mar 3 and TZ was basically constitutive which was further inducible in the presence of the substrate in the growth medium but for some unknown reason, the isolate Mar 3 could not be stably maintained in axenic culture.

Protease production is an inherent property of all organisms. These enzymes are generally constitutive but at times they are partially inducible (Beg *et al.* 2002; Kalisz, 1988). Proteases are largely produced during the stationary phase and are thus generally regulated by carbon and nitrogen stress. Different methods in submerged fermentations have been used to regulate protease synthesis, 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. As the onset of stationary phase is marked by the transition from vegetative growth to sporulation stage in spore-formers, 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-

Table 2.2 Quantitation of proteolytic activity

Isolate	Enzyme Activity (U/ ml)		
	Skimmed milk powder concentration in medium		
	0%	0.5%	1.0%
HOF1	N.D.	0.74	13.72
A6T	N.D.	N.D.	N.D.
AH2	**2.48**	**12.37**	**10.25**
7091	N.D.	5.44	3.04
Mar3	5.49	9.22	3.21
MH1	N.D.	0.92	0.99
TC-1	N.D.	10.80	12.20
TC-2	N.D.	11.10	13.10
TZ	**8.50**	**22.80**	**22.20**

N.D. = Not detectable

occurring, are not related, as spore-deficient strains of *B. licheniformis* were not found to be protease-deficient (Fleming *et al.* 1995).

Extracellular protease production is a manifestation of nutrient limitation at the onset of stationary phase. Nevertheless, the final protease yield during this phase is also determined by the biomass produced during exponential phase. Medium manipulation is therefore needed to maximize growth and hence protease yields.

The results presented in this Chapter showed that thraustochytrids are a promising alternative source of protease.

Chapter 3

Optimization of growth conditions favoring maximum protease production by selected isolates

The effect of environmental conditions on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of the enzyme by specific compounds. Appropriate cultivation conditions are essential to the successful production of an enzyme and optimization of parameters such as pH, temperature and medium composition is important in developing the cultivation process. Alkaline proteases are generally produced by submerged fermentation. In addition, solid state fermentation techniques have been exploited, albeit to a lesser extent, for the production of these enzymes (Mitra and Chakraborty, 2005; Malathi and Chakraborty, 1991). In commercial practice, the optimization of the medium composition is carried out to maintain a fine balance between the various medium components, thus minimizing the amount of un-utilized components at the end of fermentation. Research efforts have been directed mainly towards evaluating the carbon and nitrogen nutrients, cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation.

In general, no defined medium has been established for the optimum production of alkaline protease from various microbial sources. Each organism or strain has its own special conditions for maximum enzyme production. In our preliminary studies on the development of the production medium, glucose and peptone were found to be important factors in enhancing the alkaline protease production.

From the results presented in the foregoing Chapter on screening and selection of the most proteolytically active thraustochytrids, two isolates TZ and AH-2 have been ear-marked as potent alkaline protease producers. These isolates as identified up to the genus level by Dr.S.Raghukumar, NIO, Goa were found to belong to the genus *Thraustochytrium.* Optimization of growth conditions of the isolates with respect to physico-chemical parameters such as harvesting time, pH, shake *vs* static conditions, temperature as well as salt concentration and general composition of the culture medium is reported in this Chapter.

3.1 Materials and Methods

All chemicals used were of analytical grade and glass double distilled water was used for all preparations. In all experiments, the measurements were carried out with duplicated parallel cultures. Each data point plotted is a representation of mean ± S.D. of values analyzed in replicate from two independent experiments.

3.1.1 Harvesting Time

MV Broth (20ml) with 0.5% milk was inoculated with the isolates TZ or AH-2, kept on a rotary shaker at room temperature and the enzyme production was monitored every 24 h up to 96 h.

3.1.2 Growth pH

Flasks containing 20ml of sterile MV Broth with 0.5% milk and at pH (3-11) pre-adjusted using 0.1M HCl / 0.1M NaOH, were inoculated, kept on a shaker at room temperature and the enzyme production was determined after 72 h.

3.1.3 Effect of temperature

MV Broth with 0.5% milk was inoculated with isolates TZ or AH-2, kept under the above optimized conditions but at different temperatures (on a temperature controlled orbital shaker) and the enzyme production was determined.

3.1.4 Effect of crude salt concentration

MV Broth containing 0.5% milk and various concentrations of crude sea salt was used as growth medium to study the enzyme production.

3.1.5 Effect of chemical parameters

Media of different chemical compositions were used for the enzyme production under the above optimum conditions:

1. MV Broth + 0.5% milk
2. Crude sea salt (3.4%) + yeast extract (0.1%) + 0.5% milk
3. Crude sea salt (3.4%) + glucose (0.4%) + 0.5% milk
4. Crude sea salt (3.4%) + peptone (0.15%) + 0.5% milk
5. ASW + glucose (0.4%) + peptone(0.15%) + yeast extract (0.1%) + 0.5%milk
6. ASW + 0.5% milk

Unless otherwise mentioned, concentrations of specific medium components were the same as routinely used in MV medium (*vide* Appendix I). Composition of ASW (artificial sea water) is also given in Appendix I.

3.2 Results and Discussion

The various physical and chemical parameters of the production medium studied for maximum protease production were harvesting time, pH, agitation *vs* static conditions, temperature and concentration of crude salt besides composition of the medium.

3.2.1 Time course of enzyme production

Protease production was studied for different time periods at room temperature (30 ± 2°C) in MV medium supplemented with 0.5% skimmed milk powder. The results showed that the protease production by both the isolates AH-2 and TZ was initiated after 24 h, increased consistently with time and reached a maximum after 72 h (Fig 3.1). In a separate experiment (data not shown) protease production was found to peak during the stationary phase of growth. So for all further experiments, time period of 72 h was chosen for maximum enzyme production by both isolates.

Similar results were obtained by Dutta and Banerjee (2006) while studying the physical parameters affecting protease production by *Pseudomonas* sp. RAJR 044. The severe drop in activity after three days in culture was attributed to exhaustion of nutrients. Maximum protease production by *Bacillus* sp. K-30 was recorded after 96 h of incubation at 50°C (Naidu and Devi, 2005) and by the

fungus *Mucor circinelloides* at the end of 96 h of incubation (Andrade et al., 2002). In contrast, protease activity of a yeast strain, *Aureobasidium pullulans*, reached maximum within 30 h of the fermentation when the cell growth reached mid log phase (Chi *et al.*, 2007).

3.2.2 Growth pH

An important characteristic of most microorganisms has been noted to be their strong dependence on the extracellular pH for cell growth and enzyme production (Kumar and Tagaki, 1999). The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Majority of the thermophilic bacilli are found to grow at pH and temperature ranges of 5.8-8.0 and 50-65°C, respectively (Zeikus, 1979).

In the present work, the production medium was adjusted to varying pH ranging from 3.5-10 and the effect on protease production is presented in Fig.3.2. Although maximum enzyme production by the thraustochytrids was found to occur during growth at pH 7.0 for both isolates, the activity of the enzyme from isolate TZ was approximately 68% more than that from isolate AH-2. For all further experiments, a pH of 7.0 was maintained for the production medium.

Alkaline protease production by *Bacillus* spp. is found to be maximum at pH 9-13 (Borris, 1987). Chi *et al.* (2007) have reported that the yeast strain *Aureobasidium pullulans* produced the highest yields of alkaline protease when the initial pH of the production medium was 7.0. Protease production by

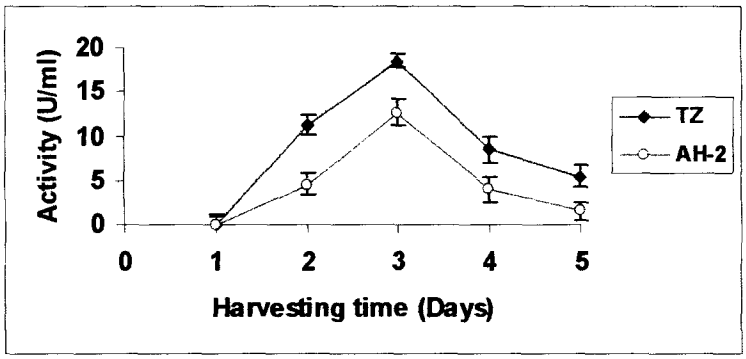


Fig.3.1 Time course of alkaline protease production

The isolates were grown in MV medium supplemented with 0.5% skimmed milk powder for 5 days and the protease activity was determined at regular intervals of 24 h.

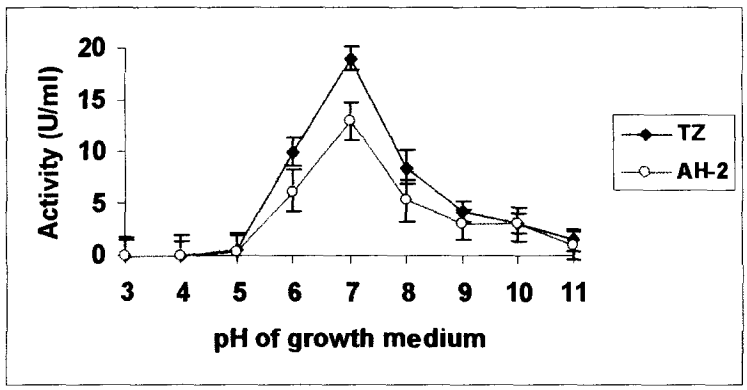


Fig. 3.2 Effect of pH on the production of alkaline proteases

Production conditions - MV medium containing skimmed milk powder (0.5%) varying pH ranging from 3.5-10, after 72 h under agitation of 120 rpm at 30 $\pm$ 2^{0}C.

Pseudomonas sp. RAJR 044 was maximum at pH 6.0 (Dutta and Banerjee, 2006). Aunstrup (1980) had reported that in general, for increased protease yields from alkalophilic microorganisms, the pH of the medium must be maintained above 7.5 throughout the fermentation period. As the metabolic activities of the microorganisms were very sensitive to pH change, protease production was affected if pH level was higher or lower than the optimum value (Tunga *et al.*, 1998; Ali and Roushdy, 1998).

3.2.3 Agitation

Alkaline proteases are generally produced by submerged fermentation. During the fermentation, different levels of dissolved oxygen in the fermentation broth can be obtained by variations in the agitation speed. This can influence greatly cell growth of the microbes and thereby production of extracellular enzymes (Chi and Zhao, 2003).

When protease production was studied under static and shake flask conditions (120 rpm) for 72 h at room temperature, the enzyme production by both the *Thraustochytrium* sp was found to be significantly high under agitation in relation to static conditions of culture (Fig.3.3). Chi *et al.* (2007) had also observed that an agitation speed 150 rpm was most suitable for protease production by the yeast strain *Aureobasidium pullulans* and by *Virgibacillus pantothenticus* (Gupta *et al.*, 2007). For all further experiments, isolates were grown under agitation for the production of protease.

3.2.4 Temperature

When grown in medium at various temperatures, it was observed that maximum production of alkaline protease occurred at 30 ± 2°C (Fig.3.4.) for both the thraustochytrid isolates. The isolate TZ showed 76 % more activity than AH-2. Both the isolates could not grow at temperatures beyond 45°C and hence there was no enzyme production either at these temperatures. For all further experiments, a temperature of 30 ± 2°C was maintained for the production medium.

Temperature is understandably one of the most critical parameters that has to be controlled in bioprocess (Chi and Zhao, 2003). Their results on temperature effects have revealed that the specific protease activity of the yeast strain *Aureobasidium pullulans* reached the highest when grown at 24.5°C (Chi *et al.*, 2007). Generally, 30°C has been the optimum temperature reported for maximum secretion of extracellular protease. Higher temperature was found to have adverse effect on the metabolic activities of the microorganism (Tunga *et al.*, 1998; Singh and Vyas, 1975).

3.2.5 Effect of crude salt concentration

When MV Broth containing 0.5% milk and various crude salt concentrations was used as the growth medium, both the isolates AH-2 and TZ were found to be able to grow over a wide range of salt concentrations (1-10%, w/v).A concentration of 3.4% crude salt resulted in maximum enzyme production although concentrations as high as 6% also elicited as much as 89 and 53 % of

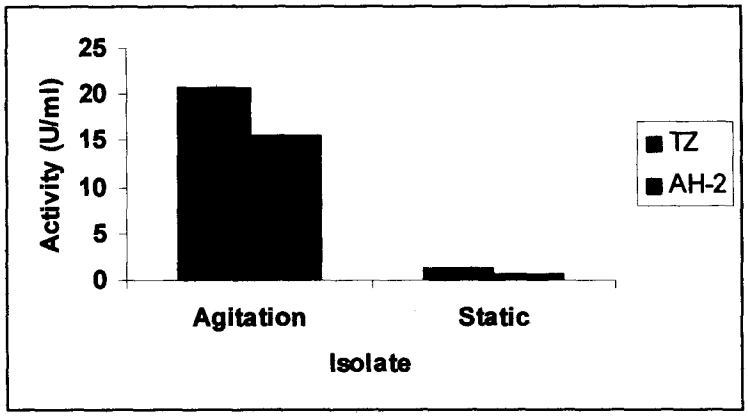


Fig.3.3 Effect of static or agitation conditions

Production conditions - MV medium containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2^0C for 72 h under shake or static culture.

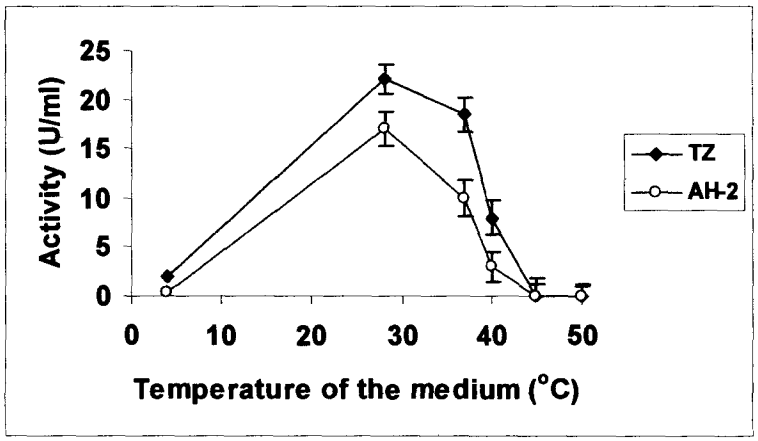


Fig. 3.4 Effect of temperature on alkaline protease production

Production conditions - MV medium containing skimmed milk powder (0.5%) pH 7.0, at different temperatures for 72 hours under agitation.

the maximal activity for the isolates TZ and AH-2, respectively (Fig.3.5). These results are in tune with the halotolerant nature of these thraustochytrids.

Studies by Mital *et al.* (2006) on the salt requirement by a group of 8 moderately halophilic and alkaliphilic bacteria for optimum enzyme secretion revealed that the enzyme production varied significantly among the isolates. The salt dependency was however; relatively lower when compared to that of extremophilic isolates such as the haloalkaliphilic Archaean ones from Soda Lake where maximum growth was at 10% salt (w/v) while for maximum protease production 15% salt was required (Sorokin *et al.*, 2003).

Similar results have also been found for the moderately halophilic and alkaliphilic coccus *Salinicoccus alkaliphilus* sp. isolated form Baer Soda Lake in Mongolia, which could grow over a range of 0-25% (w/v) NaCl with an optimum at 10% (w/v) for best growth and enzyme secretion (Zhang *et al.*, 2003).

3.2.6 Effect of chemical parameters

Investigations on protease production by many fungal cultures have shown that there were drastic variations with the composition of the medium used. For instance,the alkaline protease production by *Mucor circinelloides* was shown to be predominantly influenced by glucose and peptone concentrations (Andrade *et al.*, 2002).

When medium of different compositions was used to study the enzyme production under the above derived optimum conditions, it was seen that MV broth with 0.5% milk was the best for the alkaline protease production by both the *Thraustochytrium* spp.(Fig.3.6). Omission of any one of the ingredients resulted in poor enzyme production. Although ASW supported good growth (data not shown) it failed to elicit production of alkaline protease.

It was observed that the lack of glucose resulted in a dramatic decrease in enzyme production by both the *Thraustochytrium* spp. Glucose has been reported to suppress protease production in *Bacillus licheniformis* (Sen and Satyanarayana, 1993; Sonnleitner, 1983) but in the present study it was found to be a relatively good substrate component for enzyme production, especially when used at low concentrations (0.4%). Other workers have also reported better protease production in the presence of glucose as a substrate (Gajju, 1996).

The protease production by *Yersinia ruckeri* was influenced by the composition of the culture medium; protease production were optimum in peptone medium whereas activity was not detected when the microorganism was grown in the presence of Casamino Acids, suggesting that intact peptides are necessary for the induction process (Secades and Guijarro, 1999). A similar behavior has been observed for *Aeromonas salmonicida* (Dalhe, 1971), *Aeromonas liquefaciens* (Braun and Schmitz, 1980), *Vibrio* species (Dreisbach and Merkel, 1978) and *E. chrysanthemi* (O'Reilly and Day, 1983).

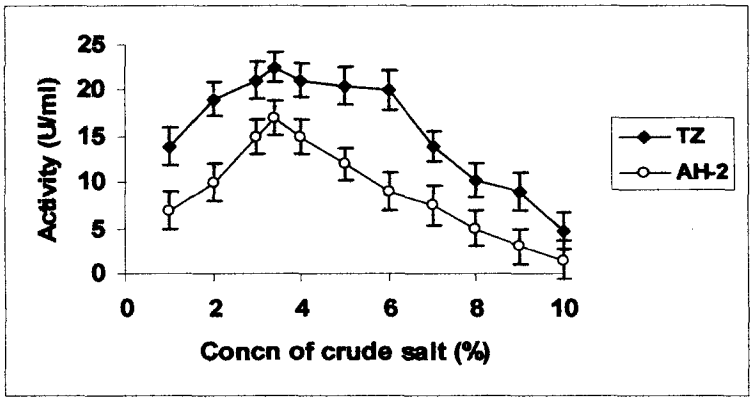


Fig. 3.5 Effect of crude salt concentration

Production conditions - MV medium at various crude salt concentrations (1-10%) and containing skimmed milk powder (0.5%) ,pH 7.0, 30 ± 2°C and agitation under 72 h.

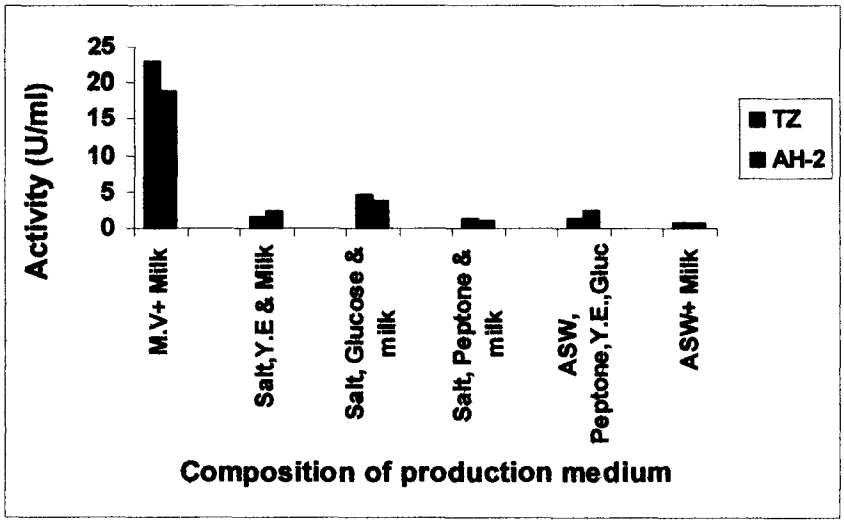


Fig. 3.6 Effect of medium composition

Production conditions – Medium containing various combinations of skimmed milk powder (0.5%) with different media components maintained at pH 7.0, 30 ± 2°C for 72 h under agitation.

The effect on protease production by our isolates of varying concentrations of glucose, peptone and yeast extract (present in MV medium) has been studied. The enzyme production was increased from 8.5 to 30 and 3.6 to 20.5 U/ml respectively, for the isolates TZ and AH-2, with an increase in glucose concentration from 0.2 to 0.4 % (w/v). Beyond 0.6 %, however, the enzyme production was highly suppressed (Fig.3.7a) suggesting a threshold level of glucose for optimum protease production. A similar relationship was reported in case of an alkaline protease from *Yersinia ruckeri* (Secedes and Guijarro, 1999) and alkaliphilic *Bacillus* sp. (Patel, 2006) where glucose repressed protease beyond the optimum level. A catabolic repression mechanism for extracellular enzyme production has been described for *V. alginolyticus* , *Pseudomonas maltophilia* and *Staphylococcus aureus* , suggesting that in the absence of the sugar (i.e., glucose) the protease plays a role in supplying peptides or amino acids as the carbon or energy source in addition to providing the nitrogen source.

Protease synthesis may hence be repressed when the energy status of the cells is high. This kind of regulatory mechanism has been postulated for proteases of other pathogens such as *P. aeruginosa* and *Vibrio* strain SA1 (Secedes and Guijarro, 1999).

Peptone and yeast extract enhanced the enzyme production when included in medium. The optimum peptone and yeast extract concentrations for maximum protease production were found to be 0.1 to 0.15 % for both the *Thraustochytrium* spp. Further increase beyond the optimum level did not

cause much change in enzyme yield from isolate TZ but resulted in decrease in the enzyme production from isolate AH-2 (Fig 3.7 b & c). A combination of carbon (glucose) and nitrogen sources (peptone and yeast extract) thus resulted in a maximum enzyme production by both *Thraustochytrium* spp. Patil *et al.*(2006) have reported similar requirement of peptone (0.5%), yeast extract (0.5%) and glucose (1.0%) for maximum alkaline protease production by *Bacillus* sp..

In summary, the optimized culture conditions for maximum production of alkaline protease by both the isolates AH-2 and TZ were a temperature of 30 $\pm$ 2^{0} C and pH 7.0 in the presence of 0.5% skimmed milk as an inducer, when grown for 72 h under agitation. Peptone (0.15%), yeast extract (0.1%) and glucose (0.4%) elicited best alkaline protease production in both the isolates.

Although the requirements of culture conditions were similar, the isolate TZ exhibited more enzymatic activity than isolate AH-2. It is noteworthy to mention that a simple and cost-effective medium has been used for the alkaline protease production by both *Thraustochytrium* spp.

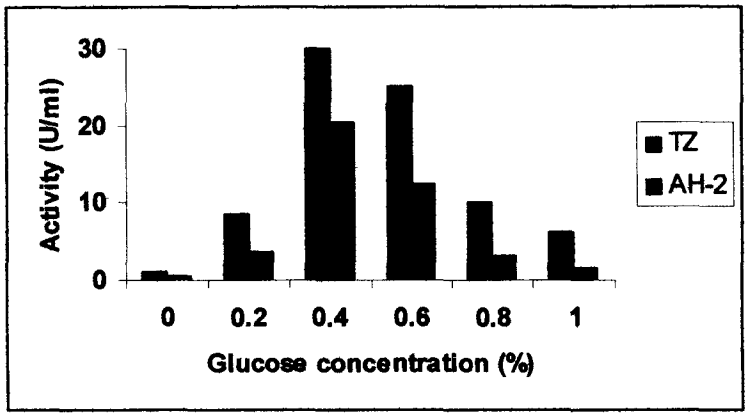


Fig. 3.7a Effect of glucose concentration

Production conditions - MV medium at various glucose concentrations containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2^{0}C for 72 h under agitation.

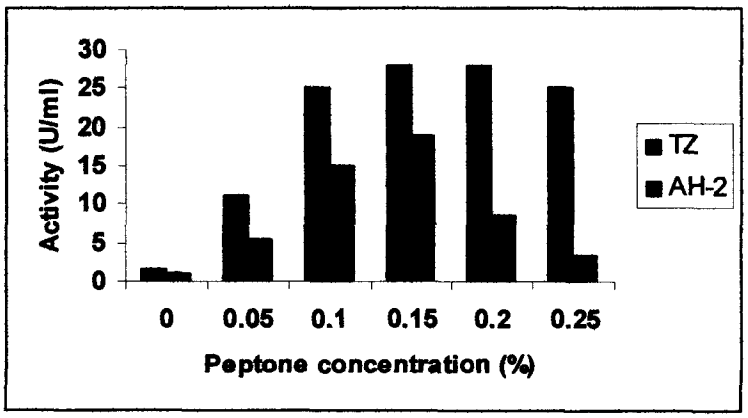


Fig. 3.7b Effect of peptone concentration

Production conditions - MV medium (at glucose 0.4%) and various peptone concentrations, containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2 ^{0}C for 72 h under agitation.

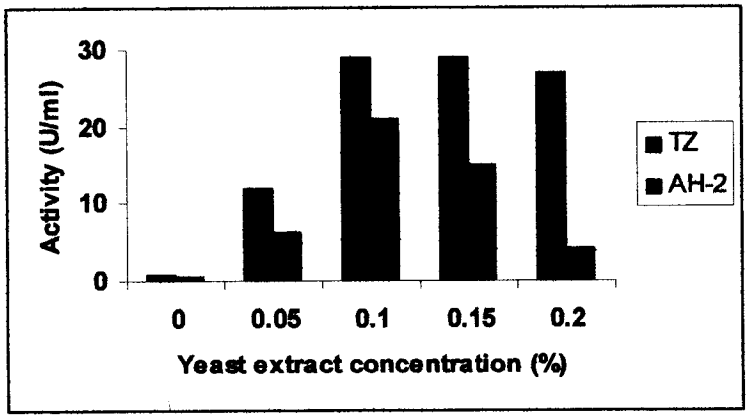


Fig. 3.7c Effect of yeast extract concentration

Production conditions - MV medium glucose 0.4 % & peptone 0.15 %) at various yeast extract concentrations and containing skimmed milk powder (0.5%), pH 7.0, 30 ± 2^{0}C for 72 h under agitation.

Chapter 4

Characterization of alkaline proteases from two isolates

Alkaline proteases from several microorganisms have been studied extensively and based on their properties, used in various industries. 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 such as V_{max}, K_m, K_{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.

In this Chapter, properties of the alkaline proteases such as optimum assay conditions, basic enzyme kinetics and biotechnologically significant characteristics (primarily pH and temperature stability) of proteolytic activity from the two isolates AH-2 and TZ are detailed.

4.1 Materials and Methods

Crude enzyme extracts from the two isolates TZ and AH-2 were obtained as described in Chapter 2. The assays were carried out in replicates and each data set is a representative of two to three independent experiments, all the results being presented as mean ± S.D.

4.1.1 Effect of enzyme concentration

Different amounts of enzyme protein were assayed in borate buffer in a final volume of 2 ml under the standard conditions as described in Section 2.1.6.

4.1.2 Time course of assay

The enzyme preparation was assayed for time periods in the range of 5-90 min from which a standard reaction time would be identified for routine assays.

4.1.3 Effect of pH on enzyme activity and stability

The protease assay was carried out in various buffers : acetate buffer (50 mM, pH 3.5 - 5.0), phosphate buffer (100 mM, pH 6.0 -8.0) or borate buffer (12.5 mM, pH 8.0 -10.0), for a reaction time of 15 or 30 min for the enzymes from isolates AH-2 or TZ, respectively.

The pH stability of the enzyme was analyzed by incubating the enzyme preparation with an equal volume of the respective buffer (pH 3.5-10) for 1 h at room temperature (30 ± 2°C). The residual activity was then measured as per the routine procedure under the standardized reaction conditions.

4.1.4 Effect of temp on enzyme activity and stability

The experiment was carried out by incubating ideal amounts of the crude enzyme protein at different temperatures (28-90°C) for 30 min with casein (0.5%) and borate buffer (12.5 mM, pH 8.0 or 9.0 for the enzymes from isolates TZ or AH-2, respectively) and assaying the proteolytic activity. Thermal stability was analyzed after subjecting the culture filtrate to heat treatment for 10 min at different temperatures from 40°C to 90°C. The precipitated protein was then centrifuged down and the residual activity of the supernatant assayed under the standard conditions. Alternatively, the crude enzyme was subjected to heat

treatment for different time periods at a selected temperature prior to assaying as above.

4.1.5 Substrate concentration studies

The enzyme extract was incubated with different concentrations of casein during the assay at 50°C and pH 8.0 or 9.0, for the enzymes from isolates TZ or AH-2, respectively, and under the above mentioned standard conditions. The kinetics of the reaction were studied by subjecting the data to Michaeleis-Menten analysis.

4.1.6 Effect of EDTA and metal ions

The enzyme from the isolate TZ was pre-incubated with varying concentrations of EDTA or metal ions at a final concentration of 1.0 and 10 mM for 30 min at 30 $\pm$ 2°C and the residual activity was calculated.

4.1.7 Substrate specificity

The crude enzyme preparation from the isolate TZ was assayed at 37°C under the standard conditions but different substrates such as gelatin, egg albumin, hemoglobin, casein and BSA at a concentration of 0.5%.

For studies using a probable natural substrate, viz., the horny protein (conchiolin) of the calcareous shells, shell fragment of marine invertebrates (which were collected from sampling site vicinity) were processed through sterilization and homogenization as described by Raghukumar and Raghukumar (1998) and used. The isolate TZ was grown in MV medium containing these shell proteins as substrate. After 72 h of growth, the crude

culture filtrate was extracted as described in Chapter 2 and the protease activity was determined using casein as substrate.

4.2 Results and Discussion

The following properties of the proteases from the selected *Thraustochytrium* spp. (isolates AH-21 and TZ) were studied in order to characterize them and to gauge their potential for various industrial applications.

When assayed at varying concentrations of enzyme protein under the standard conditions as described above, the enzyme activity was found to be linear up to at least 100 μg protein for the enzyme from the isolate AH-2 and 60 μg protein for that from isolate TZ (data not shown). All further assays were carried out using protein concentrations in this range. These results served to confirm the enzymatic nature of the reaction besides identifying the linear range of enzyme-protein to be used for the assay.

Since the activity tended to plateau off beyond 30 min of assay time (data not shown) for all further experiments assay times of 15 and 30 min respectively, were chosen for the enzymes from isolates AH-2 and TZ.

4.2.1 Effect of pH on enzyme activity and stability

The proteolytic activity from the thraustochytrids TZ and AH-2 was assayed at pH values ranging from 3.5-10 as described earlier and the results are presented in Fig 4.1 a. The pH optima were 8.0 and 9.0, respectively, for the enzyme activities from the isolates TZ and AH-2 although not much of the activity was lost even at higher pH.

Most of the commercially available subtilisin-type proteases are active in the pH and temperature ranges between 8-12 and 50-70°C, respectively. Sookkheo *et al.* (2000) had reported the production of three proteases, S, N and B from the thermophilic *Bacillus stearothermophilus* TLS33, which had pH optima of 8.5, 7.5 and 7.0, respectively. The protease S was active over a very broad pH range and about 60% activity was detectable at pH 6 and 10 in the presence of 5 mM $CaCl_2$. In contrast, proteases N and B retained relatively little activity above pH 9.0. A novel extracellular serine protease designated Pernisine purified from the archaeon *Aeropyrum pernix* K1 was active over a broad range of pH (5.0-12.0) with maximal activity between pH 8.0 and 9.0 (Catara, 2003).

Stability experiments showed that both the enzymes are highly stable at alkaline pH range, though they retain 50-55% activity at pH 7.0 also (Fig 4.1.b). The S5 protease from halophilic and alkaliphilic bacteria was highly stable when exposed to pH 9.5 for 30 min at 37°C, but at pH 7.5, the enzyme was quite unstable, the stability gradually decreasing with lowering of the pH towards 7, indicating the alkaline nature of this protease (Mital *et al.*, 2006). Similar results were also reported for *Bacillus* sp. in which enzyme was highly active and stable at pH 9-10 (Johnvesly and Naik, 2001). Likewise, the proteases from the *Thraustochytrium* spp. in the present study retained 85 and 70 % activity after incubation of the enzyme from the isolate TZ and AH-2 respectively, at room temperature for 1h at pH 9-10. At pH 6.5 only 28 or 40% of the maximum enzyme activity was obtained, which

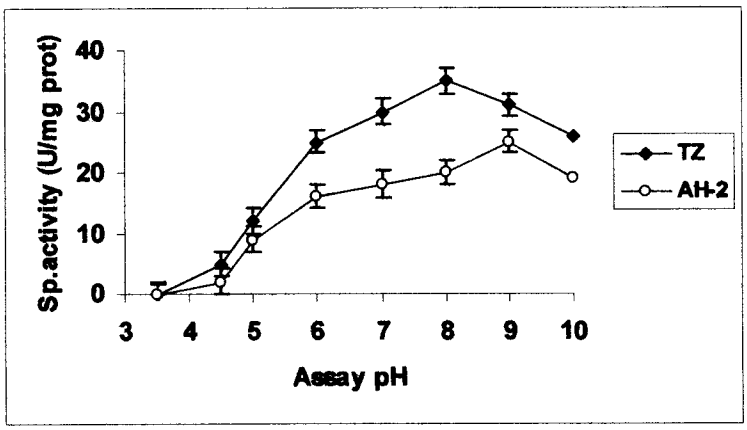


Fig. 4.1 a Effect of pH on enzyme activity

Protease assay carried out at 50°C and 15 min for isolate AH-2 and 30 min for isolate TZ at varying pH.

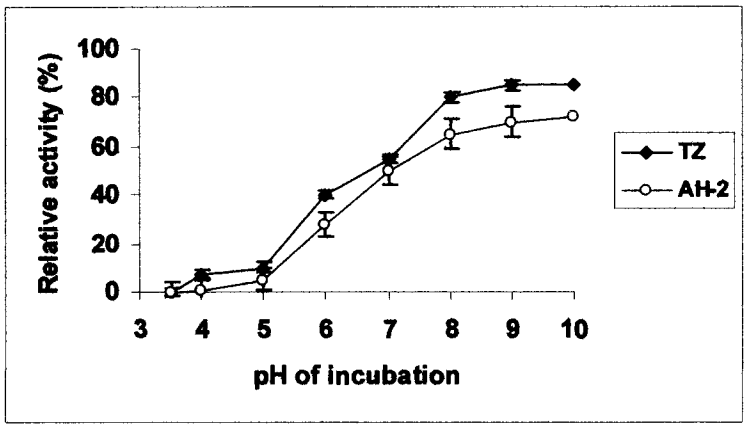


Fig 4.1 b pH stability

The crude enzyme extracts from the isolates were incubated in buffers of pH 3.5-11 for 1h at room temperature and the residual activity was assayed. The activity of untreated enzyme is taken as 100% as routine.

increased to 50 or 55 % at pH 7.0 for the enzyme from the isolate AH-2 or TZ respectively. All of these above results reconfirm the alkaline nature of the proteases under study and perhaps reflects the alkaliphilic nature of these enzymes.

