Purification, characterization and structure-functional studies of cold active lipase from *Yarrowia lipolytica* NCIM 3639

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BY
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Affectionately Dedicated

To

My Beloved Parents.

CONTENTS

DECLARATION

This is to certify that the work incorporated in the thesis entitled **"Purification, characterization and structure-functional studies of cold active lipase from *Yarrowia lipolytica* NCIM 3639.**" submitted by **Mr. Sathish Yadav, K.N.** was carried out under my supervision at NCIM Resource Centre, National Chemical Laboratory, Pune, 411008, Maharashtra, India. Material obtained from other sources has been duly acknowledged in the thesis.

Dr. K.B. Bastawde
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Declaration by the Candidate

I hereby declare that the work of the thesis entitled "**Purification, characterization and structure-functional studies of cold active lipase from *Yarrowia lipolytica* NCIM 3639.**" submitted for the degree of Doctor of Philosophy to the University of Pune, has been carried out by me at NCIM Resource Center, National Chemical laboratory, Pune 411008, Maharashtra, India, under the supervision of Dr. K.B. Bastawde (Research Supervisor). The work is original and has not been submitted in part or full by me for any other degree or diploma to any other university.

Sathish Yadav, K.N.
(Research Scholar)

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ABSTRACT

The world of microbial lipases is rapidly growing and there is an increasing demand to identify, characterize and produce specific lipases for a variety of biotechnological applications like synthesis of fine chemicals, medical and pharmaceutical, food industry, domestic applications, environmental application. Triacylglycerol hydrolases or lipases (EC 3.1.1.3) are enzymes that catalyze ester bond hydrolysis in triacylglycerols with the release of fatty acids, mono- and diglycerides and glycerol. Although naturally occurring triacylglycerols are normally the preferred substrates, these enzymes can hydrolyze a wide range of insoluble fatty acid esters. It is well established that the reaction is reversible, and that lipase can catalyze esterification as well as trans-esterification reactions often in nearly anhydrous organic solvents. Many biotechnological processes are expedited by the use of higher temperatures and this generated a lot of research into thermo-stable enzymes. However, more recently there has been a great interest in cold-adapted enzymes for transformations in which substrate and product stabilities require the use of low temperatures and energy savings. Such cold active enzymes are important in many fields including the detergent, textile and food industries, as well as for a variety of bio-catalytic reactions. Cold active lipases are largely distributed in micro organisms surviving at low temperatures. These organisms were isolated mostly from Antarctic and Polar Regions, deep sea sediments and refrigerated samples. We isolated the yeast from refrigerated tween-80 sample and identified as *Y. lipolytica.* Although much work on lipases from *Y. lipolytica* has been published, there are no reports on cold active lipases from this organism. This effort has been stimulated by the recognition that cold-active enzymes might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermo-stability and unusual specificities. We found that the location (cell bound and extracellular) of lipases in this isolated yeast is substrate dependent. We purified the extracellular lipase to homogeneity and characterized using biochemical and biophysical studies.

Chapter 1: General Introduction

This chapter deals with the literature survey on lipase, *Yarrowia lipolytica* lipases, cold active lipases with reference to their occurrence, properties and applications. More emphasis is given on microbial lipases in relation to their structure, catalytic mechanism, production and purification. Cryo-Electron microscopy, Single particle reconstruction and recent successes of cryo-EM and single particle reconstruction is also discussed.

Chapter 2: Isolation and production of cold active lipase from *Y. lipolytica*

This chapter deals with the production of cold active lipases by *Yarrowia lipolytica* NCIM 3639. A strain of *Yarrowia lipolytica* was isolated which produced cold active lipases. The results present a new finding of production of cell bound and extracellular lipase activities depending on the substrate used for growth. The strain produced only cell bound lipase activity when grown on olive oil containing medium with trace amounts of extracellular lipase. A major amount of extracellular lipase was produced when it was grown on Tween 80 as lipid source. Such strict differential localization of lipases in response to the substrates used as carbon source for growth is not reported so far. Maximum extracellular lipase activity was obtained when the strain was grown in a medium containing Tween 80 (2 %) and Peptone (0.5%). It grew profusely at 20 ^{o}C and at initial pH of 5.5 producing maximum extracellular lipase. The growth of *Yarrowia lipolytica* at 25 and 30 ^{o}C affected lipase production indicating the cold adapted nature of the enzyme.

Chapter 3: Purification, biochemical characterization of cold active lipase from *Y. lipolytica*

An extracellular cold lipase from *Yarrowia lipolytica* NCIM 3639 has been purified to homogeneity using ion exchange chromatography, Q Sepharose followed by Sepharose CL-4B gel chromatography. This protocol resulted in 14 fold purification of lipase with 11 % recovery. The purified enzyme showed a prominent single band on SDS-PAGE. The molecular weight of the lipase was estimated to be 20 kDa using SDS-PAGE. Gel permeation chromatography showed molecular mass of 400 kDa suggesting that the lipase is comprised of 20 subunits forming a multimeric native protein. Further the enzyme displayed an optimum pH of 5.0 and optimum temperature of 25 ^{o}C. The

lipase was stable over a narrow pH range (4.0-6.0). Action on *p*-nitrophenyl esters of varying fatty acid chain lengths showed that the enzyme exhibited high activity on pNP-caprylate (C_8) suggesting its preference towards medium acyl chain length esters. Peptide mass finger printing revealed that some peptides showed sequence homology (42 %) to *Yarrowia lipolytica* LIP8p.

Chapter 4: Three dimensional reconstruction of oligomeric cold active lipase from *Y. lipolytica.*

This chapter deals with the thermal and chemical deactivation and three dimensional reconstruction of the oligomeric cold active lipase by *Yorrowia lipolytica* NCIM 3639. From the gel permeation chromatography, it is evident that at 2-4 % of SDS concentration the enzyme dissociates into a lower molecular weight subunits with loss of its activity. Reconstructions from particles embedded in vitreous ice showed that lipase has a double-ringed, 20 meric structure with C20 symmetry. At 6.5 Å resolution, it was possible to unambiguously elucidate the complex barrel shape structure with the 20 individual subunits. The 3D reconstruction also revealed a subunit organization between the two rings of the complex. The density map has the area of 607.2 x 10^3 Å2 and volume of 4.314 x 10^6 $Å^3$

LIST OF ABBREVIATIONS

Å	Angstrom
2D	Two-dimensional
3D	Three-dimensional
CCD	Charge-Coupled Device
CD	Circular Dichroism
Cryo-EM	Cryo-electron microscopy
CTF	Contrast transfer function
DMSO	Dimethyl sulfoxide
DPI	Dots per inch
DTT	DiThioThreitol
EDTA	Ethylene diamine tetra acetic acid
EM	Electron microscopy
FSC	Fourier Shell Correlation
HRTEM	High Resolution Transmission Electron Microscopy
kDa	kilo Dalton
Km	Michaelis-Menten constant
NCIM	National Collection of Industrial Microorganisms
NMR	Nuclear Magnetic Resonance
O.D	Optical density
PAGE	Poly-acrylamide gel electrophoresis
PMSF	Peptide mass finger printing
*p*NPC	*p*-nitrophenyl caprylate
*p*NPP	*p*-nitrophenyl palmitate
rpm	revolution per minute
SDS	Sodium dodecyl sulphate
SNR	Signal-to-noise ratio
SOB	Synthetic oil based medium
SPR	single particle reconstruction
TEM	Transmission Electron Microscopy

CHAPTER 1

General Introduction

Beginning with the development of basic tools by our human predecessors, humans have continually searched for and utilized novel materials from the natural environment to survive and thrive. Today, our knowledge of the surrounding world extends to the molecular scale as we enter the age of genomics and systems biology, enabling previously unimaginable insight into processes that promise application in the agricultural, energy, food, medical, materials and textile industries. As environmental concerns arise, biological tools are increasingly replacing harsh chemical and physical means of processing materials and they even harbor promise for creating cost-effective sustainable energy sources. It is imperative that we continue investigating ways in which natural products can offer economical alternatives to traditional industrial processes.

As estimated, 75% of the Earth's biosphere is located in perennially cold (< 5°C) environments, including little-explored deep-sea, polar and alpine regions, shallow subterranean systems and the upper atmosphere. Organisms successfully inhabit these environments, including representatives from the *archaea* (Cavicchioli, 2006), bacteria (Christner et al., 2000; Deming, 2002), yeast (Pavlova et al., 2001; Butinar et al., 2007; Turchetti et al., 2008) and *eukarya* (Peck, 2002), in addition to viruses (Wells and Deming, 2006). These organisims surviving at low temperature must possess adaptations that enable growth and activity at low temperature. In order to overcome the detrimental effects of low temperature, cold-adapted organisms have developed numerous strategies to maintain sufficient rates of metabolic activity for their survival in cold environments. Such adaptive strategies includes alterations in membrane fluidity (Russell, 1997), expression of cold-shock and cold-acclimation proteins involved in transcription and translational processes (Michel et al., 1997), antifreeze/ice-nucleating proteins (Muryoi et al., 2004), production of compatible solutes and exopolysaccharides (Nichols et al., 2005). The necessary cold-adaptation of all aspects of these organisms suggest that a wide variety of biomolecules may find application in existing and future biotechnological processes, such as the production of polyunsaturated fatty acids for aquaculture (Russell, 1997), the use of ice-nucleating proteins for food processing (Li and Lee, 1995), and the application of cold-active enzymes for their various beneficial properties. Despite the great potential that cold environments hold for revealing diverse products and processes which may have countless industrial applications, our knowledge of the

ecology, physiology, metabolism, enzymology and genetics of cold-adapted organisms remains limited.

Lipases

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are most important kind of industrial enzymes that account for about 30% of the total worldwide sale of industrial enzymes in the world market (Jaeger et al., 1999; Hasan et al., 2006). Lipases are ubiquitous and indispensable enzymes playing a pivotal role in fat and lipid metabolism in variety of organisms. These enzymes are widely distributed in plants, animals and microorganisms and are accordingly classified as plant, animal and microbial lipases. In humans and animals, different lipases control the digestion, absorption and reconstitution of fat and lipoprotein metabolism (Desnuelle, 1986). In plants, metabolism of oil reserves during post germination provides energy and carbon skeleton for embryonic growth and this metabolism of oil reserves is controlled by action of various lipases (Huang, 1987). Microorganisms produce wide spectrum of intra- or extracellular lipid degrading enzymes to break down the insoluble lipid in to soluble components to facilitate absorption (Lie et al., 1991). Lipases catalyze the hydrolysis of esters. However, lipases preferentially catalyze the hydrolysis of water-insoluble long chain esters such as triglycerides (Figure 1.1). The *in vitro* enzymatic activity of lipases can be measured by the hydrolysis of a wide variety of carboxylic acids esters, as well as by the highly specific cleavage of long-chain acylglycerols (Jaeger et al., 1994; Beisson et al., 2000).

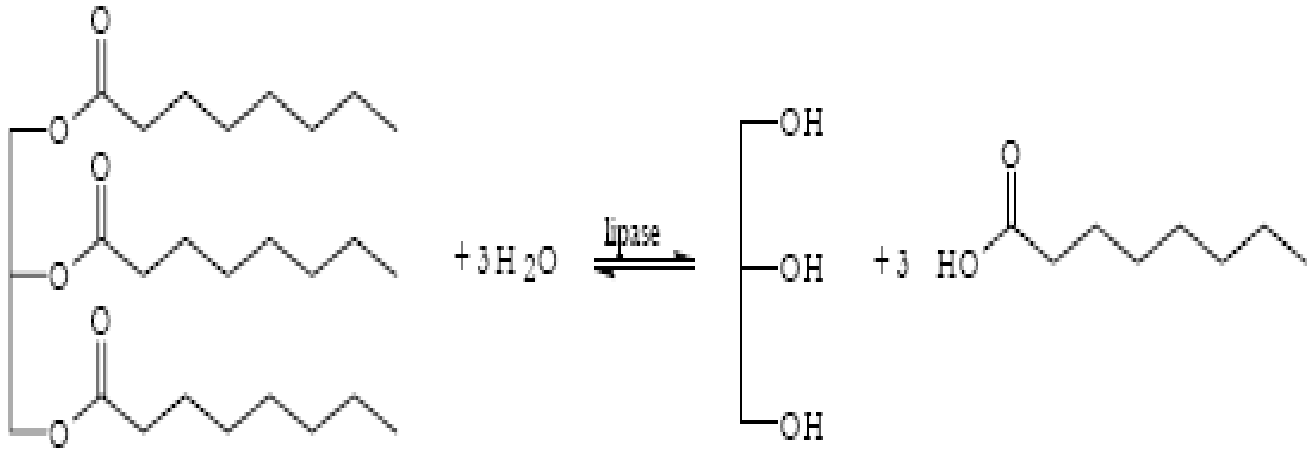


FIGURE 1.1: Lipase catalyzed hydrolysis of a triglyceride (Jaeger et al., 1994).

General lipase structure

The three dimensional structures of *Rhizomucor miehei* and human pancreatic lipases were determined in 1990 (Brady et al., 1990; Winkler et al., 1990). Since then, structures of many lipases especially from microbial origin have been solved (Cygler and Schrag, 1997). These enzymes with a molecular weight ranging from 19 to 60 kDa exhibit a characteristic folding pattern referred to as α / β hydrolase fold (Ollis et al., 1992). Hence all lipases are called as α / β hydrolases with a central core composed of upto eight β strands connected by six helices. The active site located in the central core of β strands is formed by catalytic triad consisting of serine, aspartic or glutamic acid and histidine always in order of this sequence. The nucleophilic serine residue that is conserved in lipases is located at the C-terminal end of strand β5 in a highly conserved peptide sequence Gly-X-Ser-X-Gly forming a sharp γ-like turn between β5 strand and a following α helix strand. Aspartic acid residue located in the reverse turn after the β7 strand is hydrogen bonded to catalytic histidine which is located in a loop after β8 strand (Ollis et al., 1992). Another common feature of α / β hydrolase fold is the formation of oxyanion hole which stabilizes the transition state. The first lipase structure from a thermostable organism *Bacillus stearothermophilus* is described (Tyndall et al., 2002).

Although all lipases investigated so far are structurally similar, subtle variations in the architecture of the substrate binding sites may have strong effect on catalytic properties. Human pancreatic lipase (HPL) is a major lipase involved in digestion of dietary triglycerides (Carriere et al., 1993). HPL is directly secreted as an active enzyme with 449 amino acid residues and has a molecular weight of 50 kDa. This enzyme is divided in to two distinct domains: a larger N-terminal domain comprising 1-335 residues and a smaller C-terminal domain with 336-449 residues (Winkler et al., 1990). The domains are separated by a short unstructured stretch of amino acids and are stabilized by seven disulphide bonds. The N-terminal domain is typical α / β hydrolase fold formed by a central parallel β sheet. This large domain contains the active site, a glycosylation site, Ca-binding site and possibly a heparin binding site. The active site is covered by a surface loop and is thus inaccessible to solvent (Winkler and Gubernator, 1994).The binding of co lipase to C-terminal domain through hydrophobic interaction keeps the lid in open conformation and allows the biolysis to proceed at a high rate (Lowe, 1997). Co lipase may also bind to N-terminal domain, which is necessary for reactivation of bile salt inhibited pancreatic lipase (Lowe, 1997).

Human gastric lipase (HGL) contains one major domain containing central core of β sheets and the extrusion domain composed of a "cap" and a segment consisting of 30 residues which is identified as lid. The catalytic serine is deeply buried under the extrusion domain and the displacement of lid is required for the access of substrate to the active site. HGL has only one disulphide bond in the molecule and four glycosylation sites (Cannan et al., 1999).

In most of the lipases from mammals, fungi and bacteria, the most conserved sequence is pentapeptide G-X-Ser-X-G and most lipases are active at water-oil interphase. However, lipases from *Bacillus* sp. have the sequence A-X-Ser-X-A instead of conserved sequence G-X-Ser-X-G found in most other lipases (Ransac et al., 1994).

Modifications of lipase structure for cold adaptation

Cold active lipases are structurally modified by an increasing flexibility of the polypeptide chain enabling an easier accommodation of substrates at low temperature. The fundamental issues concerning molecular basis of cold activity and the interaction between flexibility and catalytic efficiency are important in the study of structure–function relationships in enzymes. Such issues are often approached through comparison with the mesophilic or thermophilic counterparts, by site-directed mutagenesis and 3D crystal structures (Narinx et al., 1997; Wintrode et al., 2000). The molecular modeling of *Pseudomonas immobilis* lipase revealed several features of cold-adapted lipases (Arpigny et al., 1997). A very low proportion of arginine residues as compared to lysines, a low content in proline residues, a small hydrophobic core, a very small number of salt bridges and aromatic–aromatic interactions are the possible features of lipase for cold adaptation. Similarly, the weakening of hydrophobic clusters, the dramatic decrease (40%) of the Proline content and of the ratio Arg/ Arg+Lys makes lipases active at low temperature (Gerday et al., 1997). Moreover, when compared to the dehalogenase from *Xanthobacter autotrophicus*, the cold active lipase displays a very small number of aromatic–aromatic interactions and salt bridges. The location of some salt bridges which are absent in the cold lipase seems to be crucial for the adaptation to cold.

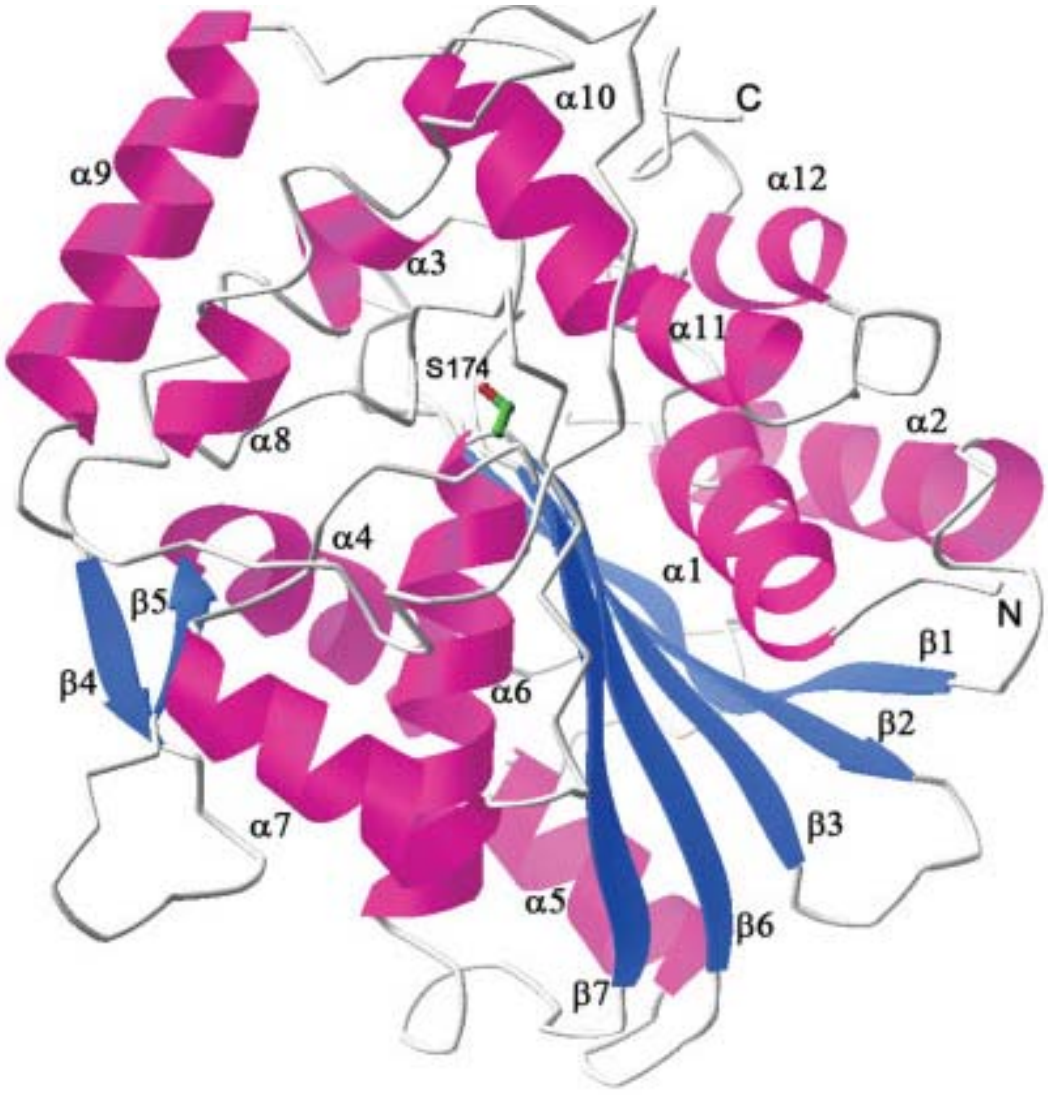
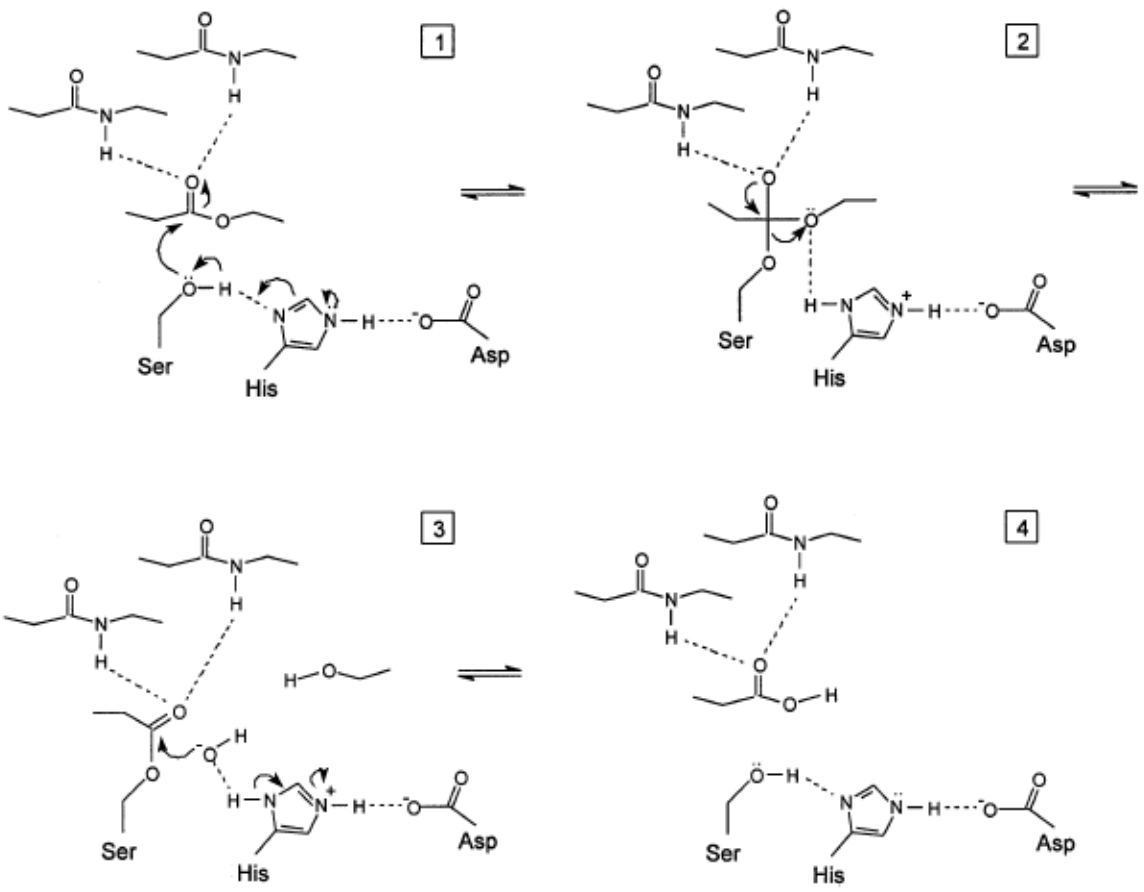


Figure 1.2: Structure of cold active lipase from *Photobacterium lipolyticum*. A: Ribbon diagram of the structure of lipase. Secondary structural elements are labeled (helix, purple; strand, blue; loop, grey). Catalytic residue Ser174 are shown as a ball-and-stick representation. The boundaries of secondary structural elements are β1 (52–61), β2 (71–78), β3(84–90), β4(110–112), β5(124–126), β 6(166–173), β7(199–206), α1(5–15), α2(30–43), α3(97–100), α4(127–138), α5(152–160), α6(175–189), α7(214–224), α8(244–247), α9(265–278), α10(304–313), α 11(315–319) and α12(328–332) (Jung et al., 2008).

A large amount of charged residues exposed at the protein surface, have been detected in the cold active lipase from *Pseudomonas fragi* (Alquati et al., 2002). They also observed a reduced number of disulphide bridges and Prolines in loop structures. Arginine residues were distributed differently than in mesophilic enzymes, with only a few residues involved in stabilizing intramolecular salt bridges and a large proportion of them exposed at the protein surface that may contribute to increased conformational flexibility of the cold-active lipase. In addition to this, the structural factors possibly involved in cold adaption are increased number and clustering of glycine residues (providing local mobility), lower number of ion pairs and weakening of charge-dipole interactions in α helices (Georlette et al., 2004; Gomes and Steiner, 2004). The substitution of Glycine with Proline by mutation caused a shift of the acyl chain length specificity of the enzyme towards short-chain fatty acid esters and enhanced themostability of the enzyme (Kulakovaa et al., 2004). A mutation in the lid region of catalytic triad of cold active lipases from *P. fragi* improved substrate selectivity and thermostability (Santarossa et al., 2005). Introduction of polar residues in the surface of exposed lid might be involved in improved substrate specificity and protein flexibility. The sequence alignment study of cold active lipase from *Photobacterium lipolyticum* showed three aminoacid residues (Ser174, Asp236 and His312) constitute an active site and RG residues (Arg236 and Gly91) making an oxyanion sequence (Ryu et al., 2006). The cold active lipase from *Pseudoalteromonas haloplanktis* TAC125 showed electrostatic surface having a large number of nonpolar residues, which make the surface highly hydrophobic (De Pascale et al., 2008). Structural analysis of *Photobacterium lipolyticum* M37 lipase (Figure 1.2) adopted a folding pattern similar to that observed in other lipase structures. However, the region beneath the lid of the M37 lipase included a significant and unique cavity that would be occupied by a lid helix upon substrate binding. In addition, the oxyanion hole was much wider in M37 lipase. These distinct structural characteristics of M37 lipase facilitate the lateral movement of the helical lid and subsequent substrate hydrolysis, which explains its low activation energy and high activity at low temperatures (Jung et al., 2008). It is understood that the catalytic cavity of the psychrophillic lipase is characterized by high plasticity. These structural adaptations may confer on the enzyme a more flexible structure, in accordance with its low activation energy and its low thermal stability. The above discussions may help obtain information for insights into the molecular mechanisms of cold adaption and thermolability of cold active lipases.

Mechanism of lipolysis

Lipases act on a variety of substrates like triacylglycerols, cholesterol esters and wax esters, which are insoluble in water. A typical reaction catalyzed by a lipase can be represented as shown in (Figure 1.1). Lipase active site consists of a Ser-His-Asp/Glu catalytic triad. This catalytic triad is similar to that observed in serine proteases and hence lipases are thought to follow essentially the same catalytic mechanism (Wrinkler and Gubernator, 1994). The catalytic reaction involves many elementary steps: 1) The formation of noncovalent Michaelis complex 2) Nucleophilic attack by the catalytic-site-serine oxygen on a carbonyl carbon atom of ester bond, leading to formation of transient tetrahedral intermediate stabilized by hydrogen bonding with two peptide NH groups belonging to so called Oxanion hole 3) An alcohol is liberated, leaving behind an acyl-lipase complex, which is hydrolyzed with the liberation of the fatty acids and the regeneration of the enzyme. This catalytic mechanism is supported by the X-ray structures of lipases covalently complexed with hydrophobic inhibitors such as alkyl phosphonates or alkyl sulphonates. These structures reveal an open lid, suggesting that the phosphonates mimic the transition state for acylation and the sulphonates for deacylation of natural triacyl glycerol ester substrates (Derewenda and Derewenda, 1991; Derewenda and Sharp, 1993). The catalytic mechanism of lipases based on catalytic triad is given in (Figure 1.3).