4.2.2 Effect of temperature

Generally alkaline proteases from various fungi have optimum activity at temperatures of 40°-50°C while those from bacteria have been reported to have higher temperature optima as high as 85°C (Litchfield, 1998). Increase in temperature above the optimum would affect important factors as protein ionization state and solubility of species in solution besides leading to protein denaturation (Zeffren and Hall, 1973) and thereby reducing enzyme activity.

When assayed at various temperatures, the enzymes from both our isolates TZ and AH-2 were found to be maximally active at 50°C (Fig 4.2a). Thermal stability studies (Fig 4.2 b) showed that the protease activity was maximally stable for at least 10 min at 50°C for the enzyme from the isolate TZ and at 45°C for the enzyme from the isolate AH-2. When heat treatment at these respective temperatures was given for different time periods ranging from 10-60 min, it was observed that that the enzymes from the isolates TZ and AH-2 could retain 85% and 55% activity respectively, following a 20 min heat treatment (Fig 4.2 c). Comparison of the data presented in Figs 4.2a and b affirms that the loss of activity at higher temperatures is due to enzyme protein degradation.

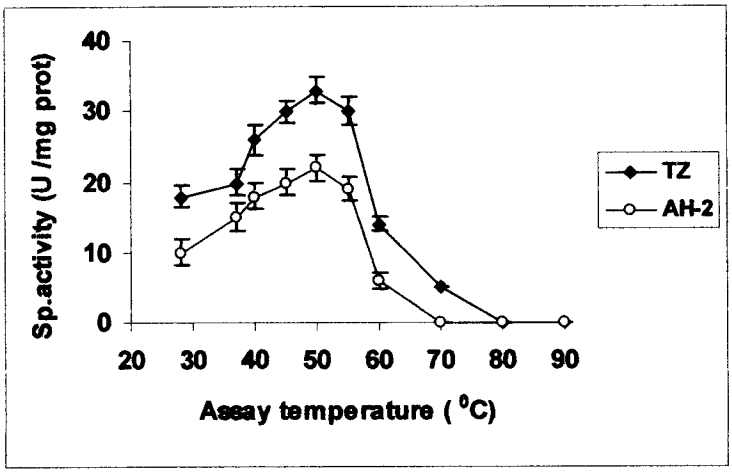


Fig. 4.2a Effect of temperature on enzyme activity

Protease assay carried out at pH 9.0 for 15 min for isolate AH-2 and 30 min for isolate TZ at varying temperature.

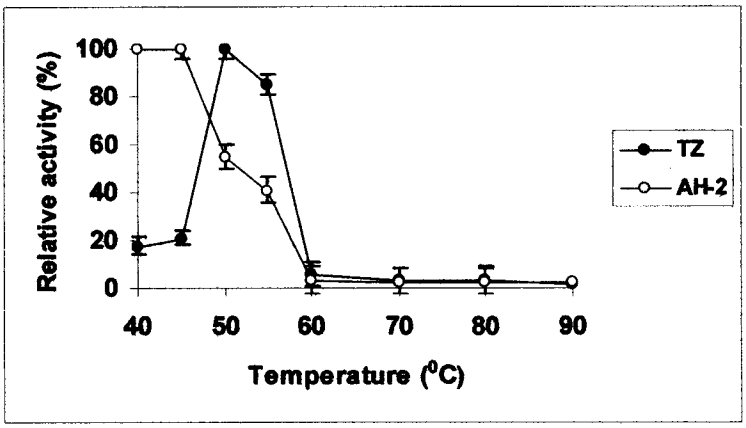


Fig. 4.2 b Thermal stability

Residual activity of enzymes from the isolates AH-2 and TZ after heat treatment for 10 min at 45°C and 50°C respectively. The activity of untreated enzyme was taken as 100%.

The optimum temperature of the protease from *Bacillus* sp strain SMIA-2 was 50°C and the enzyme was stable at 30°C for 2h while at 40°C and 80°C, 14% and 84% of the original activities, respectively, were lost (Banerjee, 1999).

As reported by Sampath *et al.* (1997) the extracellular proteases from *Streptomyces* spp G157 were stable at 40 ^{0}C but were inactivated at higher temperatures (aproximately 60% activity remained after 120 min at 50 ^{0}C or after 30 min at 80 ^{0}C).

In general, all detergent compatible enzymes are alkaline thermostable with a high pH optimum. These characteristics are important because the pH of laundry detergents is generally in the range of 8–12 and laundry temperatures may be expected to go as high as 50-60°C (Takami *et al.* 1990; Manachini and Fortina, 1988). Well-established commercial detergent proteases, namely Subtilisin Carlsberg and subtilisin BPN have a half-life of 3.4 min and 2.4 min at 50°C respectively (Durham *et al.*, 1987). The thermotolerance of the Thraustochytrium proteases discussed in this Chapter complemented by their alkaline pH optima appear to be a favorable property for possible commercial applications.

While enzyme preparations are conventionally stored at low temperature to avoid denaturation, it could be advantageous for commercial applications if they could withstand room temperatures even for short periods. Considering the practical problems during storage and transport, the ability of the crude enzyme preparation to withstand storage at ambient temperature was therefore

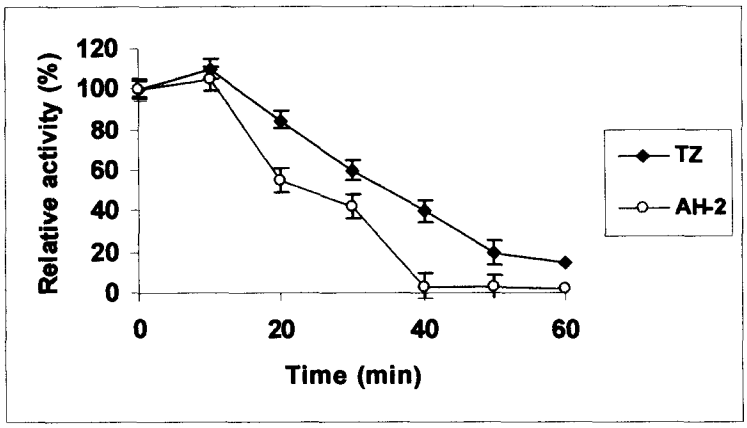


Fig. 4.2 c Time course of thermal stability

Residual activity of enzymes from the isolates AH-2 and TZ after heat treatment at 45°C and 50°C respectively for various time period. The activity of untreated enzyme is taken as 100%

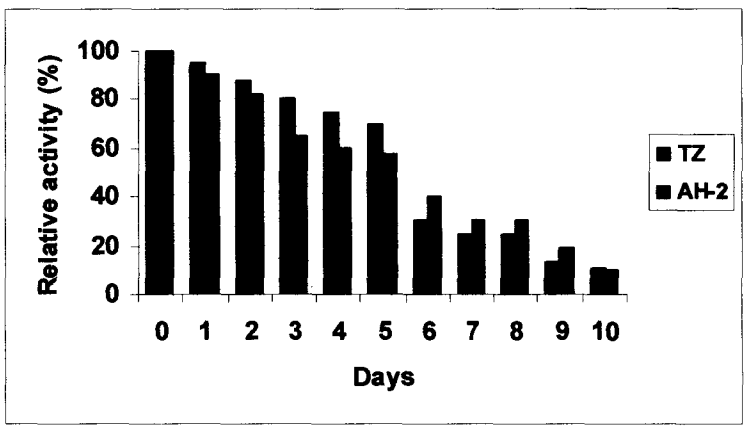


Fig.4.2 d Stability at room temperature

Protease assay carried out at 50°C (15 min or 30 min and pH 9.0 or 8.0 for the isolates TZ and AH-2 respectively), after storage at room temperature. The activity of fresh enzyme extract was taken as 100%.

studied by assaying the enzyme after being stored for 1-10 days at room temperature (30 $\pm$ 2°C). The results (Fig.4.2 d) indicate that the enzyme from the isolate TZ could be stored at room temperature for 5 days with retention of 70% activity while the preparation from the isolate AH-2 withstood storage at room temperature for 4 days with 50% residual activity.

4.2.3 Substrate concentration studies

Various complex (casein, azocasein etc.) and synthetic (para-nitroanilides esters) substrates are used for determining kinetic parameters of alkaline proteases. The synthetic substrates are much more popular than complex substrates for defining K_m and V_{max} as they are convenient (Gupta *et al.*, 2002).

From the analysis of the kinetics of the enzymes from the isolates AH-2 and TZ, it appears that both the enzyme preparations followed Michaelis-Menten kinetics. With casein as substrate, the proteases from isolates TZ and AH-2 had K_m values of 0.5mg/ml and 0.83mg/ml and could attain maximum velocities of 0.04 and 0.025 U/mg protein, respectively (Fig 4.3 a,b).

Using casein as substrate, K_m of the protease from *Streptomyces nogalator* was found to be 50 mg/ml suggesting a low affinity of the enzyme towards casein (Mitra and Chakrabartty, 2005). Ma *et al.* (2007) had reported apparent K_m for casein and V_{max} values of the enzyme from the marine yeast *Aureobasidium Pullulans* as 0.25 mg/ml and 0.0286 µmol/min/mg protein, respectively. Our results revealed that the alkaline protease from the thraustochytrids displayed a high affinity for casein.

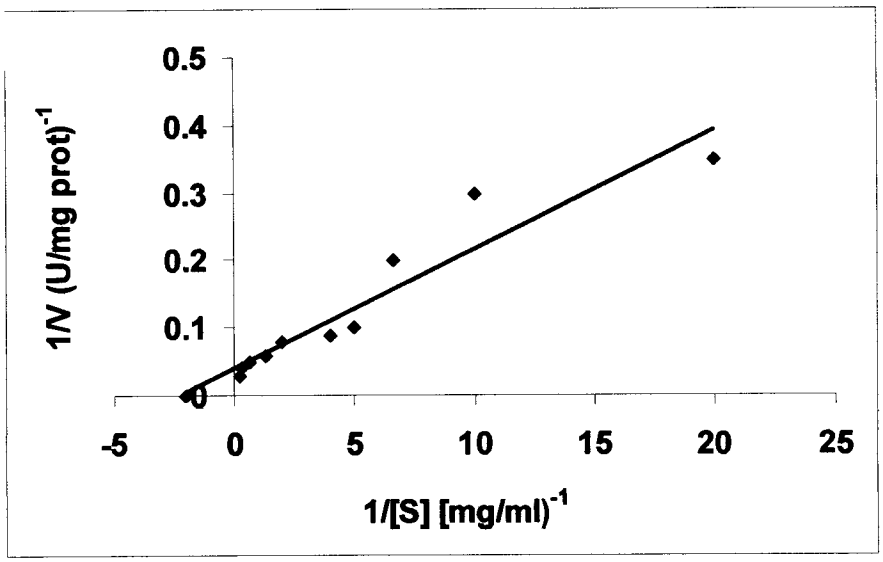


Fig.4.3 a Lineweaver- Burk plot for the enzyme from the isolate TZ

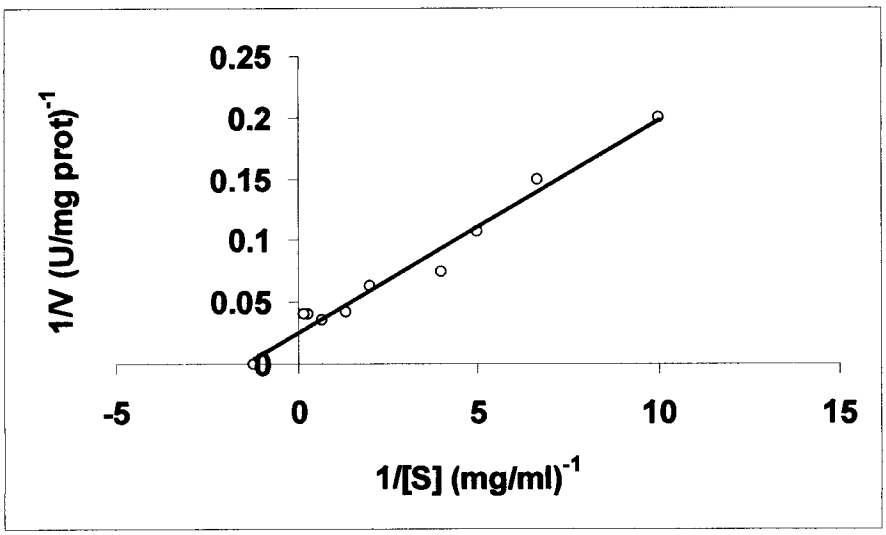


Fig.4.3 b Lineweaver- Burk plot for the enzyme from the isolate AH-2

The characteristic features studied hitherto of the two alkaline proteases are summarized in Table 4.1. The enzyme from isolate TZ thus exhibited superior properties over the one from isolate AH-2 in terms of activity, pH and thermal stability as well as kinetic parameters and hence further characterization of this enzyme was taken up.

4.2.4 Effect of EDTA and metal ions

The effect of EDTA on activity of the alkaline protease from *Thraustochytrium* sp. TZ is as shown in Fig. 4.4. Very low concentrations (up to 0.25mM) of EDTA had a stimulatory effect on the enzyme, which could probably be due to the removal of traces of some unidentified metal ion inhibitors present in the assay mixture. Higher concentrations of EDTA were inhibitory, suggesting some divalent-metal requirement for the enzyme activity.

Nascimento *et al.* (2006) had reported that the alkaline protease from *Bacillus* sp SMIA-2 showed no inhibitory effect at concentrations of 1- 10 mM of EDTA. Tunga *et al.* (2003) also reported that the protease from *Aspergillus parasiticus* was not inhibited up to 5.0 mM EDTA. In *Bacillus licheniformis* ATCC 21415, the protease was inhibited by 20 mM EDTA and lost about 62% of its original activity which was suspected to be due to the chelation of calcium ions which were necessary for enzyme activation or participated in the enzyme molecule (Mabrouk *et al.*, 1999).

The properties of alkaline proteases from isolates TZ and AH-2 are summarized in Table 4.1

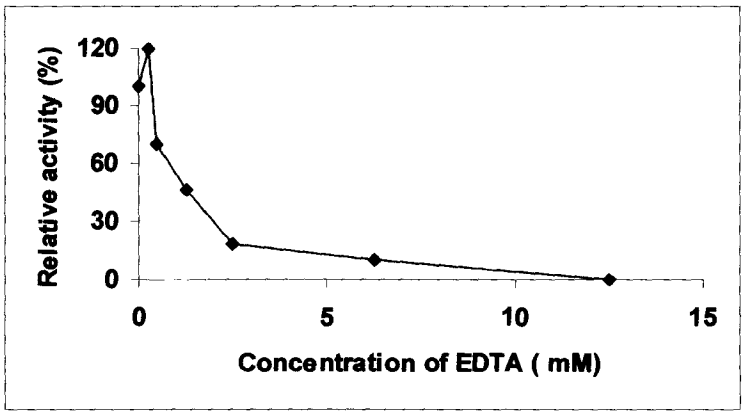


Figure 4.4 Effect of EDTA on enzyme activity from the isolate TZ

The enzyme extract from the isolate TZ was incubated in various concentrations of EDTA for 30 min at room temperature (30 ± 2°C) and the residual activity assayed. The activity of untreated enzyme was taken as 100%.

Table 4.1 Comparison of some characteristics of crude enzymes from the isolates AH-2 & TZ

Property	Enzyme from isolate AH-2	Enzyme from isolate TZ
Reaction time	15 min	30 min
Optimum temp	50°C	50°C
Optimum pH	9.0	8.0
K_m	0.83 mg/ml	0.5 mg/ml
V_{max}	0·04 U/mg	0·025 U/mg
Thermal stability	15 min at 45°C	15 min at 50°C
pH stability	stable at alkaline pH (8-10)	stable at alkaline pH(810)
Stability at RT	5 days, with 50% activity	5 days, with 70% activity

The inhibitory effect of heavy metal ions on protease activity is well documented in literature. The effect of metal ions on the alkaline protease activity from isolate TZ is presented in Table 4.2. The inhibition by mercury and copper was drastic. Although to a lesser extent, added zinc followed a similar trend. Ferric ions evoked a 30-40% inhibition at 1 mM and totally knocked out the activity at 10mM while magnesium was comparatively less inhibitory. The proteases secreted by *Brevibacillus* (*Bacillus*) *brevis* (Banerjee *et al.*, 1989) and *Bacillus* sp. SMI-2 (Nascimento *et al.*, 2004) *was* also inhibited by Hg^{2+}, Zn^{2+} and Cu^{2+}. It is known that the ions mercury, cadmium and lead react with protein thiol groups (converting them to mercaptides) as well as with histidine and tryptophan residues. Moreover, by action of silver and mercury the disulphide bonds were found to be hydrolytically degraded (Kumar *et al.*, 1999). Dahot (1994) observed similar inhibitory effect of Hg^{2+} and Ag^{2+} on alkaline protease from *Penicillium expansum* which supported the presence of -SH groups at the active site.

In the present study, calcium ions (10mM) marginally stimulated the enzyme activity. These results suggest that this metal ion could perhaps protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at higher temperatures as suggested by Beg *et al.* (2003). Similar effects of Ca^{2+} were also observed for the alkaline protease from *Bacillus stearothermophilus* F1 by Rahman *et al.* (1994) and for *Bacillus thermoruber* by Manachini *et al.* (1988).

Table 4.2 Effect of metal ions on enzyme activity from the isolate TZ

Metal ion	Conc. (mM)	Residual activity (%)
Zn^{2+}	1.0 10.0	29.8 20.6
Hg^{2+}	1.0 10.0	7.0 0
Mg^{2+}	1.0 10.0	74.6 63.9
Fe^{3+}	1.0 10.0	69.0 0
Ca^{2+}	1.0 10.0	100.0 137.0
Cu^{2+}	1.0 10.0	5.5 0

Taking into account the overall effects of EDTA and metal ions on the enzyme activity of the crude enzymeextract from the isolate TZ, it would appear that trace concentrations of certain inhibitory metals present in the enzyme preparation might have been suppressing the true activity. Such a phenomenon, if it occurred could perhaps also be responsible for the observed wide batch to batch variations in the activity of the protease. Similar effect was experienced by Aftab *et al.* (2006) who, while working with alkaline protease from *Bacillus brevis* SSA1, had reported that routine assays with casein as substrate had been showing variations in enzyme activity values from one preparation to another.

4.2.5 Substrate specificity

The ability to hydrolyze diverse protein substrates is one criterion of protease potency. In this research, the crude enzyme preparations were examined for their ability to hydrolyze various proteins. The results (Table 4.3) indicated the broad substrate specificity of the enzyme which is one of the important criteria for use in the detergent industry against a wide variety of stains. The higher activity with gelatin as substrate is also being investigated for possible applications.

A similar instance of broad substrate specificity but possessing maximum activity with casein, was also reported for proteases from *Conidiobolus coronatus* (Phadatare *et al.*, 1993), *Spilosoma oblique* (Anwar and Saleemuddin, 2000), *Nocardiopsis* sp (Moreira *et al.*, 2002) and *Pseudomonas aeruginosa* PD100 (Najafi *et al.*, 2005). In contrast, the protease from *Bacillus*

subtilis PE-11 although showing a high level of hydrolytic activity against casein, brought about poor to moderate hydrolysis of BSA and egg albumin and hardly any hydrolysis with gelatin (Adinarayana *et al.*, 2003).

4.2.6 Degradation of natural substrate proteins

The calcareous shells of marine invertebrates (Fig 4. 5) are commonly invaded by a diverse assemblage of microorganisms that have the ability to bore into and inhabit the calcium carbonate material. Such organisms are called endoliths and are ecologically important in the erosion and disintegration of calcareous shells, shell fragments and carbonate rocks that comprise a significant portion of the marine sediments and coastlines (Porter and Lingle, 1992). Microbial endoliths are known in cyanobacteria, Chlorophyta, Rhodophyta and the filamentous fungi (Porter and Lingle, 1992). The best documented endolithic marine fungus is the ascomycete, *Pharcidia balani* (winter) Bausch (Porter and Lingle, 1992). Calcareous shells have an organic matrix of conchiolin, a horny protein which is known to be used by endolithic fungi as a nutrient source. In light of this, synthesis and activity of proteases are of great significance to these endolithic fungi for survival (Raghukumar and Raghukumar, 1998).

Fig 4.5 Calcareous shells of marine invertebrates

Porter and Lingle. (1992) while working on endolithic thraustochytrids of the genus *Schizochytrium* had reported that of the three mollusk shell types used as bait, the mussel shell, with more organic material the shell was the most preferred substrate for colonization by thraustochytrids as well as various other heterotrophic endoliths. Clam shells were less frequently invaded by thraustochytrids and oyster shells were never demonstrated to have endolithic thraustochytrids.

The presence of shell matrix protein, such as conchiolin, appears to provide sufficient carbon and nitrogen sources for thraustochytrids. The apparent digestion of these proteins was suggested from the observations presented in Table 4.4. These preliminary studies indicated that this *Thraustochytrium* sp. TZ could be involved in dissolution of calcium carbonate and remineralisation of organic matter in the marine environment.

In summary, the reported alkaline proteases from thraustochytrids (specially from the isolate TZ) stands in par with that from other microbial origin with regards to pH and temperature stability and broad substrate specificity and could be exploited for its application in commercial fields.

Table 4.3 Substrate specificity of the enzyme from the isolate TZ

Substrate	Relative Activity (%)
Hemoglobin	88
Gelatin	64
Casein	100
BSA	1.2
Egg albumin	55

Table 4.4 Protease production by the isolate TZ using various substrates in the growth medium

Substrate in the growth medium	Activity (U/ml)
None	7.5
Skimmed milk powder (0.5%)	28.7
Shell fragments (0.5%)	21.5

Chapter 5

Purification of an alkaline protease

Purification of an enzyme is a prerequisite for studying its properties and structure-function relationship. Proteases in general are both extracellular and intracellular and can be purified by conventional procedures. For intracellular proteases, the cells need to be lysed by detergents or by sonication followed by the usual purification procedures employed for the extracellular enzymes.

There are no set rules for the purification of proteases. After separating the culture from the fermentation broth by filtration or centrifugation, the culture supernatant may be concentrated by means of ultrafiltration (Kang *et al.*, *1999*; Smacchi *et al.*, 1999), salting out by solid ammonium sulfate (Kumar, 2002), or solvent extraction methods using acetone (Kumar *et al.* 1999; Thangam and Rajkumar, 2002) and ethanol (El-Shanshoury *et al.*, 1995). In addition, other methods such as the use of PEG-35,000 (Larcher *et al.*, 1996), activated charcoal (Aikat *et al.*, 2001), temperature-sensitive hydrogel (Han *et al.*,1995), heat treatment of enzyme (Rahman *et al.*, 1994) and lyophilization (Manonmani and Joseph, 1993) have also been used for concentration of alkaline proteases.

Majority of the procedures involve concentration of the culture filtrate either with salt or solvent precipitation or by ultrafiltration. The concentrated culture filtrate is then further purified by a combination of chromatographic procedures such as affinity chromatography (using a bacitracin-sepharose column or sepharose bound to inhibitor or substrate or CNBr-activated sepharose), ion exchange chromatography (such as on CM-cellulose, DEAE-cellulose, phenyl-sepharose or DEAE-sepharose) and gel filtration (such as on sephadex). Apart from these

conventional procedures, more sensitive and advanced procedures such as FPLC, converging-diverging foam fractionation (Catara, 2003), crystallization (Agnihotri and Bhattacharya, 1993) and preparative PAGE (Phadatare, 1993) have been used for the purification of proteases. Other methods of choice, including aqueous two-phase systems (Sinha *et al.* 1996), dye ligand chromatography (Cowan and Daniel, 1996) and foam fractionation (Banerjee *et al.* 1993) have also been employed on a small scale but still await commercial exploitation. Identifying a simple and cost-effective procedure for any protease purification would certainly enhance its marketability.

Analysis of the characteristics of the alkaline proteases (Chapter 4) revealed that the enzyme from thraustochytrid isolate TZ was superior to that from the isolate AH-2 with respect to specific activity, thermal stability, pH stability and affinity for the substrate casein. This enzyme was therefore selected for purification and further characterization, the subject matter of which forms the content of this Chapter.

5.1 Materials and Methods

Unless otherwise specified, all the steps in the purification procedure were routinely carried out at 0 - 4°C.

5.1.1 Ion exchange chromatography

The enzyme preparation was loaded on a CM-Sephadex column (50 ml bed volume) at pH 5.5 (acetate buffer, 200 mM). The fractions (1 ml) were collected at a flow rate of 20 ml/ h by passing the same buffer down the

column. Further elution was carried out stepwise using buffer containing 0.5 M and 1 M NaCl.

5.1.2 Gel filtration

The sample was loaded on a Sephacryl S-200 gel filtration column (130 ml bed volume) equilibrated with borate buffer (25 mM, pH 8). The fractions (1 ml) were eluted out at a flow rate of 20ml/ h using the same buffer. Protein and activity were monitored during the elution.

5.1.3 Native PAGE

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out on a 10% gel at 50 mV till the dye front crossed the stacking gel and at 100 mV thereafter, following the procedure of Laemmli (1970). Staining was done by the silver staining method (Heukeshovan and Dernick, 1985).

5.1.4 *In situ* detection of enzyme activity

After the electrophoretic run, the native polyacrylamide gel was blotted over a casein gel (1% agarose gel containing 1.0% casein) and incubated for 1 h at room temperature in a moist chamber. The two gels were then stained separately with Coomassie Brilliant Blue R-250. Enzyme activity appeared as a clearance band against the blue background on the casein gel. Protein bands (blue) although faint, could be visualized on the polyacrylamide gel.

5.1.5 Molecular weight determination

Molecular weight of the pure enzyme was determined on a Sephadex G-100 gel filtration column (bed volume 130 ml) calibrated using standard protein markers. The different protein markers used were cytochrome *c* (12.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa) and BSA (67 kDa). A calibration graph of log molecular weight *vs* V_e/V_0 was plotted, where V_e was elution volume and V_0 was void volume calculated using Blue dextran (mol.wt 200,000 kDa).

5.1.6 Determination of subunit molecular weight

The subunit molecular weight of the enzyme was determined by carrying out SDS-PAGE (12% gel) with standard protein markers. The markers used were (97,400, 66,000, 43,000, 29,000, 20,100 and 14,100 Da) from Hi-Media medium molecular weight protein markers. The molecular weight was calculated based on the calibration graph of log molecular weight *vs* R_f value.

5.1.7 Characterization of the purified protease from *Thraustochytrium sp* TZ

Optimum enzyme concentration, assay time, optimum pH and temperature, pH and thermal stability as well as kinetic parameters of the purified enzyme were determined as per the methodology described in Chapter 4. Substrate specificity was studied using different protein substrates such as gelatin, egg albumin, hemoglobin, casein and BSA in the assay at 37°C for 30 min.

5.1.8 Effect of inhibitors, reducing agents, detergents and metal ions

The effect of inhibitors (PMSF and iodoacetamide) on enzyme activity was studied by incubating the enzyme with different concentration of each inhibitor for 1 h at 30°C and then measuring the residual activity as per the routine procedure. The reducing agents tested (after 30 min preincubation at 30°C) were DTT, β mercaptoethanol and sodium thioglycolate at different concentrations (0.1, 0.5 and 1.0%). The effect of these reducing agents as well as detergents (anionic detergent- SDS at 0.1%, non -ionic detergents -Tween-80 &Triton X-100 at 0.1-1.0% concentration) on protease activity was determined by pre-incubating them with the enzyme or detergents for 30 min at room temperature before carrying out the routine assay. The effect of EDTA and metal ions on protease activity was determined by the addition of the ions at final concentrations of 1.0 and 10 mM to the enzyme and then assaying under the standard conditions after pre-incubation for 30 min at room temperature.

5.2 Results and Discussion

Although most of the commercial applications like in detergent formulations do not require homogenous enzyme preparation, a certain degree of purity, however, enables efficient and successful usage (Sharma *et al.* 2001), besides giving a better insight into the characteristics of the specific protein actually responsible for the applications.

5.2.1 Purification of the alkaline protease from the isolate TZ

A number of alkaline proteases from different sources have been purified and characterized and a summary of various purification strategies adopted for purification of microbial alkaline proteases is presented in Table 5.1.

For a preliminary understanding of the binding properties of our alkaline protease to ion-exchange matrices, loading on and elution from both anion- (DEAE-Sephadex) as well as cation- (CM- Sephadex) exchange columns were studied by batch process. Despite checking out on combinations of minute buffer pH variations as well as salt concentrations for elutions, active protein could not be obtained after DEAE-Sephadex chromatography. This could be the autolysis of the enzyme protein which some other researchers (Surti and Bapat, 2002; Mane and Bapat, 2001) also had experienced or due to some reason yet unidentified. Although the binding to CM- Sephadex was extremely weak, to the extent of elution in the unbound fractions, since active preparations could be obtained, this ion-exchange was adopted as the matrix of preference.

Purification was carried out by a sequence of steps which included concentration, ion exchange chromatography and gel filtration details of which are given under Section 5.1. The crude culture supernatant was first subjected to 85% ammonium sulphate saturation, the resulting pellet was suspended in borate buffer (25 mM, pH 8.0) and dialyzed against the same buffer. The dialysate was applied to a column of CM-Sephadex. Fractions were collected first by washing with the same buffer. Elution was then carried out stepwise using 0.5M and 1M NaCl in this buffer. It was found that the enzyme got eluted

out as unbound fractions from this column. The elution profile on CM-Sephadex column showed one sharp activity peak corresponding to the unbound fractions and a few minor peaks of non-specific proteins (Fig 5.1). Fractions with high alkaline protease activity (nos 1-10) were pooled, dialyzed and concentrated by sucrose. The concentrate from the CM-Sephadex column was subjected to gel filtration on a Sephacryl S-200 column. The elution profile (Fig 5.2) showed a single peak of protease activity and one or two minor peaks of non-specific proteins. The active fractions (nos 4 to 14) obtained after gel filtration as above were pooled, concentrated by using sucrose and dialyzed. Table 5.2 summarizes the results of the purification of the alkaline protease from *Thraustochytrium* sp.TZ. After the gel filtration using Sephacryl S-200 column, the purity of the enzyme was 30- fold with a yield of 3%. The procedure need to be refined further for a better yield.

The purified protein was subjected to non-denaturing PAGE. A single protein band was observed on the native gel (Fig 5.3 a, Lane 4) and one corresponding activity band appeared on the zymogram (Fig 5.3 b, Lane 5). The homogeneity of the enzyme preparation was thus confirmed. When loaded on SDS-PAGE with or without β- Mercaptoethanol also, a single band was observed (Fig 5.4, Lanes 3&4) indicating that the protein was either monomeric or composed of identical subunits.

Table 5.1 Combination of purification techniques applied to purification of various alkaline proteases (Courtesy: Gupta *et al.*, 2002)

Microorganism	Concentration method	Column matrices
Bacteria		
Alcaligenes faecalis	Acetone	DEAE-cellulose, Sephadex G-100
Arthrobacter nicotianae 9458	Ultrafiltration	DEAE-Sephacyl, Sephacryl 200, Phenyl Sepharose, FPLC Mono Q HR 5/5
Bacillus sp. PS719	$(NH_4)_2SO_4$	DEAE-cellulose
Bacillus sp. NCDC 180	Acetone, $(NH_4)_2SO_4$	DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephacryl S-200
Bacillus sp. SSR1	$(NH_4)_2SO_4$	DEAE-Sephadex A-50, Sepharose 6B
B. pumilis MK6-5	$(NH_4)_2SO_4$	DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephacryl S-200
B. sphaericus MTCC B-0014	Ultrafiltration, $(NH_4)_2SO_4$	Phenyl agarose, Q-Sepharose
Oligotropha carboxydovorans DSM 1227	Ultrafiltration	Sephadex G-75, CM-cellulose
Pimelobacter sp. Z-483	$(NH_4)_2SO_4$	Butyl-Toyopearl 650C, Butyl Toyopearl 650 M, Phenyl Toyopearl 650 M, Toyopearl HW-50F
Pseudomonas aeruginosa MN1	$(NH_4)_2SO_4$	Sephadex G-100, DEAE-cellulose
P. aeruginosa PST-01	$(NH_4)_2SO_4$	Butyl-Toyopearl 650C, Butyl Toyopearl 650 M
Serratia marcescens ATCC 25419	$(NH_4)_2SO_4$	Q-Sepharose, Sephacryl S-200

Contd....

Fungi

Aspergillus terreus (IJIRA 6.2)	Acetone, $(NH_4)_2SO_4$	DEAE-Sephadex A25, SDS-PAGE, electroelution
Scedosporium apiospermum	$(NH_4)_2SO_4$, PEG 35,000	Sephadex G-75, immobilized phenylalanine-agarose
Oerskovia xanthineolyrica TK-1	Ultrafiltration, $(NH_4)_2SO_4$	Phenyl-Sepharose CL 4B, DEAE-Sephacel
Streptomyces cyaneus	-	Aprotinin-agarose
Streptomyces thermovulgaris	Ultrafiltration	FPLC Enono-pac Q, Seperose 12
Thermoactinomyces sp. E79	$(NH_4)_2SO_4$	DEAE-Sepharose CL-6B, Butyl-toyopearl 650 M

Moulds

Aureobasidium pullulans	Ultrafiltration	Sephadex G-75
Candida caseinolytica	-	Sephacryl S-200, DEAE-Biogel

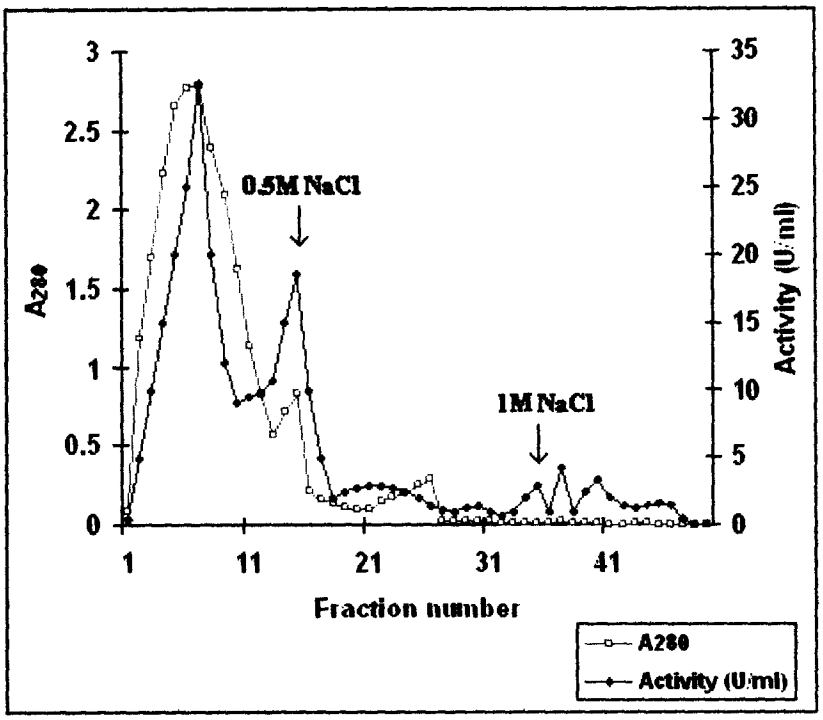


Fig.5.1 Chromatography profile of alkaline protease from isolate TZ on CM-Sephadex column

The chromatography was carried out as described in the text. The additions of salt during the elution are indicated by arrows.

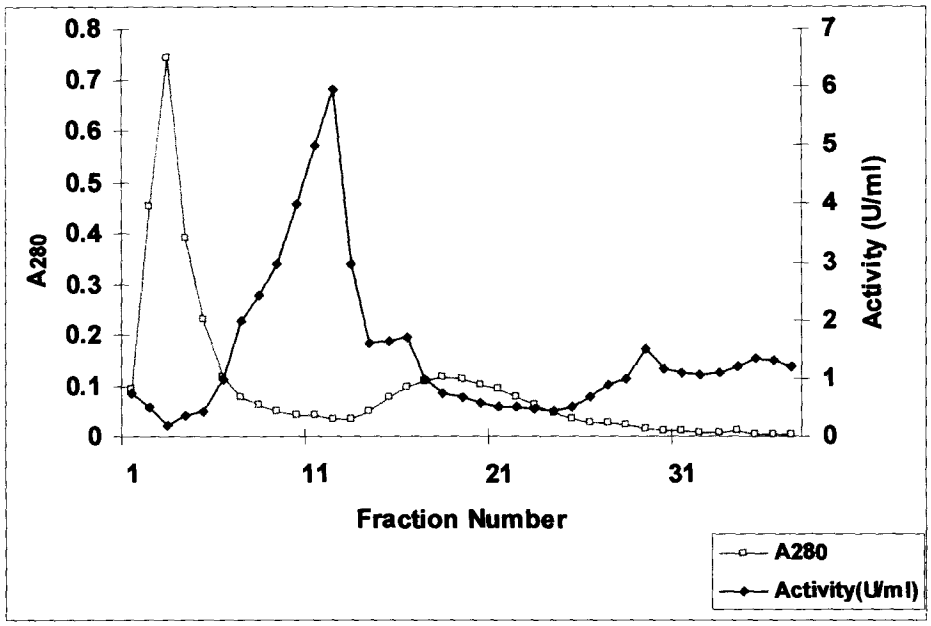


Fig.5.2 Elution profile of the alkaline protease on Sephacryl S-200 column

The column was equilibrated with borate buffer pH 8.0 Elution was carried out with the same buffer at a flow rate of 20 ml/h.

Table 5.2 Purification Table

Fraction	Volume (ml)	Protein (mg/ml)	Total activity (U)	Sp. Activity (U/mg .prot)	Yield (%)	Fold purification
Crude	900	1.35	5571	4.59	100	1.0
0-85% dialysate	33	3.40	3803	33.90	68.3	7.4
CM-Sephadex concentrate	5.5	1.40	320	41.54	5.7	9.1
Sephacryl S-200	2.2	0.54	163	138.25	2.9	30.2

5.2.2 Molecular weight determination

The molecular weight of the purified protease was determined by comparison with the mobility of standard marker proteins as described earlier. The estimate of molecular weight obtained by SDS-PAGE (26.3kD) corresponded with that obtained by gel-filtration (25.7 kDa) confirming the monomeric nature of the enzyme (Fig 5.5 a and b).

The molecular weights of most of the reported alkaline proteases are in the range of 15-40 kDa. It has been reported that the molecular masses of alkaline proteases from microorganisms range from 15 to 30 kDa with few reports of higher molecular masses of 31.6 kDa, 33 kDa; 36 kDa, and 45 kDa (Kumar and Takagi, *1999*). For example, alkaline extracellular protease from *Yarrowia lipolytica* is a 32-kDa protease of the subtilisin family (Barth and Garlardin, *1996*). Ma *et al.* (2007) had reported an alkaline protease of molecular weight 32.0 kDa from the marine yeast *Aureobasidium pullulans.*

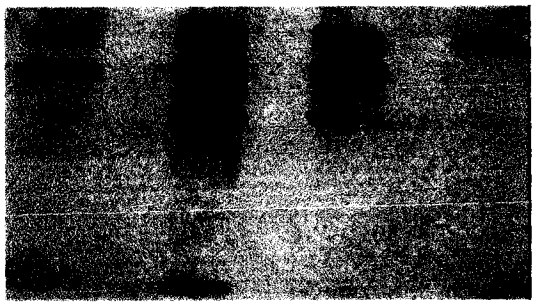


Fig 5.3 a Native PAGE

Lane 1: Crude enzyme

Lane 2: 0-85% dialysate

Lane 3: CM sephadex concentrate

Lane 4: Sephacryl S-200

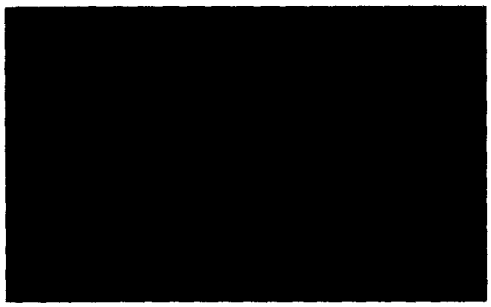


Fig 5.3 b Zymogram

Lane 1: 10-85% Dialysate

Lane 3: CM-Sephadex concentrate

Lane 5: Sephacryl S-200

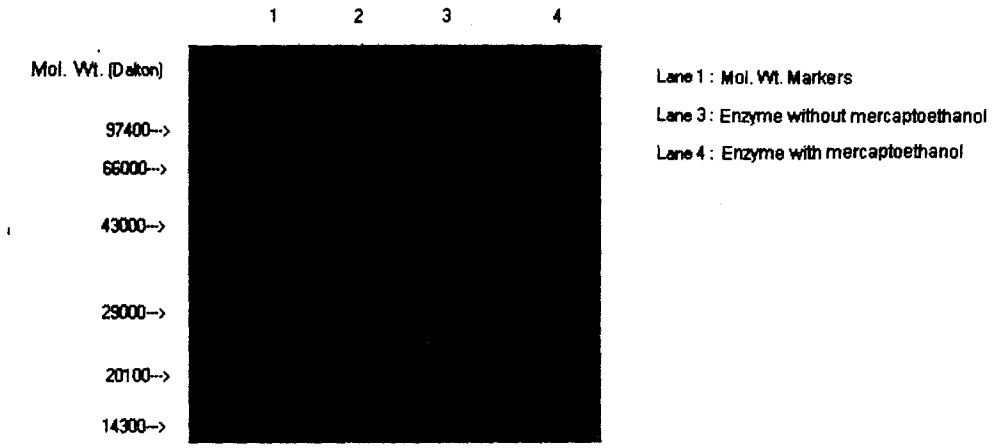


Fig 5.4 SDS-PAGE

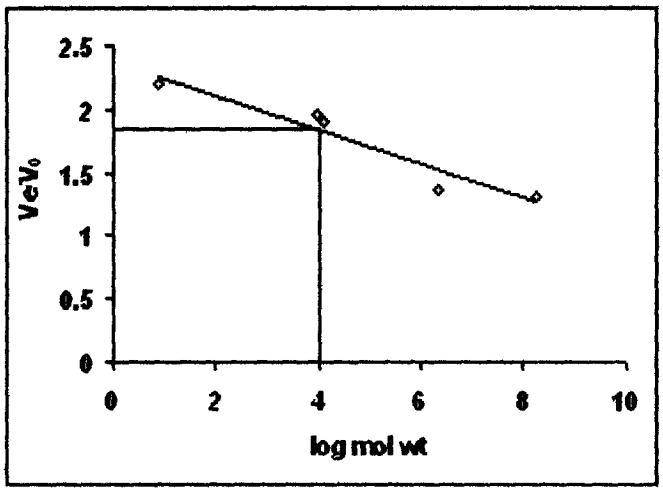


Fig 5.5 a Molecular weight determination by gel filtration

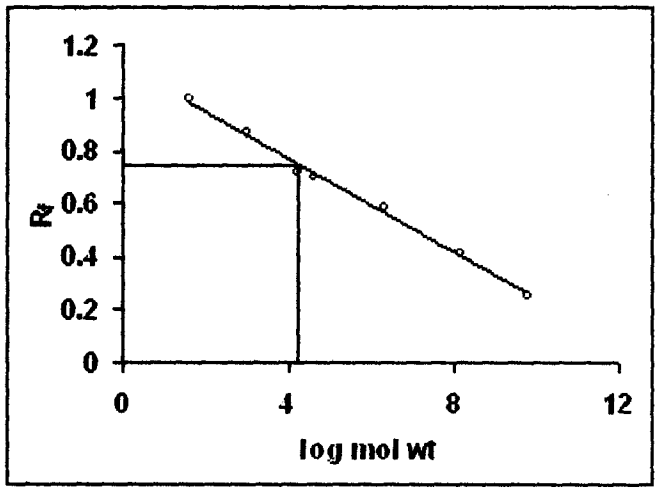


Fig 5.5 b Molecular weight determination by SDS PAGE

5.2.3 Properties of the purified enzyme from *Thraustochytrium* sp TZ

The knowledge of properties of an enzyme forms the basis for its utility in various commercial fields. The kinetic and other parameters which were studied for the purified alkaline protease are discussed in the following Section.

5.2.3.1 Effect of temperature on protease activity and stability

The temperature optimum for the assay was shifted from 50°C to 45°C upon purification of the protease (Fig 5.6). This is not unsual, considering that an enzyme would be more heat stable in crude cell-free preparations containing a high concentration of other proteins, rather than when in pure form.

Thermal stability for 10 min at different temperature ranging from 40°C-90° C has been analyzed (Fig 5.7 a). It was observed that the pure enzyme protein remained without any loss of activity with heat treatment up to 45°C. Further analysis of the stability at 45°C for different time periods revealed no loss in enzyme activity up to 20 min of heat treatment and retention of 55% activity even after 40 min heat treatment (Fig 5.7 b). The higher thermostability of the purified alkaline protease than that of the crude extract could be due to the removal of some non-specific proteins during purification. It has been reported that the optimum temperatures of alkaline proteases from bacteria range from 50 to 70°C (Kumar and Takagi, *1999*). Ma *et al.* (2007) had reported an alkaline protease from the marine yeast *Aureobasidium pullulans* .was inactivated rapidly at temperature higher than 45°C within 30 min. Liu *et al.* (1997) had reported a heat sensitive cystein alkaline protease from *Vibrio harveyi* which

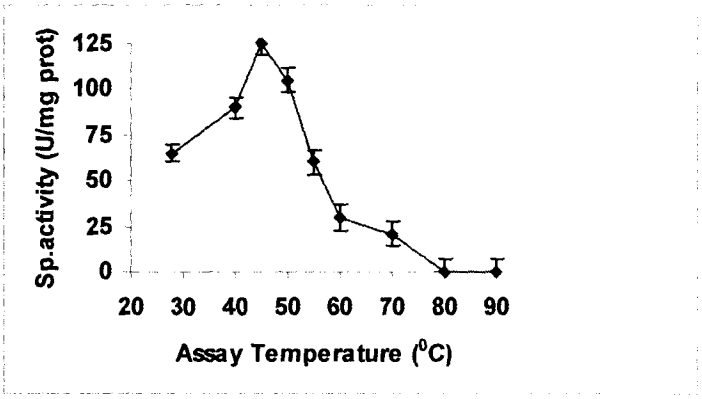


Fig 5.6 Effect of temp on enzyme protease activity

Assay conditions – pH 8.0, 30 min, temperature range 28°C-90°C

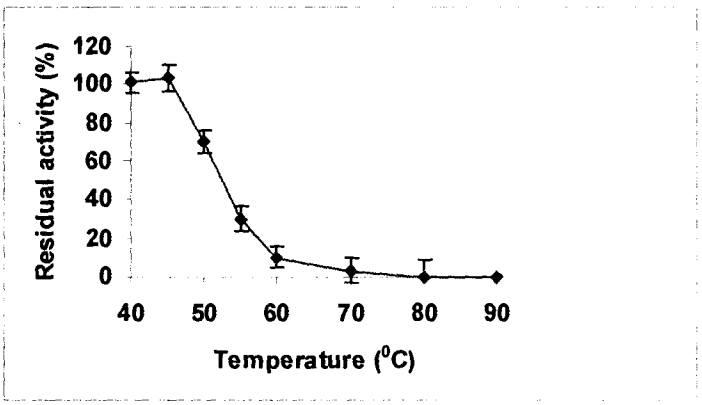


Fig 5.7 a Thermal stability

The pure enzyme was subjected to heat treatment at the specified temperatures for 10 min. The activity of untreated enzyme was taken as 100%.

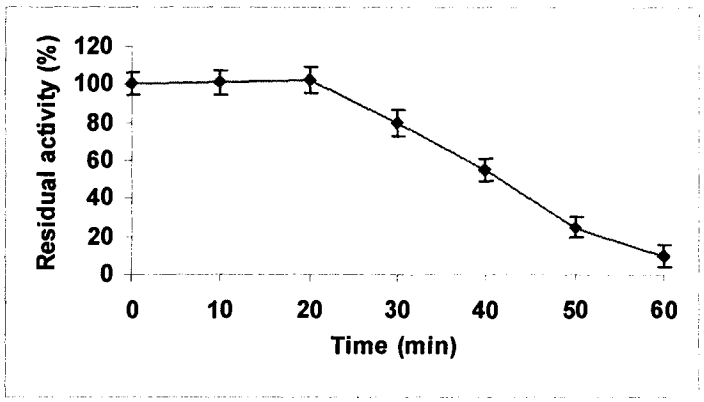


Fig 5.7 b Thermal stability at 45^{0}C

The pure enzyme was subjected to heat treatment at 45^{0}C for varying time periods and the residual activity estimated. The activity of untreated enzyme was taken as 100%.

had a temperature optimum at 50°C and was inactivated above 45°C after 120 min heat treatment.

5.2.3.2 Effect of pH on enzyme protease activity and pH stability

Upon purification the pH profile of the alkaline protease did not change at all (Fig 5.8 a). The enzyme continued to be active over the alkaline pH range (pH 8-10) for the assay. In this range about 95% of activity was retained even after one hour of incubation at room temperature in various buffers (Fig 5.8 b).

Most fungal proteases are stable in the pH range of 5.0 to 10.0, and more so at neutral pH. A protease from *Bacillus* sp. highly active and stable at pH 9-10 was reported by Zeng *et al.* (2003). Two alkaline protease producing alkaliphilic bacteria designated as AL-20 and AL-89 were isolated from a naturally occurring alkaline habitat (Gessesse *et al.*, 2003). Protease AL-20 was active in a broad pH range displaying over 90% of its maximum activity between pH 7.5 and 11.5, the peak being at pH 10. An extracellular protease purified from a psychrophilic *Pseudomonas* sp. displayed optimal activity at 40°C and pH 10 (Zeng *et al.*, 2003). A novel extracellular serine protease (designated Pernisine) purified from the archaeon *Aeropyrum pernix* K1 was active over broad range of pH (5.0-12.0) with maximal activity between pH 8.0 and 9.0. The enzyme from our isolate TZ was maximally active over an alkaline pH range of 8 to 10. The stability of the enzyme at alkaline pH and higher temperatures suggests its usefulness in industrial applications.

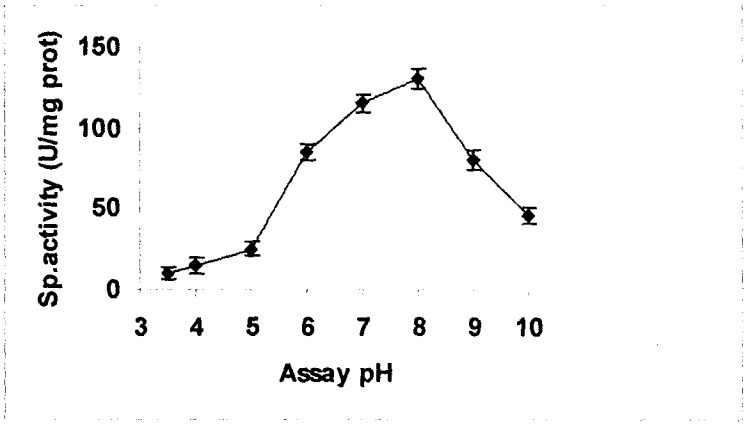


Fig 5.8a Effect of pH on enzyme assay

Assay condition–varying pH range 3.5-10.0, 45^{0}C, using casein (0.5%) as substrate

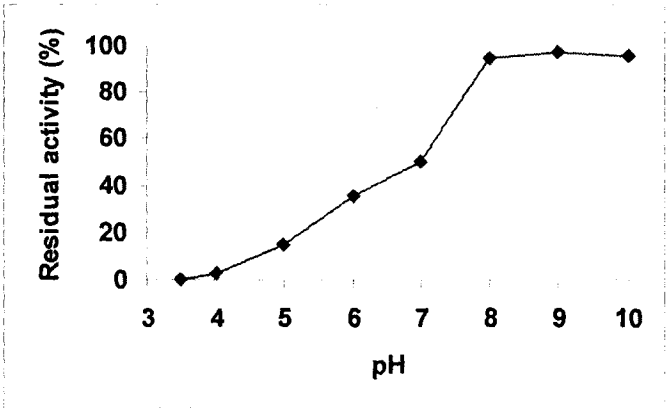


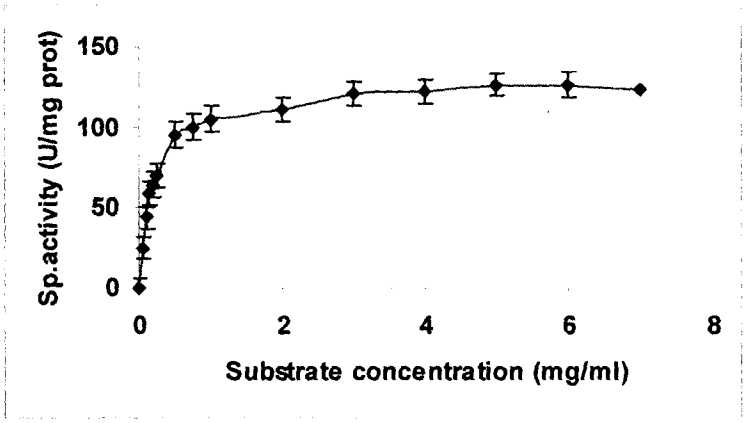
Fig 5.8b Effect of pH on enzyme stability

The pure enzyme was incubated in buffers of pH 3.5-11 for 1 h at room temperature. The activity of untreated enzyme was taken as 100%.

5.2.3.3 Effect of substrate concentration

The effect of substrate concentration on enzyme activity was studied by varying the substrate (casein) concentration in the assay at pH 8.0 and 45°C. From Fig 5.9 a, it can be observed that enzyme activity followed typical Michaelis-Menten kinetics with saturation beyond a casein concentration of 5 mg/ml and a $V_{max\ of}$ 12.5 U/mg protein. Lineweaver- Burk analysis of the data gave a K_m value of 0.178 mg/ml for casein. This was about 2.8 fold lower than that of the crude preparation of this alkaline protease from *Thraustochytrium* sp TZ (*cf* Chapter 4) indicating the higher affinity of the purified enzyme for casein.

The apparent K_m value of the enzyme from *Bacillus polymyxa* was 2.9 mg/ml (Madan *et al.*, 2002). Lower K_m values for casein have been reported from *B.alkalophilus* and *Pseudomonas*. sp. AFT-36, which had K_m values of 0.4 and 2.5 mg/ml respectively (Takii *et al.*, 1990). A very high K_m value of 9.1 mg/ml for casein was reported for alkaline protease from *B. licheniformis* (Mabrouk *et al.*, 1999). The apparent K_m value for *B.subtilis* NCIM 2713 was 2.5 mg/ml (Mane and Bapat, 2001) and 1.06 mg/ml for *Bacillus subtilis* NCIM 27110 (Surti and Bapat, 2002). An alkaline protease from the marine yeast *Aureobasidium pullulans* showed an apparent K_m for casein were 0.25 mg/ml (Ma *et al.*, 2007). Our results compare very favorably with these reported values.



Fig, 5.9 a Effect of substrate concentration on enzyme activity

Assay conditions- 45^{0}C, pH 8.0, with varying casein concentration (0.05-5mg)

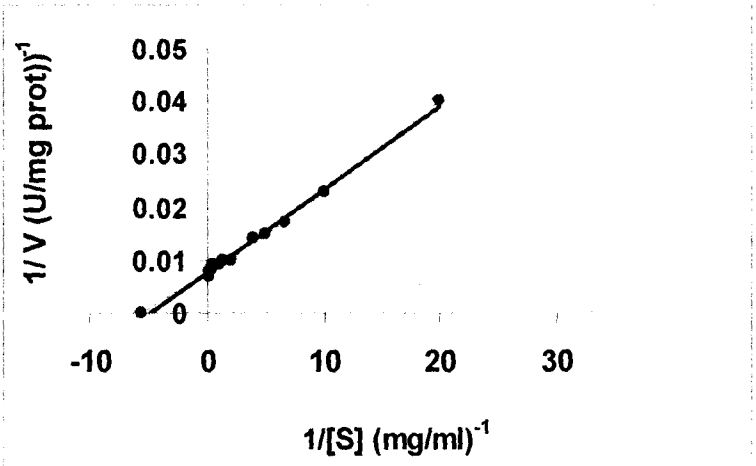


Fig 5.9 b Lineweaver- Burk plot

5.2.3.4 Effect of protease inhibitors on enzyme activity

Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements and the nature of the active center (Sigma and Mooser, 1975) The effect of various protease inhibitors such as a serine inhibitor phenylmethylsulphonylfluoride (PMSF) and a cysteine-inhibitor (iodoacetamide) were studied by pre-incubation with the enzyme solution for 60 min at 30°C before the addition of substrate. The residual protease activity was measured and the results are presented in Table 5.3. It is quite apparent that the enzyme is a cysteine protease since iodoacetamide drastically inhibited the activity probably by disturbing an –SH group at the enzyme active site which may be essential for its activity. . Liu *et al.* (1997) had reported a heat sensitive cystein alkaline protease from *Vibrio harveyi.* An alkaline cysteine protease was purified from *Penicillium expansum* (Dahot.1994)

5.2.3.5 Effect of reducing agents and detergents

The anionic detergent SDS totally denatured the enzyme at 0.1% concentration (data not shown). The non -ionic detergents tested were Tween-80 &Triton X-100 and the results are as in Fig. 5.10. The purified alkaline protease could retain more than 70% activity even at 1% concentration of these detergents.

A good detergent protease should be stable in the presence of detergents and oxidizing agents. The purified enzyme from *Thraustochytrium* sp.TZ appears to be reasonably stable towards the tested non-ionic detergents. A similar result was observed by Surti and Bapat (2002) where non-ionic detergents had little

Table 5.3 Effect of protease inhibitors

Inhibitor	Conc. (mM)	Residual activity (%)
PMSF	0.5	100
	1.0	95.3
	2.0	68.1
Iodoacetamide	0.5	18.3
	1.0	8.9
	2.0	0

Assay conditions- pre-incubation of enzyme with inhibitor for 1 h at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

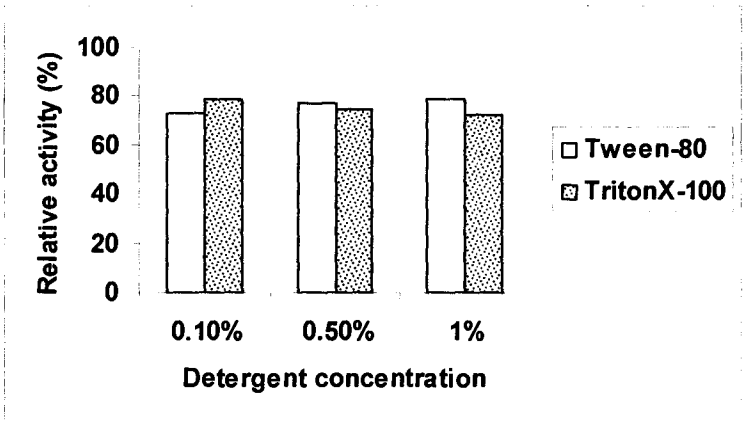


Fig 5.10 Effect of detergents

Assay conditions- pre-incubation of enzyme with detergents for 30 minutes at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

or no effect on the enzyme activity of the purified protease from Bacillus subtilis, *viz* NCIM 2711, while anionic detergent, SDS caused
loss of 98% of the enzyme activity. SDS denatures the molecule by disrupting the hydrogen bonds and hydrophobic interactions which play a crucial role in holding together the protein tertiary structure (Mane and Bapdt.2001).

5.2.3.6 Effect of EDTA

The effect of EDTA on enzyme activity is shown in Figure 5.11. Very low concentrations of EDTA had a stimulatory effect on the enzyme, which could probably be due to the removal of traces of some unidentified metal ion inhibitors present in the assay mixture. Higher concentrations of EDTA were however, inhibitory, suggesting a probable metal ion requirement for the enzyme activity.

Tunga *et al.* (1998) had reported that while the protease from *Aspergillus parasiticus* was not inhibited by 5 mM EDTA at 10 mM the enzyme retained about 84% of its activity when incubated for 30 min. The loss of about 62% of the original activity of the alkaline protease from *Bacillus licheniformis* ATCC 21415 in presence of 20 mM EDTA was attributed to chelation of calcium ions which were necessary for enzyme activation or participated in the enzyme active site conformation (Banerjee *et al.*, 1989).

5.2.3.7 Effect of metal ions

Metal ions are known to bind to the enzyme and alter its activity by stabilization or destabilization of the protein conformation. The effect of metal ions on

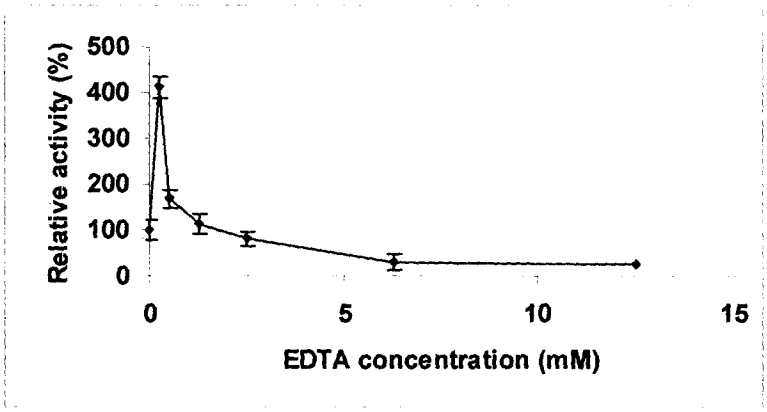


Fig 5.11 Effect of EDTA

Assay conditions- pre-incubation of enzyme with EDTA for 30 min at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

protease activity was therefore studied. Chlorides of the metals (Zn, Fe, Ca, Mg, Hg and Cu) were added at two different concentrations and the residual activity after a 30 min pre-incubation calculated the enzyme enzyme (Table 5.4). Corresponding values for crude enzyme as reported in Chapter 4 are reproduced for easy comparison. Purified enzyme resisted Hg^{++} inhibition much better than the crude preparation. Similarly, Zn^{++} and Cu^{++} also showed the same effect. The stimulation by Ca^{++} was marginally less in the purified enzyme. Four metal ions were again tested for their concentration - dependent effects on the pure enzyme. It was observed that up to 1 mM concentration, 50% of enzyme activity was retained while with higher concentration the activity declined drastically (Fig 5.12). Further work on these aspects would clarify the mechanism of these metal ion effects.

The metal ions Hg^{2+}, Cd^{2+} and Cu^{2+} had an inhibitory action on the enzyme activity from marine shipworm bacterium which was suggested as probably due to the reactions of these ions with histidine and tryptophan residues as well as with the thiol groups in the protein (Rivett, 1985). The disulphide bonds are also hydrolytically degraded by the action of Hg^{2+} leading to conformational changes in the enzyme protein and hence loss of activity (Surti and Bapat, 2002). It has been reported that thiol inhibitors have little effect on alkaline proteases of *Bacillus* spp., although they do affect the alkaline enzymes produced by *Streptomyces* sp. (Ma *et al.*, 2007).

A stronger inhibitory effect was observed in the presence of Cu^{2+}, Zn^{2+} and Hg^{2+} for the enzyme from *Bacillus* sp strain SMIA-2 at 10mM concentrations

Table 5. 4. Effect of metal ions

Metal ion	Conc. (mM)	Residual activity (%)	
		Crude enzyme	Pure enzyme
$ZnCl_2$	1	29.8	46.2
	10	20.6	25.7
$HgCl_2$	1	7.0	40.9
	10	0	15.4
$MgCl_2$	1	74.6	81.8
	10	63.9	56.3
$FeCl_3$	1	69.0	61.6
	10	0	0
$CaCl_2$	1	100.0	87.0
	10	137.0	107.7
$CuCl_2$	1	5.5	66.8
	10	0	10.1

Assay conditions- pre incubation of enzyme with the metal ions for 30 min at 28°C before the addition of substrate. The activity of untreated enzyme is taken as 100%.

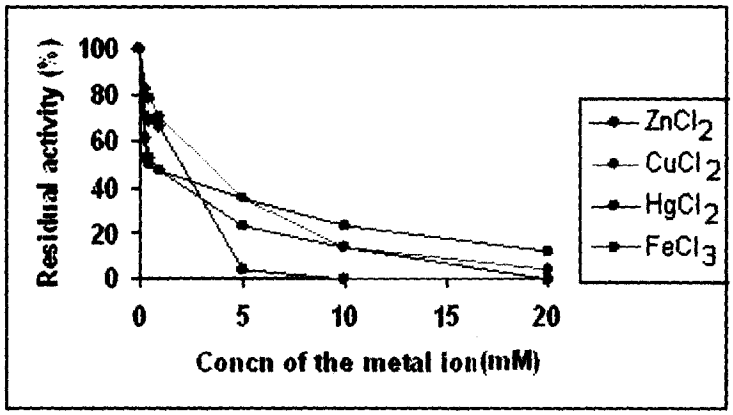


Fig 5.12 Effect of metal ion concentration

Assay conditions- pre- incubation of pure enzyme with metal ions for 30 min at 28°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

(Nascimento and Martins, 2004) and for the protease secreted by *Brevibacillus* (*Bacillus*) *brevis* (Banerjee *et al.*, 1999)). The inhibitory effect of heavy metal ions is well documented in the literature. Protease activity from *Bacillus mojavensis* was stimulated by Mn^{2+} and Ca^{2+}. These results suggest that these metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at higher temperatures (Beg *et al.*, 2003).

5.2.3.8 Substrate specificity

The ability to hydrolyze diverse protein substrates is one criterion of protease potency and its suitability for use in laundry detergents. The purified enzyme preparation was examined for its ability to hydrolyze various proteins. This was achieved using different substrates such as gelatin, egg albumin, hemoglobin, casein and BSA. Casein was found to be the most preferred substrate followed by gelatin and haemoglobin. Egg albumin could also serve as substrate albeit less efficiently (Table 5.5). The corresponding activities for the crude enzyme as reported in Chapter 4 are reproduced in this Table for easy comparison.

Proteases from *Conidio boluscoronatus* showed maximum activity with casein at pH 8.0 (Phadatare *et al.*, 1993). The broader substrate specificity of the alkaline protease from our thraustochytrid isolate TZ may be advantageous for use in detergents against a wide variety of protein stains. Other substrates were also hydrolyzed by this enzyme (haemoglobin, bovine serum albumin, gelatin and egg albumin) quite efficiently. These data confirmed that the

Table 5.5 Substrate specificity

Substrate	Relative Activity (%)	
	Crude enzyme	Pure enzyme
Egg albumin	55.0	11.6
Gelatin	64.0	67.0
Hemoglobin	88.0	71.0
BSA	1.6	7.2
Casein	100	100

Assay conditions- The purified enzyme was assayed with different protein substrates at 37^0C, pH 8.0 for 30 min. The enzyme activity using casein was taken as 100%.

cleansing effect of protease in the proteinaceous stain removal from the swatches (to be described in Chapter 6) is probably due to the action of this alkaline protease from the *Thraustochytrium* sp.TZ and not by the action of some non-specific proteins or enzymes present in the crude enzyme preparation which was used for the above mentioned stain removal experiment.

The capability to digest the protein substrates as assessed *in vitro* can also have far-reaching implications, as this feature has been exploited in the therapeutic use of enzymes in wound debridementl (Anwar and Saleemuddin, 2000).

5.2.3.9 Effect of NaCl on the purified enzyme

The purified enzyme was incubated with different concentration of NaCl for 1 h at room temperature and the residual activity was measured (Fig 5.13).The enzyme retained 75% activity up to 0.5 M and more than 50% activity at 0.75 M NaCl.

A similar kind of high salt tolerance was also observed in a moderate halophile, *Pseudoalteromona* CP1 protease. Generally, these halophilic proteins maintained their stability and activity by increased ion binding and glutamic acid content, both allowing the protein inventory to compete for water at high salt (Sanchez-Porro *et al.*, 2003). Further, in moderate halophiles, compatible solutes such as glycine, betaine and hydroxyectoine are known to protect protein, at high salt concentration (Litchfiled, 1998; Madern *et al.*, 2000; Niehaus *et al.*, 1999; Roberts, 2004). Damare *et al.* (2005) had also reported

that about 70% of the activity was retained by the deep sea fungus *Aspergillus ustus* NIOCC # 20 in the presence of sodium chloride of 0.5 M concentration which equals to 29 ppt salinity of seawater.