FFIGURE 1.3: Reaction mechanism of lipases. (1) Binding of lipid, activation of nucleophilic serine residue by neighboring histidine and nucleophilic attack of the substrate's carbonyl carbon atom by Ser Oi. (2) Transient tetrahedral intermediate, with Oi stabilized by interactions with two peptide NH groups. The histidine donates a proton to the leaving alcohol component of the substrate. (3) The covalent intermediate ("acyl enzyme"), in which the acid component of the substrate is esterified to the enzyme's serine residue. The incoming water molecule is activated by the neighboring histidine residue, and the resulting hydroxyl ion performs a nucleophilic attack on the carbonyl carbon atom of the covalent intermediate. (4) The histidine residue donates a proton to the oxygen atom of the active serine residue, the ester bond between serine and acyl component is broken, and the acyl product is released (Jaeger et al., 1999).

Activation of lipolytic enzymes by interfaces

Lipase activity is greatly increased at the lipid-water interface of miceller or emulsified substrate (Sarda & Desnuelle, 1958; Schmid and Verger, 1998; Reis et al., 2008; Reis et al., 2009), a phenomenon known as interfacial activation. It was proposed to correlate the interfacial activation with transition between the closed (inactive) form and the open (active) form of lipase (Verger, 1997). The increase in activity is due to the structural rearrangement of lipase active site region (Brady et al., 1990; Kohno et al., 1996). In absence of lipid-water interface, the active site is covered by so called lid resulting in closed (inactive) conformation. However, the presence of hydrophobic substances, the inactive enzyme is first absorbed to interface. This absorption leads to the opening of the lid (open confirmation) making the catalytic residues accessible to substrate and exposing a large hydrophobic surface. This large hydrophobic surface is presumed to interact with the lipid interface. This hypothesis was confirmed by elucidation of X-ray structures of several inhibitor bound lipases (Brzozowski et al., 1991; Nardini et al., 2000; Eydoux et al., 2008). This lid may consist of single helix in *Rhizomucor miehei* lipase (Brzozowski et al., 1991; Derewenda et al., 1992), two helices in case of *Pseudomonas cepacia* lipase (Kim et al., 1997; Schrag et al., 1997), a loop region in *Candida rugosa* lipase (Grochulski et al., 1994) or presences of two lids in case of *Staphylococcus simulans* lipase (Frikha et al., 2008). This lid hypothesis has since been used to discriminate between true lipases and esterases (Verger, 1997; Jaeger et al., 1999). In addition, the lid domain has also been identified as being important for substrate recognition, catalytic activity, substrate specificity (Carriere et al., 1998; Chahinian et al., 2002; Brocca et al., 2003; Santarossa et al., 2005; Secundo et al., 2006). This region is also important for enantioselectivity (Liebeton et al., 2000; Secundo et al., 2006; Boersma et al., 2008a) and activity in organic solvent (Mingarro et al., 1995; Fishman and Cogan. 2003). Two CALB mutants were generated by replacing the lid region of *C. antarctica* with corresponding lid regions from the CALB homologues from *Neurospora crassa* and *Gibberella zeae*. The characterization of these mutant lipases revealed the improved hydrolytic activity on simple esters, and also increased enantioselectivity in hydrolysis of racemic ethyl 2-phenylpropanoate (Skjøt et al., 2009). In summary, lid like structural element constitutes the most important structural elements of lipases (Secundo et al., 2006). Although all lipases show interfacial activation phenomenon, there are some exceptions. For example, lipase from *Bacillus subtilis*

(Lesuisse et al., 1993; van Pouderoyen et al., 2001), *Staphylococus simulans* lipase (Sayari et al., 2001), lipase from *Xanthomonas compestris* did not show interfacial activation (Yang et al., 2006). All these lipases lack a lid that covers the active site in absence of lipid-water interfaces.

Lipases of Yarrowia lipolytica

Recently attention has been turned to nonconventional yeasts (NCY). Interest in these microorganisms may be due to a variety of reasons, ranging from their use in specific technological applications to the treatment of infections caused by some of them (Flores et al., 2000). *Y. lipolytica* is an obligate aerobic dimorphic ascomycete that naturally secretes large amounts of various metabolites (such as organic acids and extracellular proteins) (Barth and Gaillardin, 1997). It is able to produce several substances of biotechnological importance, including citric acid (Finogenova et al., 2002) and enzymes (McEwen and Young, 1998). It is also being used for expression of heterologous proteins (Madzak et al., 2000) and biotransformations (Mauersberger et al., 2001). Since an increasing number of studies have clearly shown that *Y. lipolytica* occurs naturally in various kinds of food, information about this yeast is of great importance to the agro-food industry (Fickers et al., 2005a). Strains of this species have mostly been isolated from lipid-rich food such as cheese and olive oil, as well as from sewage, and this yeast grows readily on hydrophobic substrates such as alkanes, fatty acids and oils (Yamagami et al., 2001; Yamagami et al., 2004; Fickers et al., 2005a; Beopoulos et al., 2009) and thus can serve as an alternative in wastewater degradation (Scioli and Vollaro, 1997). Its substrate preferences and physiological properties have been attributed to the fact that this yeast efficiently produces and secretes proteolytic and lipolytic enzymes (Barth and Gaillardin, 1997). Several processes have been developed involving the use of *Y. lipolytica* in bioconversion reactions with hydrophobic substrates (Fickers et al., 2005a), `some of which have been patented in the fields of bioremediation, fine chemistry and the food industry. For instance, *Y. lipolytica* can be used for alkane and fatty-acid bioconversion, the production of aromas, single-cell protein (SCP), single-cell oil (SCO) and citric acid, as well as for steroid biotransformation. This yeast naturally secretes several proteins, depending on the growth conditions (Heslot, 1990). For example, if the pH is higher than 6, it secretes an alkaline extracellular protease (AEP) (Nicaud et al., 1989). Under optimal conditions, up to 1 g of AEP

per liter is secreted by this organism (Ogrydziak et al., 1993). Since *Y. lipolytica* can use fat and oils as carbon sources, increasing attention has been paid to the lipases produced by this yeast. However, factors controlling lipase synthesis and transport have been investigated only in few cases. Lipase secretion was first reported in 1948 by Peters and Nelson (Peters and Nelson, 1948a; 1948b), who described a single type of glucose repressible activity. An extracellular and two cell-bound types of activity corresponding to lipase I (39 kDa) and lipase II (44 kDa) were described by Ota and coworkers (Sugiura et al., 1976; Ota et al., 1982). The extracellular lipase required oleic acid as a stabilizer-activator, whereas the cell-bound lipases did not require such stabilizers-activators. Cell bound lipases differed in several properties from the extracellular enzyme (Ota et al., 1984). The rates of production of the extracellular and cell-bound enzymes were reported to depend on the carbon and nitrogen composition of the medium. Extracellular lipase was only detected in cultures grown with an organic nitrogen source (Novotny et al., 1988; Pereira-Meirelles, 1997), and lipase levels were shown to be modulated by cell morphology. In minimal medium supplemented with *N*-acetylglucosamine or citrate buffer, both of which promote dimorphic growth, higher levels of cell-bound lipases were detected. However, no clear relationship was established between the dimorphic state and lipase production (Novotny et al., 1994). In *Y. lipolytica*, the lip2 gene which codes for a 301-amino acid extracellular lipase YLLIP2 has been cloned and amplified and it was established that most of the extracellular lipase activity results from lip2 expression (Pignede et al., 2000a; Pignede et al., 2000b). This yeast produces a 38 kDa extracellular Lip2 lipase, encoded by the LIP2 gene. The optimal activity was found at 37 °C and pH 7 whereas K_m and k_{cat} values, were found equal to 807 mM and 30 × 103 min^{-1}, respectively (Pignede et al., 2000b). The Lip2 prefered substrates are the saturated triglyceride tricaprylin (C8: 0), the unsaturated triglyceride triolein (C18:1) and the long-chain fatty acid methyl ester (C12, C14, C16). The higher activity observed towards triglycerides compared to hydrophilic fatty acid methyl ester indicate that Lip2 is a true lipase (Yu et al., 2007). The recent studies reported that methyl oleate modulates YLLIP2 expression in *Y. lipolytica* (Fickers et al., 2005b). Although YLLIP2 seems to be involved in the use of triglycerides as a carbon source for the yeast, the finding that a lip2 knock-out strain is still able to use this source suggested the presence of other lipases in *Y. lipolytica*. The cloning and functional analysis of the lip7 and lip8 genes encoding two predicted lipases were recently performed (Fickers et al., 2005c). Unlike YLLIP2, which is

secreted into the culture medium, it was observed that YLLIP7 and YLLIP8 are mainly associated with the cell wall. Fungal lipases mainly belong to two families: (i) lipases which are homologous to acetylcholine esterase (i.e. *Geotrichum candidum* and *Candida rugosa* lipases) and (ii) triacylglycerol lipases including *Rhizopus oryzae*, *Rhizomucor miehei*, *Candida antarctica* and *Thermomyces lanuginosus* (Murzin et al., 1995). Amino acid sequence comparisons have showed that YLLIP2 belongs to the second group. The highest amino acid sequence similarity was 91.6% with *Candida deformans* lipase LIP1. Great similarities were also found to exist with the lipases from *Thermomyces lanuginosus* (30.3%), *Candida ernobii* (27.7%) and *Rhizomucor miehei* (25.1%). In spite of the great interest in both lipases and *Yarrowia*, much in respect to them is yet unknown. As a consequence, the choice of the culture conditions, and in particular of the medium composition, is important to production of *Yarrowia lipolytica* lipase preparations with defined and reproducible properties. In this context, it is necessary to obtain an exhaustive description of the effect of a wide range of substrates on the yield and quality of the enzyme.

Cold active lipases

Cold active lipases are largely distributed in microorganisms surviving at low temperatures. Although a number of lipase producing sources is available, only a few bacteria, fungi and yeast were reported for the production of cold active lipases. Attempts have been made from time to time to isolate cold active lipases from these microorganisms having high activity at low temperatures. A list of various cold active lipase producing psychrophillic and psychrotrophic bacteria is presented in Table 1. These bacterial strains were isolated mostly from Antarctic and Polar regions which represent a permanently cold and constant temperature habitat. Various studies have shown that a high bacterial count has been recorded as high as 105 ml^{-1} and 106 ml^{-1} in water column and in the sea ice respectively (Sullivan and Palmisano, 1984; Delille, 1993). Another potential source of cold active lipases is deep-sea sediments. There have been reports on biological communities present around the areas of the cold environments below a depth of 1,000 m that have a constant temperature of 4–5°C. These biological communities harbor a variety of microorganisms (Feller and Gerday, 2003) which are considered to be very unique (Gray, 2002; Levin et al., 2001). Few bacterial genera have been isolated and characterized from deep-sea sediments where temperature is below 3 °C.

They include *Aeromonas* sp. (Lee et al., 2003), *Pseudoalteromonas* sp. and *Psychrobacter* sp. (Zeng et al., 2004), *Psychrobacter* sp. 7195 (Zhang et al., 2007), *Photobacterium lipolyticum* (Ryu et al., 2006), *Pseudomonus fragi* (Aoyama et al., 1988; Alquati et al., 2002), *Pseudomonas fluorescens* (Dieckelmann et al., 1998) and *Serratia marcescens* (Abdou, 2003). Permanently cold regions such as glaciers and mountain regions are another habitat for microorganisms producing cold active lipases. The soil and ice in Alpine region also harbor psychrophillic microorganisms. In addition to all these permanently cold regions, there are many other accessible and visible soil and water regions which become cold both diurnally and seasonally from which cold active lipase producing microbes are isolated that includes, *Acinetobacter* sp. CR9 (Kasana et al., 2008), *Pseudomonas* sp. 7323 (Zhang and Zeng, 2008). The widespread use of refrigeration to store fresh and preserved foodstuff provides a great diversity of nutrient rich habitat for some well known psychrotolerant food spoilage microorganisms. A list of various cold active lipase producing psychrophillic and psychrotrophic fungi is presented in Table 2. They include *Candida lipolytica*, *Geotrichum candidum* and *Pencillium roqueforti* isolated from frozen food samples (Alford and Pierce, 1961), *Aspergillus nidulans* (Mayordomo et al., 2000) and *Mariannaea camptospora* (Peterson et al., 2009). More recently cold active lipase was isolated from uncultured microbial communities by metagenomic approach (Elend et al., 2007; Jeon et al., 2009).

Table 1: Sources of cold active bacterial lipases.

Organisms	Sources	References
Geotrichum sp. SYBC WU-3	Chinese soil	Cai et al., (2009)
Acinetobacter baumannii BD5	water on agar plates of Luria-Bertani (LB) medium	Park et al., (2009)
Pseudomonas sp. MSI057	marine sponge	Kiran et al., (2008)
Moritella sp. 2-5-10-1	Antarctic region	Yang et al., (2008)
Pseudoalteromonas haloplanktis TAC125	Antarctic seawater	De Pascale et al., (2008)
Acinetobacter sp. CR9	Chandra river in sub-alpine region of western Himalaya	Kasana et al., (2008)
Pseudomonas sp. 7323	deep-sea sediment	Zhang and Zeng, (2008)
Psychrobacter sp. 7195	Antarctic habitat	Zhang et al., (2007)
Psychrobacter sp.	Antarctic habitat	Parra et al., (2008)
Pseudomonas fluorescens	soil samples	Luo et al., (2006)
Corynebacterium paurametabolum MTCC 6841,	Naukuchiatal lake Uttaranchal	Joshi et al., (2006)
Bacillus sphaericus MTCC 7526	Gangothri Glacier (Western Himalaya)	Joseph et al., (2006)
Photobacterium lipolyticum M37	Marine habitat	Ryu et al., (2006)
Pseudoalteromonas sp.	Antarctic marine	Lo Giudice et al., (2006)
Staphylococcus epidermidis	Frozen fish samples	Joseph et al., (2006)

Psychrobacter sp. Ant300	Antarctic habitat	Kulakovaa et al., (2004)
Psychrobacter sp. wp37	Deep-sea sediments	Zeng et al., (2004)
Aeromonas sp. LPB 4	Sea sediments	Lee et al., (2003)
Psychrobacter okhotskensis.	Sea coast	Yumoto et al., (2003)
Serratia marcescens	Raw milk	Abdou, (2003)
Pseudomonas fragi strain no. IFO3458	BCCM LMG2191 bacteria collection	Alquati et al., (2002)
Acinetobacter sp. strain no. 6	Alaskan and Siberian tundra soil	Suzuki et al., (2001)
Pseudomonas sp.	Subterranean water sample	Rashid et al., (2001)
Pseudomonas fluorescens	Refrigerated human placental extracts	Preuss et al., (2001)
Pseudomonas sp. B11-1:	Alaskan and Siberia soil	Choo et al., (1998)
Aeromonas hydrophila	Marine habitat	Pemberton et al., (1997)
Psychrobacter immobilis strain B 10	Antarctic habitat	Arpigny et al., (1997)
Pseudomonas P38	Ns	Tan et al., (1996)
Morexella sp TA144	Antarctic habitat	Feller et al., (1991)
Moraxella sp.	Antarctic habitat	Feller et al., (1990)
Pseudomonas fluorescens	Refrigerated food	Andersson, (1980)

Table 2: Fungi producing cold active lipases.

Organisms	Sources	References
Mariannaea camptospora	koala faeces	Peterson et al., (2009)
Candida antarctica	Antarctic habitat	Siddiqui and Cavicchioli, (2005) Zhang et al., (2003) Koops et al., (1999) Patkar et al., (1997)
Aspergillus nidulans	Ns	Mayordomo et al., (2000)
C. lipolytica	Frozen food	Alford and Pierce, (1961)
Geotrichum candidum	Frozen food	Alford and Pierce, (1961)
Pencillium roqueforti	Frozen food	Alford and Pierce, (1961)

Production of cold active lipases

Microorganisms have been regarded as best sources of useful enzymes. In recent years, there has been a phenomenal increase in the use of enzymes as industrial catalysts. These enzymes offer advantages over the use of conventional chemical catalysts for numerous reasons**:** they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts, highly biodegradable, pose no threat to the environment, and are economically viable. Cold active lipases are mostly extracellular and are highly influenced by nutritional and physicochemical factors such as temperature, agitation, pH, nitrogen source, carbon source, inducers, inorganic sources and dissolved oxygen. Submerged fermentation is the most common method used for cold active lipase production (Dieckelmann et al., 1998; Lee et al., 2003; Kiran et al., 2008). A list of various production parameters for different cold active lipase producing microorganisms is given in Table 3. Cold-adapted microorganisms tend to have good growth rate at low temperature. *Pseudoalteromonas haloplanktis* TAC125 is isolated from Antarctic seawater (Médigue et al., 2005) which was grown in aerobic conditions at 4 ^{o}C and 15 ^{o}C (De Pascale et al., 2008). The production of cold active lipase is considered

to be temperature dependent and thermolabile (Mayordomo et al., 2000; Rashid et al., 2001). *Moraxella* sp. isolated from Antarctican habitat grows well at 25 °C and produced cold active lipolytic enzyme (Feller et al., 1990). *Staphylococcus epidermidis* isolated from spoiled frozen marine fish produced lipase at 22 °C (Joseph et al., 2006). Normally psychrotrophs have the highest enzyme production at temperatures lower than their optimal growth temperatures. In *Psychrobacter* sp., highest lipase activity was produced in the late logarithmic phase when the organism was grown at 15–20 °C (Zeng et al., 2004). In *Bacillus mycoides*, there was no growth or production of lipase below 10 °C or above 50 °C. The absence of lipase activity in the culture supernatant above 50 °C is probably due to the less thermoliability of enzymes (Joseph et al., 2006). *Pseudomonas* sp. strain B11-1 utilized yeast extract and tryptone as best carbon and nitrogen sources for growth and production of lipases. Tween 80 and Tributyrin induced production of cold active lipases at 4 °C with an optimum pH 7.6 (Choo et al., 1998). *A. nidulans* WG312 produced cold active lipase at 30 °C by utilizing olive oil as an inducer (Mayordomo et al., 2000). Soybean oil induced the production of cold active lipases from *Acinetobacter* sp. strain no.6 at 4 °C (Suzuki et al., 2001). *Aeromonas* sp. LPB 4 produced lipase at 10 °C by using tryptone and yeast extract as carbon and nitrogen source and trybutylin as an inducer (Lee et al., 2003). Another isolate of *Pseudoalteromonas* sp. wp27 produced lipases at 25 °C with olive oil and Tween 80 as inducers (Zeng et al., 2004). Trybutrin induced the production of *Staphylococcus epidermidis* cold active lipase by using peptone as nitrogen source (Joseph et al., 2006).

Table 3: Production of cold active lipases.

Organism	Temperature	pH	Carbon sources	Nitrogen Sources	References
Pseudoalteromonas haloplanktis TAC125	4 & 15 ^{o}C	Ns	Ns	Ns	De Pascale et al., (2008)
Pseudomonas sp. (MSI057)	30 ^{o}C	9.0	Tributyrin	Ammonium Sulphate	Kiran et al., (2008)
Bacillus sphaericus MTCC 7526	15 ^{o}C	8	Tributyrin	Peptone	Joseph et al., (2008)

Microbacterium phyllosphaerae MTCC 7530	15 ^{o}C	8	Tributyrin	Peptone	Joseph et al., (2008)
Corynebacterium paurometabolum MTCC 6841	25 ^{o}C	8.5	Soybean oil/olive oil	NaNO3 and KNO3	Joshi et al., (2006)
Staphylococcus epidermidis	22 ^{o}C	7.0	Tributyrin	Peptone	Joseph et al., (2006)
Psychrobacter sp. wp37	25 ^{o}C	Ns	Tween80, Tween20	Yeast extract	Zeng et al., (2004)
Pseudoalteromonas sp. wp27	25 ^{o}C	Ns	Olive oil, Tween80	Yeast extract	Zeng et al., (2004)
Serratia marcescens	6 ^{o}C	Ns	Skim milk	Ns	Abdou, (2003)
Aeromonas sp. LPB 4	10 ^{o}C	Ns	Trybutylin	Tryptone, Yeast extract	Lee et al., (2003)
Pseudomonas sp. KB700A	−5 ^{o}C	7.2	Tributyrin	Tryptone, Yeast extract	Rashid et al., (2001)
Pseudomonas sp. B11-1	4 ^{o}C	7.6	Tween80, Tributyrin	Yeast extract, Tryptone	Choo et al., (1998)
Moraxella sp.	25 ^{o}C	Ns	Ns	Ns	Feller et al., (1990)
Fungus					
Aspergillus nidulans WG312	30 ^{o}C	Ns	Olive oil	yeast extract	Mayordomo et al., (2000)

Purification and characterization of cold active lipases

Proteins are usually purified by a series of independent steps in which the various physiochemical properties of the proteins of interest are utilized to separate them progressively from other unwanted constituents. The characteristics of the proteins that are utilized in

purification include solubility, ionic charge, molecular size, adsorption properties, and binding affinity to other biological molecules (Robert, 1982). Several microbial cold active lipases have been isolated and purified to homogeneity. The significance of cold active lipases is extensively recognized in a number of applications (Houde et al., 2004). The purified lipases are needed for the synthesis of fine chemicals, cosmetics and in pharmaceutical industries. Sometimes homogenous preparations of cold-adapted lipases are not required for all industrial applications. However, some degree of purity is required for their successful and efficient uses in industries for the production of fine chemicals, pharmaceuticals and cosmetics. The traditional purification procedures are sometimes troublesome leading to low yields of purified enzymes. The purification procedures employed should be inexpensive, rapid and high yielding so that continuous product recovery of the desired product is possible at industrial operations. Therefore, alternative novel technologies are needed to increase the overall yield and to reduce the number of steps in the down streaming processes. Since lipases are different from other enzymes in terms of their hydrophobic nature and interfacial activation phenomenon, some novel purification technologies have been employed. These novel technologies include membrane processes, hydrophobic interaction chromatography with epoxy-activated spacer arm as a ligand and aqueous two phase systems (Saxena et al., 2003; Gupta et al., 2004). Techniques employed for purification of some of the microbial cold active lipases are given in Table 4.

The general purification steps involve concentration of the crude preparations containing lipases by ammonium sulphate precipitation, ultrafiltration or extraction with organic solvents. Since lipases are known to be hydrophobic in nature having large hydrophobic surfaces around the active site, the purification may be achieved by employing affinity chromatographic techniques. The widely used chromatographic technique involves columns packed with QAE sephadex, CM cellulose, DEAE cellulose, phenyl-sepharose etc. However in certain applications, further purification can be achieved by gel filtration chromatography. The usual procedures for purification of lipases are troublesome, time consuming and results in low yield. Low thermostability of these cold active lipases is also a major problem in purification. Novel purification steps are therefore needed to increase the overall enzyme yield and purification fold.

Table 4: Purification strategies employed for cold active lipases.

Organism	Purification scheme	Fold purification	% Recovery	Mol. Wt kDa	References
Geotrichum sp. SYBC WU-3	DEAE-cellulose-32 Sephadex G100	Ns	20.4%	41.1 kDa	Cai et al., (2009)
Pseudomonas sp. MSI057	Ammonium sulphate precipitation	6.62	43.67	50 kDa	Kiran et al., (2008)
Pseudomonas sp. 7323	DEAE Sepharose CL-6B Sephadex G-75 Prep. SDS-PAGE	13.7	23	67 kDa	Zhang & Zeng. (2008)
Pseudoalteromonas haloplanktis TAC125	Q-Sepharose	12	7.8	Ns	De Pascale et al., (2008)
Psychrobacter sp. 7195	DEAE Sepharose CL-4B, Sephadex G-75	35	13.7	35 kDa	Zhang et al.., (2007)
Bacillus sphaericus MTCC 7526	DEAE cellulose	17.74	4.70	40 kDa	Joseph et al., (2008)
Microbacterium phyllosphaerae MTCC 7530	DEAE cellulose	22.03	7.50	42 kDa	Joseph et al., (2008)
Serratia marcescens	CM Cellulose, DEAE cellulose Sephadex G-150	20	45	52 kDa	Abdou, (2003)
Aeromonas sp. LPB 4	QAE Sephadex	53.5	7.5	50 kDa	Lee et al., (2003)
Aspergillus nidulans WG312	phenyl-sepharose , linolenic acid-agarose	1186	10	29 kDa	Mayordomo et al., (2000)
Moraxella sp.	AcA 34 column, Ultrafiltration	Ns	Ns	Ns	Feller et al., (1990)

Table 5: Characterization of cold active lipases.