In summary, the purified alkaline protease from the T*hraustochytrium* sp TZ is a monomeric cysteine protease with a molecular weight 27 kDa, pH optimum at 8.0, temperature optimum at 45°C, stable at 45°C for at least 20 min, stable in alkaline pH, has a high affinity for casein, broad substrate specificity and is quite stable with tested detergents and H_2O_2.

Properties of some alkaline proteases from different microbial sources is presented in Table 5.6.

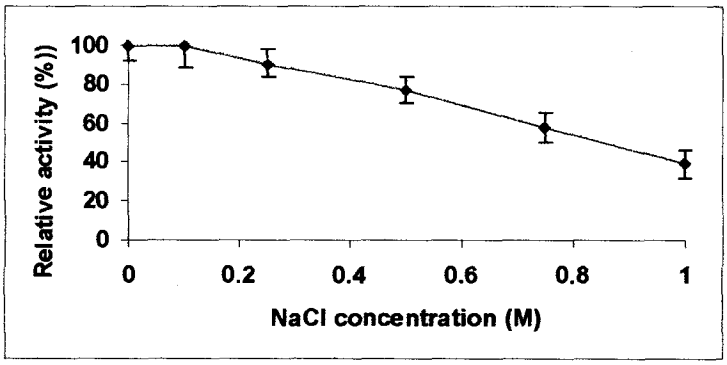


Fig 5.13 Effect of NaCl on pure enzyme

Assay conditions- pre-incubation of enzyme with varying NaCl concentrations for 60 min at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

Table 5.6. Properties of some alkaline proteases from different microbial sources (Gupta *et al.*, 2002).

Microorganism	pH optima	Temperature optima (°C)	Substrate specificity	MW (kDa)	Other properties
Bacteria					
Alcaligenes faecalis	9	55	Casein, BSA, gelatin, azocoll, azocasein	67	-
Arthrobacter nicotianae 9458	9; 9.5	55-60; 37	casein	55; 70-72	-
Bacillus sp. JB99	11	70	Casein	29	Metal ions enhance thermostability
Bacillus sp. NG-27	9.2	40	Casein	n.s.	Half life of 55 min at 90°C
Bacillus sp. KSM-KP43	11	70	Casein	n.s.	Oxidation-resistant
Bacillus sp. NCDC-180	11; 12	50; 55	Casein, synthetic *p*-nitroanilides	28; 29	Stable up to pH 13
Bacillus sp. PS179	9	75	Azocasein	42	Ca^{2+} enhances thermostability
Bacillus sp. SSR1	10	40	Azocasein	29	Ca^{2+} enhances thermostability
B. brevis MTCC B0016	10.5	37	Azocasein	n.s.	Detergent compatible
B. mojavensis	10.5	60	Casein	30	Bleach- and SDS-stable, detergent compatible
B. pumilis MK6-5	11.5	50-55	*p*-Nitroanilides	28	Ca^{2+}-independent
Oligotropha carboxydovorans DSM 1227	9	60; 50	Casein, azocasein, azocoll	23	-
Pimelobacter sp. Z-483	9	50	Casein	23	EDTA-resistant

Contd.....

Pseudomonas aeruginosa PST-01	8.5	55	Casein	38	Organic solvent stable
Serratia marcescens ATCC 25419	9.5	48	Azocasein	66.5	-
Fungi					
A. terreus (IJIRA 6.2)	8.5	37	Na-caseinate, synthetic substrates (*p*-nitroanilidines)	37	-
Beauveria bassiana	7.5-9.5	25	Azocoll, elastase	31.5	Elastase and cuticle degradation activity
Oerskovia xanthineolytica TK-1	9.5-11	50	Synthetic esters	20	Yeast-lytic activity
Streptomyces cyaneus	9	25	-*N*-*p*-Tosyl-L-arginine methyl ester	30, 120	-
Thermoactinomyces sp. E79	11	85	Casein	31	Calcium enhanced thermostability, broad pH stability (5-12)
Moulds					
Aureobasidium pullulans	9.5-10.5	41	Azocoll, casein	27	-
Candida caseinolytica	4.5-11	37	Casein	30	-

[a]Not specified

Considering the overall properties and purification strategies of different alkaline proteases of microbial origin and their evaluation, the reported alkaline protease appears superior with regard to the cumulative effects of pH and temperature stability, detergent compatibility (discussed further in Chapter 6) and above all bleach stability, for its future application in detergent formulation and possibly other industries. The simple purification procedure involving only three steps (concentration, ion-exchange chromatography and gel filtration) is an added advantage that may require the use of purified enzyme.

Chapter 6

Biotechnological applications of the alkaline protease

The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Enzymes find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile and cosmetic industries. In the above scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates.

Proteases are among the most important industrial enzymes, accounting for nearly 60% of the total world-wide enzyme sales (Kalisz, 1988; Outtrup and Boyce, 1990). In comparison with animal and fungal proteases, bacterial proteases are the most significant (Ward, 1985). This group accounts for 20% of the world market, with predominant use as additives to synthetic detergents (Wolff *et al.*, 1996). Over the past 30 years, the importance of proteases in detergents has changed, passing from their status of minor additives to become key ingredients. Alkaline proteases hold a great potential for application in the detergent and leather industries due to the increasing trend to develop environmentally friendly technologies. Important commercial detergent proteases available are Subtilisin Carlsberg, Subtilisin BPN, Alcalase, Esparase and Savinase. All these enzymes are stable in the presence of the various components of detergents and are active at washing temperatures and pH conditions (Gupta *et al.* 1999).

While bacterial alkaline proteases have a long history of application in the detergent industry, their application in the leather industry for dehairing and bating of hides is a relatively new development and has conferred added biotechnological importance to these enzymes (Rao *et al.*, 1998). In leather processing, the first step in the beam house is to remove hairs from hides and skins. The conventional dehairing method involves the use of high proportions of lime and sulfide. This process contributes to 80–90% of the total pollution in the leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime (Thanikaivelan *et al.*, 2004). Deaths due to this toxic chemical process have also been reported (Wang *et al.*, 2007). Alkaline proteases can be used as an alternative to sulfide. Although there are reports that complete hair removal was achieved through an 'enzyme-only' dehairing process and without chemical assistance (Thangam *et al.* 2001; Macedo *et al.* 2005), the enzymatic dehairing on an industrial scale is generally accompanied by the use of small amount of lime to improve the dehairing efficacy and reduce the cost (Thanikaivelan *et al.* 2004).

Various studies have been carried out over a long period of time to recover silver from the waste X-ray photographic films and most of them are patented. Recovery of silver by burning the films directly, a general method presently in use, generates such a foul smell that it is desirable to replace burning by pollution-free methods. The stripping methods using proteolytic enzymes obtained from various microorganisms are now being used more often for recovery of the silver (Nakiboglu *et al.*, 2000).

This Chapter highlights the promising roles of the *Thraustochytrium* alkaline protease in the detergent, leather and photographic industries.

6.1 Materials and Method

All measurements reported in this Chapter are representation of two to three independent experiments.

6.1.1 Enzyme stability tests

The commercial laundry detergents used were Advanced Ariel, Mr. White, Wheel, Tide, Rin Shakthi and Surf Excel. They were diluted in distilled water (~2g/l), incubated with protease for 30 min at 30°C and the residual activity was determined. The concentration dependent effects of H_2O_2 on the pure enzyme were analyzed after pre-incubation for 1 h at room temperature. The enzyme activity of a control sample (without any additive) was taken as 100%.

6.1.2 Washing Test performance with Protease Preparation

Fresh blood (50 µl) was spread uniformly on swatches of cotton test fabric (5 x 5 cm), air-dried and used after 24 h for the experiment. Four swatches were used for each detergent, to test the efficiency of the added enzyme. The concentration of the detergents used in the experiments was as per the recommended strength given by the manufacturer (~2g/l). To the petri-plate containing stained swatch, added various combinations of tap water/detergent with/without enzyme (10 U). After 30 min at room temperature, the swatches were given a gentle wash under running water, dried and the extent of stain removal visually compared. Untreated cloth pieces stained with blood were

taken as control. The above experiment was also carried out with egg stain as egg yolk contains 50% protein.

As the lysed blood sample gave an absorption peak at 416 nm, the contents of the above petri-plates were centrifuged and the clear solution monitored spectrophotometrically at 416 nm as a rough estimate of the degree of solubilization of blood.

6.1.3 Enzyme effects on goat skin

Two sets of experiments were conducted: Goat skin was obtained from the local butchery and square pieces (4 x 4 cm) were cut out and introduced into the test medium. For Experiment I, three conical flasks containing 20 ml each of MV Broth with 0.5% skimmed milk powder were prepared aseptically and inoculated. The flasks were kept on a mechanical shaker for three days and examined for dehairing of skin. Weight of the skin was noted before and after the experiment. The contents of the flasks were centrifuged and the clear supernatant analyzed for amino acids liberated. For Experiment II, the same procedure was followed except that crude enzyme extract (25 U) in buffer was used in place of inoculum and the incubation was for 24 h.

6.1.4 Gelatin degradation in used X-ray film

Used X-ray films were washed with distilled water, wiped with cotton impregnated with ethanol and cut into 4 X 4 cm pieces after drying. Each of the films was incubated the crude enzyme extract (60 U) or purified enzyme (40 U) for 1 h at room temperature and the gelatin-silver layer stripping visualized.

6.2 Results and Discussion

6.2.1 Detergent compatibility of the enzyme

The key challenge for the use of enzymes in detergents is their stability (Beg *et al.*, 2003; Mabrouk *et al.*, 1999). It is now well established that proteases exhibiting activity in the high-alkaline range have potential in detergent and stain-removing formulations. Their utility can be significant only if they also exhibit compatibility with various detergents (Phadtare *et al.*, 1993; Anwar and Saleemuddin, 1997). Taking this into consideration the compatibility of the *Thraustochytrium* lpase with commercial laundry detergents containing various additives was investigated.

The stability of the enzyme in each of six commercial laundry detergents was tested at the end of a 30 min preincubation period at room temperature. In all cases, when assayed, the enzyme retained at least 40% of its original activity (Fig 6.1). The higher activity with respect to control in presence of Surf Excel might be due to the fact that this detergent inherently has enzymes in it.

The protease from *Bacillus brevis* showed compatibility at 60°C with commercial detergents such as Ariel®, Surf Excel®, Surf Ultra® and Rin®. This enzyme retained more than 50% activity with most of the detergents tested, even after 3h incubation at 60°C (Banerjee *et al.*, 1999). Bhosale *et al.* (1995) reported that in the presence of 25 mM $CaCl_2$, the protease preparation from *Conidiobolus coronatus* showed compatibility at 50°C, with a variety of commercial detergents. This enzyme retained 16% activity in Revel, 11.4% activity in Ariel and 6.6% activity in Wheel (Wellingta and Meire, 2006) detergent.

Phadatare *et al.* (2000) while studying the compatibility of alkaline protease from *Conidiobolus coronatus* with commercial detergents, observed that the enzyme retained more than 80 % of its activity in the presence of detergents such as Snow white, Nirma, Revel and more than 56 % activity in the presence of Wheel and Surf when incubated for 1 h. Similarly, among the three proteases isolated from *Tritirachium album Limber*, proteinase R and T were reported to retain 90 and 80% activity, respectively, up to 1 h in the presence of detergents like ERA Plus and Dyanamo, while BPN from *Subtilisin* was highly unstable in all the detergents and retained just 4 % activity after 10 min (Samat *et al.*, 1990). Studies on *Bacillus polymyxa* by Madern *et al.* (2000) showed that after incubation for 10 min, maximum activity was observed in Vim Ultra(84.5%) followed by Ariel (76.9%), Nirma Super (61.5%),Wheel (50%), Nirma (42%) and Ariel Superwash (20.%). Enzyme activity was completely lost in presence of Surf. The enzyme retained 37.5 - 62.4% and 25 - 40% activity after 30 and 50 min of incubation, respectively in presence of Wheel, Avis, Vim Ultra and Ariel while the activity was completely lost in presence of Surf, Nirma Super and Ariel Super wash. A longer incubation time of 70 min resulted in loss of enzyme activity in all detergents except Vim Ultra and Ariel where the activity was 40 and 24.7 % of the original. After the supplementation of $CaCl_2$ and glycine,the enzyme from *B. subtilis* PE-11 retained more than 50% activity with most of the detergents tested even after 3 h incubation at 60°C (Adinarayana *et al.*, 2003).

In the present study, highest compatibility of the enzyme preparation was observed with Surf Excel after 30 min preincubation at room temperature followed by the 30 min assay at 50°C. As mentioned earlier, the apparently

very high residual activity could be the cumulative result of the inherent protease in the detergent. Among the other detergents tested, although with only marginal differences, the enzyme appeared to be most compatible in presence of Wheel and Mr. White with 59 and 54% residual activity respectively.

The data obtained from the experiments carried out in presence of commercial detergents as well as the broad substrate specificity of protease in cleaving most of the proteins suggest strongly that the proteolytic enzyme from the *Thraustochytrium* sp has all the potential to be used as a laundry detergent additive for improving the performance of heavy-duty laundry detergents.

6.2.2 Stability in oxidizing agent

In certain cleansing formulations, oxidizing agents may be added to enhance the brightness of and/or sterilize the washed material. A good detergent protease should therefore be stable in the presence of surfactants and oxidizing agents. In the present research, the protease from *Thraustochytrium* sp. has been shown to be stable in the presence of non-ionic detergents (Tween 20 and Tween 80) (Chapter 4). Gupta *et al.* (1999) have reported similar results for a strain of *Bacillus* sp.

The peroxide inactivation curve (Fig. 6.2) showed that the enzyme was stable even at high concentrations of H_2O_2 tested (3 % v/v), retaining 87% residual activity after 1 h. This is an important property because bleach stable enzymes are not generally available except for a few reports (Phadatare *et al.*, 1993). These results were again reminiscent of Gupta *et al.* (1999) who observed

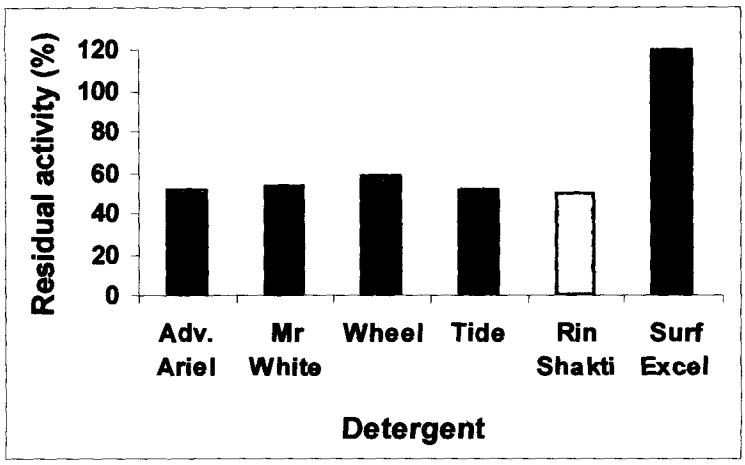


Fig 6.1 Stability of the enzyme in commercial detergents

The crude enzyme extract was incubated with the commercial detergents (~2g/l) for 30 min at 30°C and the residual activity was determined. The enzyme activity of a control sample (without any additive) was taken as 100% activity.

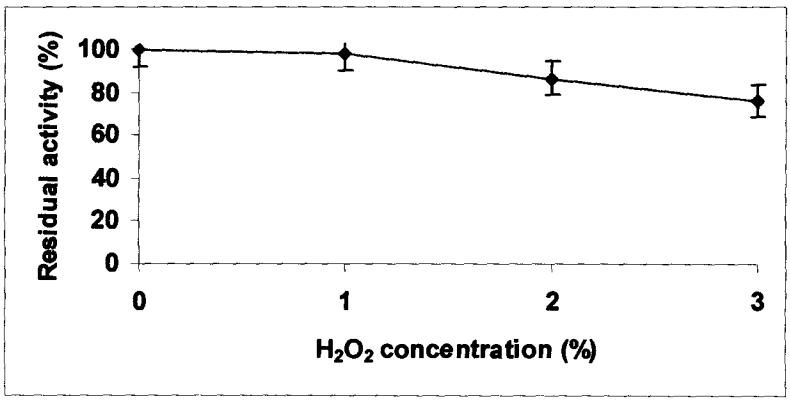


Fig.6.2 Enzyme stability in presence H_2O_2

The purified enzyme was pre-incubated with of H_2O_2 for 1 h at 30°C and the residual activity was determined. The enzyme activity of a control sample (without H2O2) was taken as 100% activity.

stability of a protease from *Bacillus* sp at high concentrations of H_2O_2 (10% v/v) for1 h, with 85% residual activity.

Manachini *et al.* (1988) while studying the alkaline protease from *Bacillus licheniformis* found that in 3% H_2O_2, the proteolytic enzyme retained 70% of activity after 1 h at 25°C and 45 % after 30 min at 40°C. Nascimento *et al.* (2006) had reported that the protease from *Bacillus* sp did not display good stability in a 5% (w/v) concentration of peroxide solution when tested for 30 min at 60°C. Bleach stability has therefore been introduced by workers either by site-directed mutagenesis or by protein engineering (Boguslawski and Shultz, 1992; Wolff *et al.*, 1996) whereas the present native protease from the wild type *Thraustochytrium* sp. showed inherent stability with H_2O_2.

6.2.3 In blood or egg stain removal

Anwar and Saleemuddin (1997) had reported usefulness of protease from *Spilosoma obliqua* for removal of blood stains from cotton cloth in the presence and absence of detergents. In the present study, it was seen that the protease was able to remove blood or egg stains very easily even in the absence of any detergent. The supplementation of the enzyme preparation in detergent could significantly improve the cleansing of the blood stains and egg stains by the detergent and one such representation of the wash test performance in presence of the detergent Wheel is shown in Fig 6.3 and 6.4. The same set of experiments when carried out at 50°C for 15 min, gave identical results. The compatibility of the enzyme with commercial detergents and the high

temperature optimum (50^0C) facilitated quicker stain removal. The thermophilic nature of the enzyme has been exploited herein.

The absorbance at 416 nm (measured as an approximation of the degree of solubilization of blood) was higher for the detergents supplemented with the enzyme as compared to unsupplemented detergents, indicating that more blood was solubilised in the presence of alklline protease (Fig 6.5). These results match with the qualitative observations described above and represented in Fig.6.3. In fact the enzyme preparation on its own could solubilize blood to an equivalent, if not higher, extent than most detergent preparations (Fig 6.5).

Kumar and Bhalla (2004) had reported that alkaline protease from *Bacillus* sp. APR-4 (100 U/ml) was efficient in removing egg stain from the test cotton fabric at 40°C after incubation for 2h and at higher temperatures of 55 and 60°C the stain removal was effected after 1h and 30 min, respectively. Our *Thraustochytrium* alkaline protease had an even better washing performance in that the time taken was only 30 min at room temperature with much less enzyme protein.

The comparative superiority of the present enzyme over a few others with respect to wash performance in blood stain removal from the test swatches is presented in Table 6.1

Blood stain **Tap water wash** **Detergent wash**

Enzyme wash **Enzyme + Detergent wash**

Fig 6.3 Wash performance of the enzyme in blood stain removal

Cotton swatches stained with blood were incubated with enzyme and/or detergent under the above mentioned experimental conditions for 30 min at room temperature, given a gentle wash with tap water and dried.

Egg stain **Tap water wash** **Detergent wash**

Enzyme wash **Enzyme + Detergent wash**

Fig 6.4 Wash performance of the enzyme in egg stain removal

Cotton swatches stained with egg yolk were incubated with enzyme and/or detergent under the above mentioned experimental conditions for 30 min at room temperature, given a gentle wash and dried.

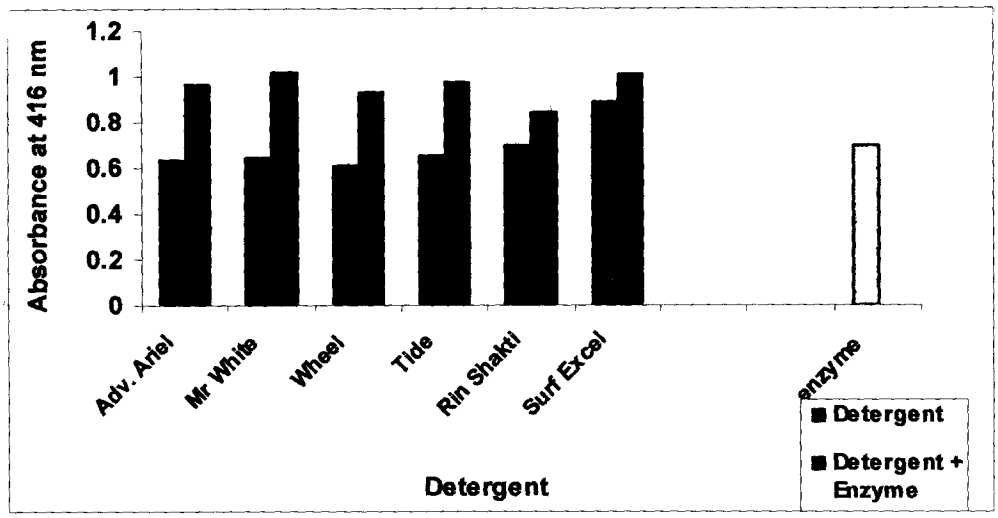


Fig 6.5 Solubilisation of blood in swatch assay

After the wash test performance on the test swatches, the washings were centrifuged and the absorbance measured.

Table 6.1 Comparison of wash performance in blood stain removal

Culture	Enzyme (U)	Temp (°C)	pH	Time (min)	Ref
Bacillus sp. APR-4	100	65	9.0	30	Kumar and Bhalla, 2004
Bacillus brevis SSA1	1600	60	8.0	15	Aftab *et al.* 2006
Pseudomonas aeruginosa PD100	5 (pure)	45-50	n.d	50	Najafi et al. 2005
Bacillus subtilis PE-11	10000	60	n.d.	15	Adinarayana et al. 2003
Thraustochytrium sp TZ	10	28	8.0	30	**Present study**

6.2.4 Dehairing of hide

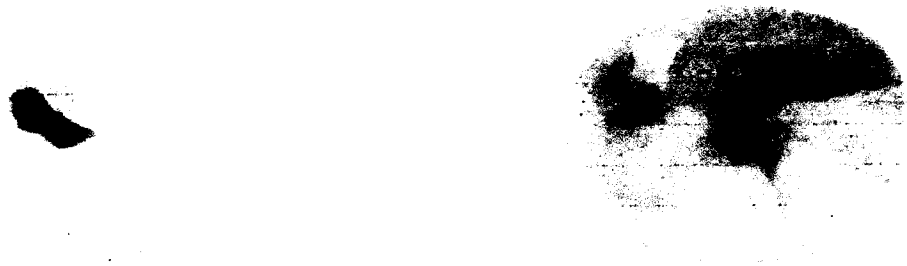
Another important area in which proteases are used is in the dehairing and smoothening of hides in the leather industry. The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards. The biotreament of leather using an enzymatic approach is thus preferable as it offers several advantages such as easy control and processing time and waste reduction, besides being ecofriendly (Anderse, 1998). Alkaline proteases with elastolytic and keratinolytic activity can be used to added advantage in leather-processing industries. The enzymatic treatment destroys undesirable pigments and increases the skin area, thereby producing clean hide. Bating is traditionally an enzymatic process involving pancreatic proteases. Recently however, the use of microbial alkaline proteases has become popular. Varela *et al.* (1997) reported the use of *B. subtilis* IIQDB32 alkaline protease for unhairing sheepskin. Hameed *et al.* (1996) used *B. subtilis* K2 alkaline protease in bating and leather processing. Alkaline proteases are able to speed up the process of dehairing because the alkaline conditions enable the swelling of hair roots and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair (Gupta *et al.*, 2002).

Two sets of experiments were carried out as described in Section 6.1.3. On the 4^{th} day dehairing was noticed in both sets of the experiments but the extent of dehairing was more when the hide was incubated with the growing culture (Fig 6.6a) rather than the enzyme extract (Fig 6.6b). The amount of amino acid

released is as given in Table 6.2.a. The decrease in dry weight of the skin as a result of the treatments is presented in Table 6.2.b.

These preliminary experiments could show that the enzyme degraded a substantial amount of protein, resulting in smoothening and dehairing of the hide and indicated a potential application in the leather industry for alkaline proteases from isolate TZ. Before exploiting its application in leather industry, the collagenolytic activity of this alkaline protease from T*hraustochytrium* sp.TZ need to be studied which will otherwise affect the texture and in turn, the quality of the leather.

From Table 6.3, it is evident that our alkaline protease from *Thraustochytrium* sp.TZ was indeed efficient in dehairing of hides, more so, considering that the experiment was performed at room temperature. Although it took 72 h for dehairing when the growing culture was used, it should be possible to bring down the time requirement from 72 h by adjusting the other parameters in the incubation.



Control **Hide + Inoculum**

Figs 6.6 a Dehairing of goat hide by the isolate TZ in culture

Goat hide was incubated with the growing culture of *Thraustochytrium* sp.TZ for 72 h. Control flask contained goat hide in the medium without inoculum.

Control **Hide + Enzyme extract**

Fig 6.6 Dehairing of goat hide by enzyme extract from isolate TZ

Goat hide was incubated in buffer (borate buffer, pH 8) with the crude enzyme extract from *Thraustochytrium* sp.TZ for 24 h. Control flask contained goat hide and buffer (borate buffer, pH8).

Table- 6.2.a Amino acids liberated (mg/ml)

Experiment I	**Control 1** (medium+ inoculum)	**Control 2** (medium + hide)	**Test** (medium+ inoculum + hide)
	0.590	0.820	3.060

Experiment II	**Control 1** (buffer)	**Control 2** (buffer + hide)	**Test** (buffer+crude enzyme extract + hide)
	0.575	0.750	1.250

Table-6.2.b Dry Weight of hide before and after the experiment

		Dry wt of the hide (g)	
		before the experiment	after the experiment
Expt I	Control 2	1.700	1.600
	Test	1.570	0.205
Expt II	Control 2	1.505	1.420
	Test	1.455	0.625

The following comparision (Table 6.3) highlights the performance of the *Thraustochytrium* protease in dehairing of hides.

Table 6.3 Comparison of hide dehairing performance

Source of enzyme extract	**Enzyme * activity added (U)**	**Temp (°C)**	**pH**	**Time (h)**	**Ref**
Bacillus pumilus 2006	2000	37	9.6	24	Wang *et al.*
Psedomonas aeruginosa PD 100	2 U/ml	50	8.0	3	Najafi *et al.2005*
Bacillus cereus MCM B-326	5860	RT	7.0	21	Zambare, 2007
Streptomyces nogalator	7.5 (pure)	RT	7.5-8.5	22	Mitra and Chakrabartty, 2005
Thraustochytrium sp TZ	25	RT	8.0	24	**(Present study)**

* unless specified all enzymes added were as crude preparations

6.2.5 Application in silver recovery from used X-ray films

The waste X-ray photographic films containing black metallic silver spread in gelatin are a very good source for silver recovery compared to other types of films. The amount of silver varies between 1.5 and 2.0% by weight. Various studies have been carried out over a long period of time to recover the silver from these wastes and most of them are patented. Since the emulsion layer containing silver contains the protein gelatin, it is possible to break it down using a proteolytic enzyme. The stripping methods using proteolytic enzymes obtained from various microorganisms (Fujiwara *et al.*, 1989; Fujiwara *et al.*, 1991) and alkali hydroxides (Nakiboglu *et al.*, 2001) for recovery of the silver have been used more often than the burning and oxidation methods. Well-known enzymes used in silver recovery from films are the alkaline proteases from *Bacillus subtilis.* It has been reported that it took 30 min at 50 to 60°C to decompose the gelatin layer when Subtilisin BPN, an alkaline protease from *Bacillus subtilis* strain N was used and treatment at 30°C increased the decomposition time to two to three hours (Fugiwara *et al.*, 1989). The enzyme from *Bacillus* 351 *subtilis* ATCC 6633 required less than 15 min at 50°C to decompose the gelatin layer while the other alkaline proteases took more than 20 min to act and the enzyme rapidly became inactive at these temperatures (Nakiboglu, 2001). The present alkaline protease from *Thraustochytrium* sp TZ showed better gelatin degrading ability even at room temperature.

The gelatin degradation from used X-ray film by alkaline protease from our isolate TZ is captured in Fig.6.7 and Table 6.4 gives the estimation of amino acids liberated. The results show that this alkaline protease can be efficiently

used for the recovery of silver from used X-ray films by degrading the gelatin layers on the films.

In summary, this Chapter highlighted the promising roles of the alkaline protease in the detergent, leather and photographic industries. The crude enzyme extract was stable in presence of several commercial detergents for at least 30 min at room temperature as well as at 50^0C. Protein stains (blood and egg yolk) could be successfully cleared from test fabrics during this period.

Actively growing cultures of the isolate brought about the dehairing of goat hide introduced into the medium. In view of its broad substrate specificity, the application of this alkaline protease in degradation of gelatin from X-ray films was also tested and the results were promising. This alkaline protease is thus an alternative enzyme for the industrialists.

Control **Test-1** **Test-2**

Fig 6.7 Gelatin stripping from used X-ray film

The X-ray film piece was incubated with crude enzyme extract (Test-2) for 1 h at 30°c. In the control, distilled water was used in the place of enzyme.

Table 6.4 Estimation of amino acids liberated during gelatin stripping

Sample	Amino acids released (mg/ml)
Control(X-ray film+ Water)	0
Test-1 (X-ray film + crude enzyme)	0.770
Test -2 (X-ray film + pure enzyme)	4.375

Chapter 7

Screening of Thraustochytrids for alkaline lipase production

Numerous species of bacteria, yeasts and moulds produce lipases with different properties and specificities but moulds are known to be more potent lipase producers (Choo *et al.*, 1998). These microorganisms produce lipases both by solid substrate and submerged fermentations (He *et al.*, 2004). In view of huge variation in applications, the commercial availability of lipases with specific characteristics is still a limiting factor. The search for new lipases with different characteristics and improving lipase production thus continue to be important.

With the advent of rapid methods for discovering novel enzymes or altering the properties of enzymes, there is paramount interest in the development of screening tools that can be used to search for the best performance with respect to a specific property. An efficient screening or selection system is thus an absolute prerequisite to identifying the lipase-producing species/clones from a diverse population as well as to pinpointing enzyme variants that display the desired properties.

Lipolytic activity is difficult to determine due to the fact that lipases are water soluble enzymes acting on water insoluble substrates. Additional factors such as the substrate concentration at the interface or the use of different detergents must therefore be taken into account to interpret the activity and the enzyme kinetics observed. For these reasons, a large number of methods for measuring the activity of lipases, or their inhibition, have been reported and reviewed (Beisson *et al.*, 1999;; Pencreac'h and Barrati., 1996). These methods differ based on the process used for substrate solubilization, on the

activity marker employed and on the detection system, all of which make any comparison of the results obtained difficult (Beisson *et al.*, 1999). Moreover, most of the methods used are not suitable for large number of assays because they are expensive, time-consuming or require larger quantities of substrates. There is thus an increasing utilization of chromogenic and fluorimetric assays, by far the simplest, the most reliable and the easiest for experiments (Sing *et al.*, 2005).

The activity of lipases can be assayed by monitoring the release of either fatty acids or glycerol from triacylglycerols or fatty acid esters. Further, since lipases act at the oil/water interface, change in the properties of the interface is an important criterion for measuring lipolysis.

While there are several methods available for lipase assay, the method to be selected would invariably depend upon the user's requirements and available resources. Of all, titrimetry is the most reliable and widely used procedure involving hydrolysis of fats and oils. Besides this, the *p*-nitrophenyl palmitate (*p*-NPP) assay is convenient when handling large numbers of samples.

a) Titrimetry This is one of the oldest and most widely used quantitative assays for lipase, on account of its simplicity, accuracy and reproducibility. Triolein (or as a cheap alternative, olive oil, which contains 70% triolein) is a generally used and internationally accepted substrate (Jensen, 1983). This pH-stat method is a highly sensitive as well as a quantitative method that can measure the release of even 1 μmol of released fatty acid/min. However, at a pH value of less than 7, where free fatty acids are not fully ionized, pH-stat

titration is either inaccurate or impossible to perform, even after introducing a correction factor (Gupta *et al.*, 2003).

b) Spectrophotometric assay *p*-Nitrophenyl esters of various chain-length fatty acids are generally used as substrates and release of *p*-nitrophenol is measured spectrophotometrically at 410 nm. Short-chain esters are water-soluble and therefore their hydrolysis provides a measure of esterase, rather than lipase, activity. The use of *p*-NPP as a measure lipase activity is, therefore, more appropriate (Gupta *et al.*, 2003).

This Chapter discusses the isolation of Thraustochytrids from mangrove ecosystems of Goa and the screening of these and other coastal isolates for alkaline lipase production.

7.1 Materials and methods

Sample collection and isolation of thraustochytrids were carried out as described in Chapter 2. All the 11 isolates which were screened for alkaline protease production were screened for alkaline lipase production also. *p*-NPP was purchased from Sigma Chemicals. All the other chemicals were of analytical grade. In all experiments, the measurements were carried out with duplicated parallel cultures.

7.1.1 Preparation of crude enzyme

MV medium supplemented with 0.5% olive oil was inoculated with the respective isolate and kept under agitation for four days. The broth culture was then centrifuged at 5°C at 10,000 rpm for 15 min and the residual oil was

removed by filtration through Whatman filter paper. The supernatant contained the crude enzyme and was stored in aliquots at -20°C until use in further experiments.

7.1.2 Qualitative detection of lipolytic activity

Qualitative screening for lipase production by thraustochytrids was performed in two ways:

i) All the isolates were checked qualitatively for lipolytic activity by spotting on MV agar medium containing 0.5% olive oil as substrate. The production of lipase was confirmed by the appearance of clearance zones on the plates. The ratio of the diameter of the clearance zone to that of colony served as the index for preliminary selection of protease high-yield strains.

ii) Chromogenic substrate plates were prepared by using Phenol Red (0.01%) along with 1% olive oil, 10 mM $CaCl_2$, and 2% agar. The pH was adjusted to 7.3–7.4 by using 0.1 N NaOH. The crude enzyme preparation was impregnated onto a small disc of filter paper (diameter 5 mm) and placed on the surface of the chromogenic substrate plates. The plates were incubated at room temperature for 15–30 min.The discs with heat-inactivated enzyme served as control (Singh *et al.*, 2005).