Organism	Temperature		Activation energy (Kcal/mole)	References
	Optima	Stability		
Acinetobacter baumannii BD5, [recombinant]	35 ^{o}C,	Ns	Ns	Park et al., (2009)
Geotrichum sp. SYBC WU-3	Lipase-A 20 ^{o}C, Lipase-B 15 ^{o}C.	both the enzymes retained 10% of their activity after 60 min at 50 ^{o}C	Ns	Cai et al., (2009)
Deep-sea sediment metagenome	25 ^{o}C	Highly stable at 10–35 ^{o}C, Retaining 80% of the initial activity	3.28	Jeon et al., (2009)
Intertidal Flat Metagenome	30 ^{o}C	Ns	Ns	Kim et al., (2009)
Activated sludge Metagenome	40 ^{o}C	showed more stability at 10 ^{o}C than 20 ^{o}C	Ns	Roh and Villatte. (2008)
Baltic Sea sediment Metagenome	35 ^{o}C	Half-life at 40 ^{o}C is 5 min	Ns	Hårdeman and Sjöling. (2007)
Psychrobacter sp. [recombinant]	20 ^{o}C	Half-life at 30 ^{o}C is 20 min.	5.5	Parra et al., (2008)
Pseudomonas sp. MSI057	37 ^{o}C	Stable up to 50 ^{o}C	Ns	Kiran et al., (2008)
Pseudomonas sp. 7323	30 ^{o}C	Half-life at 30 °C is about 4.5 h	Ns	Zhang & Zeng. (2008)
Pseudoalteromonas haloplanktis TAC125	40 ^{o}C.	Ns	Ns	De Pascale et al., (2008)

Psychrobacter sp. 7195	30 ^{o}C	Half-life at 30 ^{o}C is 5 h and at 60 ^{o}C is 15 min.		Zhang et al., (2007)
Bacillus sphaericus MTCC 7526	15 ^{o}C	Ns	Ns	Joseph et al., (2008)
Microbacterium phyllosphaerae MTCC 7530	20 ^{o}C	Ns	Ns	Joseph et al., (2008)
Photobacterium sp .strain, M37	25 ^{o}C	Complete lost of activity above 35 ^{o}C.	2.07	Ryu et al., 2006
Pseudo-alteromonas sp. wp27	20–30 ^{o}C	Ns	Ns	Zeng et al., (2004)
Psychrobacter sp. wp37	20–30 ^{o}C	Ns	Ns	Zeng et al.. (2004)
Serratia marcescens	37 ^{o}C	Ns	Ns	Abdou, (2003)
Aeromonas sp. LPB 4	35 ^{o}C	Stable up to 50 ^{o}C	Ns	Lee et al., (2003)
Pseudomonas sp. strain KB700A	35 ^{o}C	70 % of activity is retained at 60 ^{o}C after 5 min	Ns	Rashid et al., (2001)
Acinetobacter sp. strain no. 6	20 ^{o}C	Half-life at 50 ^{o}C is 30 min	9.8	Suzuki et al., (2001)
Aspergillus nidulans WG312	40 ^{o}C	Ns	Ns	Mayordomo et al., (2000)
Pseudomonas sp. strain B11-1: [recombinant]	45 ^{o}C	Loss of activity above 40 ^{o}C	11.2	Choo et al., (1998)

The effective catalytic properties of enzymes have led to introduction into several industrial products and processes (Koeller and Wong, 2001; Schmid et al., 2001). In the past decade, the technique of protein engineering has allowed investigators to create new enzymes and proteins. This new technology has not only increased our knowledge on structure-function relationships of proteins, but also has led to practical applications (Joo et al., 1998; Koeller and Wong, 2001; Eijsink et al., 2004; Demain and Adrio, 2008; Chaput et al., 2008; Boersma et al., 2008b). The characterization and kinetic study of cold active lipases were studied in terms of optimum pH and stability, optimum temperature, thermo-stability and effect of chelating agents, inhibitors, solvents and metal ions. The cold active lipases have an optimum activity at 20 ^{o}C and are stable at a wide range of temperatures. However, these cold active enzymes are unstable above 60 ^{o}C (Table 5). Lipase hiLip1 (AAZ67909) from Baltic Sea sediment Metagenome showed optimal activity at 35 ^{o}C with 44% of the activity remaining at 10 ^{o}C (Hårdeman and Sjöling, 2007); Lipase LipP (AF034088) of the psychrotrophic *Pseudomonas* sp. strain B11-1 showed apparent optimal activity at 45 ^{o}C and was unstable >44 ^{o}C (Choo et al., 1998); lipase KB-Lip (AB063391) of the psychrotrophic *Pseudomonas* sp. strain KB700A showed optimal activity at 35 ^{o}C, which decreased by 70 % after 5 min at 60 ^{o}C (Rashid et al., 2001); lipase rPFL (AJ250176) of *Pseudomonas fragi* showed optimal activity at 29 ^{o}C, which dramatically decreased at 50 ^{o}C (Alquati et al., 2002); lipo1 from uncultured micro-organism showed optimum activity at 40 ^{o}C, displaying 95 % of the activity at the optimum temperature of 10 ^{o}C (Roh and Villatte, 2008). The reactions catalyzed by the enzymes derived from cold-adapted organisms have usually lower activation energy than those catalyzed by the corresponding enzymes from their mesophilic counterparts (Feller et al., 1996). The cold active lipase from *Psychrobacter* sp. isolated from marine Antarctic origin showed activation energy of 5.5 kcal/mol in the temperature range from 5 to 20 ^{o}C (Parra et al., 2008). *Pseudomonas* sp. strain B11-1 lipase exhibited an activation energy of 11.2 kcal/mol for LipP (Choo et al., 1998); Lipase of *Acinetobacter* sp. strain no. 6 showed an activation energy of 9.8 kcal/mol (Suzuki et al., 2001) and *Photobacterium* strain, M37 lipase has an activation energy of 2.07 kcal/mol for lipase M37 (Ryu et al., 2006). The cold active lipase isolated from Deep-sea sediment metagenome showed activation energy of 3.28 kcal/mol (Jeon et al., 2009). These cold active lipases possessing stability at various physical and chemical conditions may have potentials in biotechnological and industrial applications at low temperatures. The molecular

weight of these purified proteins varied from 29–67 kDa and they have broad substrate specificity. Lipases are widely used as industrial catalyst; there are several advantages and disadvantages for industrial application of cold active lipases. They are easily deactivated when subjected to heat, extreme pH (Jensen, 1983; Longo and Combes, 1999; Matsumoto et al., 2001; Noel and Combes, 2003).

Numbers of strategies have been proposed to overcome such a limitation including the use of soluble additives, immobilization, protein engineering, and chemical modification (Kwon and Rhee, 1984; Chae et al., 1998; Park et al., 2001; Lee et al., 2002). The modification of protein surface with modifiers by chemical binding appears to be a good strategy to improve biocatalyst performance. Modified enzymes were typically macroscopic catalysts that retained in the reactor; therefore, continuous replacement of the enzyme is not necessary. The activity of the cold enzyme presents an apparent optional activity around 35 ^{o}C and retains about 20% of its activity at 0 ^{o}C. The activity of mesophilic lipase is close to zero below 20 ^{o}C and still increases at temperatures above 60 ^{o}C. Properties of cold lipase from *P. immobilis* strain B10 was compared with lipase from mesophillic bacterium, *Pseudomonas aeruginosa* (Arpigny et al., 1997). The activation energies evaluated from the Arrhenius plots are 63 and 110 kJ/mol for the cold and mesophilic enzymes, respectively underlining the cold character of lipase produced by the Antarctic bacterium. This characteristic was also illustrated by the high thermosensitivity of the cold active lipase displaying at 60 ^{o}C a half-life 2 orders of magnitude lower than that of the mesophillic enzyme. The widely characterized cold active lipase is from yeast (*C. antarctica*). The two lipase variants are *C. antarctica* lipase A (CAL A) and *C. antarctica* lipase B (CAL B) with different physiochemical properties (Kirk and Christensen. 2002). CAL B belongs to the α/β-hydrolase fold superfamily (Ollis et al., 1992), which contains enzymes that have evolved from a common ancestor (divergent evolution) to catalyze various reactions such as hydrolysis of esters, thioesters, peptides, epoxides, and alkyl halides or cleavage of carbon bonds in hydroxynitriles (Holmquist, 2000). CAL B is made up of 317 amino acids and has a molecular weight of 33 kDa. CAL B is less thermostable, smaller in size, and more acidic than CAL A (Patkar et al., 1993). CAL B is a stable enzyme that has been used at higher temperature, in organic solvents of high polarity such as acetonitrile and dimethyl sulfoxide in ionic liquids, in solid/gas systems and in supercritical carbon dioxide (Suen et al., 2004). Cold active lipolytic enzyme was produced by cloning the putative lipolytic

gene encoding lipo1 from the metagenomic library and expressed in *Escherichia coli* BL21 using the pET expression system (Roh and Villatte, 2008). The expressed recombinant enzyme was purified by Ni-nitrilotriacetic acid affinity chromatography and characterized using general substrates of lipolytic property. The gene consisted of 972 bp encoding a polypeptide of 324 amino acids with a molecular mass of 35.6 kDa. This lipolytic enzyme exhibited the highest activity at pH 7.5 and 10 ^{o}C. At thermal stability analysis, lipo1 was more unstable at 40 ^{o}C than 10 ^{o}C.

Applications of cold active lipases

Cold active lipases offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures and low thermostability and unusual specificities. Indeed, the cold active enzymes, along with the host microorganisms, cover a broad spectrum of biotechnological applications. The potential applications for a lipase with a relatively high activity at low temperatures could be detergent additives, or in the processing of volatile substances, thereby making it possible to reduce temperature and thus bring down energy costs (Margesin and Schinner, 1994). A low temperature-active lipase would be attractive for the bioremediation of low-temperature soils or waters. The low stability at moderate temperatures could be beneficial for terminating an enzyme reaction by heat inactivation in biotransformation processes. In molecular biology applications, heterologous gene expression in psychrophilic hosts to prevent formation of inclusion bodies (Feller et al., 1996). A number of reviews have already been devoted to the use of cold-adapted microorganisms and their enzymes in biotechnology (Russell et al., 1998; Margesin and Schinner, 1994; Gerday et al., 2000; Cavicchioli et al., 2002; Marx et al., 2007). Most of these are appreciated without a detailed knowledge of how cold active enzymes achieve their performance. The number of present uses is low and likely to reflect the state of the field, which has not developed as rapidly as the thermophile field. Nevertheless, despite the difficulties with prediction, important advances have been made (Cui et al., 1997).

Synthesis of fine chemicals

Key intermediates used in the synthesis of pharmaceutical, agrochemical compounds are complex or chiral compounds that are difficult to synthesize with chemical methods. It is also known that one of the two drug enantiomers is pharmaceutically functional and hence the

synthesis of enantiomerically pure building blocks has become an important task for the pharma industry (Patel, 2001). This is the major reason for biocatalysts especially lipases to expand their scope dramatically in the area of transformation of synthetic chemicals with high chemo-, regio- and enantioselectivity (Patel, 2008).

Kinetics of acyl transfer reactions in organic media catalyzed by lipase B from *C. antarctica* has been reported (Martinelle and Hult, 1995). Lipase produced by a psychrotroph, *P. fluorescens* P38, was found to catalyze the synthesis of butyl caprylate in n-heptane at low temperatures (Tan et al., 1996). Applications of lipase B of *C. antarctica* in organic synthesis has been reported (Anderson et al., 1998). The ethyl esterification of docosahexaenoic acid (DHA) for the production of ethyl docosahexaenoate (EtDHA) in an organic solvent free system using immobilized *C. antarctica* lipase was reported (Shimada et al., 2001). Use of lipase B from *C. antarctica* for the preparation of optically active alcohols has been reported (Rotticci et al., 2001). Enzymatic reactions in non-aqueous solvents offer new possibilities for the biotechnological production of many useful chemicals using reactions that are not feasible in aqueous media. The use of enzymes in non-aqueous media has found applications in organic synthesis, chiral synthesis or resolution, modification of fats and oils, synthesis of sugar-based polymers, etc. The use of lipases in esterification reactions to produce industrially important products such as emulsifiers, surfactants, wax esters, chiral molecules, biopolymers, modified fats and oils, structured lipids, and flavor esters is well documented. The interest in using lipases as biotechnological vectors for performing various reactions in both macro- and microaqueous systems has picked up tremendously during the last decade (Hari Krishna and Karanth, 2002). Crude soybean oil did not undergo methanolysis with immobilized *C. antarctica* lipase but degummed oil did (Watanabe et al., 2002). Therefore, the substance that was removed in the degumming step was known to inhibit the methanolysis of soybean triacylglycerols (TAGs). The main components of soybean gum are phospholipids (PLs), and soybean PLs actually inhibited the methanolysis reaction. Indeed, three-step methanolysis successfully converted 93.8% degummed soybean oil to its corresponding methyl esters, and the lipase could be reused for 25 cycles without any loss of the activity. Lipase from *C. antarctica* has been evaluated as a catalyst in different reaction media for hydrolysis of tributyrin as reaction model (Salis et al., 2003). To introduce polymer to cellulosic material, a new approach was developed by Gustavsson et al., (2004) using ability of a cellulose-binding

module of *C. antarctica* lipase B conjugate to catalyze ring opening polymerization of epsilon-caprolactone in close proximity to cellulose fiber surface. CAL A possess high thermostability allowing operations at temperature above 90 °C. It also has the ability to accept tertiary and sterically hindered alcohols probably due to the existence of a specific aminoacidic sequence in the active site. Furthermore, it is an excellent biocatalyst for the asymmetric synthesis of amino acids/amino esters, due to its chemoselectivity towards amine groups (Domínguez et al., 2005). Lipase B from *Candida antarctica* (CAL-B) catalyses the highly enantioselective transesterification of some optically active 1-(het) arylalkan-1-ols with vinyl acetate (Kourist et al., 2005). Cold active lipase from *C. antarctica* increased the performance of lipase B in the enantioselective esterification of ketoprofen (Ong et al., 2006). Immobilization of CALB in the EMR was able to reduce the amount of enzyme required for the enantioseparation of (R,S)-ketoprofen. Immobilized CALB in the EMR assured higher reaction capacity, better thermal stability, and reusability (Ong et al., 2008). Improvement of the enantioselectivity of lipase from *C. Antarctica* (lipase B) via adsorption on polyethylenimine-agarose has been reported by Torres et al., (2006). Structure and activity of lipase B from *C. antarctica* in ionic liquids has been studied (van Rantwijk et al., 2006). Ascorbyl fatty acid esters are good antioxidants and surfactants. These esters are prepared by acylation of ascorbic acid (vitamin C) using different acyl donors using lipases (Karmee, 2008). The selectivity of acetylation of d-functionalized secondary alcohols catalyzed by *Candida antarctica* lipase B has been Studied (Nyhlén et al., 2008). (R)-1-Arylallyl alcohols were synthesized with excellent enantioselectivities via kinetic resolution of the corresponding acetates using immobilized *Candida antarctica* lipase B (Kadnikova and Thakor. 2008). Acylation of the phenolic group of tocopherols (vitamin E) by transesterification with vinyl acetate in 2-methyl-2-butanol (2M2B) was carried out by using *Candida antarctica* lipase B (Torres et al., 2008). Synthesis of both enantiomers of 6,7-dimethoxy- 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid was carried out through dynamic kinetic resolution using CAL-B- or subtilisin (Paál et al., 2008). Ethyl hexanoate a typical fragrance compound was catalyzed by the esterification of hexanoic acid and ethanol using *Candida antarctica* lipase B (Han et al., 2009). CAL-B was found to be effective for regioselective synthesis of ascorbic acid esters (Karme,. 2009). Synthesis of *n*-butyl acetamide was carried out by using *C. antarctica* lipase B (Yadav and Borkar, 2009).

Medical and pharmaceutical applications

Cold active lipases have emerged as an important biocatalysts in biomedical applications because of their excellent capability for specific regioselective reactions in a variety of organic solvents with broad substrate recognition. Biocatalysis offers a clean and ecological way to perform chemical processes, in mild reaction conditions and with high degree of selectivity. Parmar et al., (1996) have reviewed a variety of substrates accepted by hydrolytic enzymes, including lipases to produce compounds in high enantiomeric excess. These enantiomers 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). Many lipases demonstrate high regioselectivity and/or enantioselectivity in catalyzing the reactions in aqueous and organic solvents, making them excellent candidates for development of fine chiral compounds that are used in the biopharmaceutical and agricultural industries (Jaeger and Reetz, 1998; Patel, 2001; Jaeger and Eggert, 2002). These characteristics have encouraged the search for identifying novel lipases with unique properties (Henne et al., 2000; Bell et al., 2002; Bornscheuer, 2002; Reetz, 2004; Ferrer et al., 2007; Salameh and Wiegel, 2007). The use of enzymes, especially *Candida antarctica* lipase B, in organic solvents proved an excellent methodology for the preparation of single-isomer chiral drugs (Gotor-Fernández et al., 2006a). *C. antarctica* lipase B (CAL B) has been used for the production of amines and amides due to its simplicity of use, low cost, commercial availability and recycling possibility. This lipase proved to be an ideal tool for the synthesis and resolution of a wide range of nitrogenated compounds which are the precursors for production of certain pharma products (Gotor-Fernandez et al., 2006b). An efficient protocol has been developed for synthesis of acetamides using *Candida antarctica* lipase B (Dhake et al., 2009). *C. antarctica* lipase was used to tolerate a range of functionalized vinyl esters as acyl donors in the enantioselective acylations of alcohols (Chênevert et al., 2009). 1-(heteroaryl) ethanamines had been synthesized with *Candida antarctica* lipase B lipases for the preparation of nitrogenated compounds in high optical purity (Alatorre-Santamaria et al., 2009).

Applications in food industry

In the food industry, reactions need to be carried out at low temperatures in order to avoid changes in food ingredients caused by undesirable side-reactions that would otherwise occur at higher temperatures. Cold-adapted enzymes are particularly attractive for the processing of foods due to their high catalytic activity at temperatures that minimize spoilage and alterations in taste and nutritional values. Their inherent low structural stability also facilitates inactivation once a desired product is attained. Lipases have become an integral part of modern food industry. The use of enzymes to improve the traditional chemical processes of food manufacture has been developed in the past few years. Stead, (1986) and Coenen et al., (1997) stated that though microbial lipases are best utilized for food processing, a few, especially psychrotrophic bacteria of *Pseudomonas* sp. and a few moulds of *Rhizopus* sp. and *Mucor* sp. cause havoc with milk and dairy products and with soft fruits. An example of the application of a cold-adapted enzyme in non-aqueous biotransformation is the use of a lipase from *Pseudomonas* strain P38 for the synthesis in n-heptane of the flavor compound, butyl caprylate (Tan et al., 1996). Immobilized lipases from *C. antarctica* (CAL B), *C. cylindracea* AY30, *H. lanuginosa*, *Pseudomonas sp*. and *G. candidum* were used for the esterification of functionalized phenols for synthesis of lipophilic antioxidants to be used in sunflower oil (Buisman et al., 1998; Pandey et al., 1999). Whole-cell biocatalyst of mutated *C. antarctica* lipase B (mCAL B) was constructed using a yeast molecular display system which showed more preference to short chain fatty acids (Kato et al., 2007). This mutated lipase, mCALB is displayed on the cell surface which increased its thermostability and also accessibility to the substrate. This mCALB lipase can be used for synthesis of flavor esters. Long-chain alkyl benzoates, e.g., lauryl 4- hydroxybenzoate, palmityl 4-hydroxybenzoate, and oleyl 4-hydroxy-3-methoxybenzoate, are synthesized in high to moderate conversion by lipase-catalyzed transesterification of the corresponding short-chain alkyl benzoates with fatty alcohols in an equimolar ratio. The substrates are reacted in vacuo in the absence of solvents and drying agents in the reaction mixture by Immobilized lipase B from *Candida Antarctica* (Vosmann et al., 2008). Long chain alkyl (hydroxy)phenylacetates were synthesized by transesterification of the corresponding short-chain alkyl hydroxyphenylacetates and fatty alcohols by Immobilized lipase B from *Candida antarctica* (Weitkamp et al., 2008). A recombinant *Saccharomyces cerevisiae* displaying *Candida antarctica* lipase B (CALB) on the cell surface

was used as a whole-cell biocatalyst to catalyze the esterification of hexanoic acid and ethanol for the preparation of ethyl hexanoate, a fragrance compound of liquor (Han et al., 2009). Immobilized *Candida antarctica* lipase-catalyzed was used for transesterification of triolein with cinnamic and ferulic acids (Choo and Birch, 2009) that resulted in increase in radical scavenging activity of cinnamic acid and not ferrulic acid.

Domestic applications

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. *C. antarctica* lipase was developed into recombinant enzyme used for detergent formulation (Uppenberg et al., 1994). Godfrey and West, (1996) reported that about 1000 tons of lipases are sold every year in the area of detergents. Enzymes can reduce the environmental load of detergent products, since they save energy by enabling a lower wash temperature to be used. Commercial preparations used for the desizing of denim and other cotton fabrics contain both α amylase and lipase enzymes. Lipases are stable in detergents containing protease and activated bleach systems. Lipase is an enzyme, which decomposes fatty stains into more hydrophilic substances that are easier to remove than similar non-hydrolysed stains (Fuji et al., 1986; Thirunavukarasu et al., 2008). The commercial applications of lipases includes, detergents is in dish washing, clearing of drains clogged by lipids in food processing or domestic/industrial effluent treatment plants (Bailey and Ollis, 1986). Further, it is used in a bleaching composition (Nakamura and Nasu, 1990), decomposition of lipid contaminants in dry-cleaning solvents (Abo, 1990), contact lens cleaning (Bhatia, 1990), degradation of organic wastes on the surface of exhaust pipes, toilet bowls, etc. (Moriguchi et al., 1990). Removal of dirt/cattle manure from domestic animals by lipases and cellulases (Abo, 1990), hard-surface cleaning (Jurado et al., 2007), washing, degreasing and water reconditioning by using lipases along with oxidoreductases, which allows for smaller amounts of surfactants and operation at low temperatures (Novak et al., 1990). The lipase component causes an increase in detergency and prevents scaling. The cleaning power of detergents seems to have peaked; all detergents contain similar ingredients based on similar detergency mechanisms. To improve detergency, modern types of heavy-duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes (Ito et

al., 1998). Lipases show unusual versatile substrate specificity. The tertiary structure of lipases is known, there are presently significant efforts to improve this class of enzymes by protein engineering techniques, in view of their use in detergents and other fields of industrial application (Schmid and Verger, 1998). Cold active lipase from *Microbacterium phyllosphaerae* and *Bacillus sphaericus* has a remarkable capacity to retain its activity in presence of commercially available detergents and exhibited high efficiency for the removal of lipid stains from fabrics (Joseph, 2006). The use of cold-adapted enzymes may therefore be more economical than mesophilic homologs, as smaller amounts are required to achieve the same effect. Such characteristics of cold-adapted enzymes hold promise for decreasing not only energy requirements of washing but also the costs for manufacturing and use of liquid or granular detergents, stain removers, household cleaners, and industrial cleansing applications.

Environmental application

Bioremediation for waste disposal is a new avenue in lipase biotechnology. Cold-adapted lipases have great potential in the field of wastewater treatment, bioremediation in fat contaminated cold environment and active compounds synthesis in cold condition (Buchon et al., 2000). This aspect requires more efforts in identifying and cloning novel lipase genes. Suzuki et al. (2001) identified as a psychrotrophic strain of the genus *Acinetobacter* strain no. 6 produced extracellular lipolytic enzyme that efficiently hydrolyzed triglycerides such as soybean oil during bacterial growth even at 4 °C; it degraded 60 % of added soybean oil (initial concentration,1 % w/v) after cultivation in LB medium at 4 ^{o}C for 7 days. The bacterium is potentially applicable to in situ bioremediation or bioaugumentation of fat contaminated cold environments. Belousova and Shkidchenko, (2004) isolated 30 strains capable of oil degradation at 4–6 ^{o}C. Maximum degradation of masut and ethanol benzene resins were observed in *Pseudomonas* sp. and maximum degradation of petroleum oils and benzene resins were observed in *Rhodococcus* sp. Further, they stated that the introduction of psychrotrophic microbial degraders of oil products into the environment is most important in the contest of environmental problems in temperate regions. Ramteke et al., (2005) stated that in temperate regions, large seasonal variations in temperature reduce the efficiency of microorganisms in degrading pollutants such as oil and lipids. The enzymes active at low and moderate temperature may also be ideal for bioremediation process.

Cryo-electron microscopy

Transmission electron microscopy (TEM) is a structural technique that has existed for many years in biology to study the ultra-structure of cells. However, over the last 3 decades, it has also evolved into a powerful technique for the structural study of biological macromolecules. The main difference between this molecular EM and the more conventional EM of fixed tissue sections is its ability to deliver three-dimensional (3D) structures of the studied complexes at the higher resolution necessary to visualize structural details of molecules (on the scale of nanometers) rather than of the gross architecture of cells (on the scale of micrometers). Whereas modern electron microscopes can routinely deliver images of inorganic materials at atomic resolution, biological specimens pose great difficulties for EM imaging, significantly reducing the attainable resolution. These methodological improvements lie at the level of the instrument itself, that is, better microscopes, but most significantly, more powerful algorithms and software platforms, and, importantly, dramatically increased speed of computers to deal with the noisy images of proteins obtained with the microscope.

It was the pioneering work of Taylor and Glaeser (1974) and Dubochet and colleagues (1988) that paved the way for electron cryo-microscopy. These were the first researchers to take advantage of the fact that the vapor pressure of water becomes negligible at temperatures below –100°C. Sample drying can thus be avoided by keeping the protein at liquid nitrogen temperature. However, the sample must be frozen, avoiding the formation of crystalline ice because ice crystals can destroy the delicate ultra structure of proteins. Rapid freezing technique such as plunge-freezing (Dubochet et al., 1988) is preferable as the molecules are frozen in their native environment. Vitrification of the sample using the plunge-freezing method works particularly well for individual proteins and multiprotein complexes whereas for whole cells and tissues vitrification can be achieved only by physical fixation such as in slam-freezing or high-pressure freezing (Sawaguchi et al., 2004). In cryo-EM, samples are embedded in vitreous ice on a ‘‘holey’’ carbon grid and maintained at low temperatures (100–113 K) whilst under the electron beam. Vitreous ice is essentially a supercooled liquid, produced when water is very rapidly cooled below 273K (at about 10^5 Ks^{-1}) (Slayter and Slayter, 1992). This avoids damage to the specimen by ice crystal formation. Vitreous ice forms a structureless medium in which the molecules are hydrated, despite the requirement for vacuum conditions (the low temperatures lead to a very slow rate of sublimation of the ice).

The specimen is therefore imaged under conditions more like those in its native environment. The low temperature and the use of low doses of electrons (10–20 electrons/$Å^2$) minimise beam damage (Stark et al., 1996; Baker et al., 2010; Karuppasamy et al., 2011).

Single particle analysis

Cryo-electron microscopy in combination with single particle image analysis is a rapidly progressing technique. Large multi-subunit complexes are not suitable for NMR studies and very often oppose to any attempt of crystallization. Walter Hoppe is generally credited with being one of the first to formulate the possibility of reconstructing 3D density maps from EM images of individual molecules. The first 3D reconstructions were produced by collecting a single-axis tilt series and combining the projections of an individual molecule into a 3D reconstruction (Hoppe et al., 1974). Single particle methods allow to image protein complexes in their native conformation, quick-frozen in amorphous ice (Dubochet et al., 1988), and without any crystallization constraints. Moreover, molecules in different conformational states can be isolated on the image level and dynamical interactions can be monitored. Single particle analysis assumes the presence of multiple copies of identical molecules that have different spatial orientations. Due to the low signal-to-noise ratio of cryo-electron micrographs, the evaluation of the relative orientations of the particles represents a major difficulty, especially for a completely unknown structure. Valid approaches for the determination of the spatial relationship of the projections are the methods of random conical data collection (Radermacher et al., 1986) and common line search (Crowther, 1971). Refinement of the first three-dimensional model is obtained by iterative cycles of projection matching alternating with volume reconstruction. In the last decade, methodical improvement and rapid growth of computational capacities led to the determination of numerous structures of large complexes at subnanometer resolution. Most high-resolution single particle volumes concern icosahedral virus particles (B¨ottcher et al., 1997; Conway et al., 1997), since the effective signal-to-noise ratio is considerably higher than for asymmetric particles, due to their sixty-fold symmetry. Prominent examples of lower-symmetry reconstructions include the chaperone protein GroEL at 6Å resolution (Ludtke et al., 2004). The resolution of the published structures has improved from 30- 40 °A fifteen years ago to sub-nanometer resolution in recent years, and future directions clearly aim at resolutions at near atomic level, which permit tracing of the

polypeptide backbone without the need for crystallization. Major efforts in the field regard the development of automated image acquisition, particle pickup and image processing methods, as well as the improvement of the fitting of x-ray structures into medium-resolution density maps. High-resolution cryoEM requires careful consideration of a number of factors, including proper sample preparation to ensure structural homogeneity, optimal configuration of electron imaging conditions to record high-resolution cryoEM images, accurate determination of image parameters to correct image distortions, efficient refinement and computation to reconstruct a 3D density map, and finally appropriate choice of modeling tools to construct atomic models for functional interpretation. This progress illustrates the power of cryoEM and ushers it into the arsenal of structural biology, alongside conventional techniques of X-ray crystallography and NMR, as a major tool (and sometimes the preferred one) for the studies of molecular interactions in supramolecular assemblies or machines.

Recent Successes in Single-Particle Electron Microscopy

The last 2–3 years have been characterized by a rapid increase in EM publications, which have been dealing with some challenging applications of single-particle EM. These works shed light on where the field is going in the very near future. The year 2008 has become a watershed year in single-particle EM because for the first time density maps have been produced at near atomic resolution. Three density maps have been obtained of specimens with icosahedral symmetry at resolutions that made it possible to trace the backbone of the polypeptide chains and even to build atomic models (Jiang et al., 2008; Yu et al., 2008; Zhang et al., 2008). Recently, atomic or near-atomic resolution structures of several viruses and protein assemblies have been determined by single-particle cryoEM, allowing ab initio atomic model building by following the amino acid side chains or nucleic acid bases identifiable in their cryoEM density maps. In particular, these cryoEM structures have revealed extended arms contributing to molecular interactions that are otherwise not resolved by the conventional structural method of X-ray crystallography at similar resolutions (Zhou et al., 2011). These density maps prove that single-particle EM can indeed deliver structural information at near-atomic resolution from images of noncrystalline samples, as predicted by Richard Henderson more than decade years ago (Henderson et al., 1995).