7.1.3 Quantitative estimation of lipolytic activity

The extracellular lipase activity was assayed according to Winkler and Stuckman (1979) and Sing *et al.*(2005). The substrate used was *p*-NPP. The

stock solution of *p*-NPP (8 mM) was prepared in isopropanol. The assay mixture containing *p*-NPP (0.16 mM), appropriately diluted enzyme sample and borate buffer (pH 9.0, 50 mM) in a total volume of 2.5ml was incubated at 50^0C for 10 min in a water bath. The reaction was stopped by chilling at -20°C for 5 min and the absorbance of *p*-nitrophenol released was measured at 410 nm. Appropriate enzyme blank and substrate blank were also included in the assay as controls. Enzyme activity was calculated by using the molar extinction coefficient of *p*-nitro phenol estimated under the same pH as the assay as 2.316 $\times 10^3$ $M^{-}cm^{-1}$ (Plummer, 1988). One unit of lipase activity (1 U) was defined in terms of micromoles of *p*-nitrophenol released per minute by the hydrolysis of *p*-NPP under the assay conditions. Protein was measured by the method of Lowry *et al.* (1951) using BSA as the standard.

7.2 Results and Discussion

The principle behind the qualitative detection of lipase on agar plates is the lowering of pH due to release of fatty acids upon lipolysis. A number of methods have been developed based on this principle using pH indicators such as Bromophenol blue, Bromocresol green, Victoria Blue, neutral Red, Crystal Violet, Methyl Red, etc. (Yadav *et al.*, 1998; Muller *et al.*, 2001; Snellman *et al.*, 2002). These methods, however, lack sensitivity as small pH changes go undetected due to the use of substrate at a pH range much farther from the dye end point.

In the present study, the chromogenic plates were prepared by incorporating Phenol Red along with a lipidic substrate. Phenol Red has an end point at pH

7.3–7.4 where it is pink and a slight decrease in pH turns it yellow. As soon as hydrolysis is initiated, the dye becomes yellow, indicating lipolysis and giving a fast and sensitive activity staining method. In order to avoid diffusion of the yellow zone of the fatty acids on prolonged incubation, $CaCl_2$ was introduced in the medium for quenching the fatty acids (Singh *et al.*, 2005).

In short, this technique is highly efficient, reliable, reproducible and rapid for qualitative detection of lipases and esterases on plates and gels. A simple laboratory pH indicator has been used, which does not affect the enzyme activity. The method is very sensitive and can detect as low as 0.5 *p*-NPP enzyme units within 15 min. It allows comparison between lipases and esterases.This method could thus prove to be of immense importance in the field of lipase research (Singh *et al*, 2005).

Plate assays are non-quantitative methods. Lipase activity is usually detected by the appearance of degradation haloes on culture media supplemented with mechanical emulsions of the desired substrates such as tributyrin, triolein or olive oil (Jaeger *et al.*, 1999). The release of fatty acids from these substrates can also be detected by adding pH indicators whose colour changes in response to the acidification produced by free fatty acids (Samad *et al.*, 1989) or by adding fluorophores such as Rhodamine B, which form complexes with fatty acids producing an orange–pink fluorescence under UV light irradiation (Kouker and Jaeger, 1987).

Lipolytic activity as detected on i) MV agar plate supplemented with 0.5% olive oil and ii) chromogenic agar plates are reproduced in Fig.7.1 a and b. The

result of qualitative screening for lipase production is given in Table 7.1. In order to select two of the most promising isolates with regard to lipolytic activity, after the initial qualitative screening, quantitation of alkaline lipase production was carried out using crude extracts from isolates. The results of quantitative estimation are summarized in Table 7.2.

Although qualitative screening for alkaline lipases tested positive for four isolates (A6T, AH-2, TZ and CW), the results of activity estimation confirmed that only two isolates (AH-2 and TZ of coastal and mangrove habitats, respectively) produced the enzyme in detectable quantity. The qualitative detection was performed at/near neutral pH. Since quantitative estimation was carried out at alkaline pH, the enzymes from isolates AH2 and TZ were alkaline lipases.

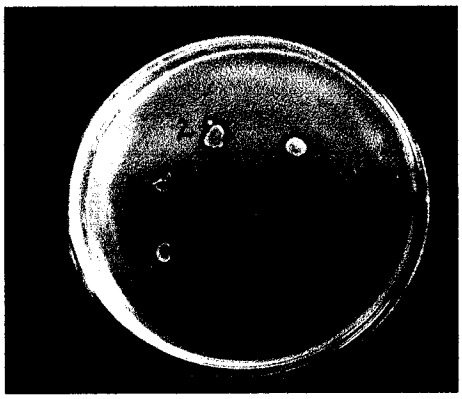


Figure 7.1a Detection of lipolytic activity on M V agar plates supplemented with 0.5% olive oil

Isolated thraustochytrid cultures were spot inoculated on MV agar plates supplemented with 0.5% olive oil showing the clearance zone.

1. Isolate A6T
2. Isolate AH-2
3. Isolate CW
4. Isolate TZ

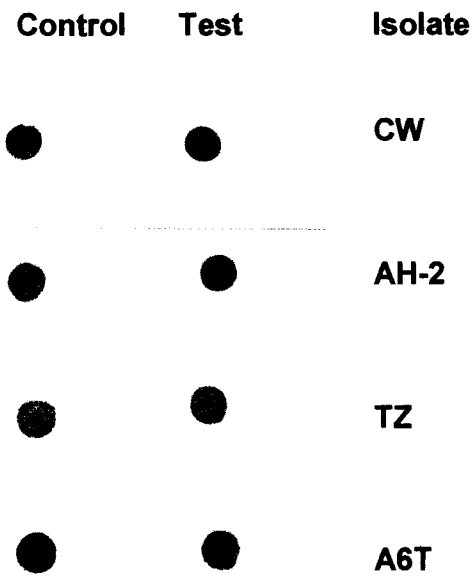


Figure 7.1b Detection of lipolytic activity on chromogenic agar plates

Filter paper discs impregnated with the crude enzyme preparations from the above mentioned isolates, placed on chromogenic substrate plate showing the lipolytic action. For each experiment, the heat-inactivated enzyme served as control.

Table 7.1 Qualitative screening for lipases

Habitat	Isolate	Ratio of clearance zone to colony size
Coastal	HOF1	0
	A6T	2.7
	AH-2	3.0
	7091	0
	Mar3	0
	MH1	0
	SL	0
	CW	2.0
Mangrove detritus	TC-1	0
	TC-2	0
	TZ	3.5

Table 7.2 Quantitative estimation of lipolytic activity

Isolate	Activity (U/ml)
TZ	0.3
AH-2	0.2
CW	N.D
A6 T	N.D

N.D. = Not detectable

Chapter 8

Optimization of growth conditions favoring maximum lipase production by selected isolates

Microbial lipases are mostly produced in submerged culture (Ito *et al.*, 2001) but solid state fermentation methods (Chisti, 1999) could also be used. Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production in submerged cultures. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and the dissolved oxygen concentration (Elibol and Ozer, 2001). Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield although a few authors have reported good yields in the absence of fats and oils. Most microbial lipases are extracellular. Optimisation of fermentation conditions for microbial lipases is of great importance, since culture conditions influence the properties of the enzyme produced. The amount of lipase produced is dependent on several environmental factors such as cultivation temperature, pH, nitrogen composition, carbon and lipid sources, concentration of inorganic salts and the availability of oxygen.

A few researchers have performed systematic medium optimization and fermentation studies for lipase production. The organisms are normally grown in a complex nutrient medium containing a carbon source (usually oil), a nitrogen source (organic/inorganic), phosphorus source (sodium or potassium phosphate) and mineral salts, supplemented with micronutrients (MgSO4 or CaCl2). The pH of the medium is generally maintained around 7.0. A pH range between 8.0-10.0 has been used for lipase production by alkalophillic bacteria (Horikoshi, 1990).

Optimization of growth conditions of the two selected thraustochytrid isolates with respect to physico-chemical parameters such as harvesting time, pH, shake *vs* static conditions, temperature and salt concentration of the culture medium as well as varying compositions of the medium is reported in this Chapter.

8.1 Materials and Methods

All chemicals used were of analytical grade and glass double distilled water was used at all times. In all experiments, the measurements were carried out with duplicated parallel cultures. Each data point plotted is a representation of mean ± S.D. of values analyzed in replicate from two independent experiments.

8.1.1 Harvesting Time

MV Broth containing 0 -1 % olive oil was inoculated with isolates TZ or AH-2, kept on a rotary shaker at room temperature and the enzyme production was monitored every 24 hr for a seven day period.

8.1.2 Growth pH

Production medium with pre-adjusted pH (3-11) with the respective buffers was inoculated with each of the isolates and kept under agitation for 4 days at room temperature. The enzyme production was then determined as described in Chapter 7.

8.1.3 Effect of temperature

MV Broth with 0.5% olive oil was inoculated with isolates TZ and AH-2, kept under the above mentioned optimized conditions at different temperatures and the enzyme production was determined.

8.1.4 Effect of crude salt concentration

MV Broth at various crude salt concentrations and containing 0.5% olive oil was used to grow the isolates under optimized conditions as above and the enzyme production was determined.

8.1.5 Effect of inducers

The enzyme production at 0.5% concentration of various oils (groundnut, sunflower, coconut, palm, gingely and olive oil) provided as inducers was studied under the above optimized culture conditions.

8.1.6 Medium composition

Different composition of media used for the enzyme production under the above optimized conditions were:

1. Crude sea salt (3.4%) + Glucose (0.4%) + 0.5% olive oil
2. Crude sea salt (3.4%) + Glucose (0.4%) + 1.0% olive oil
3. Crude sea salt (3.4%) + Peptone (0.15%) + 0.5% olive oil
4. Crude sea salt (3.4%) + Peptone (0.15%) + 1.0% olive oil
5. Crude sea salt (3.4%) + Yeast extract (0.01%) + 0.5% olive oil
6. Crude sea salt (3.4%) + Yeast extract (0.01%) + 1.0% olive oil
7. MV medium + 0.5% olive oil
8. Crude sea salt (3.4%) +Peptone (0.15%) + Yeast extract (0.01%) + 0.5% olive oil

8.2 Results and Discussion

In the present study, the various physico-chemical parameters optimized to obtain maximum alkaline protease production by the two thraustochytrids were

a) harvesting time b) growth pH c) agitation d) temperature e) concentration of crude salt f) effect of inducers and g) composition of the medium

8.2.1 Time course of enzyme production

Lipase production was studied for different time periods at room temperature in MV medium containing 0, 0.25, 0.5 and 1.0 % olive oil. The results showed that with 0.5% olive oil as inducer, the enzyme production by the isolates TZ (Fig 8.1) and AH-2 (Fig 8.2) was maximum at an optimum period of 96 h. There was no production of the enzyme in the absence of olive oil (data not shown) indicating the inducible nature of the enzyme production by both the isolates. The enzyme production which was initiated at as early as 24 h of incubation gradually reached a maximum by 96 h. So for all further experiments, time period of 96 h was chosen for maximum enzyme production by both isolates.

Similar results were reported for the extracellular lipase from *Fusarium solani* FS1 cultures where maximum activity was elicited after a 96 h incubation in medium containing 3% (w/v) peptone supplemented with different carbon sources (Maia *et al.*, 1999). In *Penecillium wortmanii* cultures, maximum lipase production was obtained in a 7-day old culture using olive oil (0.5%) as the carbon source (Costa and Peralta, 1999).

The inhibition of the synthesis of lipases at higher olive oil concentrations could be due to poorer oxygen transfer into the medium. Low oxygen supplies can alter fungal metabolism and consequently, the production of lipases (Lima *etal.*, 2003). A decline in enzyme production was observed after four days by both

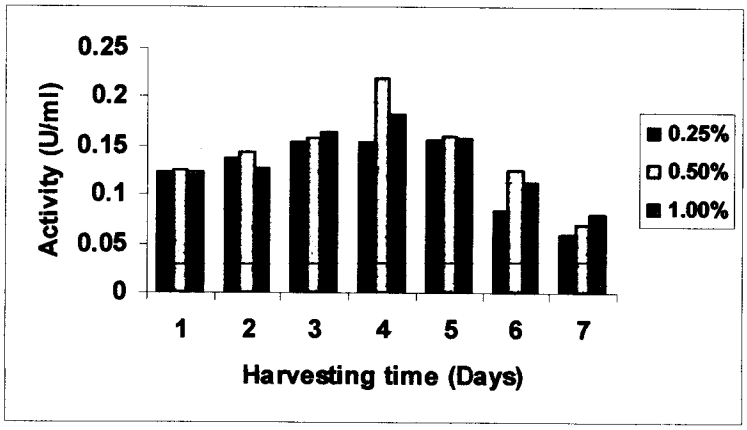


Fig. 8.1 Time course for the production of alkaline lipase by the isolate AH-2

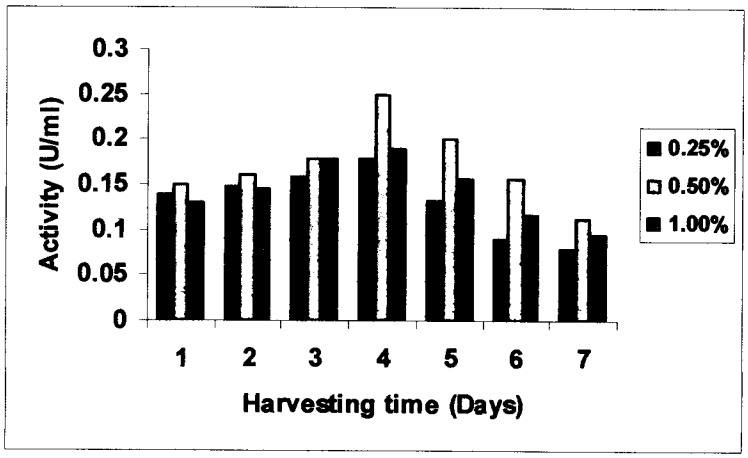


Fig.8.2 Time course for the production of alkaline lipase by the isolate TZ

Production conditions - The isolates were grown in MV medium supplemented with varying concentrations of olive oil for 7 days and the lipase activity was determined at regular intervals of 24 h.

Thraustochytrium spp. in the present study which could be attributed to end product repression by the accumulation of fatty acids. A similar kind of repression was reported in *Pseudomonas aeruginosa* EF2 (Gilbert *et al.*, 1991), *P. fragi* (Smith and Alfrod, 1966) and *Rhizopus japonicus* (Aisaka and Terada, 1979). Higher concentrations of olive oil also suppressed lipolytic activity in *Penicillium aurantiogriseum* (Lima *et al.*, 2003).

8.2.2 Optimum pH of the production medium

When the pH of the production medium was pre-adjusted to values ranging from 3.5-10, maximum lipase production was observed at pH 6.0 (Fig 8.3). On either side of this pH, the activity elicited dropped sharply by almost 50%. For all further experiments, a pH of 6.0 was maintained for the production medium.

The initial pH of the growth medium is important for lipase production. Maximum activity was observed at pH values beyond 7.0 for *P. fragi* and at pH 9.0 for *P. aeruginosa* wherein development of acidity in media reduced lipase activity (Saxena *et al.*, 1999). In contrast, maximum growth was reported for *Staphylococcus lipolytica*, *Mucor caseolyticus*, *M. racemosus*, *M. hiemalis Bacidiobolus licheniformis*, *Aspergillus wentii*, *Rhizopus nigricans*, *R. oligosporus*, and *Pseudomonas aeruginosa* EF_2 at acidic pH (4.0–7.0) (Gilbert *et al.*,1991). Lipase activity from *Aspergillus niger* MTCC 2594 was maximum at pH 6.5 (Kamini *et al.*, 1998) but differed from those from *A. niger* NCIM 1207 (Mahadik *et al.*, 2002) and from *A. carneus* (Saxena *et al.*, 2003) in that the latter were maximum at pH 2.5 and 9.0, respectively.

8.2.3 Agitation *vs* static condition

Enzyme production by the two isolates was studied for growth under static conditions or with agitation (120 rpm). The results clearly indicated superior lipase production under agitation (Fig 8.4). For all further experiments, isolates were grown under agitation for the production of lipase.

Aeration has variable effects on lipase production by different organisms. The degree of aeration appears to be critical in some cases since shallow layer cultures (moderate aeration) produced much more lipase than shake cultures (high aeration). Vigorous aeration greatly reduced lipase production by *Rhizopus oligosporus, Penicillum fragi, Pseudomonas aeruginosa* and *Mucor racemosus* (Gilbert *et al.,* 1991). However, high aeration was needed for eliciting high lipase activity by *Penicillum mephitica* var. *lipolytica*, *Aspergilus wentii* and *Mucor hiemalis*. Increasing aeration by shaking initially resulted in both increased growth and lipase production, followed by a rapid decrease of lipase activity as shaking continued (Saxena *et al.*, 1999). In contrast, Oso (1978) reported that stationary conditions in *Torulopsis emersonii* favored maximum lipase production.

8.2.4 Temperature

The effect of temperature on lipase production in culture was studied in the temperature range of 28-50°C as well as at 4°C. From Fig.8.5 it can be observed that the maximum production was at 30 ± 2°C by both isolates AH-2 and TZ. The isolates could neither grow nor produce lipases beyond 45°C.

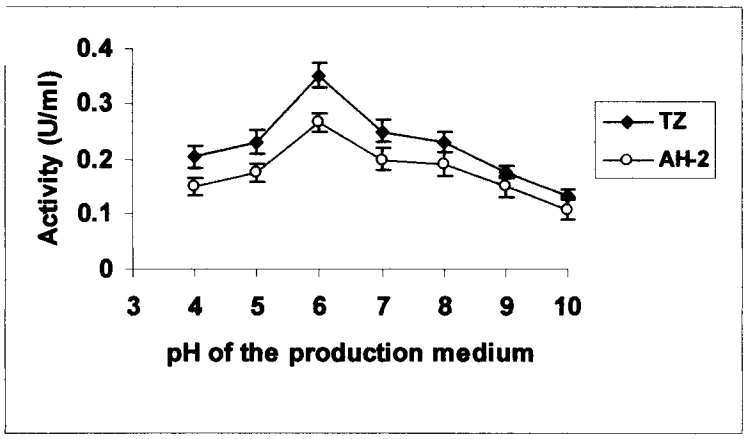


Fig. 8.3 Effect of pH on alkaline lipase production

Production conditions - MV medium containing olive oil (0.5%), varying pH ranging from 3.5-10, 30 ± 2°C for 96 h under agitation.

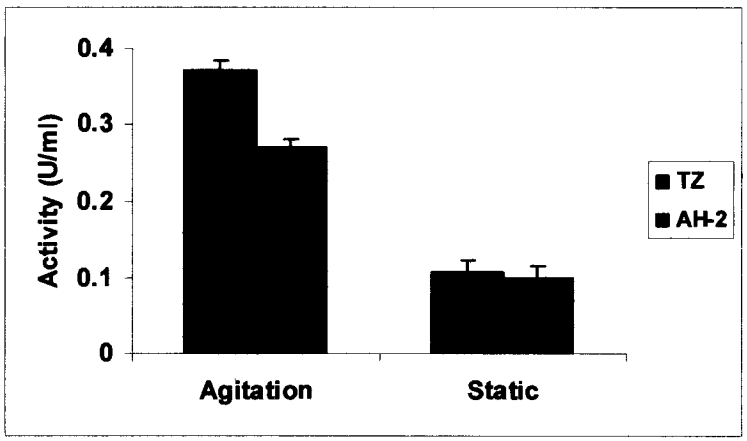


Fig. 8.4 Effect of static or agitation conditions

Production conditions - MV medium containing olive oil (0.5%) pH 6.0, 30 ± 2°C for 96 h under agitation or static culture, as indicated.

Interestingly, a low but not insignificant activity was elicited when grown at 4°C also. For all further experiments, a temperature of 30 ± 2°C was maintained for the production medium.

The influence of temperature on the production of lipases by fungi has not been extensively studied although temperature appears to be a crucial parameter. For example, for *A. niger* the optimum temperature for lipase production was 24°C and differences as little as 1°C could considerably decrease the yield (Ohnishi *et al.*, 1994). Cultures for production of lipases by fungi of the genus *Penicillium* are generally incubated between 25 and 30°C, most often at 28 °C (Lima *et al.*, 2003). Oso (1978) determined 45°C to be the best temperature for lipase production by *T. emersonii.* A broader temperatures range of 22–35°C was however reported to be conducive for maximum lipase production by *A. wentii* (Chander *et al.*, 1981), *M. heimalis* (Akhtar *et al.*, 1980), *R. nigricans* (Chander *et al.*, 1981) and *M. racemosus* (Chopra *et al.*, 1981).

8.2.5 Effect of crude salt concentration

MV broth with various concentrations of crude salt, 0.5% olive oil and was used as growth medium and the enzyme production was analyzed. The results (Fig 8.6) showed that a crude salt concentration of 3.4% resulted in maximum enzyme production although higher concentrations were also not very inhibitory. These results clearly suggested the prominent role of extracellular lipases in ecological sustenance of these organisms. Garcia *et al.* (1991) reported that maximum lipase activity in *A. niger,* occurred at an optimal salt

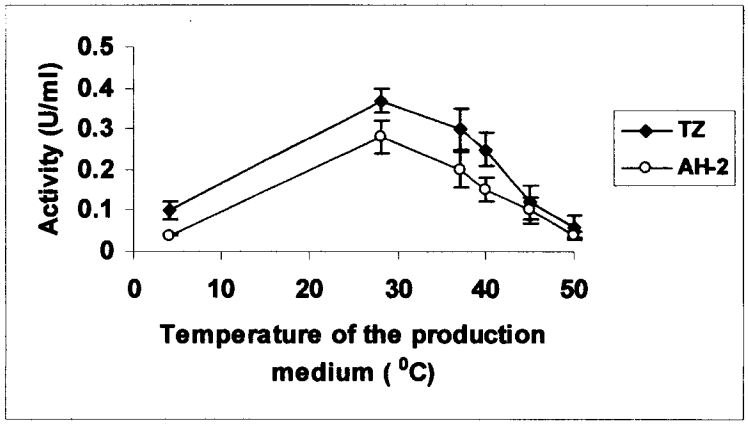


Fig. 8.5 Effect of temperature on production of alkaline lipases

Production conditions - MV medium containing olive oil (0.5%) pH 6.0, temperature range 30 ± 2°C-50°C for 96 h under agitation.

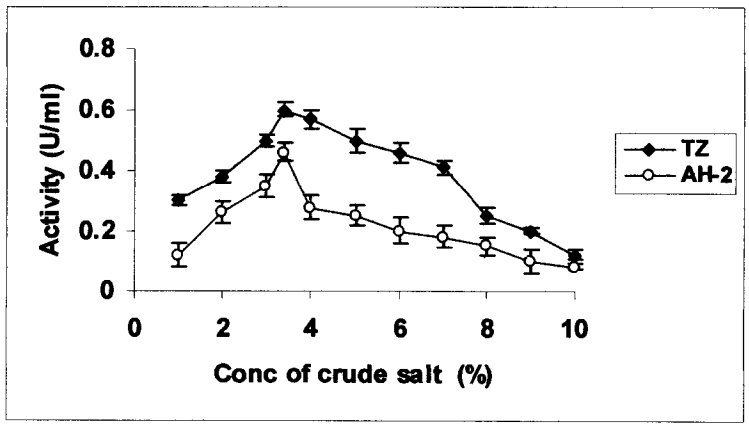


Fig. 8.6 Effect of crude salt concentration

Production conditions - MV medium at various crude salt concentrations (1-10%) containing olive oil (0.5%) pH 6.0, 30 ± 2°C for 96 h under agitation.

concentration of 2 mM and pH 7.5. Lee and Rhee (1993) and Toida *et al.* (1998) reported a similar salt requirement in the lipase production medium of *P. Putida* and *A.oryzae*. For all further experiments, a crude salt concentration of 3.4% was maintained for the production medium.

8.2.6 Effect of inducers

Extracellular lipase production by different microorganisms in medium supplemented with lipids has been extensively reported. It was demonstrated that lipase activity is induced by the presence of lipid substrates in the medium (Nutan *et al.*, 2002).The effect on enzyme production of various oils (such as groundnut, sunflower, coconut, palm, gingelly and olive, at 0.5% concentration) introduced as inducers was therefore studied and the results are presented in Fig.8.7. Olive oil at 0.5% concentration was found to be the best inducer for the production of lipase by both the thraustochytrids.

Although all the inducers tested resulted in enzyme production to varying extents, olive oil gave the maximum yield followed by sunflower, coconut, palm, gingelly and groundnut oils. Induction appears to be influenced not only by the lengths of fatty acids in these olis but also by the number of unsaturations. The manner by which these compounds influence lipase biosynthesis is yet not well understood (Lima *et al.*, 2003). The fatty acids present in the greatest proportions in these oils are oleic and linoleic acids. Better lipase production appears to be correlated with a higher content of oleic acid in the oil (Iwai and Tsujisaka, 1984). Among the above oils tested for the production of lipases by both the *Thraustochytrium* sp, olive oil was found to be the best carbon source

for lipase production probably because of its highest content of oleic acid (28%) and low linoleic acid (3 %) while those of other oils contain higher percentages of linoleic acid (~30-50%).

Similar reports on maximum lipase production in presence of inducers at optimal concentration were reported by several workers: in *Pe. Wortmanii* (olive oil) (Costa and Peralta, 1999), in *Aspergillus oryzae* (soyabean oil) and in *Candida rugosa* (olive oil) (Ohnish *et al.*, 1994). Higher oil concentrations could be affecting the aeration rate of the culture and promoting a delay in mycelial growth and lipase production in *A. niger* (Falony *et al.*, 2006).

8.2.7 Effect of different media compositions

Various combinations of components of MV medium (as outlined in Section 8.1.6) were used and the enzyme production compared. The results (Fig.8.8) showed that medium no.8 (containing peptone 0.15% and yeast extract 0.01%) was the best and led to maximum enzyme production. Presence of glucose appeared to inhibit the inductive efficiency of olive oil. Enzyme production was same (low) even at lower concentrations of glucose (up to 0.1%). Also increased amounts (up to 0.1%} of added yeast extract concentration did not show any betterment in enzyme production (data not shown).

Many researchers have reported the positive effect of sugars on lipase production in various species of bacteria and fungi (Salleh *et al.*, 1993). In present study, however, sugar substrates only favored the growth of the

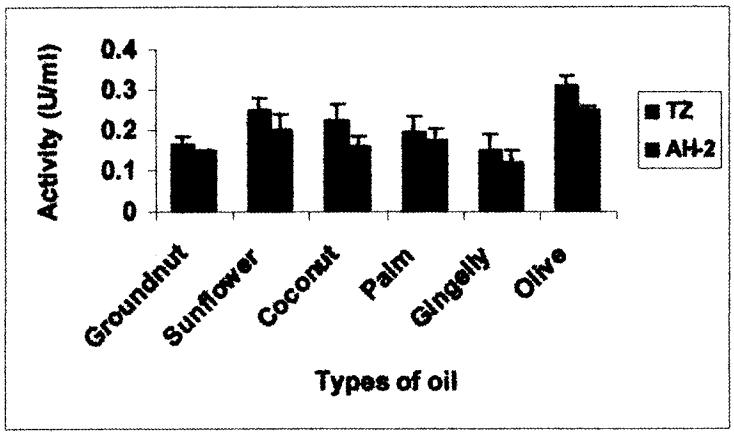


Fig. 8.7 Effect of inducers (0.5%) on production of alkaline lipases

Production conditions – MV medium containing various inducers at 0.5% concentration, pH 6.0, 30 ± 2°C for 96 h under agitation.

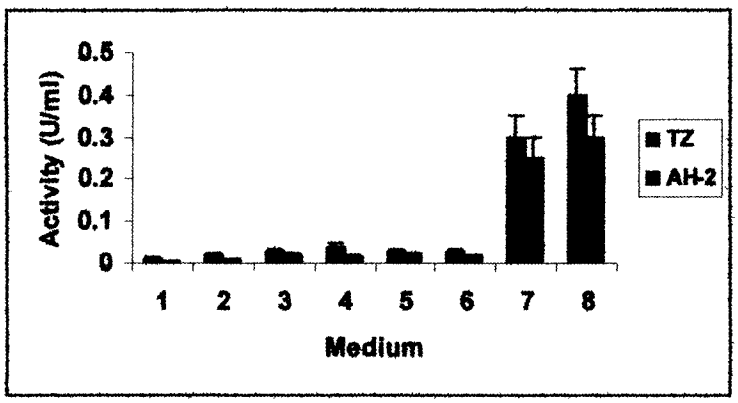


Fig. 8.8 Effect of different media on production of the alkaline lipases

Production conditions – various combinations of medium components (as in Section 8.1.6) with crude salt (3.4%), pH 6.0, 30 ± 2°C for 96 h under agitation.

microorganism but not the synthesis of lipase by both T*hraustochytrium* sp. Similar results where glucose inhibited lipase production were reported in *Pseudomonas fluorescens* 2D (Makhzoum *et al.*,1995) and in *P. aurantiogriseum* (Chahinian *et al.*, 2000).

Generally microorganisms provide high yields of lipase when organic nitrogen sources are used. Complex nitrogen sources such as yeast extract, peptones, soybean meal and corn steep liquor have traditionally been used for fungal lipase production (Sharma *et al.*, 2007). For fungi of the genus *Penicillium*, better results have been obtained with organic nitrogen sources or a combination of organic and inorganic sources (such as peptone or yeast extract with ammonium sulfate) than with inorganic compounds (such as ammonium sulfate) as the sole nitrogen source (Pimentel *et al.*, 1994; Freire *et al.*, 1997). Salleh *et al.*, (1993) obtained maximal production of extracellular lipase by the thermophilic fungus *Rhizop. oryzae* when the medium contained peptone as the nitrogen source. At a peptone concentration of 3% (w/v), the highest lipase production was obtained in medium containing 0.5% (v/v) olive oil and a decrease in peptone concentration to half caused a 2.5- fold decrease in lipase activity by *F. oxysporum* (Freire *et al.*, 1997). Thermostable lipase of *Pseudomonas sp.* KW1-56 was produced in a medium that contained peptone (2% w/v) and yeast extract (0.1% w/v) as nitrogen sources (Izumi *et al.*, 1990).

In the present study, media excluding glucose but with higher concentration of peptone (up to 1.0%) were tested, the results (Fig 8.9) showed that 0.5% peptone gave the best enzyme yield.

The harvesting time, pH and shake culture conditions were therefore again optimized with the medium composition now identified (MV medium containing 0.5% peptone, 0.01% yeast extract, 3.4% crude salt and 0.5% olive oil) for maximum enzyme production and the results are shown in Fig 8.10 a, b, c.

The optimum time for harvesting was increased from 4 to 7 days with increase in peptone concentration, while other parameters such as agitation and pH of the production medium remained the same. Iwai and Tsujisaka (1984) had noted that higher nitrogen concentrations typically used to increase lipase production resulted in prolonged growth period by fungi in general. The delayed lipase production (observed at increased peptone concentration) by both *Thraustochytium* spp in cultures appears to be of a similar nature.

In summary, MV medium containing peptone (0.5%), yeast extract (0.01%), crude salt (3.4%) and supplemented with 0.5% olive oil was found to be the most favorable culture conditions for maximum lipase production by both *Thraustochytrium* spp, under conditions of initial pH 6.0 for a time period of 7 days under agitation.

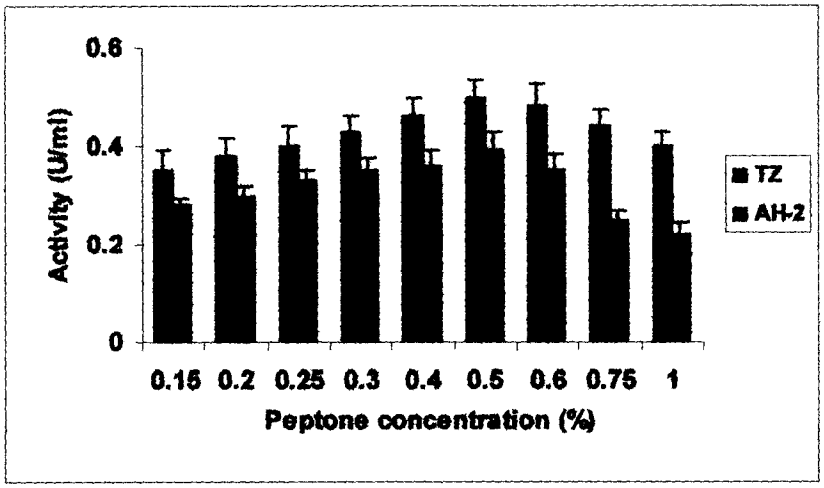


Fig. 8.9 Effect of peptone concentration

Production conditions -Medium 8 with varying peptone concentration (0.15-1.0%), olive oil (0.5%) crude salt (3.4%), pH 6.0, 30 ± 2°C for 96 h under agitation.

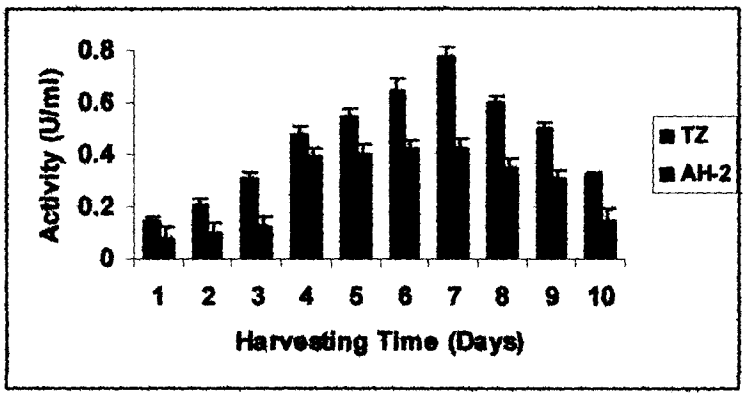


Fig.8.10 a Effect of harvesting time

Production conditions –Medium with 0.5% peptone, olive oil (0.5%) crude salt (3.4%), pH 6.0, 30 ± 2°C for 10 days under agitation.

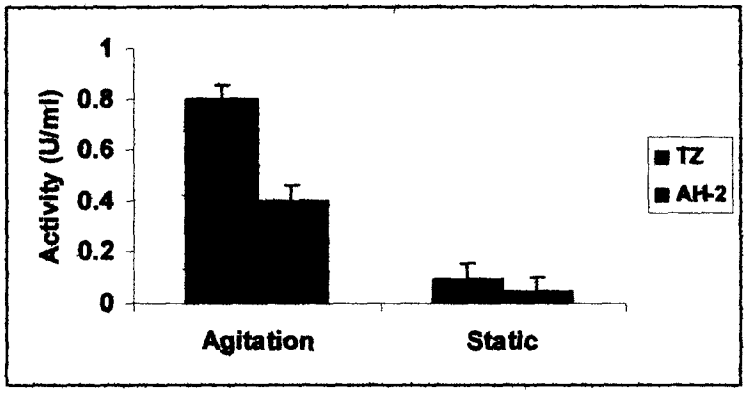


Fig.8.10 b Effect of agitation on production of alkaline lipases

Production conditions- Medium with 0.5% peptone, olive oil (0.5%) crude salt (3.4%), pH 6.0, 30 ± 2°C for 7 days under static conditions or agitation.

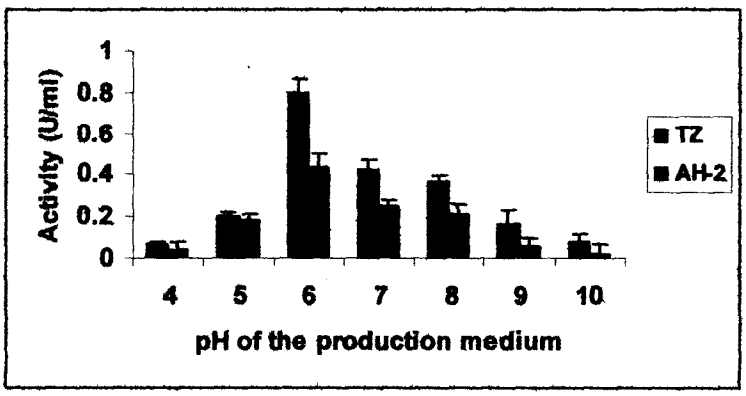


Fig.8.10 c Effect of pH on production of alkaline lipases

Production conditions- Medium with 0.5% peptone, olive oil (0.5%) crude salt (3.4%), pH 3.5-10, 30 ± 2°C for 7 days under agitation.

Chapter 9

Characterization of alkaline lipases from two isolates

Lipases possess a wide range of catalytic properties which are mostly strain-dependent. They have frequently been used in the form of a crude extract for synthesis of chiral building blocks and enantiomeric compounds. Catalytical properties such as specificity, enantioselectivity and operational parameters like thermostability and optimum pH, among others, are relevant because they define the enzyme application range and type of process (Pera *et al.*, 2006)

Although a large number of bacterial lipases with many different enzymological properties have been produced (Jaeger and Reetz, 1998), very few lipases with optimum activity under alkaline conditions were reported so far (Winkler and Stuckmann, 1979). The knowledge and expertise in new microorganisms capable of producing lipases as well as a greater understanding of their properties would be very useful in the application of such systems for the purpose of finding use in industries.

In this Chapter, alkaline lipases from the two *Thraustochytrium* spp. AH-2 and TZ have been characterized. Their properties such as optimum assay temperature, pH, basic enzyme kinetics and biotechnologically significant characteristics (pH and temperature stability metal tolerance and substrate specificity) are detailed. Based on the results, the more active of the two lipases would be selected for further studies.

9.1 Materials and Methods

Crude enzyme extracts from the two isolates TZ and AH-2 were produced as described in Chapter 7. All experiments were carried out in duplicate and each

data set is representative of two to three independent experiments. Each parameter is reported as the mean ± S.D.

9.1.1 Effect of enzyme concentration and time

Different amounts of enzyme protein were assayed in borate buffer in a final volume of 2.5 ml under the standard conditions as described in Section 7.1.3. The enzyme preparation was assayed for time periods up to 40 min in order to select the most appropriate assay time.

9.1.2 Effect of temperature on enzyme activity and stability

The experiment was carried out by incubating ideal amounts of the crude enzyme protein at different temperatures (28°-90°C) for 10 min with *p*-NPP in the presence of borate buffer (50 mM, pH 9.0) for assaying the lipolytic activity. Thermostability was analyzed after subjecting the culture filtrate to heat treatment for 10 min at various temperatures ranging from 40 to 90°C. The precipitated protein was then centrifuged down and the residual activity of the supernatant assayed under the standard conditions. The time course of thermostability was studied by subjecting the crude enzyme to heat treatment for different time periods at a selected temperature, prior to assaying under the standard conditions.

9.1.3 Effect of pH on enzyme activity and stability

The enzyme reaction was carried out in various buffers *viz,* acetate buffer (50 mM, pH 3.5- 5.0), phosphate buffer (100 mM, pH 6.0- 8.0) or borate buffer (15 mM, pH 8.0-10.0), other assay conditions being maintained as above.

The pH stability of the enzyme was analyzed by incubating the preparation with an equal volume of the respective buffer (pH range 3.5-10) for 1 h at room temperature (30 ± 2°C). The residual activity was then measured as per the routine assay procedure.

9.1.4 Substrate concentration studies

The enzyme extract was incubated with different concentrations of *p*-NPP during the assay under the above standard conditions. The kinetics of the reaction were studied by subjecting the data to Michaelis-Menten analysis.

9.1.5 Effect of EDTA and metal ions

The enzyme preparation from the isolate TZ was pre-incubated with varying concentrations of EDTA or metal ions (30 min, 30°C) and the residual activity of the enzyme were then assayed.

9.2 Results and Discussion

Each application of the enzyme requires unique properties with respect to activity, stability, temperature and pH dependence. The following properties of the lipases from the selected isolates AH-2 and TZ were thus studied with a view to characterize them and to understand their potential for industrial applications.

9.2.1 Effect of enzyme concentration and time

When different amounts of enzyme protein were assayed under the standard conditions as described above, the activity was found to be linear up to about

0.04 mg protein for lipases from both the isolates TZ and AH-2 (Fig 9.1a, b). All further assays were carried out using protein concentrations in this linear range. This dependence of the measured release of *p*-nitrophenol on enzyme protein concentration also confirmed that the estimation was an enzymatic process and ruled out any artifacts of measurement procedures.

This displacement from the linear curve as seen in Figs 9.1a and b could be due to i) the presence of small amounts of some impurity in one of the components of the incubation mixture other than the enzyme solution itself. This would poison the first amounts of added enzyme and it is only when enough of the enzyme is added to combine with the whole of the impurity that further amounts of enzyme will remain active or ii) the presence of a dissociable activator or coenzyme in the enzyme preparation (Dixon & Webb,1979).

The time course of lipase activity (Fig 9.2) showed that the activity from both isolates tended to plateau off beyond 10 min. An assay time of 10 min was hence chosen for all further experiments.

9.2.2 Effect of temperature

The effect of assay temperature on enzyme activity was studied by carrying out the assay at different temperatures under the standard experimental conditions. From Fig 9.3, it is seen that the enzyme was maximally active at 50°C beyond which the activity started declining. This temperature of 50°C was thus chosen as the standard assay temperature for the lipases from both the *Thraustochytrium* spp. being studied.

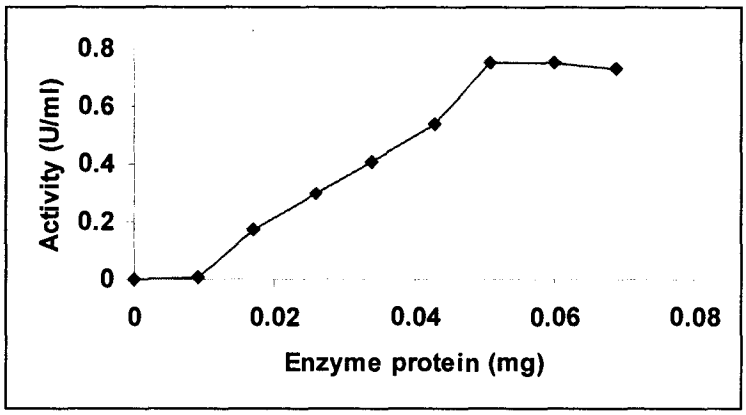


Fig 9.1a Enzyme concentration curve for lipase from isolate TZ

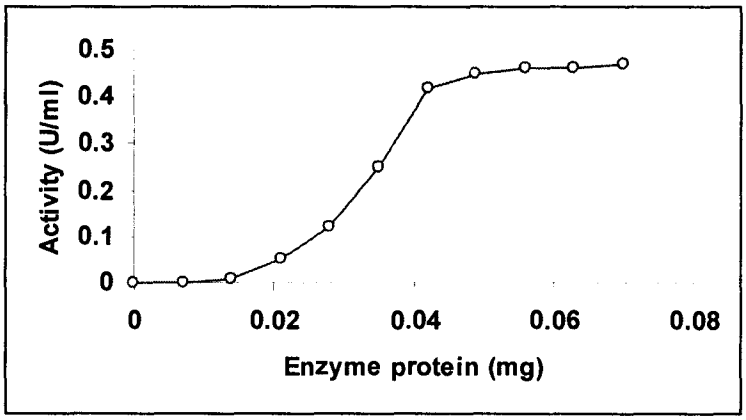


Fig 9.1 b Enzyme concentration curve for lipase from isolate AH-2

Lipase assay carried out at 50^{0}C,10 min, pH 9.0 using *p*-NPP as substrate and varying concentration of enzyme protein from isolates TZ and AH-2.

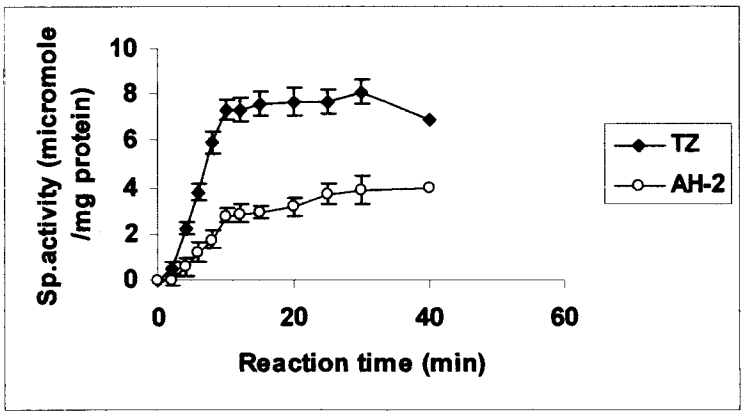


Fig. 9.2 Time course of enzyme activity

Assay conditions – pH 9.0, 2-40 min, temperature range 50°C using *p*-NPP as substrate and varying assay time as indicated.

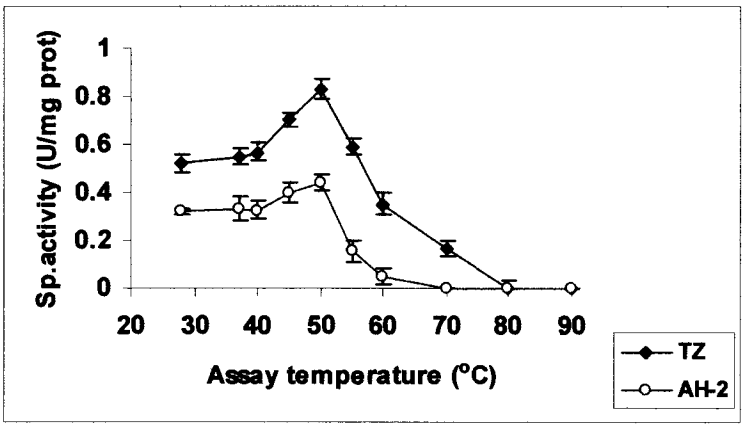


Fig. 9.3 Effect of temperature on enzyme activity

Lipase assay was carried out at pH 9.0 for 10 min at varying temperatures

The lipases produced by *Fusarium oxysporum* f. sp. *line* and *F. oxysporum* f. sp. *vasinfectum* showed optimum activity at 42°C and 45°C, respectively (Hoshino, 1992; Rapp, 1995). Toida *et al.* (1998) found the maximum lipase activity of *A.oryzae* at 40^0C and at higher temperatures, particularly above 55^0C, a marked fall in enzyme activity was observed. The lipase from *P. simplicissimum* lost its activity completely at 60^0C (Sztajer *et al.*, 1992). On the contrary, lipases from our *Thraustochytrium* sp.retained 71% (for isolate TZ) or 35% (for isolate AH-2) activity at 55^0C. Even at 60°C the lipase from isolate TZ was 42% active.

According to Razak *et al.*, (1997) very few fungal lipases exhibit temperature optima above 40°C. Generally lipases from *A. niger* strains have been reported to be active between 40 and 55°C (Kamini *et al*, 1998). Saxena *et al.* (2003) reported that *Aspergillus carneus* lipase showed optimum activity at 37°C. The lipase activity from *Rhizopus* sp.had a 50°C temperature optimum (Maria and Gláucia, 2006).

The optimal reaction temperature for the lipases from *Thraustochytrium* spp.reported in this study is also higher than that reported for many bacterial lipases (Snellman et al., 2002) under similar experimental conditions. Activity at high temperature is a useful characteristic for lipases that are used in detergent formulations.

Thermal stability results (Fig 9.4a) showed that the lipase activity was maximally stable for at least 10 min up to 50°C or at 45°C for the enzymes from

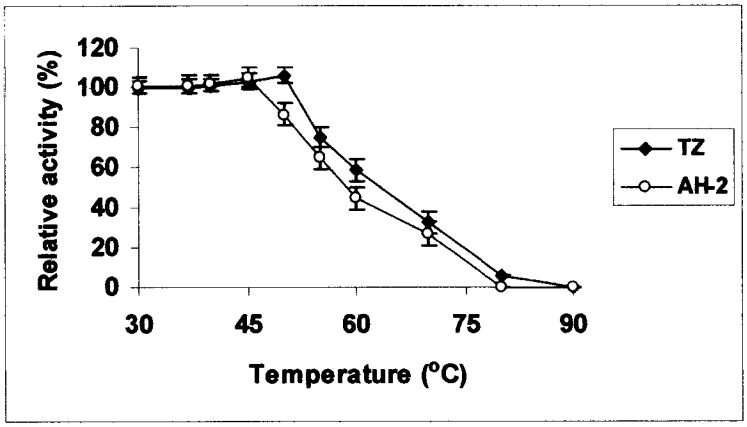


Fig.9.4a Thermal stability

Residual activities of enzymes from the isolates AH-2 and TZ after heat treatment for 10 min at 45°C and 50°C respectively. The activity of untreated enzyme was taken as 100%.

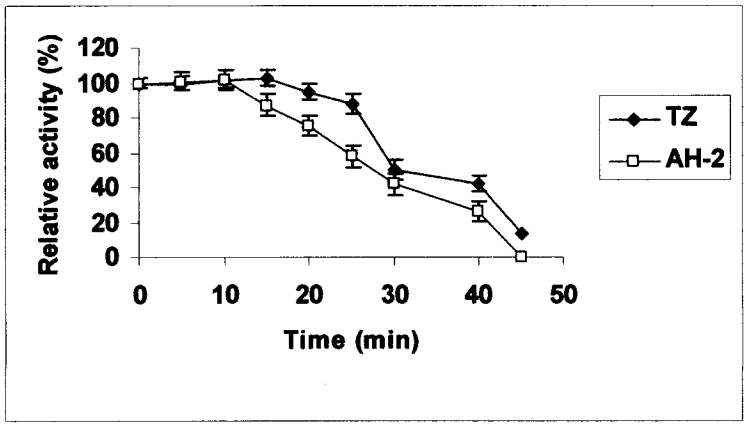


Fig. 9.4 b Time course of thermal stability

Residual activities of enzymes from the isolates AH-2 and TZ after heat treatment at 45°C and 50°C respectively for various time period. The activity of untreated enzyme was taken as 100%.

the isolates TZ and AH-2, respectively. When heat treatment at these respective temperatures was given for different time periods, it was observed (Fig 9.4b) that the enzymes from the isolates TZ and AH-2 could retain about 95 and 75% activity respectively, following a 20 min heat treatment.

The comparatively better thermotolerance during the assay (Fig.9.3) especially for the lipase from isolate AH-2, attributed to partial protection afforded by the presence of the substrate.

In recent years there has been a great demand for thermostable enzymes in industrial fields. Thermal stability of an enzyme is obviously related to its structure and is also influenced by environmental factors such as pH and the presence of metal ions. At least in some cases, thermal denaturation appears to occur through intermediate states of unfolding of the polypeptide. Attempts are being made to protein engineer lipases for improved thermal stability (Zhu *et al.*, 2001). Thus the observed thermotolerance of the extracellular lipase from *Thraustochytrium* sp. may be of significance.

9.2.3 Effect of pH on enzyme activity and stability

The lipolytic activity from isolates TZ and AH-2 was assayed at pH values ranging from 3.5-10 as described earlier and the results are presented in Fig 9.5a. The optimum pH value was found to be 9.0 for the enzymes from both the isolates TZ and AH-2 and more than 50% of the maximum activity was retained even at pH 11. Stability experiments showed that both the enzymes were highly stable at alkaline pH range, retaining 70 and 92% activity of the

enzymes, from the isolates AH-2 and TZ, respectively, after 1 h incubation (Fig 9.5b). These results reconfirm the alkaline nature of the lipases under study.

Most of the extracellular lipases have been known to possess neutral or alkaline pH optima (Sugiura *et al.*,1977; Yamamoto and Fujiwara,1988; Shabtai and Daya-Mishne,1992; Lesuisse *et al.*,1993; Schuepp *et al.*,1997; Lee *et al.*,1999). The optimal pH for the crude lipase activity were 7.0 for *Pe wortmanii* (Costa and Peralta,1999), 8-9 for *P. putida* 3SK (Lee and Rhee 1993), 6.5 for *P. burtonii* (Sugihara *et al.* 1995), 9-10 for *B. stearothermophilus* (Kim *et al.* ,1998), 5.5 for *A. oryzae* (Toida *et al.*, 1998), 7.0 for *M. hiemalis* (Hiol *et al.*,1999) and 5-6 for *A. niger* (Namboodiri and Chattopadhyaya, 2000).

The enzyme from the fungus *Cunninghamella verticillata* was stable up to pH 9.0 and lost its activity when the pH was raised above 9 (Gopinath *et al.*, 2002). Extracellular lipase of *F. oxysporum* f. sp. *vasinfectum* showed stability over the pH range of 4 to 10 during 1h incubation at 30°C (Rapp, 1995). On the other hand, others strains of *F. oxysporum* showed stability only at alkaline pH (Hoshino *et al*, 1992).

Triglycerides present in stains on fabrics are difficult to remove because they are hardly saponified compared to fatty acids. Lipases functioning would be useful in this regard. The pH studies on the lipase from the *Thraustochytrium* spp. AH-2 and TZ revealed that these enzymes could work best in alkaline environments and this characteristic of the enzyme would therefore have many

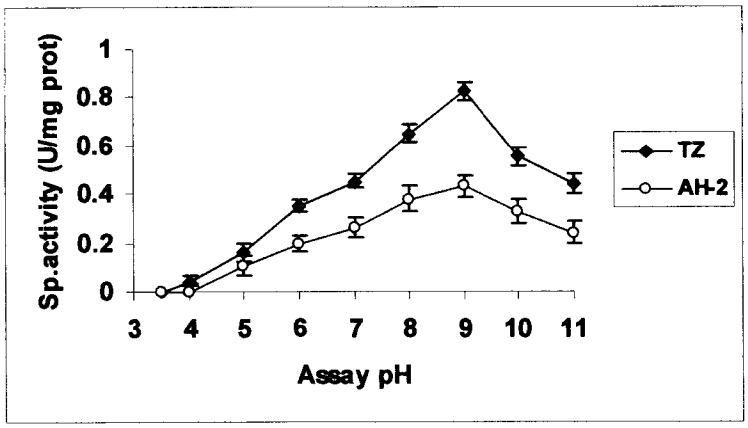


Fig. 9.5a Effect of pH on enzyme activity

Lipase assay carried out at 50^{0}C and 10 min at varying pH

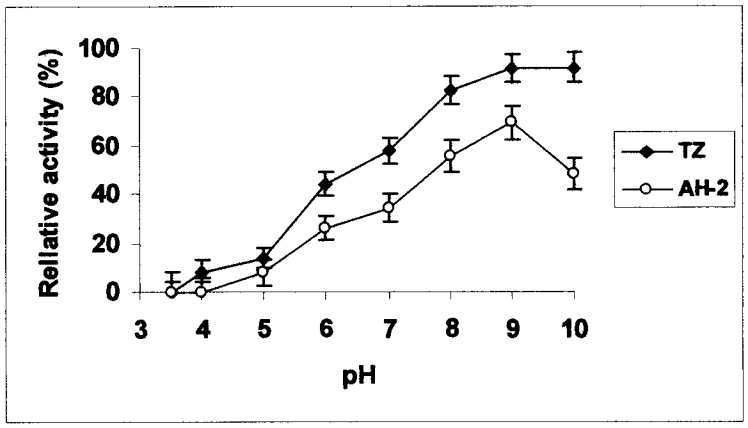


Fig.9.5b Effect of pH stability on enzyme activity

The crude enzyme extracts were incubated in buffers of pH 3.5-10 for 1 h at room temperature and the residual activity was calculated. The activity of un incubated enzyme was taken as 100%.

applications in industry and also in reactions performed under alkaline conditions.

A lipase that is stable at high alkaline conditions and high temperature is however rare (Savitha *et al.*, 2007) while in the present study we have isolated *Thraustochytrium* spp. which produced an inducible, extracellular, alkalophilic and thermotolerant lipase. There have been very few reports available to date with molds having alkalophilic and thermostable lipase. The lipases from the *Thraustochytrium* sp, reported in this research work could therefore be exploited for commercialization as enzymes of these characteristics find immense application as additives in washing powders.

9.2.4 Substrate concentration studies

Varying concentrations of *p*-NPP were used for the assay and the kinetics of the reaction were studied. The enzymes from both *Thraustochytrium* spp. showed saturation kinetics and the K_m values were 0.182 and 0.5 μmol/ml for the enzymes from the isolates TZ and AH-2, respectively (Fig 9.6a, b).

The K_m of lipase from *Fusarium solani* was 1.8 mM (Maia et al., 2001), from *Bacillus coagulans* MTCC-6375 was 28 mM (Kanwar *et al.*, 2006) and from *Acinetobacter* sp.B2 was 21.8 mM (Korean medical database, 2004).

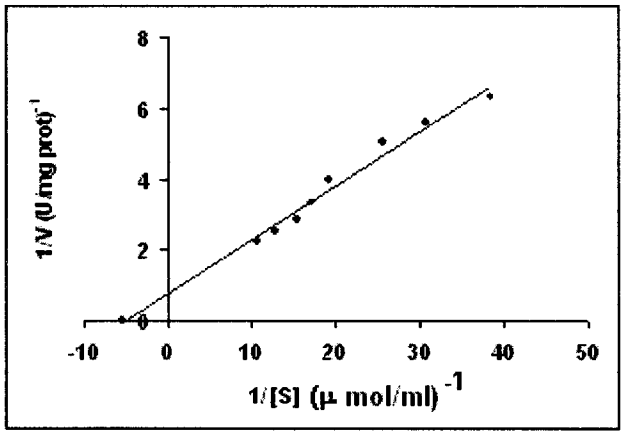


Fig.9.6a Lineweaver- Burk plot for the lipase from isolate TZ

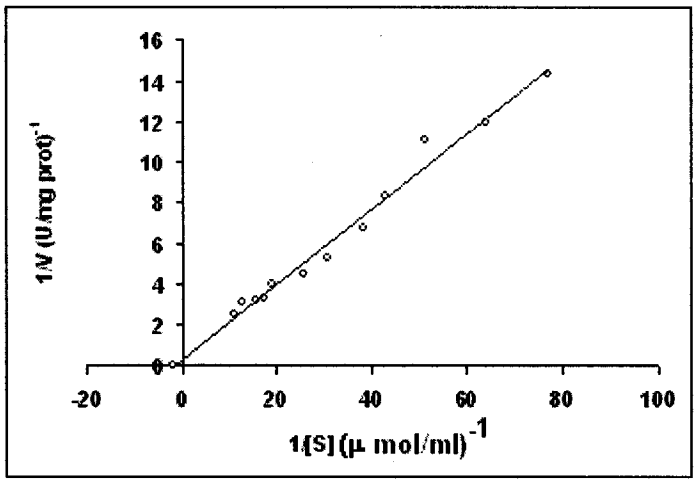


Fig.9.6b Lineweaver- Burk plot for the lipase from isolate AH-2

9.2.5 Effect of EDTA and metal ions

The effect of EDTA on lipase activity as shown in Fig.9.7 revealed that even at the lowest concentration (0.25mM) of EDTA tested, 50% of the activity was lost and as the concentration increases complete loss of activity was observed. Inhibition by EDTA probably results from its access to Ca^{2+} or Mg^{2+} binding site and ion removal. A similar inhibition by EDTA has been reported for a few other lipases also (van Oort *et al.*, 1989, Baral and Fox, 1997, Sharon *et al.*, 1998). An extracellular lipase, LipA, of *Acinetobacter* sp. RAG-1 was totally inactivated at 1mM EDTA. (Snellman *et al.*, 2002).

The effect of metal ions on lipase activity is shown in Table 4.1. Added magnesium ions marginally stimulated the enzyme activity. With calcium ions also, the activity was stable although no stimulation of activity was observed. There was total inhibition of activity by mercury, zinc, manganese, ferric and copper ions even at low concentrations of 1 mM.

Cofactors are generally not required for lipase activity, but divalent cations such as calcium and magnesium are known to often stimulate enzyme activity in microorganisms. This has been suggested to be due to the formation of the calcium salts of long-chain fatty acids. Rathi *et al.* (2001) observed stimulation in lipase production from *Burkholderia sp.* in the presence of Ca^{2+} and Mg^{2+}. Sharma et al. (2002) also reported stimulation of lipase production from *Bacillus* sp. RSJ1 in the presence of calcium chloride. Calcium- stimulated lipases have been reported in the case of *B. subtilis* 168 (Lesuisse *et al.,* 1993), *B. thermoleovorans* ID- 1 (Lee *et al.*, 1999), *S. hyicus* (van Oort *et*

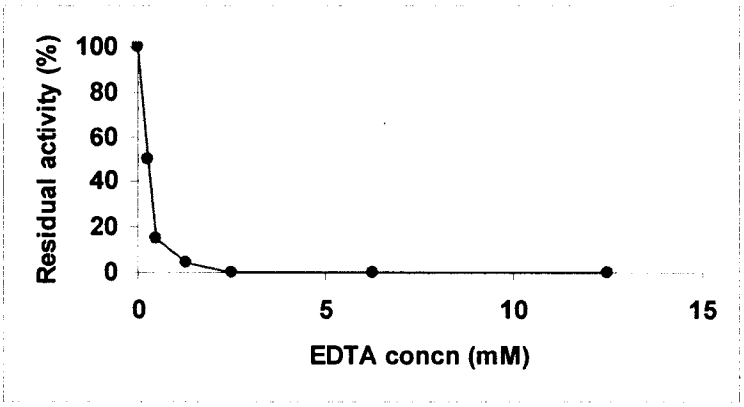


Figure 9.7 Effect of EDTA on enzyme activity from the isolate TZ

The crude enzyme extract from the isolate TZ was incubated in various concentrations of EDTA for 30 min at 30°C and the residual activity was assayed. The activity of untreated enzyme was taken as 100%.

Table 9.1 Effect of metal ions

Metal ion	Concentration (mM)	Relative activity (%)
Zn^{2+}	1	9.0
	10	0
Fe^{3+}	1	1.3
	10	0
Hg^{2+}	1	0
	10	0
Cu^{2+}	1	0
	10	0
Mn^{2+}	1	0
	10	0
Mg^{2+}	1	113.4
	10	100.4
Ca^{2+}	1	90.5
	10	92.4

al., 1989) and *Acinetobacter* sp. RAG-1 (Snellman *et al.*, 2002). In contrast, the lipase from *P. aeruginosa* 10145 (Finkelstein *et al.*. 1970) was inhibited by the presence of calcium ions.

Further, lipase activity is in general drastically inhibited by heavy metals (Kanwar et al. 2002) and metal ions like Zn^{2+} and Cu^{2+} are reported by several workers to have slight inhibitory effect on *Pseudomonas* lipases (Yamamato and Fujiwara, 1988, lizumi *et al.* 1990, Kumura *et al.* 1993, Chartrain *et al.* 1993). The present study also yielded similar results. Metal ions tested may have variable affects on lipase aggregation and on the substrate–water interface through interaction with free fatty acids (Snellman et al., 2002).

The characteristic features of the two alkaline lipases studied are summarized in Table 9.1

Table-9.2 Comparison of characteristics of crude enzymes from the isolates AH-2 &TZ

Property	Enzyme from AH-2 isolate	Enzyme from TZ isolate
Reaction time	10 min	10 min
Optimum temp	50°C	50°C
Optimum pH	9.0	9.0
K_m (μ mol/ml)	0.5	0.182
Thermal stability	at 45°C for 15 min	at 50°C for 15 min
pH stability	Stable at alkaline pH (≥70%)	Stable at alkaline pH (≥90%)

Between the two enzymes, the superior one (the lipase from isolate TZ) was selected for further studies in the following Chapters.

Chapter 10

Purification of an alkaline lipase from the isolate TZ

Industrial fermentations for the production of extracellular enzymes usually yield large volumes of fermentation broth. Efficient downstream processing and purification of these fermentation products is hence a key component affecting the economics of the process. Further, although enzymes are not always needed as homogeneous preparations, a certain degree of purity or quality is required in usages such as fine chemicals, pharmaceuticals and cosmetics. In addition, purified enzymatic preparations are also involved in applications such as drug targeting, identifying the primary amino acid sequence and more recently, determining the three-dimensional structure (Taipa *et al.*, 1992; Aires-Barros *et al.*, 1994). In the field of lipase research, innumerable examples are available wherein these enzymes have been purified and characterized.

Traditionally, the purification of a microbial lipase to homogeneity with a high yield and purity requires at least four to five steps. A critical overview on purification procedures (Antonian, 1988; Taipa *et al.*, 1992; Aires-Barros *et al.*, 1994; Palekar *et al.*, 2000; Saxena *et al.*, 2003; Gupta *et al.*, 2004) shows that the purification of lipases was, in most cases, achieved by a traditional multi-step chromatographic methodology, such as hydrophobic interaction and ion exchange. Alternative modern technologies such as aqueous two-phase systems and reversed micellar systems, which are comparatively less cumbersome and involve only one to two steps, are gradually coming to the forefront in the purification of lipases (Palekar *et al.* 2000; Saxena *et al.* 2003). The use of these techniques is however, limited because of the disadvantages of low yields, inefficient back-extraction protocols and difficult scale-ups. Today's industries thus look for purification strategies that are inexpensive,

rapid, high-yielding and amenable to large-scale operations. One such approach is the adsorption of an enzyme on a suitable matrix followed by its desorption.

Many reports on procedures that follow multi-step purification protocols have many a times shown yields of purified protein in the range of 50–60%. The fold-purification is highly variable, from as low as 1.7- and 2.6-fold, respectively, in the case of *Chromobacterium viscosum* (Vicente *et al.*. 1990) and *Bacillus* sp. THL027 (Dharmsthiti and Luchai, 1999) to as high as 7,760-fold in the case of *Bacillus* sp. (Sugihara *et al.*. 1991; Palekar *et al.*. 2000). This is because the final purification depends on the initial concentration of the contaminating proteins, which varies in different culture broths.

Most of the lipase purification schemes described in literature focused on purifying small amounts of the enzyme to homogeneity to characterize it. Little information has been published on large-scale processes for commercial purification of lipase. Most commercial applications of lipases do not require highly pure enzyme. Excessive purification is expensive and reduces overall recovery of the enzyme (Chisti, 1998).

This Chapter describes the sequence of steps through which the lipase enzyme from *Thraustochytrium* sp (TZ) was purified to homogeneity. The purified enzyme is then characterized in terms of its pH optimum and stability, temperature optimum and stability as well as enzyme kinetics, besides

analyzing the effect of inhibitors, metal ions, reducing agents, detergents and organic solvents.

10.1 Materials and Methods

Unless otherwise mentioned all procedures were carried out at 0 - 4°C. Each data set is representative of two to three independent experiments.

10.1.1 Heat treatment

The crude enzyme preparation was subjected to heat treatment at 50^0C for 10 min, chilled rapidly in ice, centrifuged and the supernatant was used for subsequent steps.

10.1.2 Gel filtration

A Sephadex G-100 column (130ml bed volume) equilibrated with borate buffer (25 mM, pH 9) was used. One ml fractions were collected at a flow rate of 20ml/ h by passing the same buffer down the column. Protein in each fraction was monitored spectrophotometrically at 280 nm and selected fractions were analyzed for lipolytic activity. The active fractions were pooled and concentrated using sucrose.

10.1.3 Native PAGE

Non-denaturing PAGE using a 10% gel was carried out at 50 mV during the stacking and at 100 mV for resolving the proteins. Coomassie Brilliant Blue R-250 was used to stain the protein bands (Laemmli, 1970).

10.1.4 *In situ* detection of lipase activity

Zymogram analysis was carried out according to Singh *et al.* (2005). Chromogenic substrate gel was prepared by using phenolphthalein (0.01%), 1% olive oil, 10 mM $CaCl_2$ and 2% agar. The pH was adjusted to 9.3 -9.4 by using 0.1 M NaOH.

Native PAGE (10% gel) was performed according to Laemmli (1970). The electrophoresed gel was rinsed three times with distilled water and equilibrated in 25 mM borate buffer (pH 9.0) for 30 min at room temperature. It was then overlaid with the chromogenic substrate gel and incubated at 30°C. Depending on the amount of lipase, the activity was observed within 5 -15 min as a colorless band over a pink background indicating lipolysis.

10.1.5 Molecular weight determination

Molecular weight of the pure enzyme was determined using a Sephadex G-100 gel filtration column (bed volume 130 ml) which was calibrated using standard protein markers. The different protein markers used were cytochrome *c* (12.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa) and BSA (67 kDa). A calibration graph of log molecular weight *vs* V_e/V_o was plotted, V_e being elution volume and V_o the void volume. Void volume of the column was calculated using Blue dextran.

10.1.6 Determination of subunit molecular weight

The subunit molecular weight of the enzyme was determined by running SDS-PAGE (12%) with standard protein markers. The markers used (97,400,

66,000, 43,000, 29,000, 20,100 and 14,100 Da) were the Hi-Media medium molecular weight range protein markers. The molecular weight of the enzyme protein was calculated based on the calibration graph of log molecular weight *vs* R_f value.