Near-atomic resolution has so far only been accomplished with viruses and virus-like particles, which are ideal objects for single-particle EM, because they are large, stable, and have high symmetry. Density maps of molecules that are smaller, or more labile, or have less symmetry have not yet reached near-atomic resolution, but over the past few years, many 3D reconstructions have been produced at subnanometer resolution, resolving α-helices and occasionally even β-sheets and loops. Examples include the transferrin-transferrin receptor complex (Cheng et al., 2004), the D6 clathrin cage (Fotin et al., 2004), Gro-EL (Ludtke et al., 2004; Stagg et al., 2006), the GroEL-GroES complex (Ranson et al., 2006), the ribosome (Halic et al., 2006; Chandramouli et al., 2008), and the proteasome (Rabl et al., 2008). The 3D reconstruction of GroEL made it possible to trace the protein backbone (Ludtke et al., 2008). The helical reconstruction of TMV in its calcium-free, metastable assembling state at 3.3 angstrom resolution by cryo electron microscopy, revealed both protein side chains and RNA bases (Ge and Zhou, 2011). Density maps of GroEL complexes containing substrate also revealed the locations and topologies of unfolded protein substrates (Elad et al., 2007). EM density maps at any resolution provide the most exciting insights if they can be interpreted on the basis of available crystal structures. By fitting the crystal structures of the subunits into the EM map of a macromolecular complex, pseudoatomic models of the complex can be generated. With low-resolution density maps, placing of crystal structures can provide information on the orientation of the proteins with respect to each other, providing unique insights. For example, by docking the individual domains of the crystal structure of the soluble form of the bacterial toxin pneumolysin into EM density maps, it was possible to deduce the conformational changes that are associated with pore formation by cholesteroldependent cytolysins (Tilley et al., 2005). If the density map has subnanometer resolution, the models resulting from docking are quite reliable, and interactions between proteins can be interpreted on the amino acid level, although the deduced interactions still benefit from experimental testing by mutational analysis (Cheng et al., 2004). For example, by docking the crystal structure of the proteasome into a density map of the proteasome in complex with a peptide derived from an activator, it was possible to visualize the conformational changes associated with gate opening in the proteasome (Rabl et al., 2008). Protein folding by chaperones is another example illustrating the insights that can be obtained by combining EM maps with crystal structures (Saibil, 2008). The prime example of what can be learned from fitting crystal

structures into EM density maps is, however, the ribosome, which helped to understand the complex process of protein translation (Mitra and Frank, 2006; Frank et al., 2007; Valle, 2011; Jomaa et al., 2011).

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CHAPTER 2

Isolation and production of cold active lipase from *Yarrowia lipolytica* NCIM 3639

Abstract

This chapter deals with the production of cold active lipase by *Yorrowia lipolytica* NCIM 3639. A strain of *Yarrowia lipolytica* was isolated which produced cold active lipases. The results present a new finding of production of cell bound and extracellular lipase activities depending on the substrate used for growth. The strain produced only cell bound lipase activity when grown on olive oil containing medium with trace amounts of extracellular lipase. A major amount of extracellular lipase was produced when it was grown on Tween 80 as lipid source. Such strict differential localization of lipases in response to the substrates used as carbon source for growth is not reported so far. Maximum extracellular lipase activity was obtained when the strain was grown in a medium containing Tween 80 (2 %) and Peptone (0.5%). It grew profusely at 20 ^{o}C and at initial pH of 5.5 producing maximum extracellular lipase. The growth of *Yarrowia lipolytica* at 25 and 30 ^{o}C affected lipase production indicating the cold adapted nature of the enzyme.

Introduction

Triacylglycerol hydrolases or lipases (EC 3.1.1.3) catalyze ester bond hydrolysis in triacylglycerols with the release of fatty acids, mono- and diglycerides and glycerol. As this substrate presents a very low solubility in water, the catalytic reaction takes place at the lipid–water interface (Sarda and Desnuelle, 1958). Although naturally occurring triacylglycerols are the preferred substrates, these enzymes can hydrolyze a wide range of insoluble fatty acid esters. It is well established that the reaction is reversible, and that lipases can catalyze esterification as well as trans-esterification reactions often in nearly anhydrous organic solvents. Many biotechnological processes are expedited by the use of higher temperatures and this generated a lot of interest in thermo-stable enzymes. However, more recently there has been a great interest in cold-adapted enzymes for transformations in which substrate and product stabilities require the use of low temperatures leading to energy savings. Such cold active enzymes are important in many fields including the detergent, textile and food industries, as well as for a variety of bio-catalytic reactions (Alquati et al., 2002; Zimmer et al., 2006).

Cold active lipases are largely distributed in microorganisms surviving at low temperatures. Although a number of lipase producing sources is available, only a few bacteria and yeast were exploited for the production of cold active lipases (Joseph et al., 2008). The organisms producing cold active lipases were isolated mostly from Antarctic and Polar regions which represent a permanently cold and constant temperature habitat. Another potential source of cold active lipases is deep-sea bacteria. A marine bacterium *Aeromonas hydrophila* growing at a temperature range between 4 and 37 °C produced cold active lipase enzyme (Pemberton et al., 1997). Few bacterial genera have been isolated and characterized from deep sea sediments where temperature is below 3 °C. They include *Aeromonas* sp. (Lee et al., 2003); *Pseudoalteromonas* sp. and *Psychrobacter* sp. (Zeng et al., 2004) *Photobacterium lipolyticum* (Ryu et al., 2006). Bacteria including *Pseudomonas fragi* (Aoyama et al., 1988; Alquati et al., 2002), *Pseudomonas fluorescens* (Dieckelmann et al., 1998) and *Serratia marcescens* (Abdou, 2003) which produce cold active lipases were isolated from refrigerated milk and food samples.

Recently, attention has been turned to non-conventional yeasts (NCY). Interest in these microorganisms may be due to a variety of reasons, ranging from their use in specific technological applications to the treatment of infections caused by some of them (Flores et al., 2000). *Yarrowia lipolytica*, one of the non conventional yeasts, is used as a model to study dimorphism (Herrero et al., 1999) and secretion (Flores et al., 2000). It is able to produce several substances of biotechnological importance, including citric acid (Finogenova et al., 2002) and proteases (McEwen et al., 1998), and is increasingly being used for expression of heterologous proteins (Madzak et al., 2000; Mauersberger et al., 2001). An extracellular and two cell-bound activities corresponding to lipase I (39 kDa) and lipase II (44 kDa) were described (Ota et al., 1982; Sugiura et al., 1976). The cell-bound lipases differed in several properties from the extracellular enzymes (Ota et al., 1984). Although lot of work on lipase has been published from *Yarrowia lipolytica*, there are no reports of cold active lipases. There is still a greater interest in new enzymes with commercial useful properties. This effort has been stimulated by the recognition that cold-active enzymes might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermo-stability and

unusual specificities. These properties are of interest in different fields such as detergents, textile and food industry, bioremediation and bio-catalysis under low water conditions (Gerday et al., 2000).

In this chapter, we concentrated our research on the production of cold active extracellular and cell bound lipases by *Yarrowia lipolytica* isolated from refrigerated Tween 80 samples with more stress on extracellular lipase production.

Materials and methods

Materials

Peptone, yeast extract, malt extract, glucose, agar were purchased from Hi-Media Laboratories Limited Mumbai, India. Olive oil samples used were obtained from local market. Tween–80, Tween–20 and Triton X-100 were obtained from Merck India Ltd. *p*-Nitrophenylpalmitate (*p*NPP), Nonidet P-40 (NP-40) and 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), Sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Co. (USA). All other chemicals were of analytical grade.

Methods

Microorganisms and growth media

Yarrowia lipolytica NCIM 3639 was isolated from refrigerated solution of 5 % Tween 80 in 50 mM phosphate buffer, pH 6.0. The strain was identified by National Collection of Yeast Cultures (NCYC), England using 26S rDNA D1/D2 sequencing approach. The strain was maintained on MGYP agar slopes containing malt extract 0.3%, glucose 1.0%, yeast extract 0.3%, peptone 0.5% and agar 2.0%. Synthetic oil-based (SOB) medium was used as production medium which contained $NaNO_3$ 0.05%, $MgSO_4.7H_2O$ 0.05%, KCl 0.05%, KH_2PO_4 0.2%, yeast extract 0.1%, bacto-peptone 0.5% and olive oil 1.0%. The initial pH of the medium was adjusted to 5.5 with 0.1 N NaOH or HCl prior to sterilization.

Inoculum preparation and lipase production

Cells of *Yarrowia lipolytica* were inoculated in 10 ml MGYP liquid medium and allowed to grow on a rotary shaker with shaking at 150 rpm at 20 ºC for 48 h. This grown culture (5 ml) was transferred to 250 ml shake flask containing 50 ml of MGYP medium and allowed it to grow for 48 h at 20 ºC. This was used as an inoculum (5 %) for lipase production in 250 ml conical flasks containing 70 ml of SOB medium. The Conical flasks were incubated on a rotary shaker at 150 rpm at 20 ºC for 72 h. The samples were removed after specific time intervals to determine lipase activity, soluble protein and biomass. The cells were harvested by centrifugation at 5000 g for 10 min at 4 °C and the supernatant was used as extracellular lipase preparation. Cells were washed twice with 50 mM citrate phosphate buffer (pH 5.0) and re-suspended in known amount of buffer solution. This cell suspension was used as a source of cell bound lipase.

Enzyme assay, Biomass and protein estimation

The spectrophotometric lipase assay was performed as described earlier (Mahadik et al., 2002). The substrate solution was prepared by adding solution A (30 mg of *p*NPP in 10 ml of propane-2-ol) to 9.5 ml of solution B (0.1 g of gum arabic and 0.4 g of Triton X-100 in 90 ml of distilled water) drop wise with intense stirring. The assay mixture consisted of 0.9 ml of substrate solution, 0.1 ml of citrate phosphate buffer (0.5 M, pH 5.0) and 0.1 ml of suitably diluted enzyme. The assay mixture was incubated at 20 ^{o}C for 30 min and the *p*-nitrophenol released was measured at 410 nm in Spectronic-117 spectrophotometer. One unit of activity was expressed as the amount of enzyme that released 1µmoles of *p*-nitrophenol per min under the assay conditions. Cell biomass was determined by measurement of the absorbance of cells at 660 nm, after being washed and re-suspended in saline. Dry weight (DW) was calculated from the absorbance value using a standard curve (18 OD corresponds to 10.2 g/l dry weight). Protein concentration was estimated by the method of Lowry with bovine serum albumin as a standard (Lowry et al., 1951).

Intracellular and cell bound lipase preparations

Wet cells (0.5 g) were washed with 20 ml of citrate phosphate buffer (50 mM, pH-5.0) and suspended in the same buffer at a final volume of 5 ml. The cell suspension was homogenized at 4 °C with a Sonicator (Sonics, Vibra cell) for ten periods of 30 seconds each. The disrupted cells were centrifuged at 5,000 g at 4 °C for 10 min. The supernatant was used as intracellular lipase source. The cell debris, after washing with the buffer, was used as cell bound lipase enzyme. For lipase extraction, cell debris was suspended in 10 ml citrate phosphate buffer (50 mM, pH-5.0) with detergents such as 0.5% (v/v) of Triton X-100, Tween 80, NP-40, SDS and CHAPS and the suspension was shaken at 150 rpm, 20 °C for 4h. The debris was removed by centrifugation at 5000 g at 4 °C for 10 min and the lipase activity in the filtrate as well as debris was measured.

Results

Cell growth and lipase production

The mode of lipase induction in *Y. lilolytica* NCIM 3639 is shown in (Table 1). Tween 80 was found to be the best inducer for the production of extracellular lipase. Maximum extracellular lipase activity was obtained in a medium with 2% Tween 80 with small amount of cell bound lipase activity. Greater or lesser concentrations of Tween 80 resulted in decreased extracellular lipase production. Other substrates such as Tween 20 induced low levels of both cell bound and extracellular lipases, whereas Triton X-100 inhibited both growth and lipase production. The growth of *Y. lipolytica* in olive oil containing media led to the production of high amounts of cell bound lipase with traces of extracellular lipase. Combination of Tween 80 and olive oil produced both extracellular and cell bound lipases.

Table 1. Effect of Tween 80 and olive oil on lipase production by *Yarrowia lipolytica*

Substrate	Biomass (g/l)	Extracellular activity (IU/ml)	Cell-bound activity (IU/g)
Tween 80 (0.5%)	7.5 ± 0.4	3.3 ± 0.2	3.4 ± 0.25
Tween 80 (0.75%)	7.3 ± 0.5	5.0 ± 0.3	4.2 ± 0.30
Tween 80 (1.0%)	7.4 ± 0.3	8.5 ± 0.6	6.0 ± 0.50
Tween 80 (2.0%)	10.8 ± 0.6	15.2 ± 0.8	24.0 ± 1.3
Tween 20 (2.0%)	8.5 ± 0.6	3.0 ± 0.2	6.0 ± 0.4
Triton X-100 (2.0%)	2.0 ± 0.2	ND	ND
Olive oil (1.0%)	8.0 ± 0.4	<0.05	101.3 ± 7.5
Olive oil (1.0%) + Tween 80 (2.0%)	13.3 ± 0.6	2.8 ± 0.2	69.3 ± 4.5

The culture was grown in SOB medium with lipid substrates and incubated for 48 h at 20°C with shaking at 150 rpm. The values given in the table are the average of three independent experiments. ND –Not detected.

Time course of lipase production

Time-coursed production of *Y. lipolytica* lipase was examined in SOB medium containing 2 % Tween 80 (Figure. 1). Extracellular Lipase production began to increase after 24 h of incubation, corresponding to the late exponential growth phase, and reached a maximum at 48h. Majority of the lipase activity remained cell bound throughout the period of incubation when the organism was grown in 1 % olive oil based medium for 72 h. We were not able to extract the cell bound lipase when the cells were treated with different surfactants such as Triton X-100, NP-40, CHAPS and even Tween 80 (data not shown) indicating the cell bound nature of the lipase.

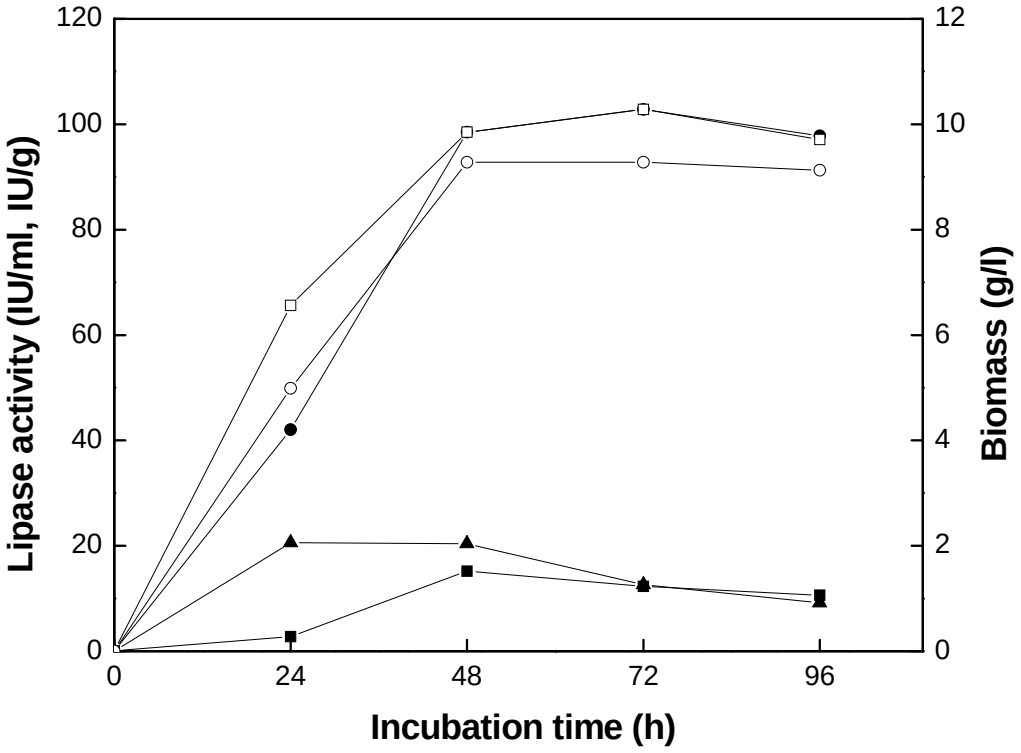

Figure 1. Time course of lipase production in Tween 80 and Olive oil containing media. Olive oil biomass (□), olive oil cell bound lipase (●), Tween 80 biomass (○), Tween 80 extracellular lipase (■), Tween 80 cell bound lipase (▲).

Effect of carbon and nitrogen sources on extracellular lipase production

We determined the effect of various carbon and nitrogen sources on lipase production. The influence of different carbon sources are shown in Table 2. Among the carbon sources tested, sucrose was comparable to control and glucose and lactose individually repressed lipase production. Sucrose at higher concentrations (>0.5%) affects lipase production. Different nitrogen sources were evaluated for lipase production (Table 3). Cell growth and lipase activity were highest when 0.5% peptone was used in the medium. Yeast extract did not prove to be a good source of nitrogen. Higher concentrations of peptone were not effective for lipase production. The pH of the fermentation medium increased above 7.0 when the organism was grown in a medium with higher concentrations of peptone.

Table 2. Effect of addition of various sugars on lipase production by *Yarrowia lipolytica*

Sugars	Biomass (g/l)	Extracellular activity (IU/ml)
Control	10.7± 0.7	15.0 ± 1.1
Glucose (1.0%)	12.2± 0.8	5.8 ± 0.4
Lactose (1.0%)	8.5 ± 0.6	8.7 ± 0.5
Sucrose (0.5%)	13.7 ± 1.0	14.3 ± 1.1
Sucrose (1.0%)	11.2 ± 0.7	12.8± 0.8
Sucrose (2.0%)	12.0 ± 0.8	12.6 ± 0.7

The culture was grown in SOB medium with Tween 80 (2%) and various sugars at 20 °C with shaking at 150 rpm for 48 h. The values are the average of three independent experiments.

Table 3. Effect of addition of various nitrogen sources on lipase production by *Yarrowia lipolytica*

Nitrogen source	Biomass (g/l)	Extracellular activity (IU/ml)
Control	10.2 ± 0.7	15.01± 1.0
Malt extract (0.5%)	1.2 ± 0.1	< 0.1
Soya meal (0.5%)	6.2 ± 0.4	5.0 ± 0.3
Beef extract (0.5%)	7.2 ± 0.5	12.3 ± 0.7
Tryptone (0.5%)	11.6 ± 0.7	10.3 ± 0.6
Casein (0.5%)	8.6 ± 0.5	12.6 ± 0.6
Yeast extract (0.1%)	7.2 ± 0.4	3.0 ± 0.2
Yeast extract (0.25%)	10.3 ± 0.6	12.1 ± 0.7
Yeast extract (0.5%)	12.2 ± 0.7	8.3 ± 0.5
Peptone (0.5%)	10.1 ± 0.6	15.37 ± 1.1
Peptone (1.0%)	11.0 ± 0.5	4.8 ± 0.3
Peptone (2.0%)	9.8 ± 0.6	2.0 ± 0.1

The culture was grown in SOB medium with Tween 80 (2 %) and various nitrogen sources at 20 °C with shaking at 150 rpm for 48 h. The control contained SOB medium with yeast extract (0.1 %) and peptone (0.5 %). The values are the average of three independent experiments.

Effect of temperature and pH on extracellular lipase production

We determined the optimal temperature and initial pH of the medium for cell growth and lipase production. Maximum activity and biomass were obtained at 20 ^{o}C within 48 h of incubation (Figure 2). The lipase production was delayed when the organism was grown at 15 ^{o}C and the maximum activity was obtained after 96 h of incubation. Growth of organism at higher temperatures (25 and 30 ^{o}C) resulted in significant decrease in lipase production without affecting the cell growth. Effect of initial pH on biomass and lipase production was evaluated in the pH range from 3 to 8 (Figure 3). The lipase production increased up to pH 5.5 and decreased sharply thereafter at pH 7.0.

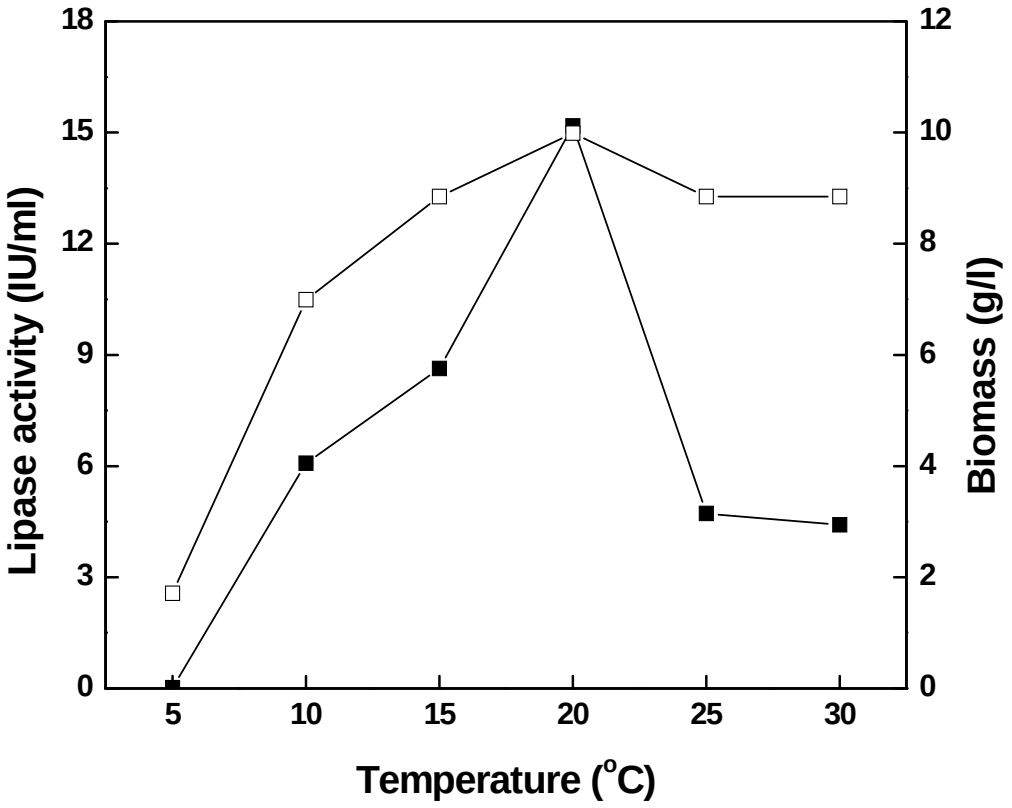

Figure 2. Effect of Temperature on growth and extracellular lipase production by *Y. lipolytica* NCIM 3639. The strain was grown in SOB basal medium containing 2 % Tween 80, incubated at different temperatures with shaking at 150 rpm. Lipase activity was measured after 48 h. Biomass (□), Extracellular lipase (■).

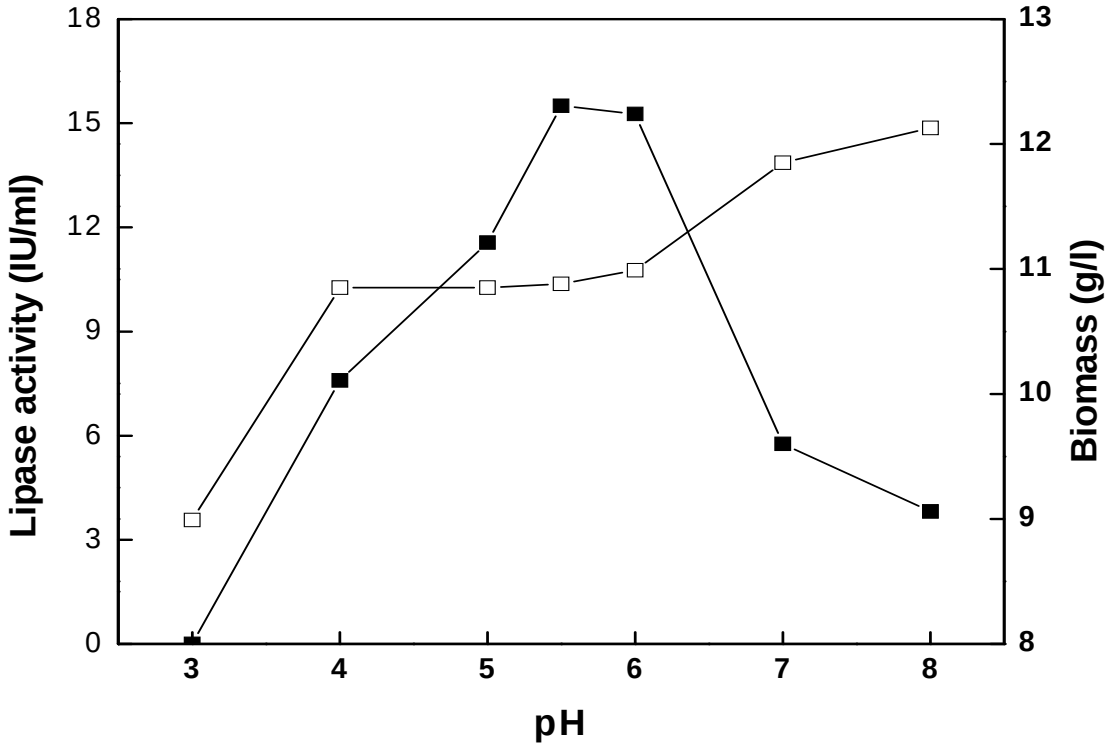

Figure 3. Effect of pH on growth and extracellular lipase production by *Y. lipolytica* NCIM 3639. The yeast strain was grown in SOB basal medium containing 2 % Tween 80, incubated at various initial pH with shaking at 150 rpm. Lipase activity was measured after 48 h. Biomass (□), Extracellular lipase (■).

Discussion

The stain, *Yarrowia lipolytica* NCIM 3639, was isolated from refrigerated Tween 80 samples in our laboratory and we feel that this could be a good source of cold active lipase. Cold active lipases function effectively at cold temperatures with higher catalytic rates in comparison to mesophilic lipases. Although many lipase producing organisms are reported, only some bacteria and a few yeasts are known for the production of cold active lipases. Among the psychrophilic fungi and yeasts, *Candida lipolytica, Geotrichum candidum Candida antarctica, Penicillium roqueforti* (Joseph et al., 2008), and *Aspergillus nidulans* (Mayordomo et al., 2000) are the potent producers of cold active lipases. Interest in the enzymes produced by cold-adapted microbial strains has recently increased (Margesin and Schinner, 1994). However, most of the studies have focused on the properties of these enzymes (Feller and Gerday, 1997) and few have investigated the regulation of their production according to the physiology of the microorganism (Gounot, 1991). Cold active lipases are mostly extracellular and are highly influenced by nutritional and physicochemical factors such as temperature, agitation, pH, nitrogen source, carbon source, inducers, inorganic sources and dissolved oxygen. Submerged fermentation is the most common method used for cold active lipase production (Dieckelmann et al., 1998; Lee et al., 2003).

Several lipases have been detected in *Y. lipolytica*, including intracellular, membrane-bound, and extracellular enzymes (Fickers et al., 2005a). In the present study, it is shown that *Y. lipolytica* NCIM 3639 was found to produce either cell bound or extracellular lipase depending upon the carbon source used for its growth. It produced predominantly cell bound lipase when olive oil is used in the medium. The growth of *Y. lipolytica* on Tween 80 containing medium resulted in the production of significant amount of extracellular lipase. Such differential induction pattern of cell bound and extracellular lipase is not reported so far in the literature. Dominguez et al. (2003) reported that *Y. lipolytica* was found to produce only extracellular lipase when it was grown in olive oil and sunflower oil. Dominguez et al. (2003) also reported that addition of surfactants such as Tween 80 decreased the extracellular lipase production in *Y. lipolytica*. Ota et al. (1982) reported cell bound lipase from *Saccharomycopsis lipolytica* which was extractable using Triton X-100. Fickers et al. (2004) found that the lipase

remained cell bound during the growth phase before being released in the media. Lipases YlLip7 and YlLip8 are two cell bound lipases which can easily be extracted with phosphate buffer (Fickers et al., 2005b). Bacteria such as *Aeromonas* (Lee et al., 2003), *Bacillus sphaericus* (Joseph et al., 2008) and *Pseudomonas* sp. (Rashid et al., 2001) produced cold active lipases using tributyrin as carbon source. *Moraxella* sp. from Antarctic habitat grew well at 25 ^{o}C and produced cold active lipase (Feller et al., 1996). *A. nidulans* WG 312 produced cold active lipase at 30 ^{o}C utilizing olive oil as an inducer (Mayordomo et al., 2000). Fickers et al. (2004) reported that tryptone and oleic acid are the most suitable nitrogen and carbon sources for production of extracellular lipase by *Y. lipolytica* mutant. They also found that the lipase remains cell bound during the growth phase before being released in the media. We observed that peptone at low concentration (≤0.5%) and Tween 80 (2%) are the most suitable nitrogen and carbon sources respectively for extracellular lipase production by *Y. lipolytica* NCIM 3639. The growth of yeast in media containing higher concentrations of peptone resulted in increase in pH of the medium which could be attributed to decreased levels of lipase activity.

Cold-adapted microorganisms tend to have good growth rate at low temperatures. The production of cold active lipase is considered temperature dependent and thermolabile (Rashid et al., 2001). The optimal temperature for lipase production by *Y. lipolytica* is comparable to the Psychrotrophic *Pseudomonas* sp. Strain KB700A (Rashid et al., 2001). *A. nidulans* WG 312 produced extracellular cold active lipase at 30 ^{o}C utilizing olive oil as an inducer (Mayordomo et al., 2000). Tween 80 and Tween 20 were the best inducers for cold active lipase production by *Pseudoalteromonas* sp. wp37 at 25 ^{o}C (Zeng et al., 2004). *Y. lipolytica* NCIM 3639 produced significant lipase activity in a pH range of 5-6 with maximum activity at pH 5.5. This observation is consistent with that of reported for *Pseudomonas* sp. (pH 5.5) (Sarkar et al., 1998). The maximum lipase production by *A. nidulans* was observed at pH 6.5 (Mayordomo et al., 2000).

The use of cell bound lipases is potentially cost effective because the biomass can be directly utilized. Furthermore, the cell structure may act as natural matrix which protects the enzymes from the possible negative action of external agents, providing an effect analogous to that exerted by common matrix used for enzyme immobilization. The

cell bound lipase of *Yarrowia lipolytica* could act as a good source of naturally matrix bound lipase for various reactions and bio-transformations.

In conclusion, cold active lipases are promising enzymes useful for chemical synthesis and bio-transformations. These enzymes could replace the conventional enzyme processes operative in biotechnological industries. The yeast, *Yarrowia lipolytica* NCIM 3639 isolated in our laboratory, produced cold active extracellular as well as cell bound lipase. The properties of these lipases appear to be quite different from other *Yarrowia* lipases. Tween 80 and olive oil were used as efficient carbon sources for production of extracellular and cell bound lipase respectively. Such differential induction of lipases depending on the nature of carbon source used for the growth of an organism is not reported so far. Peptone alone was found to be a good nitrogen source for lipase production by *Yarrowia lipolytica* NCIM 3639. These results suggested that both extracellular and cell bound lipases produced by our yeast strain could be the potential biocatalysts for carrying out lipase mediated reactions at low temperatures to get higher product yields and to avoid undesired products that are liberated at high temperatures. The substrates for induction of extracellular and cell bound lipases are different and hence it is worth studying these enzymes in detail. Hence the third chapter deals with the purification and characterization of the extracellular lipase of *Y. lipolytica* NCIM 3639.

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CHAPTER 3

Purification, biochemical and biophysical characterization of cold active lipase from *Yarrowia lipolytica*

Abstract

An extracellular cold lipase from *Yarrowia lipolytica* NCIM 3639 has been purified to homogeneity using ion exchange chromatography, Q Sepharose followed by Sepharose CL-4B gel chromatography. This protocol resulted in 14 fold purification of lipase with 11 % final recovery. The purified enzyme showed a prominent single band on SDS-PAGE. The molecular weight of the lipase was estimated to be 20 kDa using SDS-PAGE. Gel permeation chromatography showed molecular mass of 400 kDa suggesting that the lipase is comprising of 20 subunits forming a multimeric native protein. Further the enzyme displayed an optimum pH of 5.0 and optimum temperature of 25 ^{o}C. The lipase was stable over a narrow pH range (4.0-6.0). Action on *p*-nitrophenyl esters of varying chain lengths suggested the high preference of the enzyme towards medium and long acyl chain length esters. Peptide mass finger printing revealed that some peptides showed homologues sequence (42 %) to *Yarrowia lipolytica* LIP8p.

Introduction

Lipases are triacylglycerol acylhydrolases (E.C. 3.1.1.3) capable of catalyzing many reactions on ester bonds with preference on water insoluble substrates. They are widely found in animals, plants and microorganisms. One of the unique properties of lipases is the ability to hydrolyze the ester bonds at oil-water interfaces releasing mono and diacylglycerols, glycerol and fatty acids from triglycerides (Raclot et al., 2001). Lipases are also able to catalyze ester synthesis and trans-esterification in organic media containing minute amount of water (Pandey, 1999; de-Oliveira and Aves 2000). Lipases find many promising applications in food, chemical, pharmaceutical industries. These enzymes are also used for the synthesis of structured triglycerides, surfactants, agrochemicals and polymers. Microbial lipases are diversified in their properties and substrate specificities, which improve their biotechnological importance and justify the search for novel lipases possessing entirely new properties and specific substrate specificities depending on their applications.

Lipases have been purified from animal, plant, fungal, yeast and bacterial sources using variety of methods involving ammonium sulphate precipitation, ion exchange chromatography followed by gel filtration. In recent years, new techniques like affinity chromatography have come in to practice which help in decreasing the number of steps necessary for lipase purification with greater yields of purified enzymes. Techniques like reversed micellar (Yadav et al., 1998) and aqueous two-phase systems (Queiroz et al., 1995), membrane processes (Sztajer and Bryjak, 1989), immunopurification (Bandmann et al., 2000) have been used for lipase purification. Since lipases are different from other enzymes in terms of their hydrophobic nature and interfacial activation phenomenon, some novel purification technologies have been employed. These novel technologies include hydrophobic interaction chromatography with epoxy-activated spacer arm as ligands (Saxena et al., 2003). The usual procedures for purification of lipases are troublesome, time consuming and results in low yield. Low thermostability of these cold active lipases is also a major problem in purification. Novel purification steps are therefore needed to increase the overall enzyme yield and purification fold.

The yeast *Yarrowia lipolytica*, the only species recognized so far in the *Yarrowia* genus, is quite different from the other frequently studied yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* in terms of its phylogenetic evolution, physiology, genetics, and molecular biology. *Y. lipolytica* serves as an excellent model to study the non-conventional lipolytic yeasts (Fickers et al., 2005b). Consequently, the lipase produced by the yeast *Y. lipolytica* is currently the best studied example of biocatalyst for creating an enantioselective esterification (Cambon et al., 2011; Bordes et al., 2009; Cancino et al., 2008). An extracellular and two cell-bound activities in *Yarrowia lipolytica* corresponding to lipase I (39 kDa) and lipase II (44 kDa) were reported (Ota et al., 1982; Sugiura et al., 1976). The cell-bound lipases differed in several properties from the extracellular lipases (Ota et al., 1984). Although much work on lipases from *Yarrowia lipolytica* has been published (Fickers et al., 2011), there are no reports on cold active lipases from this organism. This effort has been stimulated by the recognition that cold-active enzymes might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermo-stability and unusual specificities. This chapter deals with the purification and

biochemical characterization of extracellular cold active lipase of Y. lipolytica NCIM 3639.

Materials and methods

Materials

p-Nitrophenyl acetate, *p*-nitrophynyl butyrate, *p*-nitrophenyl caprylate, *p*nitrophenyl decanoate, *p*-nitrophenyl laurate, *p*-nitrophenyl myristate, *p*-nitrophenyl palmitate, *p*-nitrophenyl stearate , Sodium Dodecyl Sulphate (SDS) and gel filtration markers, Coomassie Brilliant Blue R-250, Bromophenol Blue and Q Sepharose were purchased from Sigma Chemical Company, U.S.A. Sepharose CL-4B were obtained from Pharmacia, Sweden. All other chemicals used were of analytical grade.

Methods

Enzyme assay

Enzyme activity was assayed as described in Chapter 2. The amount of *p*-nitrophenol liberated following the hydrolysis of *p*-nitrophenyl palmitate , at pH 5.0 and 25 ^{o}C, was determined at 410 nm. One unit of the enzyme is defined as the amount of enzyme required to liberate 1µmol of *p*-nitrophenol /min under the assay conditions.

Protein determination

Protein concentration was determined according to Lowry et al (1951) using BSA as standard.

Purification of Y. lipolytica NCIM 3639 extr acellular lipase

All procedures were performed at 4 °C. Cell free culture broth (500 ml) was concentrated to minimum volume by an Amicon Ultrafiltration apparatus equipped with YM-30 membrane (30000 Da cut-off). The Concentrated enzyme solution was applied to a Q Sepharose column (15 x 1.5 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7.0). The column was then washed with 100 mM NaCl in 50 mM citrate phosphate buffer (pH 5.0) till the flow through fractions showed no lipase activity. Enzyme elution was done with 1M NaCl in 50 mM citrate phosphate buffer (pH 5.0) at the flow rate of 12

ml/h. Active fractions were pooled and concentrated by Ultrafiltration. This concentrated fraction was then applied to a Sepharose CL-4B column (150 x 1 cm) pre-equilibrated with 20 mM citrate phosphate buffer (pH 5.0) and eluted at a flow rate of 3.6 ml/h. Fractions with high specific activity were pooled, concentrated by Ultrafiltration and the purity of the enzyme was analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

Native molecular weight determination

The native molecular mass of lipase was estimated by gel filtration. The gel filtration column (Sepharose CL-4B, 50 x 1 cm), was equilibrated in 50 mM citrate phosphate buffer (pH 5.0) and calibrated using gel filtration standard molecular weight markers**:** Bovine serum albumin (*M*r 66 kDa); Alcohol dehydrogenase (*M*r 150 kDa); β-amylase (*M*r 200 kDa), and Apoferetin (*M*r 443 kDa). The column void volume was determined with Blue dextran (*M*r 2000 kDa). About 2 mg (500 µl) of samples were applied to the column, which was operated at a flow rate of 10 ml/h. In order to achieve optimal calibration, the standards were individually chromatographed and the calibration graph was plotted as log *M*r versus K_{av} (partition coefficient).

Effect of pH and temperature on enzyme activity and stability

The optimal pH of the enzyme was determined by measuring the lipase activity at 25 °C at various pH levels (pH 3-8): citrate phosphate buffer (50 mM, pH 3.0-6.0) and phosphate buffer (50 mM, pH 7.0-8.0). The pH stability was studied by incubating the purified enzyme (20 ug) in various buffers with pH ranging from 3-8 for 30 min at 25 °C. The residual activity was then assayed under standard assay conditions. The optimal temperature of the enzyme was determined by measuring the enzyme activity at various temperatures (5-45 °C) in 50 mM of citrate phosphate buffer, pH 5.0. Thermal stability was determined by incubating the purified enzyme in 50 mM citrate phosphate buffer (pH 5.0) for 24 h at the desired temperatures (5-45 °C) followed by measuring the residual activity.

Substrate specificity

The substrate specificity was determined using *p*-NP esters of varying acyl chain lengths from C4 to C18. The following substrates were used: *p*-NP butyrate (C4:0), *p*-NP caprilate (C8:0), *p*-NP deconate (C10:0), *p*-NP laurate (C12:0), *p*-NP myristate (C14:0), *p*-NP palmitate (C16:0), and *p*-NP stearate (C18:0). The substrates were prepared in 2-propanol at final concentration of 100 μM. Reaction was carried out under standard assay conditions.

Effect of metal ions, EDTA and other reagents on enzyme activity

For determining the effect of metal ions, EDTA and various reagents, enzyme assays were performed in presence of various metal ions, EDTA, and other reagents at various concentrations using *p*NPP as substrate. The enzyme activity without addition of these compounds was taken as 100%. The metal ions were used at 5 mM concentration. The reagents β-mercaptoethanol, dithiothreitol (DTT), ethylenediamine-tetraacetic acid (EDTA) were used at a concentration of 10 mM. Sodium dodecyl sulfate (SDS), Tween 20, tween 80 and triton X 100 were used at a concentration of 10% (v/v).

Effect of organic solvents on enzyme stability

Lipase stability in water miscible and water immiscible organic solvents was determined by incubating the enzyme solution prepared in citrate phosphate buffer (50 mM, pH 5.0) with 20% organic solvents for 1 h at 30 °C. In case of water immiscible solvents, the enzyme was incubated with shaking at 30 °C for 1 h. The residual activity was determined under standard assay conditions.

Kinetic and temperature dependence

The K_m and K_{cat} values of the enzyme were determined under standard assay condition using 20-100 μM of *p*NPP substrate. The constant values were calculated by fitting data to linear regression using Lineweaver-Burk plot. The effect of temperature on K_m and K_{cat} of the enzyme was determined by varying the substrate concentration in the range of 20-100 μM at different temperatures ranging from 5-35 °C. All the assays were

performed under standard assay conditions. Kinetic constants were calculated using Lineweaver-Burk plots.

Peptide mass finger printing of lipase

Gel bands were manually excised and subjected to automated in-gel chemical modification of cysteine residues with dithiothreitol and iodoacetamide followed by tryptic digestion and peptide extraction (MassPrep robotic system, Waters/BioRad). The extracted peptide mixture was run on a Waters QTOF2 hybrid quadrupole mass spectrometer incorporating an integrated capillary liquid chromatography (lc) system. A total of 5µl of the digest was introduced via an autosampler. This was initially loaded onto a small C18 packed pre-column for desalting and then products of the tryptic digest were eluted onto an analytical capillary C18 column (100mm X 0.75mm id). The lc system incorporated a flow splitting device to give a final flow through the column of 200nl per minute. Typically, a solvent gradient was run over a total of 1 hour to elute peptides from the column and re-equilibrate prior to loading the next sample. Where samples were observed to contain high levels of peptides a blank run was usually incorporated between sample runs to establish that there was no significant carry-over of one sample to the next.

Eluted peptides from the analytical column were directly submitted into the mass spectrometer via a nanosprayer device attached to the outflow from the lc system and operating at 3kV. In addition, a reference solution containing a peptide of known mass was sprayed into the mass spectrometer from a separate sprayer. This ion source was sampled at regular intervals throughout the run to assist in maintaining accurate mass measurements of the ionized peptides from the analyte spray. Data dependent switching was incorporated so that whenever a peptide with an associated charge of 2+ or 3+ was detected above a pre-set threshold signal the mass spectrometer would automatically switch to ms-ms mode to generate fragmentation data from the detected peptide. The software was set up to scan over multiple channels in order to simultaneously fragment up to 3 co-eluting peptides and collect the fragmentation data from each one individually. A preset range of collision voltages was set up in the method in order to fragment each peptide as efficiently as possible.

Raw data files were analysed using MassLynx 4.0 (incorporating BioLynx) and ProteinLynx Globalserver 2 (Waters) in order to assess the identities of proteins present in the digest. The peak list file generated from PLGS2 (fig. 1a, b, c) analysis was also used in an alternative search program accepting this format of data file (Mascot, Matrix Science).

Results

Purification of Y. lipolytica extracellular lipase

The *Y. lipolytica* lipase was purified from the culture medium; the supernatant was concentrated by ultrafiltration with 30 kDa membrane, yielding 25 ml of concentrated lipase solution with 86% yield and a 3.5-fold specific activity. After anion exchange chromatography, a specific activity of 101 IU/mg was obtained with 7.23-fold purification (Table 1). Further purification using gel filtration chromatography (Sepharose CL-4B) resulted in separation lipase activity. The lipase was obtained with maximum specific activity of 195 IU/mg and 14 fold purification. SDS-PAGE analysis of purified lipase showed single protein band corresponding to a molecular mass of around 20 kDa (Figure 1). The apparent molecular weight of the purified lipase estimated by a Sepharose CL-4B, gel filtration column was 400 kDa (Figure 2). These results indicated that the enzyme is comprised of 20 subunits each with same molecular mass (20 kDa).

Table 1. Summary of steps of purification of extracellular lipase from *Y. lipolytica* NCIM 3639

Purification Steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Fold Purification	Yeild (%)
Culture broth	7000	500	14	1.0	100
Ultrafiltration	6066	125	48.58	3.47	86.6
Q sepharose	2026	20	101.3	7.23	28.9
Sepharose CL-4 B	780	4.0	195	13.9	11.14

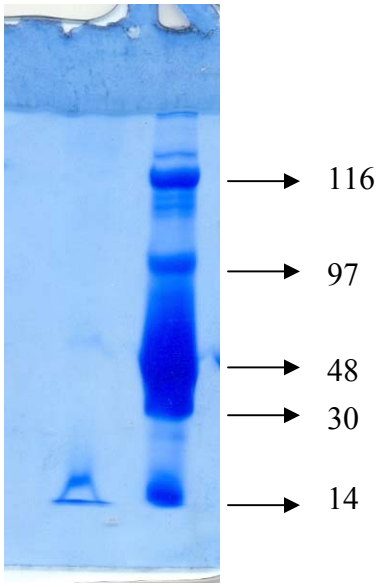

Figure 1. Molecular weight of lipase by SDS- PAGE, **Lane 1:** purified enzyme, **Lane 2:** molecular weight markers. b) Gel permeation chromatography.

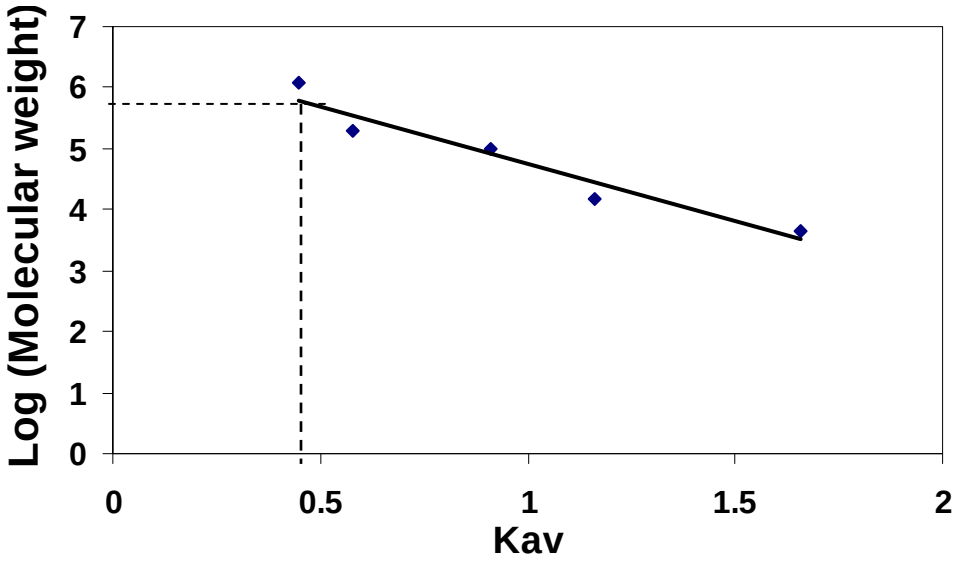

Figure2. Native molecular mass of lipase estimated by gel filtration column : Sepharore CL-4B, column (1x150 cm) calibrated using standard gel filtration molecular weight markers as described in Methods. Partition coefficient (Kav) of the proteins was determined and values are plotted against the log molecular mass (Mr).

Effect of pH and temperature on lipase activity and stability

Maximum lipase activity obtained at pH 5.0 at 25 ^{o}C and decreased significantly (50 %) when the pH was increased to 6.5 (Figure 3). The enzyme showed stability over a narrow pH range 4 to 6 with complete loss of activity at pH 7.0 (Figure 3). The purified enzyme exhibited maximum activity at 25 ^{o}C with retention of 40% of activity at 5 ^{o}C. The activity decreased sharply at temperatures above 30 ^{0}C (Figure 4). The enzyme was found to be stable at 10 and 30 ^{o}C. It lost about 40 % of activity after 6 h of incubation at 35 ^{o}C and the enzyme was completely inactivated at 45 ^{o}C after 4 h of incubation (Figure 5).

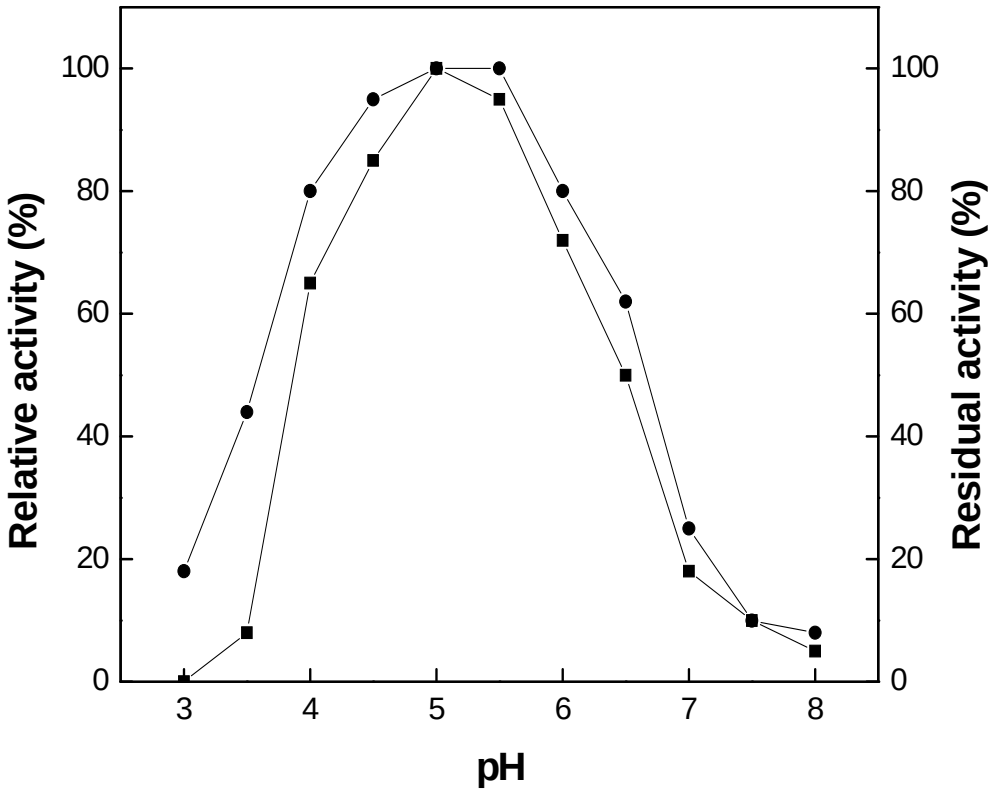

Figure 3. Influence of pH on extracellular lipase activity and stability. Optimum pH (■); pH stability (●).

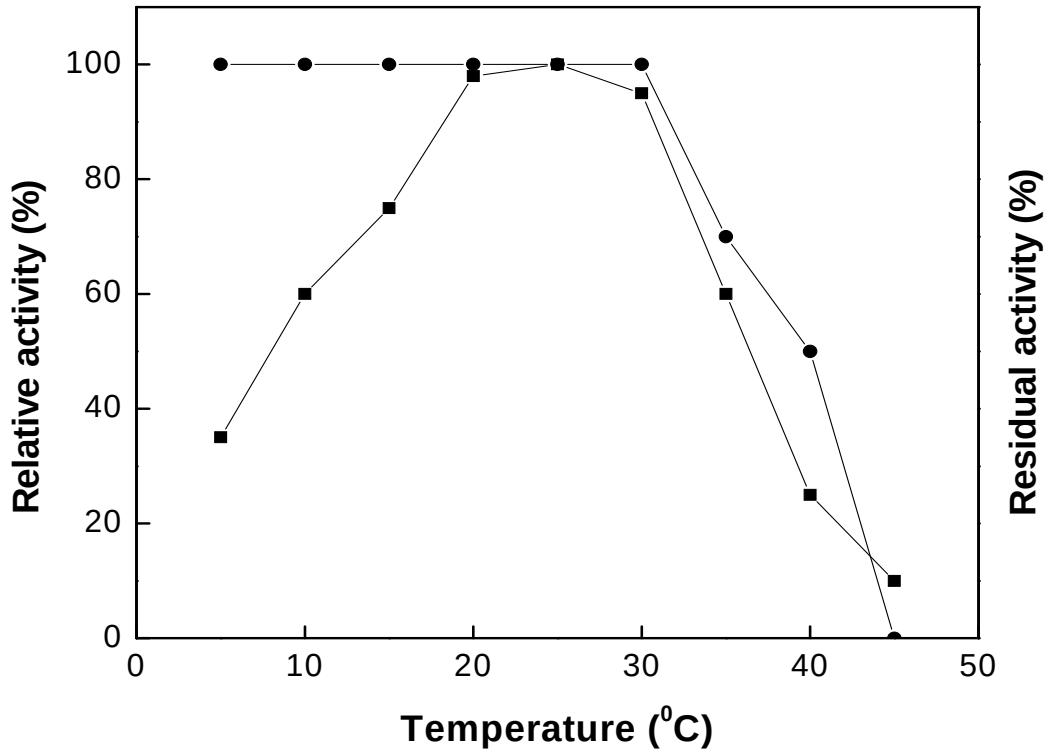

Figure 4. Influence temperature on extracellular lipase activity and stability. Optimum temperature (■); temperature stability (●). For temperature stability the enzyme was incubated at different temperature ranging from 5 to 45 °C for 6 h.

Substrate specificity of Y. lipolytica lipase

The lipase is active against a wide range of *p*-nitrophenyl esters of fatty acids with highest hydrolytic activity with *p*-NP caprylate (C8). The results indicated the clear preference of the enzyme for medium chain length fatty acids (Figure 6).

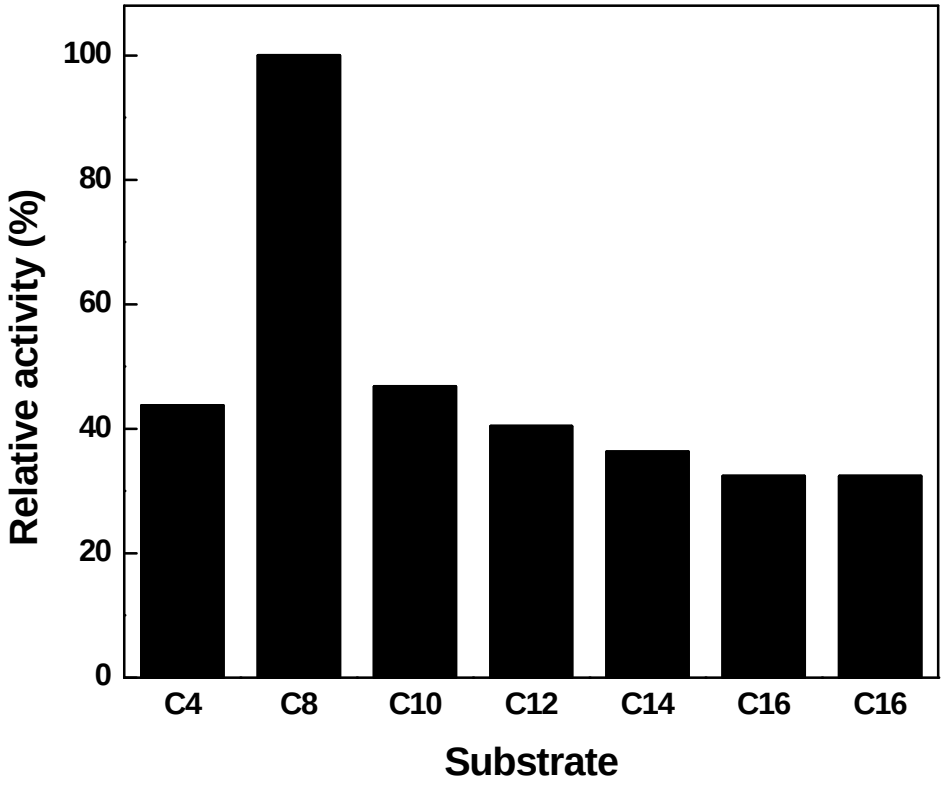

Figure 6 Substrate specificity of the *Y. lipolytica* lipase. Activity on *p*NP-caprylate is taken as 100%.