10.1.7 Characterization of purified lipase from *Thraustochytrium* sp TZ

Optimum pH and temperature, pH and thermal stability, reaction kinetics as well as the effect of EDTA and metal ions were studied using procedures as described in Chapter 9.

10.1.8 Effect of inhibitors and other additives

The effect of inhibitors (PMSF and iodoacetamide) on enzyme activity was studied by incubating the enzyme with different concentration of each inhibitor for 1 h at 30°C and then measuring the residual activity as per the routine procedure. The reducing agents tested were DTT, β mercaptoethanol and sodium thioglycolate at different concentrations (0.1, 0.5 and 1.0%). The effect of these reducing agents as well as detergents (anionic detergent- SDS at 0.1%, non-ionic detergents-Tween-80 &Triton X-100 at 0.1-1.0% concentration) on lipase activity was determined by pre-incubating the enzyme with the reducing agents or detergents for 30 min at room temperature before carrying out the routine assay. The effect of EDTA and metal ions on lipase activity was determined by the addition of the ions at final concentrations of 1.0 and 10 mM to the enzyme and then assaying under the standard conditions after pre-incubation for 30 min at room temperature.

10.1.9 Effect of organic solvents

Solvents such as acetone, methanol, ethanol, 2-propanol, acetonitrile, glycerol, diethyl ether, n-butanol or n-hexane (20%, v/v) were added to the reaction mixture and the lipase activity was determined under the standard assay conditions. A sample without organic solvents was taken as control (ie, 100% activity).

10.1.10 Positional specificity

Positional specificity of the lipase was examined by thin-layer chromatography of the reaction product obtained using triolein as a substrate. The reaction mixture composed of 0.5 g triolein, 5 ml borate buffer (50 mM, pH 9.0) and 20 U of the enzyme was incubated at 30°C for 60 min with magnetic stirring. The reaction product was then extracted with 20 ml of ethyl ether. Aliquots of the ether layer were applied on to a Silica Gel 60 plate (Merck) and developed with a 95:4:1 (v/v) mixture of chloroform, acetone and acetic acid. The spots were visualized with iodine vapour (Rashid *et al.*, 2001).

10.2 Results and Discussion

Many lipases have been extensively purified and characterized in terms of their activity and stability profiles relative to pH and temperature and effects of metal ions and chelating agents. The purification methods used have generally depended on nonspecific techniques such as precipitation, hydrophobic interaction chromatography, gel filtration and ion exchange chromatography.

Affinity chromatography has been used successfully in some cases to reduce the number of individual purification steps needed (Sharma *et al*, 2001).

Purification of the alkaline lipase from Thraustochytrium sp. TZ was carried out by a simple two-step procedure as detailed below.

10.2.1 Purification of the alkaline lipase from *Thraustochytrium* sp. TZ

The crude enzyme extract (50ml) was subjected to heat treatment at 50^0C for 10 min and then concentrated using sucrose. This sample was loaded on a Sephadex G-100 gel filtration column equilibrated with borate buffer (25 mM, pH 9). The elution profiles of protein and enzyme activity are shown in Fig 10.1. The profile showed one major protein peak which coincided with the lipase activity peak. The active fractions were pooled, concentrated using sucrose and used for further characterization. As seen from Table 10.1, after the gel filtration step, there was about 24-fold purification with a yield of 53%.

The above purified sample was then subjected to non-denaturing polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. A single band was observed on native gel (Fig 10.2, Lane 4) and one activity band on the zymogram (Fig 10.3, Lanes 3 & 4) confirming the homogeneity of the alkaline lipase preparation. As evident from fig 10.2, the crude enzyme showed at least three distinct protein bands on native gel stained with Coomasie Blue (Lanes 1 and 2). The heat treated sample showed two bands (Lane 3) with the total

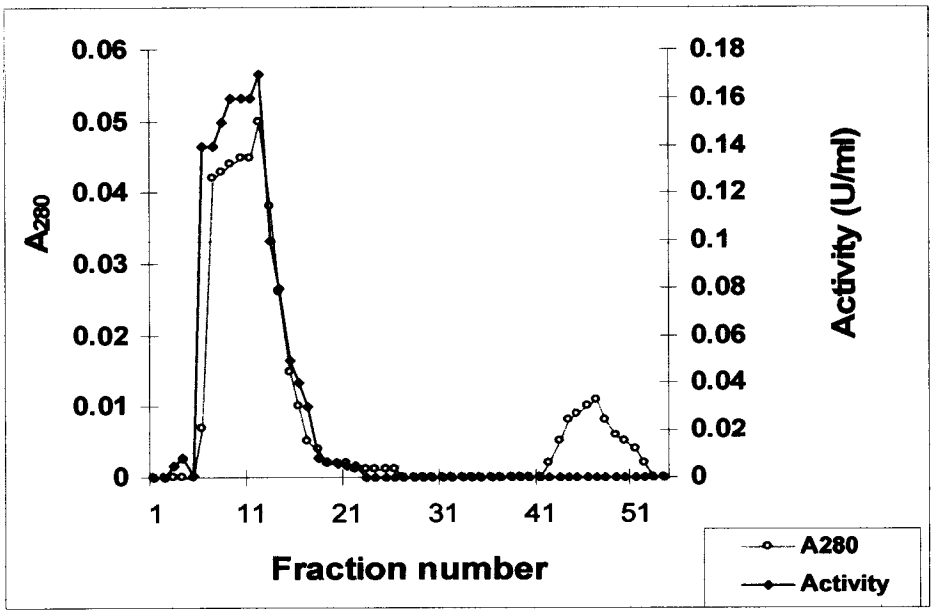


Fig.10.1 Elution profile of alkaline lipase on Sephadex G-100 column

The column was equilibrated with borate buffer (25 mM, pH 9.0). Elution was carried out with the same buffer at a flow rate of 20 ml/h.

Table 10.1 Purification table

Step	Volume (ml)	Protein (mg/ml)	Total activity (U)	Sp. Activity (U/mg prot)	Yield (%)	Fold purification
Crude	50	0.900	29.4	0.653	100	1.0
Heat treatment 50°C,10 min	50	0.838	38.0	0.906	129	1.39
Sephadex G-100	1	1.0	15.6	15.6	53	23.89

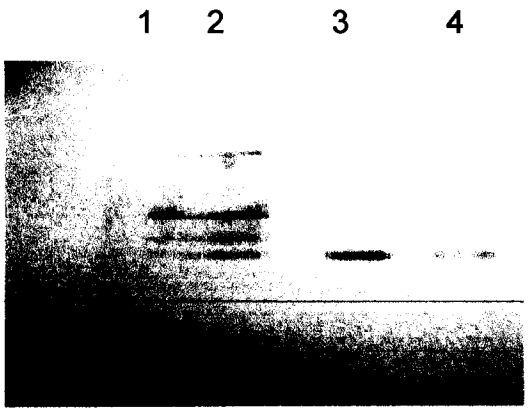


Fig. 10.2 Native PAGE

Lane 1 & 2 : Crude Enzyme

Lane 3 : Heat treated (50^0C, 10 min) enzyme

Lane 4 : Purified enzyme

Fig. 10.3 Zymogram

Lane 1 : Crude enzyme

Lane 2 : Heat treated (50° C, 10 min) enzyme

Lane 3 & 4 : Purified enzyme

elimination of one protein band. Further purification step using Sephadex G-100 resulted in a single protein band (Lane 4).

When electrophoresed on SDS-polyacrlamide gel with or without β-mercaptoethanol a single band was obtained (Fig 10.4, Lanes 3 and 5).

10.2.2 Molecular weight determination

The molecular weight of the purified alkaline lipase was determined by comparison with the mobility of standard marker proteins during molecular exclusion chromatography on a matrix as detailed in Section 10.1.5. The native protein appeared to have a molecular weight of 66 kDa (Fig 10.5).

Comparison with the mobilities of the protein markers (Fig 10.4, lane 1) on SDS-PAGE yielded a subunit molecular weight of 66 kDa (Fig 10.6) confirming the monomeric nature of the lipase, the calculated molecular weight of the native as well as SDS-treated protein being identical.

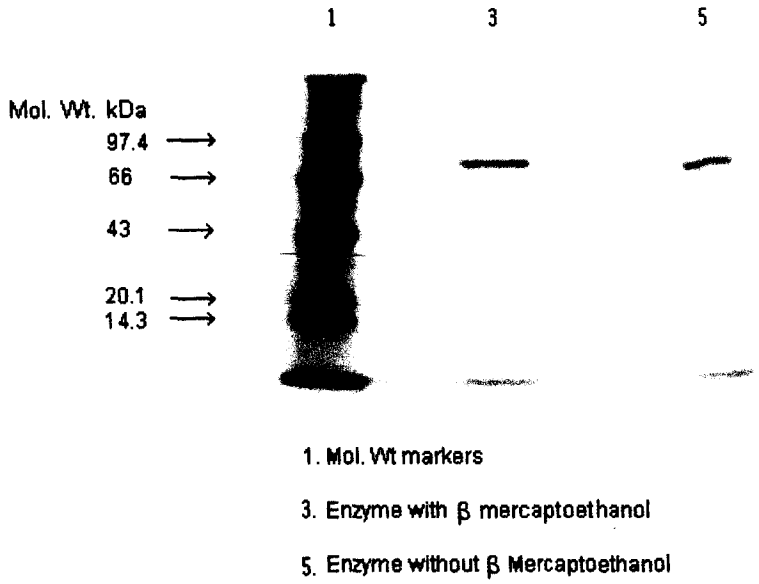


Fig.10.4 SDS PAGE

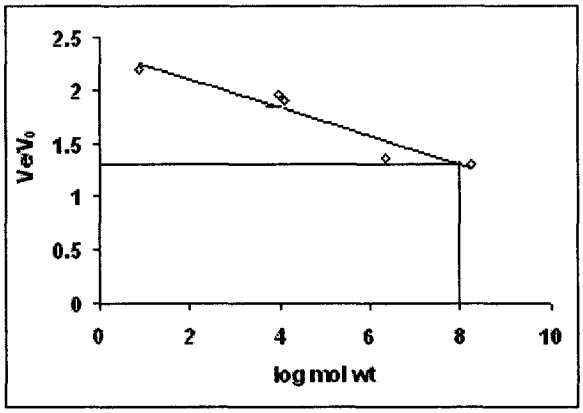


Fig. 10.5 Molecular weight determination by Gel filtration

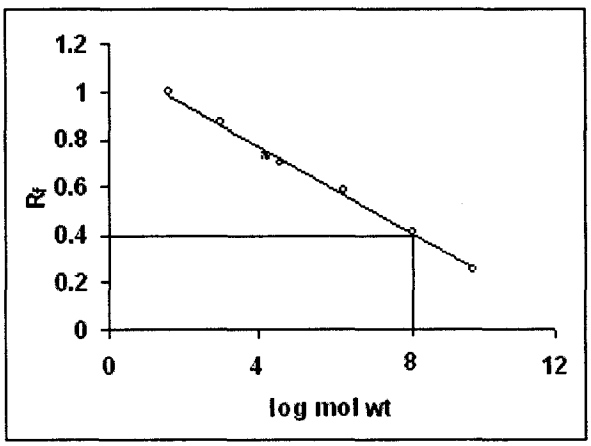


Fig. 10.6 Molecular weight determination by SDS PAGE

10.2.3 Properties of the purified enzyme from isolate TZ

The different properties studied for the purified alkaline lipase are discussed in this Section.

A initial lag period was noticed while studying the enzyme concentration dependent kinetic reaction which was similar as that of the crude lipase preparations. This initial lag in the enzyme concentration curve should not be confused with allostearism. This could be due to the same reason as discussed for crude lipases in Section 9.2.1. Alternatively, this lag could also be due to the special characteristics of the catalysis of lipolytic enzymes as proposed by Verger *et al.*(1980), described in Section1.3.2.1.

10.2.3.1 Effect of temperature on enzyme activity and stability

The optimum temperature of the purified enzyme was studied by carrying out the assay at different temperatures. The temperature optimum (50°C) did not change after the purification, remaining the same as for the crude enzyme (Fig 10.7).

Although high temperature optima have been reported for several lipases from *Pseudomonas* spp. (Gilbert, 1993), the optimal reaction temperature observed for the T*hraustochytrium* sp.TZ lipase is higher than that reported for many bacterial lipases under similar experimental conditions. Activity at high temperature is a useful characteristic for lipases that are used in detergent formulations and biotransformations. Such optima of around 50-60 °C have

been reported for lipases of other *Pseudomonas* spp. also (Yamamato and Fujiwara, 1988; Iizumi *et al.*, 1990; Kulkarni and Gadre, 1999).

When enzyme stability at different temperatures was studied by incubating the lipase at different temperatures for 10 min, the activity was maximally stable at 50°C 9Fig 10.8 a). Further when subjected to heat treatment for different time periods at 50°C, it was observed that the enzyme could retain full activity at the end of a 15 min heat treatment (Fig.10.8 b) as was the case for the crude enzyme preparation also.

The lipases from thermophilic bacilli are relatively more stable at higher temperatures (above 60°C) than those from mesophilic organisms. Lipases from mesophilic *Pseudomonas* sp. are generally stable up to 50°C, although there are a few reports of more thermostable lipases from *them* (Iizumi *et al.*, 1990; Lin *et al.*, 1996). Most of the lipases from *Penicillium* strains showed thermal stability up to 40± 45°C (Ferrer *et al.*, 2000). In comparison, the present reported lipase from thraustochytrid TZ appears to have a superior thermal stability.

10.2.3.2 Effect of pH on lipase activity and stability

When assayed over a range of pH, optimum activity of the lipase was detected at pH 9.0 (Fig.10.9a). Most of the bacterial lipases are reported to have pH optima on the alkaline side. The lipases from *Staphylococcus aureus*, *S. epidermis* and *Propionibacterium acnes* were found to be exceptions to other

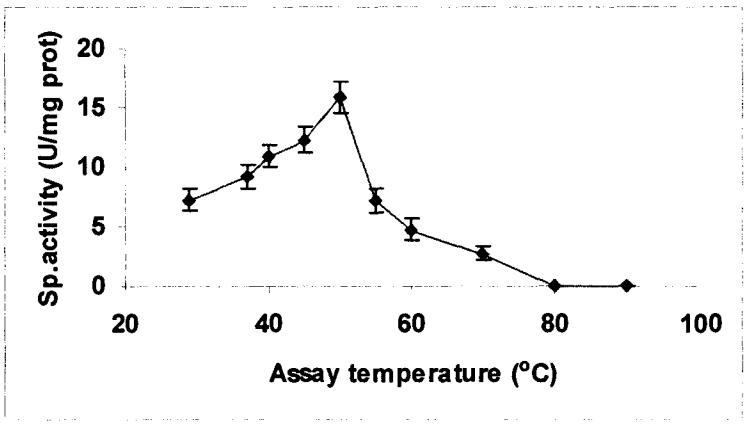


Fig. 10.7 Effect of temperature

Assay conditions – pH 9.0, 10 min, temperature range 28°C-90°C

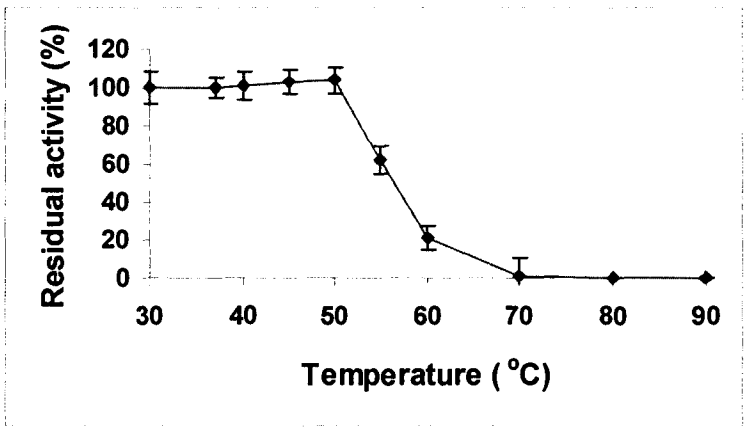


Fig.10.8 a Thermal stability

The pure enzyme was subjected to heat treatment at the specified temperatures for 10 min. The activity of untreated enzyme was taken as 100%.

bacterial lipases, with pH optimum around 6 (Ingham *et al.* 1981, Simons *et al.* 1996 and 1998). Talon *et al.* (1995) have reported a pH optimum of 9 for *S.warneri.* S. *haemolyticus* and *S. hyicus* also have alkaline pH optima (Rosenstein and Götz, 2000). *Pseudomonas* lipases are reported to have optimum activity in acidic (Iizumi *et al.* 1990) as well as alkaline (Sztajer *et al.* 1991, Kojima *et al.* 1994, Castellar *et al.* 1997) environment.

Studies on pH stability after 1 h incubation of the enzyme at 30°C in buffers of different pH (3.5 to 10) indicated stability towards alkaline pH, where more than 90% of the original activity was retained at least up to pH 10 (Fig.10.9b) as in the case of the crude enzyme preparation (Fig 9.5b).

The lipases reported from *Pseudomonas* and *Bacillus* spp.are generally stabler in the alkaline pH range. The *Staphylococcus aureus*, *S. epidermidis* and *Propionibacterium acnes* lipases were again an exception to this, being more stable in the acidic range (Ingham *et al.* 1981). Sztajer *et al.* (1988) had reported that a lipase from *Pseudomonas fluorescens* retained more than 80% of its activity following treatment at a pH range of 7-11.5 for 1h. Prazeres *et al.* (2006) had reported a lipase from *F.oxysporum* to be stable over the pH range 7-9.5. A lipase from *Pseudomonas* sp. was found to be stable over the pH range 5-9 with more than 70% activity retention for 2 h (Kulkarni and Gadre 1999). Our lipase from *Thraustochytrium* sp TZ was found to be stable in the pH range 8-10, with more than 90% activity retained which is an attractive property in relation to detergent industry.

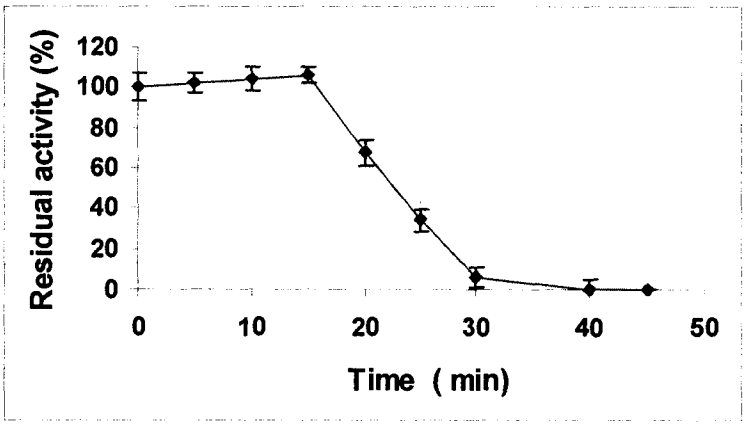


Fig.10.8 b Thermal stability at 50^{0}C

The pure enzyme was subjected to heat treatment at 50^{0}C for varying time periods and the residual activity estimated. The activity of untreated enzyme was taken as 100%.

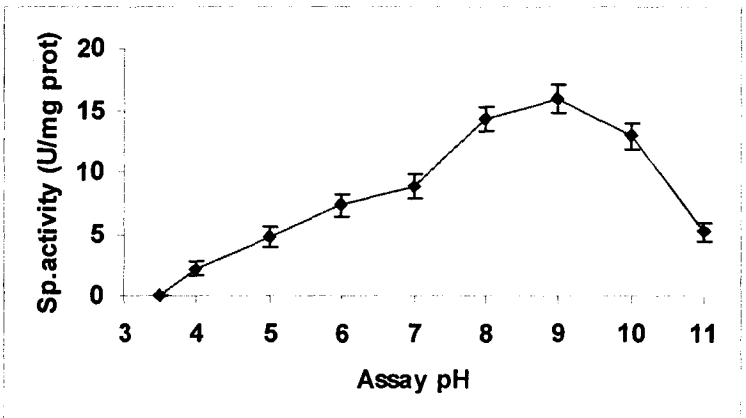


Fig.10.9 a Effect of pH on enzyme activity

Assay condition–varying pH range 3.5-10.0, 50^{0}C, using p-NPP (0.16 mM) as substrate

10.2.3.3 Effect of substrate concentration

The effect of substrate concentration on enzyme activity was studied by varying the substrate (*p*-NPP) concentration in the assay at optimum pH and temperature. The activity typically followed Michaelis–Menten kinetics (Fig.10.10a). A maximum reaction velocity of about 20 U/mg was attained and the K_m was determined as 0.019 μmol/ml (Fig. 10.10 b). The purified lipase showed approximately 17-18 fold increase in affinity towards the substrate *p*-NPP as compared to the crude enzyme from *Thraustochytrium* sp TZ.

In many cases, lipases appear to obey Michaelis–Menten kinetics (Guit *et al.*, 1991; Malcata *et al.*, 1992). The K_m values of the enzyme, in general, range widely but for most industrially relevant enzymes, K_m ranges between 10^{-1} and 10^{-5} M (Fullbrook, 1996). Pabai *et al.* (1995) reported K_m and V_{max} of a purified lipase of *P. fragi* CRDA 323 as 0.7 mg/mL and $0.97X10^{-3}$ U/min, respectively. For a *P. cepacia* lipase, Pencreac'h and Baratti (1996) reported K_m and V_{max} values of 12 mM and 30 mmol/min, respectively, when the substrate was *p*-NPP. The purified enzyme from *Rhizopus* sp. showed a K_m value of 2.4 mML and a V_{max} of 277.8 U for the substrate *p*-nitrophenyl laurate (Maria and Gláucia, 2006). The lipase from *Bacillus coagulans* MTCC-6375 had a Vmax and K_m of 0.44 mmol $mg^{(-1)}$ $min^{(-1)}$ and 28 mM, respectively, for hydrolysis of pNPP (Kanwar *et al.*,2005).

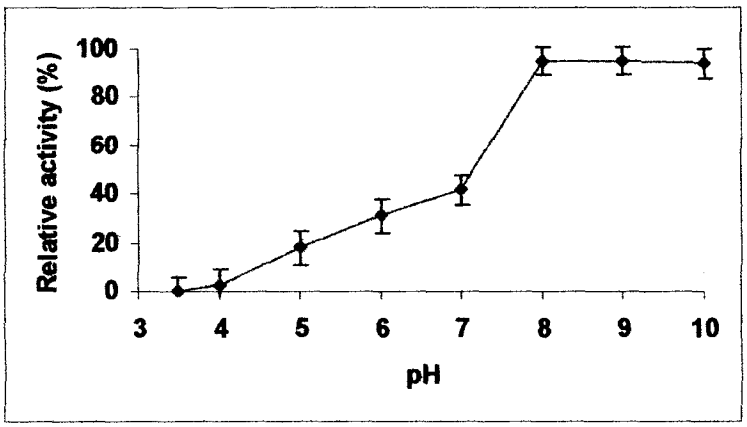


Fig.10.9 b pH stability profile

Assay conditions- The pure enzyme was incubated in buffers of pH 3.5-10 for 1 h at room temperature. The activity of untreated enzyme was taken as 100%.

10.2.3.4 Effect of inhibitors

Lipase inhibitors have been used in the study of structural and mechanistic properties of lipases. Lipases belong to the class of serine hydrolases with the catalytic triad as Ser-His-Asp/Glu. Therefore, serine inhibitors are potential

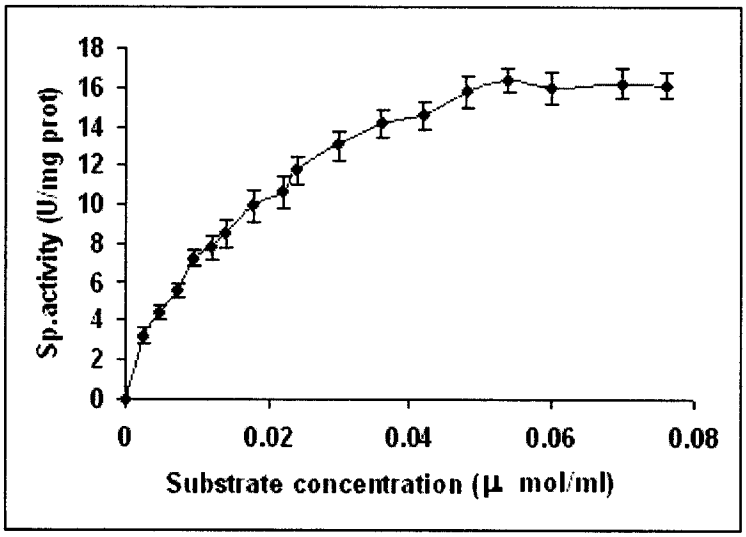


Fig.10.10 a Substrate concentration curve

Assay conditions- 50^{0}C, pH 8.0, with varying *p*-NP concentration(0.05-5 μmol/ml)

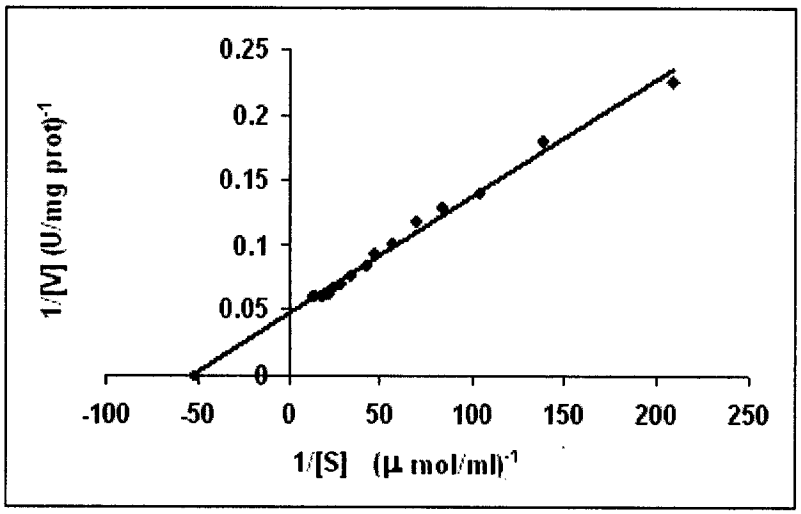


Fig.10.10 b Lineweaver - Burk plot

irreversible active-site lipase inhibitors. Generally, lipases are not sulphydryl proteins and thus in most lipases neither free –SH nor S–S bridges are important for their catalytic activity. Gupta *et al.* (2004) had also reported that the use of 2-mercaptoethanol and iodoacetate had no significant effect on the lipase from *C. viscosum*, *S. aureus* 226 and *A. calcoaceticus* LP009.

The lipase activity was totally knocked out by PMSF, suggesting the presence of serine residues at or near the active site. As expected cysteine residues did not appear to be directly significant in the catalysis. Such inhibition by PMSF was also exhibited in some *Pseudomonas* sp (Heshsmm *et al.*, 2005; Schdig *et al.*, 1991; vanOort *et al.*, 1989; Svendser *et al.*, 1995) and in *Bacillus* sp (Nawani et al., 1998).

10.2.3.5 Effect of metal ions and EDTA

EDTA at 1mM, strongly inhibited lipase activity in *Cunninghamella verticillata* which was attributed to its access to the Ca^{2+} binding site and ion removal (Gopinath *et al.*, 2002). The activity of a few other lipases has also been reported to be inhibited by EDTA (van Oort *et al.*, 1989; Baral and Fox, 1997; Sharon *et al.*, 1998).

When the enzyme from isolate TZ was incubated in presence EDTA followed by estimation of activity, it was observed that even at the lowest tested concentration (0.25mM) of EDTA, 50% activity was lost and by 1mM EDTA complete loss of activity occurred (Fig.10.11).

In the experiment on the effect of metal ions on the purified lipase, added magnesium ions appeared to stimulate or stabilize the enzyme activity while Ca^{++} did not have much effect. Near total inhibition by mercury, zinc, manganese, ferric and cupric ions was observed (Table 10.3) as in the case of crude enzyme preparation.

Metal ions may have variable affects on lipase aggregation and on the substrate–water interface through interaction with free fatty acids (Lin *et al.*, 1996; Nawani and Kaur, 2000). Salts of heavy metals (Fe^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}) strongly inhibited the lipase, suggesting that they were able to alter the enzyme conformation (Kanwar *et al.*, 2006). Hg^{+2} strongly inhibited the lipase activity in *Rhizopus* sp. while Na^{+} lightly enhanced it (Maria and Gláucia,, 2006). This behavior was also observed for the *Aspergillus carneus* lipase (Saxena *et al.*, 2003).

Metal ions like Hg^{2+}, Zn^{2+} and Cu^{2+} have been reported by several workers to have inhibitory effects on *Pseudomonas* lipases (Yamamato and Fujiwara, 1988; Kumura *et al.*, 1993; Chartrain *et al.*, 1993). Several workers have found that calcium ions are able to stimulate lipase activity (Chartrain *et al.*, 1993; Lee and Rhee, 1993).

With the above discussion in view and referring back to the lipase from our isolate, it now appears most likely that the inhibition by EDTA might have been a direct consequence of its removal of Mg^{++} or Ca^{++} which were required for the lipase activity.

Table 10.2 Effect of inhibitors on enzyme activity

Inhibitor	Concn (mM)	Residual activity (%)
PMSF	0.5	0
	1.0	0
	2.0	0
Iodo-acetamide	0.5	88
	1.0	82
	2.0	81

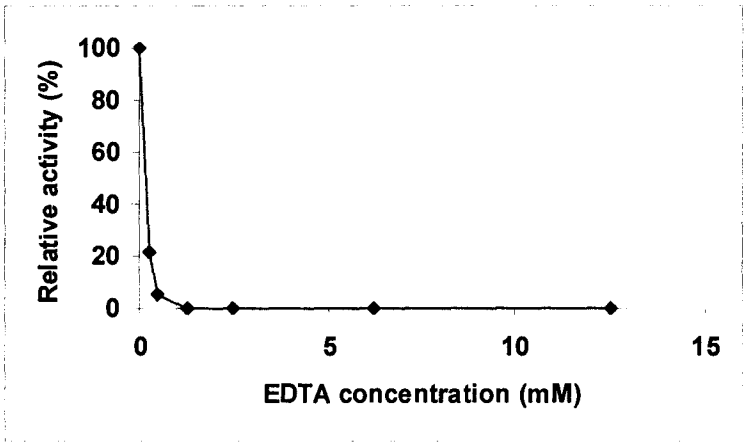


Fig.10.11 Effect of EDTA

Assay conditions- pre incubation of enzyme with EDTA for 30 min at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

Table 10.3 Effect of metal ions

Metal ion	Concentration (mM)	Relative activity (%)
Zn^{2+}	1	12.5
	10	0
Fe^{3+}	1	2.2
	10	0
Hg^{2+}	1	0
	10	0
Cu^{2+}	1	0
	10	0
Mn^{2+}	1	0
	10	0
Mg^{2+}	1	104
	10	105
Ca^{2+}	1	102
	10	100

Assay conditions- pre incubation of enzyme with divalent metal ions for 30 min at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

10.2.3.6 Effect of reducing agents & detergents

The lipase activity was not significantly affected in the presence of the reducing agents DTT, β- mercaptoethanol and sodium thioglycolate at concentrations as high as 1% (Fig.10.12) implying that either the enzyme did not have disulphide linkages or if present, they were not essential for enzyme activity. The absence of interchain S-S linkages was, in any case, ruled out earlier with the confirmation of the enzyme as a monomeric molecule.

The tested detergents (Tween-20, Tween-80, Triton-100 and SDS) completely inhibited the lipase activity from isolate TZ even at the lowest tested concentration of 0.1% (data not shown). Similar results were reported for the lipase from *P. chrysogenum*, *Aspergillus caneus* (Saxena *et al*, 2003) and in *Bacillus* sp (Nawani *et al.*, 1998) where the highest denaturing effect was observed with SDS (0.5% w/v). The inhibition was attributed to the conformation changes in the active site that result in inhibition, partial reversible unfolding and subsequent inactivation (Prazeres et al., 2006).

In *H. lanuginosa* S-38, sulphydryl-reducing agents such as DTT did not alter the enzyme activity. Reducing compounds (cysteine, 2-mercaptoethanol) and thiol group inhibitors (*p*-chloro mercuric benzoate, monoiodoacetate) did not show a detectable effect on lipase in *M. pusillus*, suggesting that it did not require either a free -SH group or an intact S–S bridge for its activity. Similar results have been reported for *Chromobacterium viscosum* (Sugiura and Isobe, 1974) and *Staphylococcus aureus* 226 lipases (Muraoka *et al.*, 1982). The LipA

of *Acinetobacter* sp. RAG-1 also was not affected by dithiothreitol or 2-mercaptoethanol at 10 mM (Snellman *et al.*, 2002).

10.2.3.7 Effect of NaCl

After pre-incubation with NaCl for 1 hour, the purified enzyme retained 75% activity up to 0.5 M and more than 50% activity at 0.75 M (Fig. 10.13). Lipase activity was totally abolished in the presence of 5% NaCl in *Corynebacterium paurometabolum*, MTCC 6841 (Joshi *et al.*, 2006). Na^+ ions have been reported to be essential for lipase activity of *Pseudomonas pseudoalkaligenes* (Kanwar *et al.*, 2006). The high stability of the lipase from *Thraustochytrium* sp.TZ in presence of NaCl could possibly be an evolutionary strategy of the organisms for survival under changing environmental conditions of salinity.

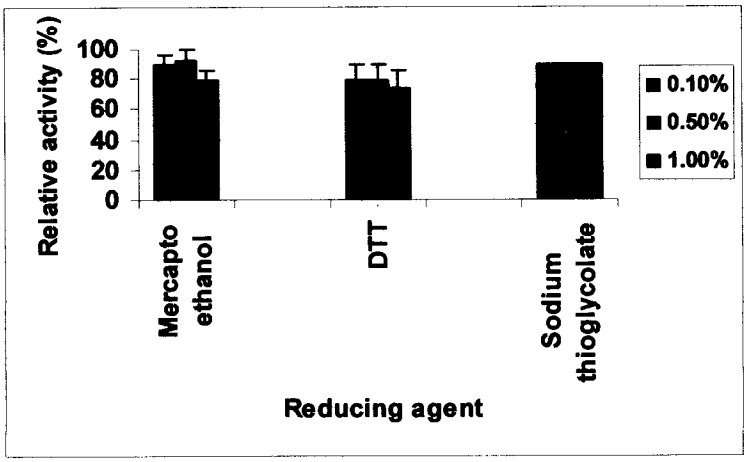


Fig.10.12 Effect of reducing agents

Assay conditions- pre incubation of enzyme with reducing agents for 30 min at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

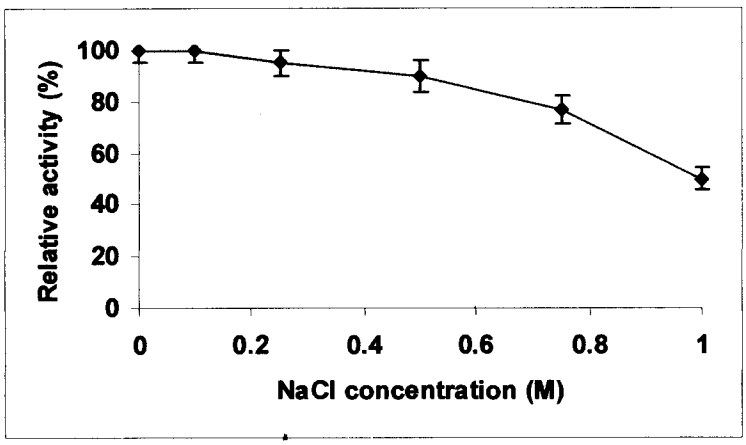


Fig.10.13 Effect of NaCl on pure enzyme

Assay conditions- pre incubation of enzyme with varying NaCl concentrations for 1 h at 30°C before the addition of substrate. The activity of untreated enzyme was taken as 100%.

10.2.3.8 Effect of organic solvents

Stability in organic solvents is an important character of protein catalysts used in organic synthesis reactions. The effect of solvents such as acetone, methanol, ethanol, 2-propanol, acetonitrile, glycerol, diethyl ether, n-butanol and n-hexane (20%, v/v) on enzyme activity was therefore studied (Table 6). The inhibition of lipase activity in presence of acetone, ethanol and methanol while retaining good stability in water-immiscible solvents confirms the general statement that polar water miscible solvents are more destabilizing than the water immiscible solvents. These water-immiscible solvents solvents may (i) modify the oil-water interface to make the enzymatic action easier without causing protein denaturation (ii) enhance the enzyme activity *via* deaggregation of the lipase (iii) activate the lipase by affecting its conformation. Stability against organic solvent is important when using enzymes for synthesis of esters. These features, along with the thermostability of the enzyme, make it a very attractive enzyme for future application in biotechnology.

The *Rhizopus* sp. lipase also had good stability in hexane but was highly denatured in hydrophilic solvents, especially methanol (Maria and Gláucia, 2006). Such behaviour was observed by Hiol *et al.* (1999, 2000) for *M. hiemalis* and *R. oryzae* lipases also. That lipase from *Thraustochytrium* sp TZ showed a high optimum temperature and also good stability in hydrophobic solvents should make it a good candidate for esterification reactions in organic media.

Table 6 Effect of organic solvent on enzyme activity

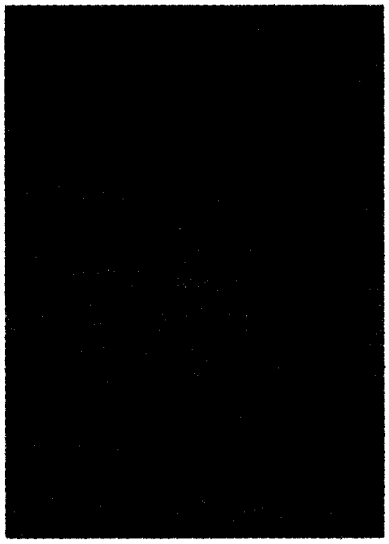
Organic solvent (20% v/v.)	Residual activity (%)
None	100
Acetone	25
Methanol	33
Ethanol	35
2-Propanol	42
Acetonitrile	50
Glycerol	100
Diethyl ether	90
n-Butanol	98
n-Hexane	100

10.2.3.9 Positional specificity

Based on the substrate specificity, microbial lipases may be divided into three categories, *viz* nonspecific, regiospecific and fatty acid-specific. Nonspecific lipases act at random on the triacylglyceride molecule and result in the complete breakdown of triacylglyceride to fatty acid and glycerol.

The positional specificity of the purified lipase was examined by analyzing the hydrolysis products of trioleine by the enzyme. The TLC studies showed that the lipase from *Thraustochytrium* sp is non specific (Fig 10.14). Most of the bacterial lipases are non specific in nature. Examples of this group of lipases include those from *S. aureus*, *S. hyicus*, *Corynebacterium acnes* and *Chromobacterium viscosum* (Jaeger *et al.*, 1998). However, regiospecific lipases have been reported from various *Bacillus* species (Dharmsthiti and Luchai, 1999) and also from several fungi and moulds (Ghosh *et al.*, 1996).

Lipases from *S. aureus* and *S. hyicus* show similar type of non-specificity. Since non-specific lipase can synthesize both primary and secondary alcohol esters, the newly obtained lipase may be useful for various ester synthesis in selected organic media such as n-hexane (Nawani and Kaur, 2000).



1 2 3 4 5

Lane 1 : Triolein

Lane 2 : Products of triolein hydrolysis by purified lipase from isolate TZ

Lane 3 : 1,2-Diolein

Lane 4 : 1,3-Diolein

Lane 5 : monoolein

Fig.10.14 TLC triolein hydrolysis purified lipase from isolate TZ

Thin layer chromatogram of the enzyme reaction products obtained with triolein as the substrate.

In summary, an alkaline lipase produced from *Thraustochytrium* sp.TZ was purified to homogeneity by two- step purification process involving heat treatment and gel filtration chromatography. The purified lipase was found to belong to serine hydrolase group with a molecular weight of 66 kDa. The enzyme was active at alkaline pH 9.0 and stable over pH range 8-10. It had temperature optimum at 50°C and retained more than 90% activity at 50°C for 20 min even in the absence of the substrate. This lipase could thus find application in the detergent industry. At the lowest tested concentration (0.25mM) of EDTA, 50% activity was lost and by 1mM EDTA complete loss of activity occurred. Added magnesium ions appeared to stimulate or stabilize the enzyme activity. The stability of lipase in hydrophobic solvents is an added advantage to be used in esterification reactions in organic media.

Chapter 11

Biotechnological applications of the alkaline lipase

Enzymes have been used to improve the cleaning efficiency of detergents for more than 35 years and are now well accepted as ingredients in powder and liquid detergents, stain removers / laundry pre-spotters, automatic dishwashing detergents and industrial/institutional cleaning products. Detergent enzymes account for about 30% of the total worldwide enzyme production and represent one of the largest and most successful applications of modern industrial biotechnology.

The performance of enzymes in detergents depends on a number of factors, including the composition of the detergent, type of stains to be removed, wash temperature, washing procedure and wash-water hardness. Enzymes designed for use in cleaning applications need to be compatible with all commonly used detergent components such as nonionic and most anionic surfactants, builders, anti-redeposition agents, optical brighteners and oxygen-releasing bleaching agents.

The most commercially important field of application for hydrolytic lipases is their addition to detergents which are used mainly in household and industrial laundry and in household dishwashers. Enzymes can reduce the environmental load of detergent products since they save energy by enabling the use of a lower wash temperature, allow the content of other (often less desirable) chemicals in detergents to be reduced, are biodegradable leaving no harmful residues, have no negative impact on sewage treatment processes and do not present a risk to aquatic life. Enzyme sales in 1995 have been estimated to be US$ 30 million, with detergent enzymes making up 30% of it. An estimated 1000 tons of lipases are added to the approximately 13 billion tons of

detergents produced each year. In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase, which originated from the fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced - Lumafast from *Pseudomonas mendocina* and Lipomax from *Pseudomonas alcaligenes*, both produced by Genencor International.

The removal of fatty food stains, cosmetics and sebum from garments by laundry detergents formulated for use at lower washing temperatures and more neutral pH's can be enhanced by addition of lipase enzyme. Problematic stains such as those of lipstick, salad oil, animal fat and butter could be removed from clothes after washes with laundry detergents containing lipase enzyme.

Microbial lipases have already established their vast potential regarding their usage in different industries. The interest in microbial lipase production has increased in the last decade because of its large potential in industrial applications as additives for foods (flavor modification), fine chemicals (synthesis of esters), waste water treatment (decomposition and removal of oily substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skins) and medical (blood triglyceride assay) (Prazeres *et al.*, 2005). Nevertheless, the biggest market of their use continues to be in the detergent formulation (Rathi et *al*, 2001). Regarding applications in detergents, new challenges for lipases producers can be pointed (1) the high variation in the triglyceride content of fat stains, demanding lipases with low substrate specificity (2) the relatively harsh washing conditions (pH 10-11 and 30-60°C) requiring stable enzymes and (3)

the effects of chemical denaturation and/or proteolytic degradation caused by detergents additives (Sharma *et al.*, 2001).

This Chapter discusses a probable role for the alkaline lipase from *Thraustochytrium* sp.TZ in the detergent industry.

11.1 Materials and Methods

11.1.1 Enzyme Stability in commercial detergents

The commercial laundry detergents prepared as described in Section 6.1.1 were incubated with crude lipase enzyme for 30 min at 30°C and the residual activity was determined under the standard assay conditions. The enzyme activity of a control sample (without any detergent) was taken as 100%.

11.1.2 Washing Test performance of lipase in oil-based stain removal

The test fabric was stained with the respective stains, air dried and used for the study. The stained swatches were incubated with solutions of either enzyme alone or detergent alone or in combination, for 1 h at room temperature. They were then given a gentle wash with water and observed for stain removing capacity of the enzyme. The same set of experiments was also carried out at 50°C (which was the optimum temperature for the enzyme) for 15 min to study the effect of higher temperature in removing the stain.

11.2 Results and Discussion

The use of enzymes in household laundry detergents is environment friendly since all enzyme products are non-toxic completely biodegradable and help reduce clothes-washing energy consumption (Gulati *et al.*, 2005).

Since the alkaline protease from our isolate TZ (identified as T*hraustochytrium* sp.) was found to be successful in protein stain removal from soiled fabrics (Chapter 6), it was interesting to look for a possible complementary lipolytic action on soiled fabrics by the alkaline lipase from the same isolate.

The effect of commercial detergents on enzyme stability was studied and the results are given in Table 11.1. The efficacy of the lipase enzyme in removing the common oil based stains such as fro pickle oil stain, lipstick and grease was also tested. The results of wash performance in test swatches are as shown in Fig 11.1. The enzyme, on its own, was equally or more effective than the commercial detergent in removing the stains. When added with the detergent, the wash performance was even more superior.

a) Grease stain removal

Grease stain **Tap water wash** **Detergent wash**

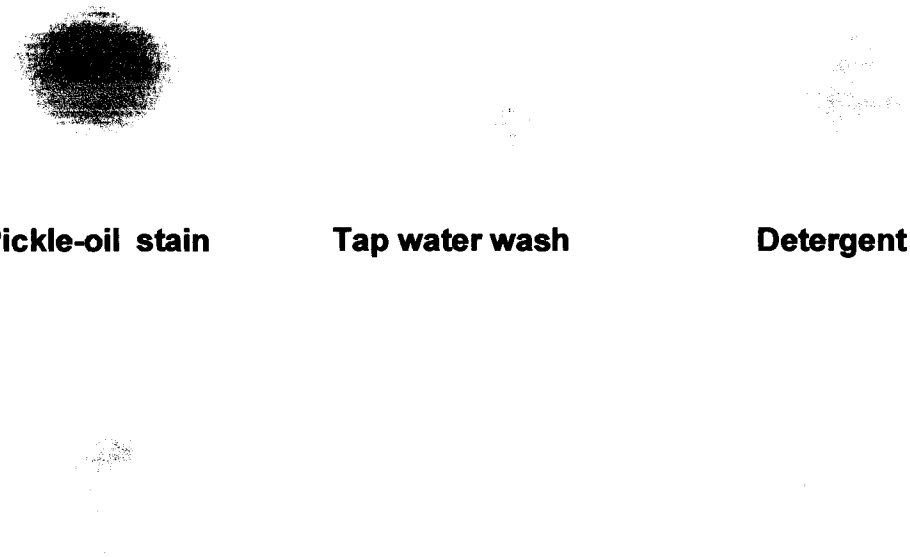
Enzyme wash **Enzyme + detergent wash**

b) Lipstick stain removal

Lipstick stain **Tap water wash** **Detergent wash**

Enzyme wash **Enzyme + detergent wash**

c) Pickle-oil removal



Pickle-oil stain **Tap water wash** **Detergent wash**

Enzyme wash **Enzyme + detergent wash**

Figure 11.1 Wash performance of the lipase in oil-based stain removal

Cotton swatches stained with the respective oil-based stain (grease, lipstick or pickle-oil) were with detergent (Wheel)and/or/enzyme preparation incubated for 30 min at 30°C, given a gentle wash with tap water and dried.

Table 11.1 Stability of the lipase in commercial detergents

Detergent	Concentration (%, w/v))	Relative activity (%)
Rin	0.1	53.0
	0.5	32.3
	1.0	29.4
Surf	0.1	41.3
	0.5	33.5
	1.0	18.0
Tide	0.1	47.0
	0.5	47.0
	1.0	3.0
Ariel	0.1	29.4
	0.5	25.2
	1.0	14.8
Wheel	0.1	74.0
	0.5	59.0
	1.0	40.7
Mr. White	0.1	61.6
	0.5	41.3
	1.0	21.2

Very few workers have been successful in characterizing micribial lipases from which showed remarkable stability in the presence of commercial detergents (Gulati *et al.*, 2005). The residual activities of lipase from *Corynebacterium paurometabolum*, MTCC 6841 in the presence of Surf Excel, Rin Shakti, Arial, and Tide brands of detergents were 65, 52, 42, and 46%, respectively (Gopal, 2006).

Rathi *et al.* (2001) studied the effect of commercial detergents as Ariel, Wheel, Nirma, Fena, Surf Ultra and Rin Supreme on lipase activity from *Burkholderia cepacia* comparing with Lipolase® (Novo Nordisk, Denmark). They found that the lipase from *B. cepacia* exhibited better resistance to commercial detergents (57-80% residual activity) than Lipolase® (40-80% residual activity) after 1h of incubation at 37°C and pH 11.0. In the present study, the lipase showed 39-74 % residual activity, comparable with that presented by Lipolase®.

In the present study, preliminary experiments on the destaining of oil, grease and lipstick stains from test fabrics have shown satisfactory results. This research, therefore, is an attempt to unravel the microbial diversity of local resources in terms of some functional attributes. The lipase produced by *Thraustochytrium* sp TZ owing to its alkaline nature and ability to work in the presence of various detergents and solvents, could prove to be of significance for applications in industrial processes such as detergent formulations, sewage treatment and leather processing.

Summary and Conclusions

The marine environment is subject to increasing human disturbances such as sewage and industrial effluent discharge, oil spills and leachates containing pesticides. Coastal areas are most vulnerable to the above disturbances and mangroves are another ecosystem that is particularly exposed to stress factors. To withstand such harsh conditions, microbes inhabitating in those environments are induced to produce certain enzymes by which they could make their survival (Kathiresan and Bingham, 2001). Most of these enzymes are of industrial importance. One such microbial community which was never explored properly for enzyme production in terms of nature, purification and applications are the marine protists, the thraustochytrids.

The great demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. They find great use in a large number of fields such as the food, dairy, pharmaceutical, leather, detergent, textile and cosmetic industries. Research has been carried out on plants and animals as sources of these enzymes. The inability of these sources to meet the current world demand has led to an increased interest in microbial sources which find additional favor by virtue of their multifold properties, easy extraction procedures and virtually unlimited supply. Although a lot of work has been carried out on bacteria, fungi and yeast as sources of these enzymes, the tireless search for new and better sources continues.

Proteases represent the class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications. They play a critical role in many physiological and pathophysiological processes. Alkaline proteases hold a great potential for application in the detergent and leather industries due to the increasing trend favoring environmentally friendly technologies (Rao *et al.*, 1998).

Lipases are an important group of biocatalysts with an unsurpassed role in swiftly growing biotechnology that is based mainly on their remarkable ability to carry out novel reactions both in aqueous and non-aqueous media. Lipolytic reactions occur at the lipid/water interface where the targetted substrates usually form equilibria between monomeric, micellar and emulsified states (Saxena *et al.*, 1999). Owing to their immense importance, these multi-faceted enzymes have tremendous potential in areas such as food technology, the detergent as well as the chemical industry and in biomedical sciences. Hence for several years, reviews on lipases have focussed on their biotechnological impetus.

With the advent of rapid methods for discovering enzymes with novel properties or altering the properties of known enzymes, there is paramount interest in the development of screening tools that could be used to search for the best performance with respect to a specific property, especially in relation to industrial applications. An efficient screening or selection system is an absolute prerequisite to identifying the enzyme-producing species from a diverse population.

The present work introduces a new marine microbial source for the alkaline protease and lipase production – the thraustochytrid protists.

Modern taxonomy classifies thraustochytrids in the phylum Heterokonta, which is in turn placed in the kingdom Chromista, based on 18S rDNA sequencing. They are a group of non-photosynthetic, heterotrophic, marine protists. This group has a typical structure consisting of an ectoplasmic net and a noncellulosic, sulphated cell wall. The ectoplasmic net is thought to be the site of organic degradation using excreted enzymes.

Studies performed to date give reason to believe that thraustochytrids perform ecological functions in the degradation of marine plants similar in character and magnitude to those of fungi and bacteria on land and in the sea (Raghukumar *et al.,* 1994). However, to our knowledge, thraustochytrids are still an untouched bio-resource for enzyme production. In the light of the above, we have carried out a study on the production of alkaline proteases and lipases from thraustochytrid protists isolated from mangrove and coastal habitats of Goa, India, with the following primary objectives in view:

1. Isolation of Thraustochytrids from the mangrove and other coastal habitats of Goa and screening for proteases and lipases.
2. Selection of promising isolates and optimization of culture conditions for maximum production of alkaline proteases and lipases.
3. Characterization of two of the most active alkaline proteases and lipases in terms of nature and properties.

4. Purification of one alkaline protease and one alkaline lipase activity.

5. Exploring the biotechnological potential of the selected enzymes

Based on the experiments carried on the production, purification, characterization and biotechnological applications of the proteolytic and lipolytic enzymes from the marine protists, thraustochytrids presented and discussed in detail in the respective Chapters of this thesis, the following points are listed in summary:

- On the basis of qualitative and quantitative screening of 11 isolates (8 of coastal and 3 of mangrove habitats, two thraustochytrids isolates designated AH-2 and TZ identified as belonging to the genus *Thraustochytrium* were found to be potent alkaline protease and lipase producers and were selected for further studies. It was noticed that both these isolates could produce alkaline proteases as well as lipases under specially derived culture conditions.

- Media optimization results showed that the production medium (glucose 0.4%, peptone 0.15%. yeast extract 0.1%, crude salt 3.4% and skimmed milk powder 0.5%) at pH 7.0, 30±2°C for 72 h incubation under shake flask culture yielded maximum alkaline protease activity by both the selected isolates. For maximum alkaline lipase production by both the selected isolates, production medium (peptone 0.5%, yeast extract 0.01%, crude salt 3.4% and olive oil 0.5%) at pH 6.0, 30±2°C for 7 days of incubation under shake flask culture was found to be the best. The

presence of glucose in the medium was found to inhibit the production of lipase by both the thraustochytrid isolates.

- Although protease production by these two isolates was basically constitutive, the production was enhanced in presence of the substrate at 0.5% level. Lipase production was, however, strictly inducible, with the inducer at 0.5% level.

- The alkaline proteases exhibited pH optima of 8.0 and 9.0 for the isolates TZ and AH-2 respectively, were stable at alkaline pH, had temperature optimum at 50°C and retained 85% or 55% activity at 50°C or 45°C for 20 min from the isolates TZ and AH-2, respectively.

- The pH optima of both alkaline lipases were found to be 9.0 and the enzymes exhibited stablity towards alkaline pH. The temperature optima for both lipases were at 50°C and the activity was maximally stable for 15-20 min at 50°C for TZ and 45°C for AH-2.

- In view of the superior characteristics of both lipase and protease enzymes isolated from *Thraustochytrium* sp.TZ over those from AH-2, these enzymes from isolate TZ were taken up for purification and studies on potential applications in industries.

- The crude enzyme preparations from *Thraustochytrium* sp.TZ showed broad substrate specificity and compatibility in presence of commercial

detergents and hence could find application as an additive in detergent powder or solution.

- Preliminary experiments on dehairing of goat hide by the alkaline protease from isolate TZ indicated a potential application in the leather industry.

- The results of gelatin degradation from used X-ray films by alkaline proteases from isolate TZ indicated that this enzyme could be efficiently used for the recovery of silver from such films.

- Purification of the alkaline protease from the isolate TZ was carried out by a sequence of steps which included ammonium sulphate precipitation, ion exchange chromatography (CM-Sephadex column) and gel filtration (Sephacryl S-200 column). The enzyme was purified about 30-fold starting from the culture filtrate, with a 3% yield.

- Purification of the alkaline lipase from the isolate TZ was carried out by a two step process of heat treatment of the crude enzyme followed by gel filtration. The enzyme was purified 24-fold starting from the culture filtrate, with a yield of 53 %.

- The purified alkaline protease from the T*hraustochytrium* sp TZ was a monomeric cysteine protease with a molecular weight 27 kDa, pH optimum at 8.0, temperature optimum at 45°C and was stable at 45°C

for at least 20 min and also at alkaline pH. It had a high affinity for casein, broad substrate specificity and was quite stable with tested detergents and H_2O_2.

- The purified lipase was found to belong to the serine hydrolase group and was monomeric, with a molecular weight of 66 kDa. The enzyme was most active at alkaline pH 9.0 and stable over a pH range of 8-10. It had a temperature optimum at 50°C and retained more than 90% activity at 50°C for 20 min even in the absence of the substrate. This lipase could thus find application in the detergent industry. At the lowest tested concentration (0.25mM) of EDTA, 50% activity was lost and by 1mM EDTA complete loss of activity occurred. Added magnesium ions in the assay appeared to stimulate or stabilize the enzyme activity. The stability of lipase in hydrophobic solvents is an added advantage for use in esterification reactions in organic media

- The lipase activity was hardly altered much in the presence of the reducing agents tested which imply that either the enzyme did not have disulphide linkages or if present, they were not essential for enzyme activity. The absence of interchain S-S linkages was, in any case, ruled out earlier with the confirmation of the enzyme as a monomeric molecule.

- The peroxide inactivation curve showed that the protease enzyme was stable even at 3% (v/v) of H_2O_2 for 1 hr, with 80% residual activity. This

is an important property because bleach-stable enzymes are not generally available except for a few reports.

- The above results appear to be the first reports of alkaline thermotolerant enzymes from marine protists, thraustochytrids.

Considering the overall property of different alkaline proteases of microbial origin and their evaluation, the reported alkaline protease is superior with regards to pH and temperature stability, detergent compatibility and broad substrate specificity, for a possible future application in detergent formulation.

The enzymatic properties of both the crude and purified alkaline protease from the *Thraustochytrium* spp. are summarized briefly in the following table:

Properties of the alkaline protease	**Crude enzyme-AH-2**	**Crude enzyme-TZ**	**Pure enzyme-TZ**
Reaction time (min)	15	30	30
Optimum temp(^{0}C)	50	50	45
Optimum pH	9.0	8.0	8.0
K_m for casein (mg/ml)	0.83	0.5	0.18
Thermal stability	at 45^{o}C for 15min	at 50^{0}C for 15 min	45^{0}C for 20 min
pH stability	stable at alkaline pH	stable at alkaline pH	stable at alkaline pH
Stability at RT	upto 5 days with 50% activity	upto 5 days with 70% activity	(no data)

In addition to the above mentioned kinetic properties, both the crude and purified alkaline protease exhibited a few biotechnologically relevant properties such as a broad substrate specificity, stimulation in activity by low concentration (0.1%) of EDTA, marginal stimulation by calcium ions, stability in commercial detergents (crude enzyme) and H_2O_2 (purified protease) which makes it an attractive novel enzyme source. The potential applications in the leather industry and also for silver recovery from used X-ray films have also been explored.

The properties of the crude as well as purified alkaline lipase from the *Thraustochytrium* sp are summarized below:

Properties of the alkaline lipase	**Crude enzyme-AH-2**	**Crude enzyme-TZ**	**Pure enzyme-TZ**
Reaction time (min)	10	10	10
Optimum temp (^oC)	50	50	50
Optimum pH	9.0	9.0	9.0
K_m for *p*-NPP (µmol/ml)	0.5	0.182	0.019
Thermal stability	at 45°C for 15 min	at 50°C for 20 min	50°C for 20 min
pH stability	stable at alkaline pH	stable at alkaline pH	stable at alkaline pH

The purified lipase also showed stability in organic solvents making it a good candidate for esterification reactions in organic media.

A self-explanatory sample comparison of enzymatic activities of alkaline protease and lipase from *Thraustochytrium* sp TZ with those of other microbes is highlighted below:

Alkaline protease activity in crude extract

Microbes	Specific activity* (U/mgprotein)	Reference
Bacillus species	1.1	Braz.J.Microbiol. Vol. 35, 2004
Alternaria alternata	1.4	J. Biosci. Vol. 9, 1985
Penicillum expansum	0.6	J. Islamic Academy of Science Vol. 7,1994
Pseudomonas aeruginosa PW100	7.0	Electronic J. Biotechnology Vol. 8, No.2, 2005
Mucor pusilius	7.2	App. Env. Microbial. Vol. 45, 1983
Bacillus subtilis	10.2	AAPS Pharm. Scitech. Vol 4 ,2003
Bacillus SS 103	21.0	J. Medicinal Food Vol. 8, 2005
Aspergillus oryzae AWT 20	25.7	The Internet Journal of Microbiology Vol. 2, No. 2, 2006
Bacterial	24.5	I.J.Expt.Biol. Vol. 37, 2001
Bacillus subtilis NCIM 2711	33.7	I.J.Expt.Biol. Vol. 41, 3003
Streptomyces nogalator (MTCC 2505)	1.9	J. Sci & Ind. Res. Vol. 64, 2005
Thraustochytrid-TZ	**37.0**	**(present study)**

*using casein as substrate

Alkaline lipase activity of purified protein

Microbes	Specific activity* (U/mg protein)	Reference
P. aeruginosa MTCC-4713	5.1	Wiley InterScience,2005
Citrobacter freundii IIT-BT L139	8.8	I.J. Expt.Biology,2006
Pseudomonas cepacia	1.5	Appl.Microbiol.Biotechnol. 1997 (47); 630-635
Pseudomonas aeruginosa BTS-2	5.4	World Journal of Microbiology and Biotechnology (2005)
Streptomyces rimosus	12.0	Food Technol. Biotechnol. (2003)
sAspergillus niger ATCC MYA-135,	1.1	Food Technol. Biotechnol. (2006)
Penicillium aurantiogriseum	13.0	Food Technol. Biotechnol. (2003)
Thraustochytrid- TZ	**16**	**(Present study)**

* using *p*-nitro phenyl palmitate as substrate

FUTURE PROSPECTS.....

- Since the same strain of the *Thraustochytrium* sp produced both protease and lipase under different culture conditions, it would be interesting but challenging if the conditions could be optimized further in such a way that the said organism would produce both the enzymes using a single medium. The combined lipolytic and proteolytic action could prove beneficial in the detergent as well as the leather industry.

- In the present study, the lipase and protease were purified to homogeneity. They could be further studied with respect to their amino acid sequence and three-dimensional structure. Amino acid sequencing analysis may help in deriving evolutionary relationships of this thraustochytrid with others.

- The production of the enzymes could be further improved to a pilot scale. Commercial exploitation of the enzyme activities may be pursed.

- The enzymes may be suitably immobilized to get a formulation favoring incorporation in laundry detergents.

- With respect to properties of the protease and its capability for degradation of different protein sources, this alkaline protease may find a potential application in waste treatment.

- Enzyme mediated oil extraction from different oil seeds could be looked into.

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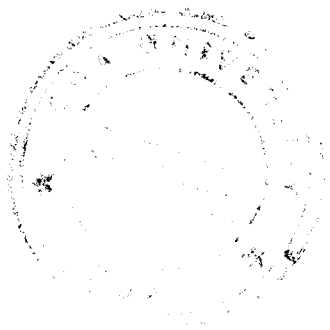

APPENDIX

APPENDIX-I

Growth medium

Modified Vishniacs medium (M.V. medium)

Glucose	0.4%
Peptone	0.15%
Yeast extract	0.1%
Crude salt	3.4%
Distilled water	100 ml

M.V. agar medium

Glucose	0.4%
Peptone	0.15%
Yeast extract	0.1%
Crude salt	3.4%
Agar	0.8%
Antibiotics	1.0%
(Streptomycin & Penicillin)	
Distilled water	100 ml

Protease production medium

Glucose	0.4%
Peptone	0.15%
Yeast extract	0.1%
*Skimmed milk powder	0.5%
Crude salt	3.4%

Distilled water	100 ml
pH	7.0

* autoclaved separately for 10 min and then added to sterile M.V. medium.

M.V. agar medium for qualitative protease detection

Glucose	0.4%
Peptone	0.15%
Yeast extract	0.1%
*Skimmed milk powder	1.0 %
Crude salt	3.4%
Agar	1.0 %
Distilled water	100 ml
pH	7.0

* autoclaved separately for 10 min and then added to sterile M.V. medium

Lipase production medium

Peptone	0.5%
Yeast extract	0.01%
Olive oil	0.5%
Crude salt	3.4%
Distilled water	100 ml
pH	6.0

The medium is emulsified before autoclaving.

ASW (artificial sea water g/l))

Tris base	6.05
Mg_2SO_4	12.32
KCl	0.74
$(NH_4)_2HPO_4$	0.13
NaCl	17.52
$CaCl_2$	0.14
pH	7.0

APPENDIX-II

Reagents for protein estimation

Folin Lowery reagents Stock solution

Solution A (100ml)

$CuSO_4 5H_2O$ 1.0 g -I

Sodium potassium tartarate 2.0 g - II

Before experiment, mix 500ul each of I & II to get 1 ml of solution A

Solution B

Na_2CO_3 20 g

NaOH 4 g

D/W 100 ml

Solution C

1 ml solution A

49 ml solution B

Solution D

Folin Ciocalteus phenol reagent

1:1 dilution with distilled water

Buffers

Phosphate buffer (KH_2PO_4) 100mM

Add KOH to get desired pH6, 7and 8

Borate buffer ($Na_2 B_4 O_7$ 10 H_2O) (50mM)

Dissolve 19.05 g in 500 ml D/W

Adjust the pH to 8 and 9 with 0.1N HCl and make up the volume to 1000 ml with D/W.

For pH 10 and 11, adjust the pH with 0.1N NaOH and make up the volume to 1000 ml with D/W

Sodium acetate buffer (200mM)

Solution A: 0.2M acetic acid

Solution B: 0.2M sodium acetate

X ml of solution A and Y ml of solution Bare mixed and the volume was made up to 100 ml with distilled water.

Solution A (ml)	Solution B (ml)	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
14.8	35.2	4.8
10.5	39.5	5.0
8.8	41.2	5.2
4.8	45.2	5.6

Reagents for PAGE

Tank buffer

Glycine	14.4 g
Tris	3.0 g

Make up volume to 1000 ml with D/W.

For SDS-PAGE, add SDS to a final concentration of 0.1%.

Separating gel buffer (4X)

Tris base	36.3 g
pH (adjusted with HCl)	8.8

Make up volume to 200 ml with D/W.

Stacking gel buffer (4X)

Tris base	3.0 g
pH (adjusted with HCl)	6.8

Make up volume to 50 ml with D/W

Ammonium persulphate (APS) (10%)

APS	0.1 g
D/W	1.0 ml

Prepare fresh before use.

Gel loading buffer for native PAGE (4X)

Glycerol	2.0ml
4X Separating gel buffer	2.5 ml
Bromophenol blue (1%)	1.0ml

Make up volume to 10 ml with D/W.

Gel loading buffer for SDS-PAGE (2X)

Glycerol	2.0ml
4X Stacking gel buffer	2.5 ml
SDS (10% stock)	4.0 ml
Bromophenol blue (1%)	1.0ml

Make up volume to 10 ml with D/W.

Acrylamide stock solution (30%)

Acrylamide	29.2 g
N,n- methylene bis Acrylamide	0.8 g

Make up volume to 100 ml with D/W

Store at 4°C in dark.

Staining solution

Coomassie Brilliant blue R-250	0.25 g
Methanol	45 ml
D/W	45 ml
Acetic acid	10 ml

Filter through a Whatman No.1 filter to remove any particulate matter.

Destaining solution

Methanol	45 ml
D/W	45 ml
Acetic acid	10 ml

Reagents for silver staining

1. Fixing solution

   Acetic acid 10%

   Keep the gel in fixing solution for 30 min

2. Sodium thiosulphate 0.02%

   Add, shake for 1 min and wash the gel with D/W for 20 sec

3. $AgNO_3$ 0.2%

Formaldehyde 0.075 ml

D/W 100 ml

Incubate for 20 min in dark and wash the gel with D/W

4. Developing solution

Na2CO3 6 g

Sodium thiosulphate 0.0004 g

Formaldehyde 0.05 ml

D/W 100 ml

LIST OF ABBREVIATIONS

A_{nm}	Absorbance
APS	Ammonium persulphate
BSA	Bovine serum albumin
cm	Centimeter
°C	Degree centigrade
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra acetic acid
FA	Fatty acid
g	Gram (S)
h	Hour(S)
IA	Iodoacetamide
K_m	Michaelis-Menten constant
kDa	Kilo Dalton
L	Litre
mg	Milligram
min	Minute(S)
mL	Milliliter
µ	Micron
µg	Microgram
mM	Millimolar
M	Molar
N	Normal
p	Para
%	Percent
PAGE	Polyacrylamidegel electrophoresis
PMSF	Phenylmethyl sulphonic fluoride
psi	Pounds per square inch
rpm	Revolutions per minute
SDS	Sodium dodecyl Sulphate
TEMED	NNN'N'- tetramethyl ethylene diamine
TLC	Thin layer chromatography
U	Unit
UV	Ultra-violet
V_{max}	Maximum velocity
v/v	Volume/volume
w/v	Weight/volume