Effect of metal ions and other reagents on Y. lipolytica lipase

As reported in Table 2, divalent and monovalent cations showed an inhibitory effect on lipase activity at various extents. Amongst the tested metal ions, Hg^{2+} was found to be a strong inhibitor. Inhibition by Fe^{3+} ions was also observed, whereas Ni^{2+}, Mg^{2+}, Ca^{2+} had minor effect on enzyme activity. Interestingly, the presence of Fe^{2+}, k+ and Cu^{2+} increased the lipase activity by 10 -20 %. A concentration of 10% (v/v) of the detergents Tween 20, Tween 80 and SDS drastically decreased activity of the enzyme, while DTT, 2-Mercaptoethanol and EDTA had no effect on catalytic activity and Triton X 100 had a limited affect on the lipase (reduction down to 90%).

Table 2. Effect of metal ions, EDTA, and other reagents on *Yarrowia lipolytica* lipase

Compound	Concentration	Residual activity (%)
Control	--	100
$Cacl_2$	5 mM	97
$Cacl_2$	10 mM	89
$MgCl_2$	5 mM	87
$NiCl_2$	5 mM	71
$ZnSO_4$	5 mM	78
NaCl	5 mM	98
$CoCl_2$	5 mM	102
$CuCl_2$	5 mM	114
KCl	5 mM	111
$FeSO_4$	5 mM	117
$HgCl_2$	5 mM	0
$FeCl_3$	5 mM	17
$CuCl_2$	5 mM	98
2-Mercaptoethano l	10 mM	100
DTT	10 mM	100
EDTA	10 mM	105
SDS	10 %	0
Triton X 100	10 %	87
Tween-80	10 %	9
Tween-20	10 %	16

The residual activity was measured with pNPP at 25 °C

Effect of organic solvents on Y. lipolytica lipase

The effect of organic solvents on *Y. lipolytica* lipase activity was also investigated. The lipase was incubated with various water-miscible and water-immiscible organic solvents at a concentration 20% (vol/vol) at 25 °C for 1 h and the residual activity was measured under standard conditions. As reported in Table 3, the lipase was not inhibited when the enzyme was incubated with 20 % of DMF, DMSO, methanol and n-hexane. Some solvents such as 2-propanol, isoamyl alcohol, ethanol, butanol, acetonitrile, 1,4 dioxane had an inhibitory effect on lipase activity. Amongst the solvents tested, butanol appeared to be a strong inhibitor. The presence of acetonitrile, 1-propanol and 2-propanol at 20 % concentration resulted in 90 %, 91 % and 85 % loss of activity, respectively.

Table 3. Effect of various Solvents on *Yarrowia lipolytica* lipase

Solvents (20 % Conc)	Residual activity (%)
Control	100
2- Propanol	15.6
Prona-1 nol	11.20
Isoamyl alcohol	34.33
n-Hexane	82.64
DMF	98.03
DMSO	94.40
1,4- Diaxane	34.33
Ethanol	68.01
Methanol	88.40
Acetonitrile	10.12
Butanol	6.42

The residual activity was measured with pNPP at 25 °C

Temperature dependent kinetics

The determination of the kinetic parameters for *p*NPP hydrolysis revealed an interesting phenomenon of kinetic optimization at environmental temperatures. The lipase displayed highest K_{cat} at 25 ^{o}C (Table 4), the difference in catalytic efficiency between 5 and 25 ^{o}C was 7.7 X 10^3 min^{-1}. In contrast, the K_m values did not show significant variation between 5 and 25 ^{o}C (Table 4). At temperatures greater than 25 ^{o}C, both k_{cat} and K_m values decreased.

Table 4. Kinetic parameters of the hydrolysis of *p*NPP by *Y. lipolytica* lipase as a function of temperature

Temperature (^{o}C)	K_m	V_{max}	K_{cat}	$K_{cat/Km}$
5	28.30	0.033	3.3 X 10^3	132.0
10	27.57	0.05	5.0 X 10^3	175.0
15	22.22	0.066	6.6 X 10^3	297.0
20	21.10	0.1	1.0 X 10^4	473.9
25	20.40	0.11	1.1 X 10^4	539.2
30	18.18	0.09	9.0 X 10^3	495.0

Assays were carried out under standard assay conditions as described in methods. Kinetic parameters are expressed as Km = µM, Vmax = µmoles min^{-1} mg^{-1}, Kcat = min^{-1}, Kcat/km = μM^{-1} min^{-1}. Incubation time was 30'.

Peptide mass finger printing of lipase

Gel bands were manually excised and subjected to automated in-gel chemical modification of cysteine residues with dithiothreitol and iodoacetamide followed by tryptic digestion and the digestion mixture was analyzed directly by QTOF2 hybrid quadrupole mass spectrometer. This mass spectrometric peptide mapping was compared with the theoretical maps of known proteins, twenty three peptides in the spectrum (Appendix 1) had a mass that was homologous for peptides from YlLip8 of *Y. lipolytica*, resulting in a sequence coverage of 46.10% (172 aa for a total size of 371 aa for the mature YlLip8 lipase). Six peptides matched with triacylglycerol lipase precursor of *Candida deformans,* three peptides are homologous to the tryptase inhibitor of chain E - medicinal leech (fragments). Further sixty four of the peptide peaks did not show any match to the known proteins.

Discussion

Several lipases from *Y. lipolytica* have been reported with different forms (Pignede et al., 2000). *LIP1* and *LIP3* genes from *Y. lipolytica* encoding two lipases were identified by Dominguez et al., (2003) that are similar to the lipases of the fungi *C. cylindracea* and *G. candidum.* These two lipases were reported to be belonging to the carboxylesterase family. An extracellular and two cell-bound types of activities corresponding to lipase I (39 kDa) and lipase II (44 kDa) were described by Ota and coworkers (Sugiura et al., 1976; Ota et al., 1982). Yu et al. (2007a) expressed lipase Lip2 in *Pichia pastoris* and the molecular weight of rYlLip2 was found to be 39 kDa which is similar to parent enzyme. Song et al. (2006) expressed YlLip7 and YlLip8 genes encoding two lipases from *Yarrowia lipolytica* AS 1216 in *P. pastotis.* These expressed proteins corresponding to two lipases were purified to homogeneity. The purified lipases exhibited molecular weight of 41 kDa each and the optimum temperature of 40 and 45 ^{o}C respectively.

Cold-adapted enzymes are mainly established in manifold applications, which offer potential economic benefits by saving energy (Feller and Gerday, 2003). Furthermore, they also minimize undesirable chemical side-reactions that occur most probably at higher temperatures (Yang et al., 2008). *Y. lipolytica* NCIM 3639 lipase displayed a temperature optimum of 25 ^{o}C. The enzyme is unstable at temperatures above 30 ^{o}C. Similar cold-adapted lipases showing temperature optima varying between 25 and 45 ^{o}C have been reported from the other microbial strains such as *Photobacterium* strain (25 ^{o}C), two cold active lipase from *Geotrichum* sp. SYBC WU-3 (20 and 15 ^{o}C), *Acinetobacter baumannii* BD5 (35 ^{o}C), *A. nidulans* (40 ^{o}C), *Pseudomonas* sp. Strain KB700A (35 ^{o}C) (Mayordomo et al., 2000; Rashid et al., 2001; Ryu et al., 2006; Cai et al., 2009; Park et al., 2009). Moreover, a further psychrophilic lipase Lip 3 obtained from a metagenomic DNA library from deep sea sediment has been described to be highly active at 25 ^{o}C (Zhang and Zeng, 2006). A lipase from *Aeromonas* sp. LPB 4 exhibited maximum activity at 10 ^{o}C (Lee et al., 2003).

Lipases are carboxylesterases displaying maximal activity towards water-insoluble long chain acylglycerols (C8-C18), whereas esterases hydrolyze partially water-soluble ester substrates with short-chain fatty acids (below C8). The lipase produced from

a psychrophilic *Photobacterium* strain showed maximum activity towards *p*-NP caprylate (Ryu et al., 2006). The other cold active lipases which showed substrate preference towards medium acyl chain are from *Pseudomonas* sp. strain KB700A (Rashid et al., 2001). Our cold active lipase from *Y. lipolytica* NCIM 3639 showed preference towards medium chain length fatty acid esters. Lipase of *Y. lipolytica* NCIM 3639 was not affected by DTT, EDTA, and 2-mercaptoethanol but was strongly inhibited by Fe^{3+}, Hg^{2+} ions and SDS. Hormone-sensitive lipase of rat adipose tissue was also inhibited by Hg^{2+} ions, indicating the occurrence of an essential thiol group of this family of lipases (Fredrikson et al., 1981). Both Fe^{2+} and Fe^{3+} ions were found to inhibit the lipases from *Aspergillus niger* and metagenomic derived cold active lipase respectively (Iwai et al. 1970; Elend et al., 2007). Metal ions like Ca^{2+}, Mg^{2+} are well known activators of lipases (Kim et al., 2000; Yu et al., 2007a) because Ca^{2+} or Mg^{2+} form complexes with ionized fatty acids which facilitated the removal of free fatty acids formed in the reaction at the water oil interface and changing their stability and behaviors at the interfaces (Gulmova, 1993). Activity of lipase from *Y. lipolytica* NCIM 3639 was neither inhibited nor enhanced in presence of Ca^{2+} and Mg^{2+} activity suggesting that this lipase is neither metal requiring enzyme. In addition, EDTA did not inhibit the enzyme activity thus confirming that our cold active lipase is not metallo enzyme.

Lipases are known to catalyze the reactions in both aqueous as well as organic solvents. They show remarkable stability in organic solvents which helps carry out synthetic reactions leading to production of pharmaceutically important products. The lipase of *Y. lipolytica* NCIM 3639 was stable in water miscible solvents such as DMF, DMSO and methanol, the enzyme was inactivated in acetonitrile, 1-propanol and 2-propanol. The lipase from *Fusarium heterosporum* is also a solvent-resistant enzyme but was completely inactivated in 50 % acetonitrile (Shimada et al., 1993). A metagenomic derived cold active lipase was found to be completely inhibited in the presence of 2-butanol (15%) and acetonitrile (30%) (Elend et al., 2007). The enzyme from *Pseudomonas* sp. Strain B11-1 was completely inhibited by acetonitrile (Choo et al., 1998). Majority of lipases show enhancement in activity in presence of these solvents. Sharon et al., (1998) reported that lipase from *Pseudomonas aeruginosa* KKA-5 was stable in presence of 50% ethanol, methanol and acetone. Addition of ethanol, methanol

and acetone up to 20% enhanced the lipase activity from *Pseudomonas sp.* AG-8 (Sharma et al., 2001). Castro-ochoa et al. (2005) have reported that lipases from *Bacillus thermoleovorans* CCR11 showed high stability in presence of water miscible organic solvents, since it retained almost 100% activity after exposure for 1h in 70% methanol, ethanol, 2-propanol and acetone. Some lipases showing resistance to such water miscible solvents are reported recently (Karadzic et al., 2006; Yu et al., 2007b).

. It has been shown that enzymes from thermophilic and psychrophilic microorganisms show the lowest K_m values for their substrates at the physiological temperatures of the source organisms (Hochachka and Lewis, 1970). To examine whether this is also the case for lipase from *Y. lipolytca*, the K_{cat} values for *p*NPP substrate were determined at different temperatures. The highest K_{cat} value was observed at around 25 ^{o}C which corresponded to the optimal growth and optimal activity of *Y. lipolytica* lipase with decline of K_{cat} value at 30 ^{o}C. At 35 ^{o}C, the lipase gets inactivated.

The molecular weight of *Y. lipolytica*, YlLip8 (Fickers et al., 2005) is 42 kDa and our purified native lipase from *Y. lipolytica* NCIM 3639 is of 400 kDa. SDS-PAGE indicated single band with molecular weight of 20 kDa which indicated that the native lipase is comprised of 20 subunits to form oligomeric native protein. This shows that the cold active lipase from *Y. lipolytica* NCIM 3639 is different from *Y. lipolytica* YlLip8 protein. Such oligomeric lipase with 20 subunits is not yet reported so far. Ota et al., (1982) purified two cell-bound lipases (lipases I and II) with the molecular weights of 39 and 44 kDa, respectively. Both of them had the similar substrate specificity, optimum pH and pH stability. Fickers et al. (2005) isolated gene LIP7 and LIP8 encoding two cell-bound lipases from *Y. lipolytica* (YlLip7 and YlLip8) which have 366 aa and 371 aa, respectively. Three extracellular lipases (YlLip2, YlLip7, and YlLip8) have been reported in *Y. lipolytica*. Lipase YlLip2 was the main extracellular lipase and secreted at the end of the growth phase (Fickers et al., 2004), while YlLip7 and YlLip8 are mainly associated to the cell wall and easily released by washing the cells with phosphate buffer (Fickers et al., 2005).

In conclusion, an extracellular cold active lipase was purified which exhibited a native molecular mass of 400 kDa. SDS-PAGE showed the molecular mass corresponding to 20 kDa indicative of the oligomeric nature of a lipase comprising of 20

subunits. The enzyme displayed an optimum pH of 5.0 and optimum temperature of 25 ^{o}C. The lipase was stable over a pH range between 4.0-6.0.The *Y. lipolytica* lipase prefers medium to long chain fatty acid esters, It is neither metal requiring nor metallo-enzyme. Lc-MS-MS mass spectrometric analysis showed about 46% homology to LIP8p lipase from *Y. lipolytica*. and some peptides did not show any homology to the any known proteins. Since this lipase was found to be unique in relation to its multimeric structure, the further studies on elucidation of its structure were performed which are given in the next chapter.

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CHAPTER 4

Three dimensional reconstruction of oligomeric cold active lipase from *Yarrowia lipolytica.*

Abstract

This chapter deals with the thermal and chemical deactivation and three dimensional reconstruction of the oligomeric cold active lipase by *Yorrowia lipolytica NCIM* 3639. From the gel permeation chromatography, it is evident that at 2-4 % of SDS concentration the enzyme dissociates into a lower molecular weight subunits and loosing its activity. Reconstructions from particles embedded in vitreous ice showed that lipase has a double-ringed, 20 meric structure with C20 symmetry. At 6.5 Å resolution, it was possible to unambiguously delineate the 20 individual subunits in the complex with barrel shape structure. The 3D reconstruction also reveals a subunit organization between the two rings of the complex. The density map has the area of 607.2 x 10^3 Å2 and volume of 4.314 x 10^6 $Å^3$.

Introduction

Lipases (EC 3.1.1.3) hydrolyze the ester bonds of long-chain acylglycerides. Biotechnologically, they have gained considerable importance as biocatalysts that are able to catalyze not only hydrolysis but also synthesis reactions, with the latter occurring in nearly water-free organic solvents (Jaeger et al., 2002) and often with high regio- and enantioselectivity (Reetz et al., 2000). A characteristic feature to distinguish lipases from esterases is called interfacial activation and describes the observation that lipase activity sharply increases as soon as the monomolecular substrate starts to form a micellar emulsion (Reetz et al., 1997). An obvious explanation was provided by several lipase crystal structures that revealed the presence of a surface-exposed α -helical polypeptide chain termed the "lid" which covers the active site and moves away upon contact with the micellar substrate interface (Brzozowski et al., 2000). However, exceptions have also been observed of lipases that possess a lid and still do not show interfacial activation (Verger, 1997).

The important regulatory role of multi-subunit proteins and multi-protein complexes in biological systems is widely recognized. Multimeric enzymes provide an attractive system for investigating spontaneous self-assembly of protein structures and for

examining regulatory interactions between subunits, and subunit association can manifest in complex ways. Several studies show clearly that quaternary structure plays a fundamental role in the stabilization of the active protein form (Gloss et al., 2009; Rumfeldt et al., 2008; Wu et al., 2008). Understanding the self-assembly processes of such macromolecules remains a major problem in protein chemistry. Although inter- and intra-subunit interactions in oligomeric and monomeric proteins are of same physical nature, the denaturation/renaturation reactions in oligomeric proteins are more complex than those of smaller monomeric proteins and involve many local and global processes that occur by sequential mechanisms (Jaenicke et al., 2000). Hence, the elucidation of the hierarchy of events occurring during the denaturation of an oligomeric protein provides an important means for delineating such a process.

Oligomeric proteins appears to be a strong selection pressure for the evolution of monomeric proteins into oligomeric complexes, driven by benefits such as reduction of surface area, increased stability and novel function through inter subunit communication (Goodsell and Olson, 1993). Over the past ten years, several laboratories have undertaken surveys of the growing structural database of oligomeric proteins, attempting to define the general structural principles of protein–protein interaction. On the basis of three structures— insulin, trypsin-BPTI and hemoglobin, Chothia and Janin (1975) defined the basic tenets: “Hydrophobicity is the major factor stabilizing protein–protein association, while complementarity plays a selective role in deciding which proteins may associate”. These tenets have been repeatedly confirmed in analyses of larger data sets (Sperry et al., 2011; Banerjee et al., 2010; Xu et al., 1997; Tsai et al., 1997).

One of the most important physicochemical characteristics of the proteins is their stability at elevated temperatures. The denaturation of proteins possessing the quaternary structure has certain peculiarities because the overall mechanism of denaturation can include the step of reversible dissociation of the protein oligomer into separate subunits. The dissociative mechanism of thermal and chemical denaturation has been demonstrated for glyceraldehyde-3- phosphate dehydrogenase from lobster muscle (Lin et al., 1990), β-galactosidase from *Escherichia coli* (Edwards et al., 1990) and *Streptococcus thermophilus* (Chang et al., 1994), glutamate dehydrogenase from *Sulfolobus solfataricus* (Consalvi et al., 1993) and bovine liver (Singh et al., 1996), β-lactoglobulin (Qi et al.,

1995), aminoacylase I from porcine kidney (Poltorak et al., 1998), and adenylate kinase from *Sulfolobus acidocaldarius* (Backmann et al., 1998).

The yeast *Yarrowia lipolytica*, the only species recognized so far in the *Yarrowia* genus, is quite different from the other frequently studied yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* in terms of its phylogenetic evolution, physiology, genetics, and molecular biology. *Y. lipolytica* serves as an excellent model to study the non-conventional lipolytic yeasts (Fickers et al., 2005).Consequently, the lipase produced by the yeast *Y. lipolytica* that currently constitutes the best studied example of creating an enantioselective, esterification (Cambon et al., 2010; Bordes et al., 2009; Cancino et al., 2008). *Y. lipolytica* produces an extracellular cold active lipase. Its production was found to be stimulated when the standard growth medium was supplemented with Tween-80 (chapter 2). It was shown that this enzyme is an oligomer comprised of 20 mers (chapter 3).

Cryo-electron microscopy (cryo- EM) of ‘‘single particles’’ is an emerging technique for determination of protein structure under native environment. The amounts of material required for cryo-EM are minute and, moreover, some degree of heterogeneity is tolerable. Although high resolution is often elusive, the medium resolution (1–2 nm) structures afforded by cryo-EM provide a platform for hybrid approaches which, in turn, can provide useful insights into the structure and mechanism of macromolecular assemblies (Robinson et al., 2007). Recently, crystal structures have been reported for *Y. lipolytica* Lip2 in its closed conformation. The Lip2 structure is highly homologous to known structures of the fungal lipase family (*Thermomyces lanuginosa*, *Rhizopus niveus*, and *Rhizomucor miehei* lipases) (Bordes et al., 2010). Furthermore, there is no structure of oligomeric lipases in the literature. Therefore, we have determined the sub nanometer resolution solution structure in the native form of oligomeric cold active lipase from *Y. lipolytica* using cryo EM and single particle reconstruction derived with an imposing of C20 symmetry among the twenty subunits. The dissociation of oligomeric lipase was studied at different SDS concentration using gel exclusion chromatography experiments. CD spectroscopy was used to investigate changes produced in the protein secondary structure upon increase of temperature.

Materials and methods

Circular dichroism (CD)

CD spectra of lipase (0.5 mg/mL) in 5 mM phosphate buffer, pH 6.0 were used to measure secondary structure and stability. Spectra were recorded between 260 and 190 nm, with a 1.0 cm path length cuvette and a Jasco J-815 circular dichroism spectrophotometer equipped with a Peltier temperature control system (Jasco Inc., USA). Scan speed of 100 nm/min, response time of 1 sec, bandwidth of 1 nm and data pitch of 0.2 nm was used for measurement. All spectra were corrected for buffer baseline by subtracting the respective blank spectra recorded identically without protein. Thermal denaturation were monitored by heating the purified protein from 10 to 90 ^{o}C upon incubation for 10 min, followed by three CD scans at each temperature were recorded and averaged, and the signals are reported as mean residue ellipticities. For refolding studies the enzyme was cooled to room temperature and CD scan was monitored.

Dissociation analysis by gel permeation chromatography

Purified enzymes (0.5 mg) were incubated in 20 mM citrate phosphate buffer (pH 5.0) at different constrictions of SDS, between 0 and 4.0 % for 1 h at 25°C and loaded onto a Sepharose CL-4B column which had been previously equilibrated with 20 mM citrate phosphate buffer (pH 5.0). The proteins were eluted at a flow rate of 12 ml/h. The protein elution profile was monitored at 280 nm at room temperature. Lipase activity was measured before loading onto the gel permeation chromatography and activity was also checked for the eluted enzyme.

Electron Microscopy

Electron micrographs were recorded at 2048×2048 slow-scan charge-coupled device (CCD) camera, with FEI Tecnai HRTEM which was operated at 200 kv. During the focusing stage of this procedure, astigmatism and defocus were corrected by inspection of the real-time power spectra calculated from the images. The samples were prepared for Cryo EM, the copper grids were mounted on a cryo plunger, 10µl of sample (0.5mg/ml) was applied to the carbon coated grids and excess amount was blotted using bloating paper. Then the grids were plunged in to the liquid ethane which was previously

maintained at -170 ^{0}C, and then the grids were transferred to the cryo-holder and to the cryo EM, JEOL Cryo EM which was observed at 200 kv. The images were recorded on negatives of Kodak SO 163 at 50,000X.

Image processing and single particle reconstruction

The films were developed and scanned on a UMAX power loop 2100XL scanner at 3000X4000 dpi. The images were CTF corrected on a per-micrograph basis using the EMAN 2 suite program Ctfit, Approximately 24,000 particle projection images were obtained from 72 micrographs. From this data set, we stringently omitted a large number of particles with poor contrast, and other defects, resulting in a ‘‘clean’’ data set of 12,400 particles used for all subsequent image processing. And 582 particle images from 20 micrographs from HRTEM were used for the initial model building reconstruction; all particles were picked interactively using Boxer 100 x 100 pixel boxes from the EMAN 2 suite. The contrast transfer function (CTF) and envelope function parameters were determined for the particles in each micrograph by using ctfit and fitctf (Ludtke et al., 1999, 2001). Particle orientations and 3D reconstruction were determined as previously described (Ludtke et al., 2004). Briefly, the entire data set of phase-flipped particles was subjected to a multirefine- based procedure (with the multirefine program in EMAN 2) by using three starting models created with startnrclasses (Ludtke et al., 1999). The final reconstruction was obtained by building a starting model from the reference-free class averages prior to stepwise structure refinement of C20 symmetry of HRTEM images. Iterations were carried out at successively finer angular sampling, The usefilt option in EMAN’s refine routine allowed two analogous data sets to be used: one band pass filtered for the classification and alignment routines, and a second unfiltered data set for the 3D reconstruction. The primary structures were further refined by using the standard EMAN iterative reconstruction algorithm (Ludtke et al., 2004), i.e., reference-based classification of particles, class averaging with CTF correction, and 3D model construction. The iterative reconstruction process continued until convergence was achieved, as assessed by observing the Fourier shell correlation (FSC) between successive rounds of refinement.

Results

Effect of temperature on secondary structure

In order to gain further insights into conformational changes during unfolding of lipase denaturation, change in the secondary structures were studied using CD spectroscopy with increasing the temperature. During temperature-induced unfolding, dramatic structural changes were observed, both the shape and intensity of CD spectra changed as temperature increased. The far UV-CD spectrum of oligomeric *Y.lipolytica* lipase at 10 and 20 ^{o}C exhibit minima at 208 and 222 nm (Figure 1), characteristic of proteins that contain significant amounts of α-helix. As the temperature increased to 30 ^{o}C, the CD signal at 222 nm increased, while the signal at 208 nm does not show much change. When the temperature was raised above 40 ^{o}C, it started losing its the secondary structure which was observed by an increase in the CD signal. The loss in signal at 222 nm was greater than that at 208 nm. As the temperature reached 90 ^{o}C, there was not much change in the 208 peak in CD signal indicating the enzyme is partially unfolded at this temperature. Enzyme refolding studies revealed that after cooling to room temperature the enzyme is partially refolded but still into an inactive form.

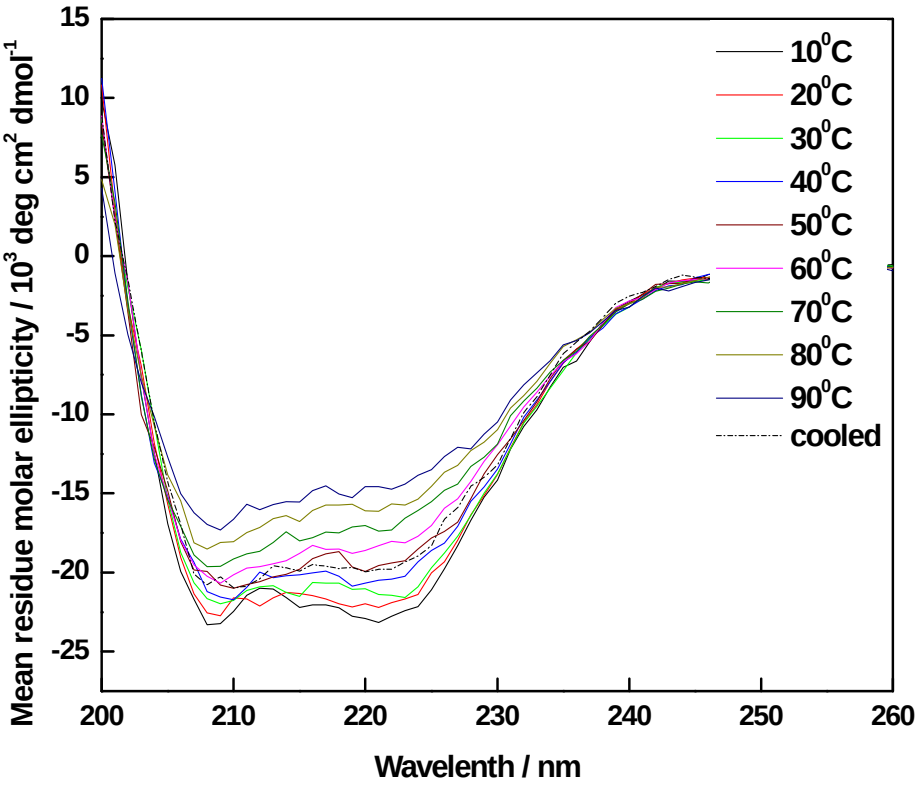


Figure 1. Effect of temperature on secondary structure of oligomeric cold active lipase

Dissociation analysis by gel filtration chromatography

Purified oligomeric lipase was incubated in increasing concentrations (0-4 %) of SDS in 50 mM citrate phosphate buffer at pH 5.0 for 1 h, and the residual activity was measured under the standard assay conditions. The enzyme activity of SDS incubated samples was measured, and the residual activity of lipase was plotted along with SDS concentration. The results showed that oligomeric form is catalytically active but with increased SDS concentration, the enzyme was deactivated (Figure 2A). The SDS treated (1-4%) enzyme preparations were subjected to gel filtration chromatography on a Sepharose-CL 4B column (Figure 2B and 2C). The native enzyme and 0.5 % SDS treated enzyme were eluted at the exclusion volume of the column giving a single sharp peak (Figure 2B). However, with increasing concentrations of SDS, the lower molecular

weight subunit fractions were observed (Figure 2C). Experiments were also performed in which enzyme was incubated with NaCl (10 M) or β-mercaptoethanol (200 mM) in the citrate phosphate buffer (50 mM, pH 5.0) to see the dissociation. The results showed that the presence of salt and β-mercaptoethanol did not have any effect on activity suggesting that the enzyme did not dissociate in presence of high salt concentration and β-mercaptoethanol.

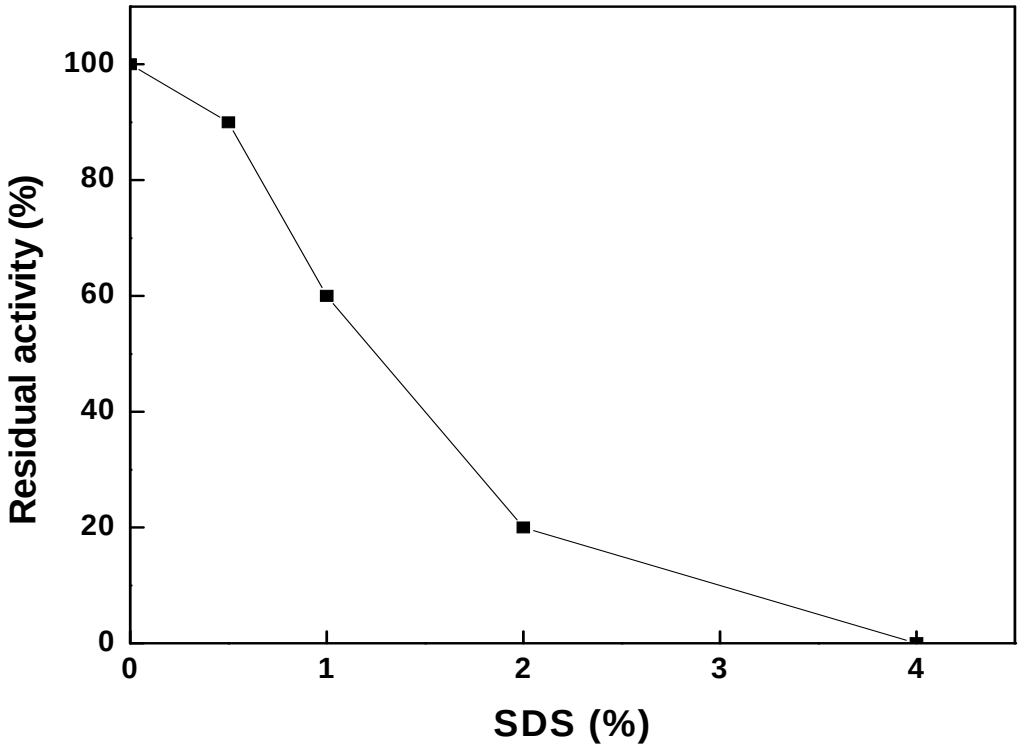
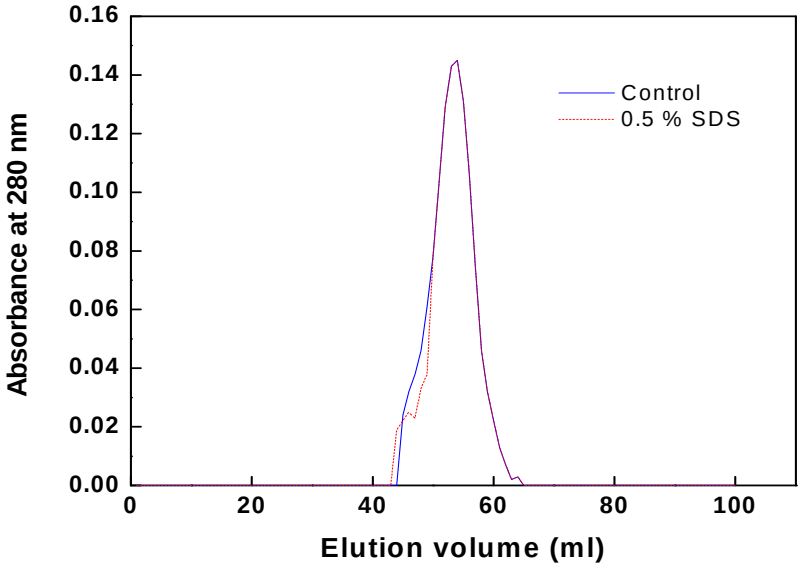


Figure 2 A Effect of various concentration of SDS on oligomeric cold active lipase activity. Lipase activities were measured using the *p*NPP as substrate at 25 °C. The relative lipase activity was calculated by considering the activity of control (without SDS) as 100%.

B



C

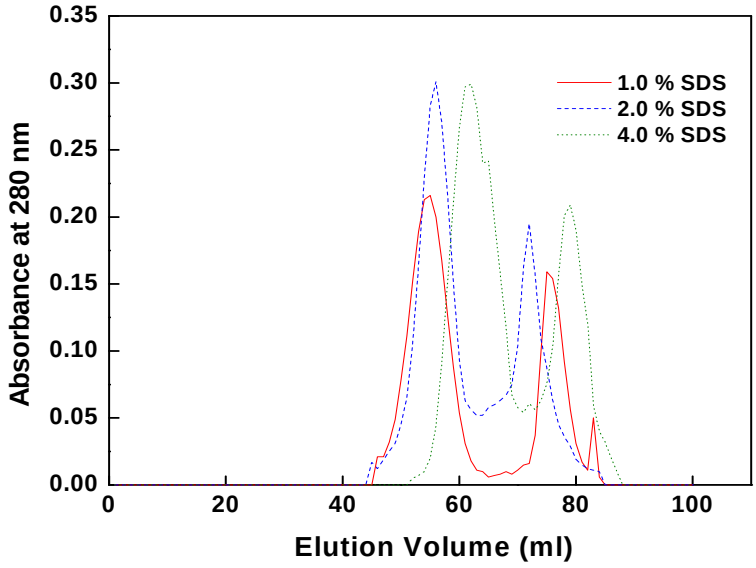


Figure 2B and C Effect of SDS on oligomeric lipase. (*B and C)* Elution profiles of native and SDS incubated oligomeric lipase as analyzed with a size-exclusion chromatography column Sepharose CL-4B.

Cryo-EM and single particle reconstruction

CryoEM micrographs of lipase embedded in vitreous ice, this biochemical preparation generated a homogeneous and spherical shape in all these particles (Figure. 3A). The reconstruction was computed from 12,500 particle images of lipase particle images that were computationally selected from an original pool of 24,200 particle images. The image particles were sorted into 74 class averages (Figure 3B). The convergence of the structure from these particle images provides a statistically defined and robust density map displaying the most prominent and reliable structural features of this oligomeric lipase (Figure. 4A). We calculated a density map by imposing C20 symmetry employing EMAN 2, a single particle reconstruction package. At 6.5 Å resolution of this map (Figure. 4A), it was possible to unambiguously delineate the 20 individual subunits in the complex with barrel shape structure. To avoid any initial arrangement bias in our analysis, we have arbitrarily named the encompassing subunits 1–20 labeled clockwise looking down on either end of the map. The map reveals two rings structure with 20 subunit boundaries and domain structures of individual subunits region of the complex which are running parallel from top to bottom of the protein (Figure. 4A). The map has the area of 607.2 x 10^3 Å2 and volume of 4.314 x 10^6 $Å^3$. Two EM bottom views, solid and mesh form are presented in Fig. 4 B, a tilted side view (Figure. 4C), side view (Figure. 4D).

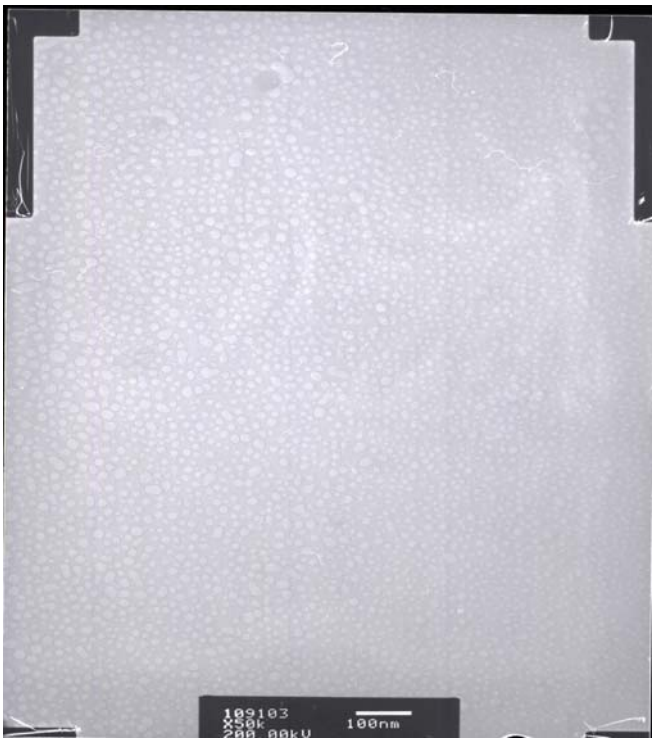
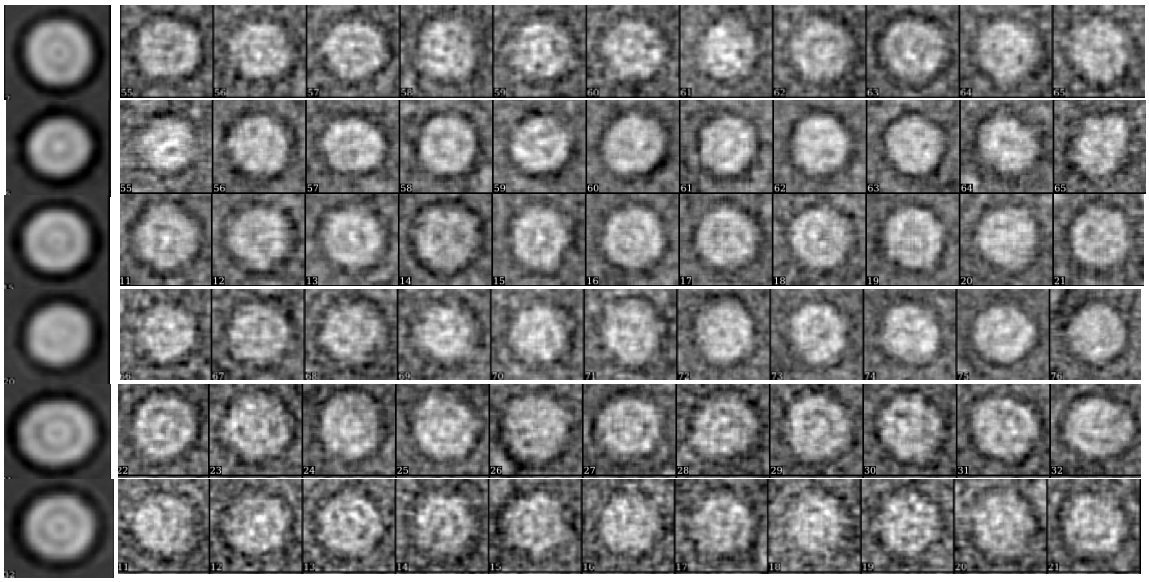


Figure 3A. Cryo-electron micrograph of lipase. The scale bars in micrograph represent 100 nm.



Projection **Class average**

Figure 3B. Gallery containing a representative collection of single molecules. Particles from the micrographs were extracted, normalized, centred and filtered using different commands found in the EMAN 2 software (Ludtke et al., 1999). Left Panel containing the projections and their corresponding class averages.

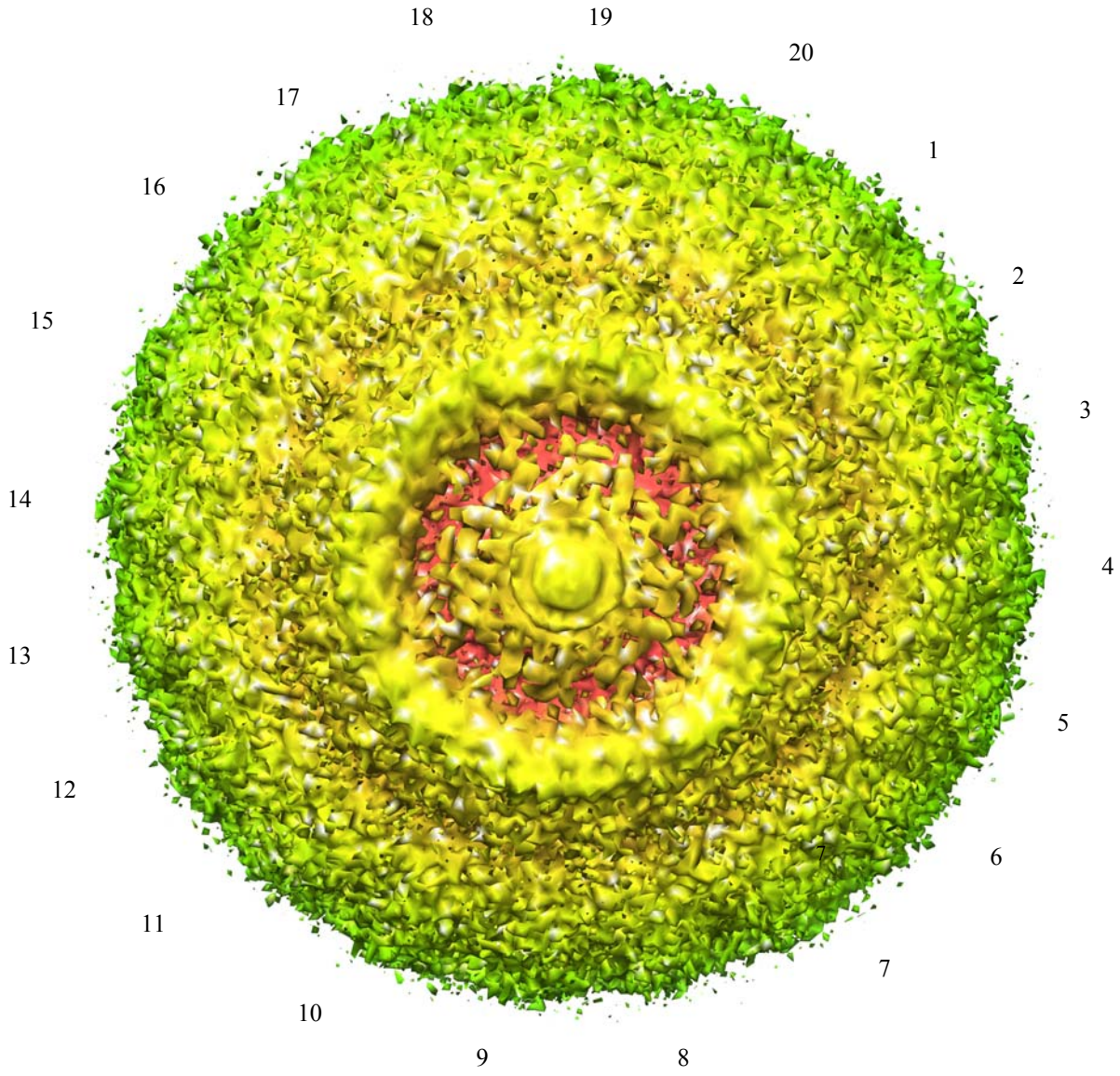


Figure 4A. Final 3D reconstruction from cryo-EM images of oligomeric cold active lipase. Surface representation of the 6.5 Å resolution cryo-EM map as viewed from the top.

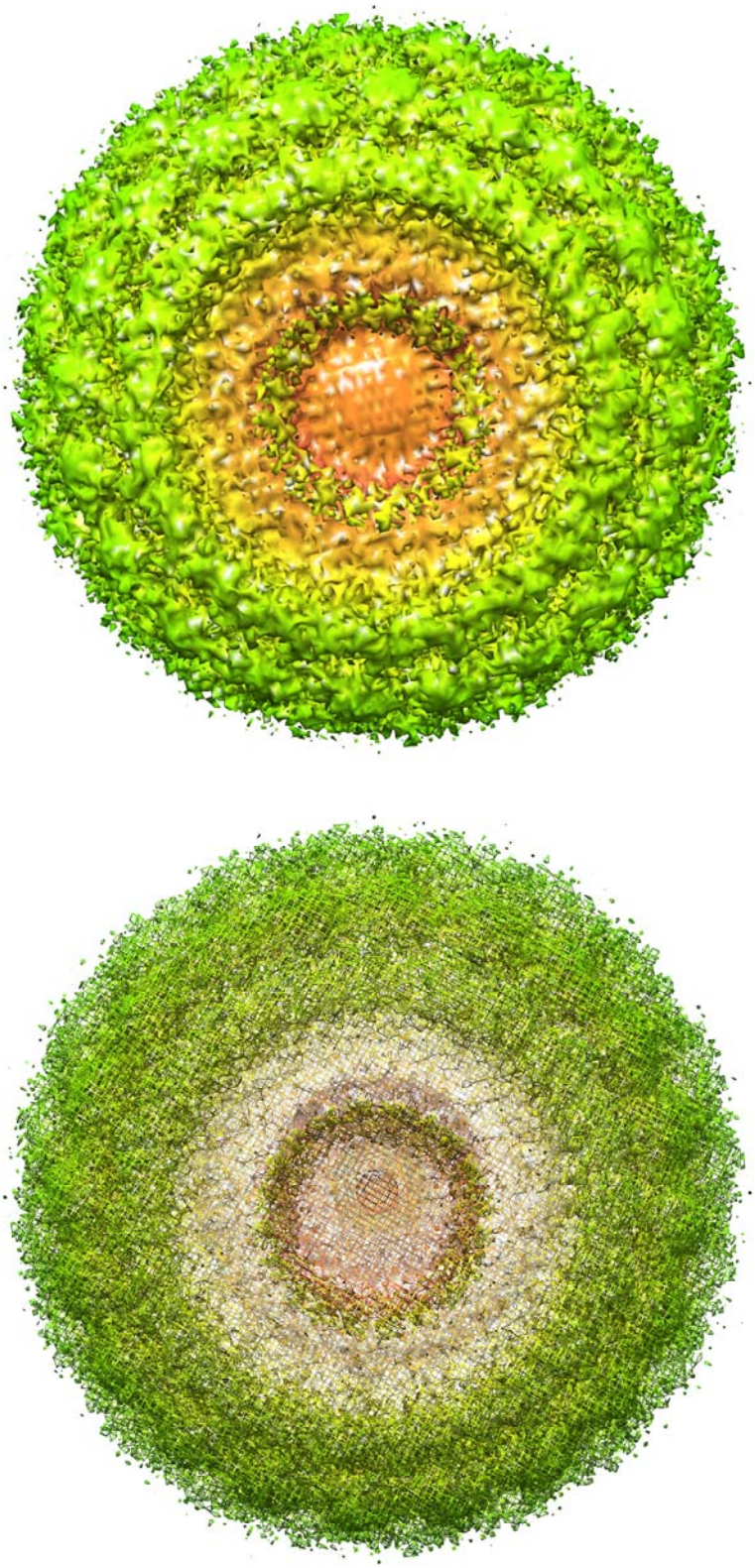


Figure 4B. Final 3D reconstruction from cryo-EM map as viewed from the bottom, top panel is in solid and bottom panel is in mesh form.

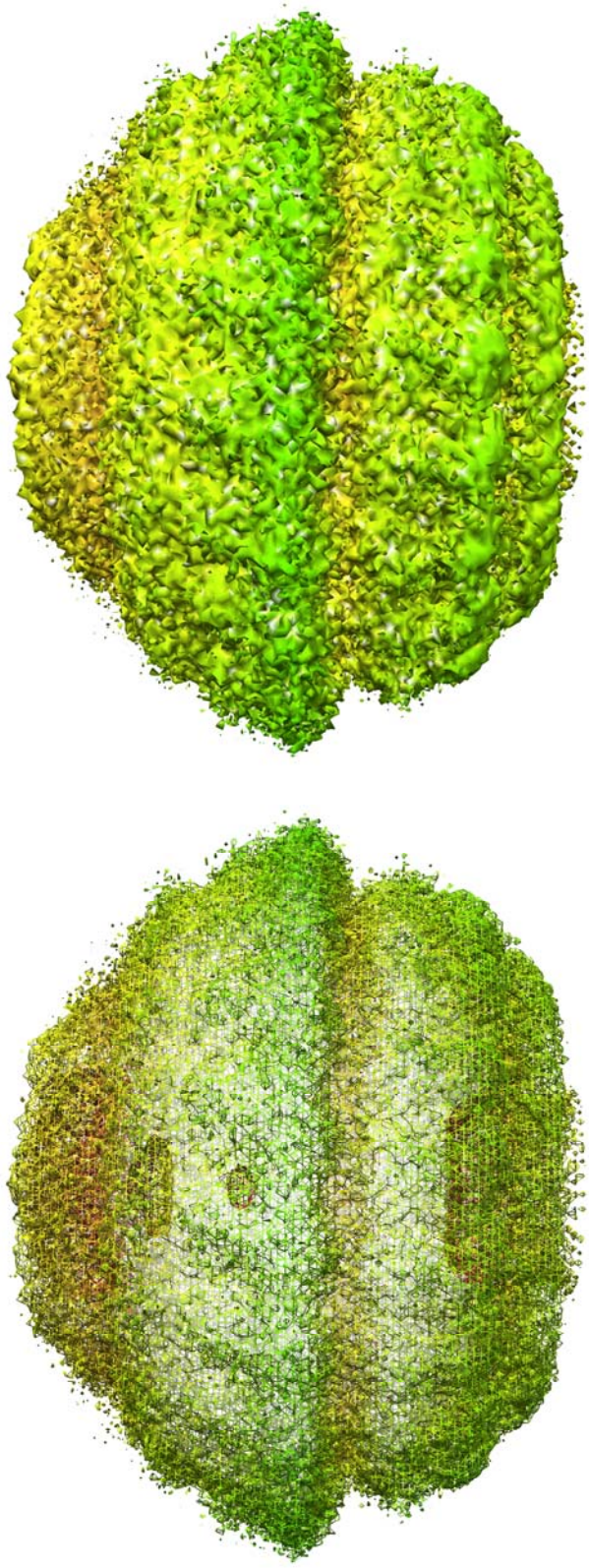


Figure 4C. Final 3D reconstruction from cryo-EM map as viewed from the tilted side view, top panel is in solid and bottom panel is in mesh form.

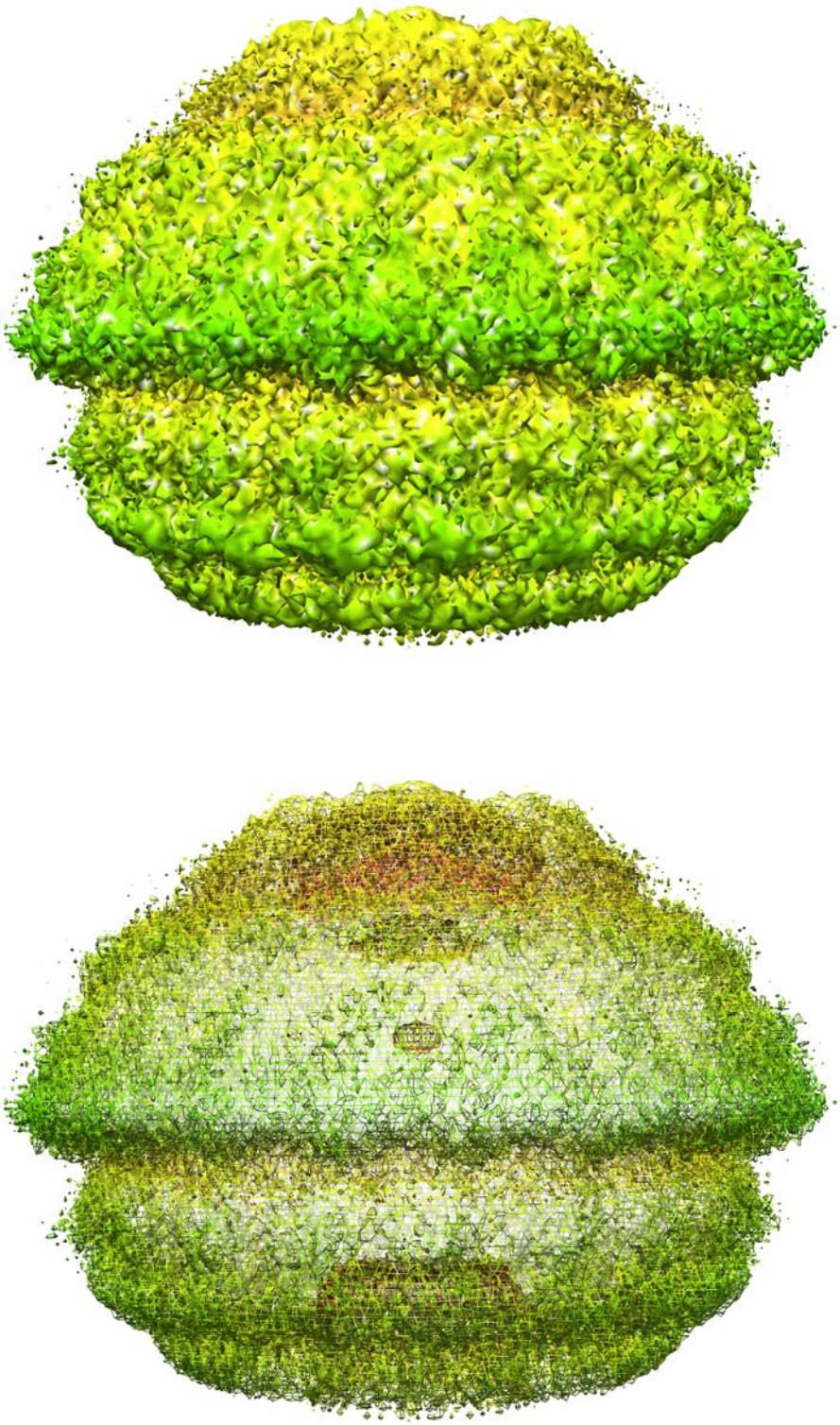


Figure 4D. Final 3D reconstruction from cryo-EM map as viewed from the side view, top panel is solid and bottom panel is in mesh form.

Discussion

Structural studies and unfolding transitions of proteins under different solvent conditions provide information about the conformation of protein molecules and the role of various stabilizing and destabilizing forces responsible for the proteins' unique three-dimensional structure (Timasheff, 1993). The unique three-dimensional structure is a basic requirement for proper functioning of a protein. It has been demonstrated in many studies that the ability to keep this native and functional structure over a particular range of temperatures, pH levels, and salinities is an intrinsic property of the protein molecule itself, which is determined by the amino acid sequence (Anfinsen, 1973). Outside of these conditions, the protein starts to unfold. Surprisingly this range of experimental conditions differs for different proteins from different sources.

As reported earlier cold active lipase of *Y. lipolytica* NCIM 3639 is active in the temperature range 10-30 ^{o}C. The results reported here show that the conformational changes coincide with the changes in functional activity. A loss in enzymatic activity for lipase at temperature grater than 30 ^{o}C, which is at first seen as a slight change in the far-UV CD signal at 30 ^{o}C and it is followed by significant change in the CD signal, this can be ascribed to the partial denaturation of lipase followed by the loss of enzyme activity.

From the results of gel permeation chromatography, it is evident that at 2-4 % of SDS concenteration the enzyme dissociates into lower molecular weight subunits. These findings suggest that the hydrophobic interactions at the subunit interface could have a greater effect on the stability of the oligomeric lipase. The results also show that the oligomeric from is active, as the enzyme dissociates it is losing its activity. Oligomerization is well established as a mechanism for controlling enzyme activity. Hormone-sensitive lipase, the mammalian enzyme responsible for liberating fatty acids from adipose tissue has been shown to become 40 times more active as a dimer than as a monomer (Shen et al., 2000), and pro-caspase 8, a member of the pro-apoptotic cascade of proteases only becomes active as a homodimer, presumably as a defence against aberrant apoptosis (Chang et al., 2003). HIF (hypoxia-inducible factor) is an $\alpha\beta$ transcription factor that modulates the hypoxic response is catalytically active only as a homodimer (Lancaster et al., 2004). Endothelial lipase (EL) is a member of a subfamily of lipases that act on triglycerides and phospholipids in plasma lipoproteins is active as a

homodimer (Griffon et al., 2009). Lipoprotein lipase (LPL) is a principal enzyme responsible for the clearance of chylomicrons and very low density lipoproteins from the bloodstream. The active form of LPL is a noncovalent homodimer with the subunits associated in a head-to-tail manner, and the dissociation of its dimeric form leads to the formation of a stable inactive monomeric conformation and irreversible enzyme inactivation (Yau et al., 2009; Sukonina et al., 2006; Osborne et al., 1985)

Since many enzymes consist of oligomeric structures for maintaining their activities, it is important to elucidate the factors involved in stabilization of their oligomeric states. Unfortunately, our knowledge concerning these aspects is still far from complete. Generally, the oligomeric structure of proteins is formed by various subunit-subunit interactions, including disulfide bonding, hydrogen bonding, and hydrophobic and electrostatic interactions. To understand how to increase the stability of these oligomeric organizations, conditions which increase or decrease the stability of an oligomeric structure have to be investigated.

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Conclusions

1. The organism was isolated from refrigerated Tween-80 and was identified as *Yarrowia lipolytica* using 26S rDNA D1/D2 sequencing approach.

2. We found different localization of lipase either cell bound or extracellular based on substrate used for production. When organism was grown in olive oil based medium, it produced cell bound lipase and when the organism grown in Tween-80 containing medium, it produced significant amount of extra cellular lipase. Such differential induction of lipases depending on the nature of carbon source used for the growth of an organism is not reported so far. The optimal temperature for growth and production is 20 ^{o}C. The optimal pH for the production is 5.5.

3. The extracellular cold active lipase from *Y. lipolytica* has been purified to homogeneity by ion exchange followed by gel permeation chromatography with specific activity of 195 and 11.14 % yield. The purified lipase showed the molecular mass of 20 kDa on SDS-PAGE. The native enzyme showed a molecular mass of 400 kDa which is confirmed by gel permeation chromatography. These results showed that the enzyme is oligomeric in nature with 20 subunits.

4. The purified enzyme showed optimum temperature and pH of 25 ^{o}C and 5.0 respectively. Lipase is active towards medium acyl chain esters. The kinetics parameters showed that its maximum K_{cat} at 25 ^{o}C.

5. Peptide mass finger printing studies showed that the lipase showed 42% homology to *Yarrowia lipolytica* LIP8p.

6. The CD experiments revealed that the enzyme is partially denatured at 90 ^{o}C

7. From the gel permeation chromatography, it is evident that at 2-4 % of SDS concentration, the enzyme dissociates into a lower molecular weight subunits with the loss of its activity.

8. The three dimensional reconstructions from particles embedded in vitreous ice showed that lipase has a double-ringed, 20 meric structure with C20 symmetry. At 6.5 Å resolution, it was possible to unambiguously delineate the 20 individual subunits in the complex with barrel shape structure.

9. The 3D reconstruction also revealed a subunit organization between the two rings of the complex. The density map has the area of 607.2 x 10^3 Å2 and volume of 4.314 x 10^6 $Å^3$

List of Publications

K.N. Sathish Yadav, M.G. Adsul, K.B. Bastawde, D.V. Gokhale (2011). Differential induction, purification and characterization of cold active lipase from *Yarrowia lipolytica* NCIM 3639. Bioresources Technology. (Communicated).

Three dimensional reconstruction of oligomeric cold active lipase from *Yarrowia lipolytica* NCIM 3639. **Manicurist under preparation.**

Annexure-1

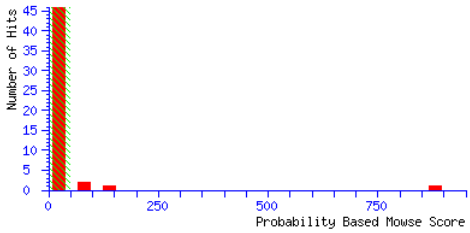
Mascot Search Results

```
User            : K Bailey
Email           : kevin.bailey@nottingham.ac.uk
Search title    : Lipase 20kda band
MS data file    : X:\QTOF DATA ARCHIVE\2009\APR 09\D V Gokhale\090408 lipase 20kda.raw\090408 lipase 20kda.pkl
Database        : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Timestamp       : 23 Apr 2009 at 08:26:41 GMT
Protein hits    : Q3HRV6_YARLI LIPY8p.- Yarrowia lipolytica (Candida lipolytica).
                  Q875G8_9ASCO Triacylglycerol lipase precursor (EC 3.1.1.3).- Candida deformans.
                  1AN1E        tryptase inhibitor, chain E - medicinal leech (fragments)
                  Q6CCX8_YARLI Similarity.- Yarrowia lipolytica (Candida lipolytica).
                  Q3F6Q1_9BURK Peptidase M50 precursor.- Burkholderia ambifaria AMMD.
                  Q6N4B7_RHOPA Possible transcriptional regulator, MarR family.- Rhodopseudomonas palustris.
                  O68872_MYXXA Hypothetical protein.- Myxococcus xanthus.
                  A30769       regulatory protein trpI - Pseudomonas aeruginosa
                  Q2DMU6_9DELT Helix-turn-helix, Fis-type.- Geobacter uraniumreducens Rf4.
                  Q6CLF7_KLULA Similar to sp|P53944 Saccharomyces cerevisiae YNL063w singleton.- Kluyveromyces lactis (Yeast) (Candida
                  Q310N7_DESDG Atrazine chlorohydrolase (EC 3.8.1.8).- Desulfovibrio desulfuricans (strain G20).
                  Q61XM8_CAEBR Hypothetical protein CBG03920.- Caenorhabditis briggsae.
                  Q8XT74_RALSO Putative alpha-amylase-related protein.- Ralstonia solanacearum (Pseudomonas solanacearum).
                  PS17_PINST   Putative cytochrome c oxidase subunit II PS17 (Fragments).- Pinus strobus (Eastern white pine).
                  Q22IV2_TETTH Cyclic nucleotide-binding domain containing protein.- Tetrahymena thermophila SB210.
                  Q1Z8J1_PHOPR Hypothetical transcriptional regulator, TetR family protein.- Photobacterium profundum 3TCK.
                  Q2T3S3_BURTA Lipopolysaccharide biosynthesis protein (O-antigen-related) (EC 4.2.1.-).- Burkholderia thailandensis (s
                  Q3JH39_BURP1 CDP-6deoxy-D-xylo-4-hexulose-3-dehydrase (EC 4.2.1.-).- Burkholderia pseudomallei (strain 1710b).
                  Q63N74_BURPS Lipopolysaccharide biosynthesis protein (O-antigen-related).- Burkholderia pseudomallei (Pseudomonas pse
                  Q9ZTY0_EMIHU Putative calcium binding protein.- Emiliania huxleyi.
```

	MSDB	Decoy	False discovery rate
Peptide matches above identity threshold	13	1	7.69 %
Peptide matches above homology or identity threshold	21	1	4.76 %

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 49 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As: Peptide Summary — Help

Significance threshold p< 0.05 — Max. number of hits 20

Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Sort unassigned: Decreasing Score — Require bold red

Select All | Select None | Search Selected | Error tolerant

1. Q3HRV6_YARLI **Mass:** 41335 **Score:** 879 **Queries matched:** 23 **emPAI:** 1.60
   LIPY8p.- Yarrowia lipolytica (Candida lipolytica).
   Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2	402.2057	802.3968	802.3974	-0.0005	0	9	8.8e+02	2	K.IHDGFSK.A
9	414.7268	827.4390	827.4905	-0.0515	0	30	5.3	1	R.FINPPLK.D
27	549.3047	1096.5948	1096.5917	0.0032	0	19	61	1	K.HFPDIELVK.T
31	618.7792	1235.5438	1235.5452	-0.0014	0	58	0.0074	1	K.DVISCAGGENSK.C + Carbamidomethyl (C)
35	682.3275	1362.6404	1362.7296	-0.0891	0	92	3.1e-06	1	R.VGNKPFAEFINK.L
36	455.2310	1362.6712	1362.7296	-0.0584	0	(62)	0.0038	1	R.VGNKPFAEFINK.L
41	682.3790	1362.7434	1362.7296	0.0139	0	(11)	4.8e+02	3	R.VGNKPFAEFINK.L
52	702.3815	1402.7484	1402.7456	0.0028	0	42	0.33	1	K.TSITGYLAVDHVK.K
56	753.3294	1504.6442	1504.7674	-0.1232	0	(45)	0.14	1	K.GYDPILINYGQPR.V
57	753.3857	1504.7568	1504.7674	-0.0106	0	(24)	20	1	K.GYDPILINYGQPR.V
60	753.3969	1504.7792	1504.7674	0.0118	0	51	0.038	1	K.GYDPILINYGQPR.V
61	753.4006	1504.7866	1504.7674	0.0192	0	(7)	1e+03	1	K.GYDPILINYGQPR.V
65	854.8875	1707.7604	1707.8104	-0.0500	0	79	5.2e-05	1	K.AFTETWGNIGEDLQK.H
73	859.4225	1716.8304	1716.8471	-0.0167	0	103	2e-07	1	K.LWFGEGNGLEITPER.K
74	859.4285	1716.8424	1716.8471	-0.0047	0	(65)	0.0015	1	K.LWFGEGNGLEITPER.K
75	859.4327	1716.8508	1716.8471	0.0037	0	(45)	0.13	1	K.LWFGEGNGLEITPER.K
76	859.4335	1716.8524	1716.8471	0.0053	0	(17)	88	1	K.LWFGEGNGLEITPER.K

☑	77	859.4337	1716.8528	1716.8471	0.0057	0	(44)	0.16	1	K.LWFGEGNGLEITPER.K
☑	78	859.4345	1716.8544	1716.8471	0.0073	0	(43)	0.21	1	K.LWFGEGNGLEITPER.K
☑	88	1023.5150	2045.0154	2045.0252	-0.0097	1	30	3.8	1	R.FINPPLKDVISCAGGENSK.C + Carbamidomethyl (C)
☑	95	1032.5411	3094.6015	3094.6087	-0.0073	0	228	3.7e-20	1	K.HLDANPDYQLYVTGHSLGAAVALLGATSIK.L
☑	96	774.6610	3094.6149	3094.6087	0.0062	0	(88)	3.4e-06	1	K.HLDANPDYQLYVTGHSLGAAVALLGATSIK.L
☑	100	1160.5275	3478.5607	3478.5789	-0.0182	0	136	3.8e-11	1	R.MTHWNDIFVGLPNWEGYTHSNGEVYINNR.F + Oxidation (M)

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2. Q875G8_9ASCO **Mass:** 41514 **Score:** 140 **Queries matched:** 6 **emPAI:** 0.19
Triacylglycerol lipase precursor (EC 3.1.1.3).- Candida deformans.
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
	2	402.2057	802.3968	802.3974	-0.0005	0	9	8.8e+02	2	K.IHDGFSK.A
	56	753.3294	1504.6442	1504.7674	-0.1232	0	(45)	0.14	1	K.GYDPILINYGQPR.V
	57	753.3857	1504.7568	1504.7674	-0.0106	0	(24)	20	1	K.GYDPILINYGQPR.V
	60	753.3969	1504.7792	1504.7674	0.0118	0	51	0.038	1	K.GYDPILINYGQPR.V
	61	753.4006	1504.7866	1504.7674	0.0192	0	(7)	1e+03	1	K.GYDPILINYGQPR.V
	65	854.8875	1707.7604	1707.8468	-0.0863	1	79	5.2e-05	1	K.AFTETWGNIGEDLKK.H

---

3. 1AN1E **Mass:** 23457 **Score:** 96 **Queries matched:** 3 **emPAI:** 0.35
tryptase inhibitor, chain E - medicinal leech (fragments)
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	14	421.7533	841.4920	841.5022	-0.0101	0	54	0.021	1	R.VATVSLPR.S
	23	530.2791	1058.5436	1058.5720	-0.0284	0	23	33	2	K.LSSPATLQSR.V
☑	92	767.0671	2298.1795	2298.1678	0.0117	0	20	36	1	K.IITHPNFNGNTLDNDIMLIK.L + Oxidation (M)

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4. Q6CCX8_YARLI **Mass:** 23525 **Score:** 58 **Queries matched:** 2 **emPAI:** 0.16
Similarity.- Yarrowia lipolytica (Candida lipolytica).
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	72	856.4991	1710.9836	1710.9953	-0.0117	0	11	2.6e+02	1	K.ILMSPLLNNIVDILK.N + Oxidation (M)
☑	93	1177.1688	2352.3230	2352.3264	-0.0033	0	46	0.057	1	K.AITDLSLNELGQAISNALLGVLK.Y

---

5. Q3F6Q1_9BURK **Mass:** 26223 **Score:** 50 **Queries matched:** 1 **emPAI:** 0.15
Peptidase M50 precursor.- Burkholderia ambifaria AMMD.
☐ Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
☑	17	428.7619	855.5092	855.5178	-0.0085	0	50	0.054	1	R.ITAVLSPR.I

Proteins matching the same set of peptides:
Q4LJV1_9BURK **Mass:** 26191 **Score:** 50 **Queries matched:** 1
Peptidase M50 precursor.- Burkholderia cenocepacia HI2424.
Q8XWF0_RALSO **Mass:** 26466 **Score:** 50 **Queries matched:** 1
Putative zn-dependent protease transmembrane protein.- Ralstonia solanacearum (Pseudomonas solanacearum).
Q39AR9_BURS3 **Mass:** 26303 **Score:** 50 **Queries matched:** 1
Peptidase M50.- Burkholderia sp. (strain 383) (Burkholderia cepacia (strain ATCC 17760 / NCIB 9086 / R18194)).

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6. Q6N4B7_RHOPA **Score:** 50 **Queries matched:** 1
Possible transcriptional regulator, MarR family.- Rhodopseudomonas palustris.
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4926	0.0166	1	50	0.055	2	K.IDRLSPR.W

---

7. O68872_MYXXA **Score:** 48 **Queries matched:** 1
Hypothetical protein.- Myxococcus xanthus.
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4814	0.0278	0	48	0.083	3	R.VEGVLSPR.R

---

8. A30769 **Score:** 44 **Queries matched:** 1
regulatory protein trpI - Pseudomonas aeruginosa
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4637	0.0456	0	44	0.2	4	R.MGPVLSPR.L

---

9. Q2DMU6_9DELT **Score:** 44 **Queries matched:** 1
Helix-turn-helix, Fis-type.- Geobacter uraniumreducens Rf4.
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4749	0.0344	1	44	0.21	5	-.MPRLSPR.I

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10. Q6CLF7_KLULA **Score:** 44 **Queries matched:** 1

Similar to sp|P53944 Saccharomyces cerevisiae YNL063w singleton.- Kluyveromyces lactis (Yeast) (Candida sphaerica).
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4749	0.0344	1	44	0.21	5	-.MPRISPR.I

---

11. Q310N7_DESDG **Mass:** 47077 **Score:** 44 **Queries matched:** 1
Atrazine chlorohydrolase (EC 3.8.1.8).- Desulfovibrio desulfuricans (strain G20).
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4814	0.0278	0	44	0.22	7	R.DLGVLSPR.T

---

12. Q61XM8_CAEBR **Score:** 38 **Queries matched:** 2
Hypothetical protein CBG03920.- Caenorhabditis briggsae.
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
35	682.3275	1362.6404	1362.6887	-0.0482	0	38	0.8	2	K.VEVLAMEAMINK.I + Oxidation (M)
36	455.2310	1362.6712	1362.6887	-0.0175	0	(24)	21	2	K.VEVLAMEAMINK.I + Oxidation (M)

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13. Q8XT74_RALSO **Score:** 37 **Queries matched:** 1
Putative alpha-amylase-related protein.- Ralstonia solanacearum (Pseudomonas solanacearum).
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
14	421.7533	841.4920	841.4658	0.0263	0	37	1.1	2	R.VATDAPLR.A

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14. PS17_PINST **Mass:** 1707 **Score:** 37 **Queries matched:** 1
Putative cytochrome c oxidase subunit II PS17 (Fragments).- Pinus strobus (Eastern white pine).
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
18	435.7726	869.5306	869.4971	0.0336	0	37	1.2	1	R.VVEALSPR.-

---

15. Q22IV2_TETTH **Mass:** 90782 **Score:** 37 **Queries matched:** 1
Cyclic nucleotide-binding domain containing protein.- Tetrahymena thermophila SB210.
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
18	435.7726	869.5306	869.4971	0.0336	0	37	1.2	1	K.TPISLSPR.I

---

16. Q1Z8J1_PHOPR **Score:** 36 **Queries matched:** 1
Hypothetical transcriptional regulator, TetR family protein.- Photobacterium profundum 3TCK.
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
18	435.7726	869.5306	869.5335	-0.0028	0	36	1.2	3	M.VTVVLSPR.S

---

17. Q2T3S3_BURTA **Score:** 35 **Queries matched:** 1
Lipopolysaccharide biosynthesis protein (O-antigen-related) (EC 4.2.1.-).- Burkholderia thailandensis (strain E264 / ATCC 700388
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4814	0.0279	0	35	1.5	8	K.IEAAISPR.T

---

18. Q3JH39_BURP1 **Score:** 35 **Queries matched:** 1
CDP-6deoxy-D-xylo-4-hexulose-3-dehydrase (EC 4.2.1.-).- Burkholderia pseudomallei (strain 1710b).
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4814	0.0279	0	35	1.5	8	K.LEAAISPR.T

---

19. Q63N74_BURPS **Score:** 35 **Queries matched:** 1
Lipopolysaccharide biosynthesis protein (O-antigen-related).- Burkholderia pseudomallei (Pseudomonas pseudomallei).
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
17	428.7619	855.5092	855.4814	0.0279	0	35	1.5	8	K.LEAALSPR.T

---

20. Q9ZTY0_EMIHU **Score:** 34 **Queries matched:** 1
Putative calcium binding protein.- Emiliania huxleyi.
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
14	421.7533	841.4920	841.5021	-0.0101	0	34	2	3	K.VASILSPR.K

Peptide matches not assigned to protein hits: (no details means no match)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
44	686.4081	1370.8016	1370.6877	0.1139	1	31	3.8	1	AWAAVPLRCDGR + Carbamidomethyl (C)
7	412.7531	823.4916	823.5028	-0.0112	1	26	11	1	VPRSLPR
23	530.2791	1058.5436	1058.6448	-0.1011	1	26	16	1	LSIRTITAGK
79	860.4271	1718.8396	1718.8376	0.0020	1	26	12	1	GGDAFWDRAEQNLLK
20	486.8097	971.6048	971.5400	0.0649	0	24	21	1	VAIITGAGDR
21	514.2737	1026.5328	1026.5822	-0.0493	0	23	27	1	IVGDNGIALR
64	850.9240	1699.8334	1699.8127	0.0207	0	23	24	1	LVSCVLEIDYEFDR
39	682.3767	1362.7388	1362.6779	0.0609	0	22	31	1	GAGGFEGILATNEK
83	872.4406	1742.8666	1742.9930	-0.1264	1	22	29	1	YLKPLEIVEGEGLKR
41	682.3790	1362.7434	1362.6779	0.0655	0	22	38	1	GAGGFEGILATNEK
19	441.2132	880.4118	880.5382	-0.1263	0	21	38	1	SILNPPIK
46	686.4155	1370.8164	1370.8068	0.0097	1	20	45	1	SLQQLLLRCLK + Carbamidomethyl (C)
68	854.9170	1707.8194	1707.8574	-0.0379	1	19	64	1	SALTEGGRQSLSISMR + Oxidation (M)
48	686.4161	1370.8176	1370.7518	0.0659	1	19	62	1	LSKAVADALVNDR
12	415.2541	828.4936	828.3072	0.1864	0	18	1e+02	1	NMNNYGT + Oxidation (M)
26	544.2945	1086.5744	1086.6033	-0.0289	0	18	98	1	EISGVIISNR
85	880.4384	1758.8622	1758.9112	-0.0489	1	17	86	1	SADSKGTPIGSAELASLR
47	686.4160	1370.8174	1370.8068	0.0107	1	16	1.2e+02	1	SLQQLLLRCLK + Carbamidomethyl (C)
50	686.4192	1370.8238	1370.7340	0.0899	1	15	1.5e+02	1	SIKIGLAGNPNCGK
67	854.9136	1707.8126	1707.9090	-0.0963	1	14	2e+02	1	VLSHLRTPDEAAMLR
45	686.4144	1370.8142	1370.7518	0.0625	1	13	2.3e+02	1	LSKAVADALVNDR
11	415.2108	828.4070	828.3687	0.0383	0	12	4e+02	1	LYDGMSK + Oxidation (M)
84	875.9213	1749.8280	1749.7848	0.0433	0	12	2.6e+02	1	EMCELNLTAPMQTGR + Carbamidomethyl (C)
10	414.7518	827.4890	827.3483	0.1407	0	12	3.6e+02	1	MNEYSGK
63	826.4596	1650.9046	1650.8981	0.0065	0	12	2.7e+02	1	DFLTNVAAFIINVSK
22	523.2911	1044.5676	1044.5815	-0.0139	0	12	3.9e+02	1	LSSPASGSVLK
8	414.2324	826.4502	826.4086	0.0417	1	12	3.8e+02	1	HHDKYK
55	737.3850	1472.7554	1472.8351	-0.0797	0	11	3.6e+02	1	TLLSATPGAAVFAVR
81	866.4409	1730.8672	1730.7669	0.1004	0	11	3.7e+02	1	EIGDDDVDAIADGAMPK
1	401.6903	801.3660	801.3150	0.0511	0	11	6.6e+02	1	CGGFTCSK
69	854.9188	1707.8230	1707.8216	0.0014	0	10	4.6e+02	1	WLEGEEIGSVEPHAR
66	854.9130	1707.8114	1707.8766	-0.0652	0	10	4.9e+02	1	LADAMLDAHFHIGGLK
2	402.2057	802.3968	802.4185	-0.0216	0	10	7.7e+02	1	ISASPNSK
3	403.2544	804.4942	804.4494	0.0449	1	9	8.3e+02	1	KFPAASGK
30	583.2866	1164.5586	1164.6866	-0.1280	1	9	6.6e+02	1	EAHEVILKVK
80	865.4376	1728.8606	1728.7308	0.1298	0	9	5.6e+02	1	ASQGDEGCVHCPINSR + Carbamidomethyl (C)
16	425.7706	849.5266	849.4192	0.1075	1	9	9.4e+02	1	QKSSADSK
42	682.3799	1362.7452	1362.6263	0.1190	1	9	7.3e+02	1	EKSIGDTEENNK
86	880.9702	1759.9258	1759.9118	0.0140	0	9	6.2e+02	1	HLEHLGGLGTAGAGAFPR
82	871.9070	1741.7994	1741.8159	-0.0164	1	8	6.1e+02	1	LDPSLYQSDLPDKDH
34	674.8125	1347.6104	1347.6857	-0.0752	0	8	8.7e+02	1	QIPDMINIFNK + Oxidation (M)
43	682.3835	1362.7524	1362.7255	0.0269	1	7	1e+03	1	QKVADFIAASSAR
29	563.7883	1125.5620	1125.6295	-0.0674	1	7	1e+03	1	VVHEFRNII
13	418.2037	834.3928	834.5076	-0.1147	1	6	1.5e+03	1	KVIPNHK
51	694.7818	1387.5490	1387.5359	0.0131	0	6	1.1e+03	1	HCSSCGFPMFEK + Oxidation (M)
33	634.7680	1267.5214	1267.6561	-0.1346	1	5	1.3e+03	1	FVFRGTGIDEK
49	686.4166	1370.8186	1370.7704	0.0483	1	5	1.4e+03	1	ATRAMEVPVLLR + Oxidation (M)
58	753.3905	1504.7664	1504.6942	0.0723	0	5	1.7e+03	1	FGMLELSFSESMK
38	455.2523	1362.7351	1362.7732	-0.0381	1	4	1.9e+03	1	NVLGHLRDKPSK
40	455.2544	1362.7414	1362.6245	0.1169	1	4	1.9e+03	1	AGAGQGCMATGQRR
71	854.9240	1707.8334	1707.8034	0.0300	0	4	1.8e+03	1	SLAFMLTMMNGFAVK + 3 Oxidation (M)
91	762.0605	2283.1597	2283.1277	0.0320	1	4	1.5e+03	1	AQADTIANPNLTNCENRIPTK
32	625.7883	1249.5620	1249.5972	-0.0352	0	3	3e+03	1	TLISCAGDSEVR
15	423.2128	844.4110	844.4225	-0.0115	1	2	4.6e+03	1	KHSGTGMK
24	536.2987	1070.5828	1070.5833	-0.0004	1	2	3.8e+03	1	AELVGVRDGR
53	707.4135	1412.8124	1412.7129	0.0996	1	2	3.3e+03	1	KCCSSLHRPLR + 2 Carbamidomethyl (C)
104	1112.2969	4445.1585	4445.0995	0.0590	1	2	7e+02	1	QMMQNLSFLGMFLAALSMSLGHCVGMCGGIVSAFSQIRFSK + 3 Oxidation
5	408.7035	815.3924	815.4250	-0.0325	0	1	6.3e+03	1	LGGGGSVGGR
37	455.2471	1362.7195	1362.7152	0.0043	1	1	4.3e+03	1	KAVGCCGGFPLALK
62	782.4356	1562.8566	1562.7188	0.1379	0	0	4.8e+03	1	QYDNFPIEICGHK
6	409.1980	816.3814	816.3978	-0.0163	0	0	6.5e+03	1	SGVVSNSAP
87	940.9897	1879.9648	1879.9457	0.0191	1	0	3.8e+03	1	AMDVAMSLMVPLKVDTK + 2 Oxidation (M)
4	405.2268	808.4390	808.3385	0.1005	0	0	5.6e+03	1	GSGAMDAGK + Oxidation (M)
25	*539.2858*	*1076.5570*							
28	*555.2753*	*1108.5360*							
54	*710.8696*	*1419.7246*							
59	*753.3936*	*1504.7726*							
70	*854.9208*	*1707.8270*							
89	*1045.5221*	*2089.0296*							
90	*761.7446*	*2282.2120*							
94	*1411.7423*	*2821.4700*							
97	*1037.1987*	*3108.5743*							
98	*1037.2053*	*3108.5941*							
99	*1048.5513*	*3142.6321*							
101	*927.2629*	*3705.0225*							
102	*965.4064*	*3857.5965*							
103	*1043.0027*	*4167.9817*							

Search Parameters

```
Type of search         : MS/MS Ion Search
Enzyme                 : Trypsin
Variable modifications : Carbamidomethyl (C),Oxidation (M)
Mass values            : Monoisotopic
Protein Mass           : Unrestricted
Peptide Mass Tolerance : ± 0.2 Da
Fragment Mass Tolerance: ± 0.2 Da
Max Missed Cleavages   : 1
Instrument type        : ESI-QUAD-TOF
Number of queries      : 104
```

Mascot: http://www.matrixscience.com/