Isolation, Purification and Characterization of Acidic Lipase From Aspergillus niger NCIM 1207

A THESIS

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BY
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DEDICATED TO

MY LATE MOTHER

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DECLARATION

Certified that the work incorporated in the thesis entitled: "**Isolation, purification and characterization of acidic lipase from Aspergillus niger NCIM 1207**" submitted by Ms. Nutan D. Mahadik for the Ph. D degree was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in this thesis.

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Research Co-guide

Dr. D.V. Gokhale
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Declaration by the Candidate

I declare that the thesis entitled "Isolation, purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207" submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me during the period from May 2003 to August 2007 under the guidance of Dr. D.V. Gokhale and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institution of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Signature of the Candidate Date

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Nutan D. Mahadik

ABSTRACT

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. Microorganisms produce wide spectrum of intra- or extracellular lipid degrading enzymes to break down the insoluble lipid in to soluble components to facilitate absorption.

Lipases catalyze hydrolysis of triacylglycerols to yield diacylglycerols, monoacylglycerols and free fatty acids under natural conditions. They also catalyze synthetic reactions such as esterification, inter-esterification, alcoholysis and acidolysis Because of their versatility; they find diverse industrial applications in food, chemical and pharmaceutical industries. Current applications of lipases in dairy industry include cheese flavor enhancement, acceleration of cheese ripening, the manufacture of cheese like products and the lipolysis of fats and cream. They have also been found useful for resolution of racemic alcohols in the preparation of prostaglandins, steroids and carboxylic nucleoside analogues. Research has been carried out on plant lipases, animal lipases and microbial lipases particularly bacterial and fungal. Even then, the first lipase whose X-ray structure was determined is from the fungus, *Geotrichum candidum*. It is also well established that microbial lipases are preferred for commercial applications due to their multifold properties, easy extraction procedures and unlimited supply. Additionally, development of new lipases with desired properties through continuous screening of microorganisms and improving lipase properties through protein engineering has attracted much research interest in microbial lipases. Now most of the commercially available lipases are from fungal sources.

We have undertaken a program on screening of microorganisms available at National Collection of Industrial Microorganisms (NCIM), NCL, Pune. Screening was taken up to look for culture producing lipase. The screening was based on the formation of butyl ester from butter oil in presence of butanol. Among the fungi screened, we found that *A. niger* NCIM 1207 produced butyl esters under extremely acidic condition. Such lipase active at acidic pH (2.0) has not been reported so far in the literature and hence further studies were carried on this unique lipase.

Chapter 1. General Introduction

This chapter deals with the literature survey on plant, mammalian and microbial 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.

Chapter 2. Production of acidic lipase by *Aspergillus niger* NCIM 1207 in solid state fermentation

This chapter deals with the production of the lipase in submerged and solid state fermentation using *Aspergillus niger* NCIM 1207. Strains of *Rhizopus, Mucor, Geotrichum, Penicillium* and *Aspergillus* were screened for their ability to form butyl esters from butter oil in presence of butanol. *Rhizopus* strains showed major intracellular activity while *A. niger* NCIM 1207 produced mainly extracellular activity. We selected *A. niger* strain for lipase production using both submerged fermentation (SmF) and solid state fermentation (SSF). Denovo biosynthesis of lipase occurred only in the presence of lipid substrate and was completely repressed by glucose. Addition of Triton X-100 (0.5%, w/v) in fermentation medium resulted in enhanced lipase activity (18 IU/ml). The pH and temperature optima for lipase were 2.5 and 50^{o}C, respectively. The crude enzyme is also stable over a wide pH range (pH 2.5 to 9.0) for 24 h at room temperature. The enzyme retained 63% of its original activity on incubation at 70^{o}C for 5 h. Lipase production was studied in SSF and the highest yields of enzyme were obtained using wheat bran as solid substrate in combination with olive oil as lipid substrate. Maximum lipase activity (630 IU/g dry solid substrate) was recovered when fermented wheat bran was extracted with NaCl (1%) supplemented with Triton X-100 (0.5%). The crude enzyme was stable in organic solvents tested except 1,4-dioxane and propane-1-ol.

We obtained enhanced production of lipase in SSF. However, the broth obtained was colored and also mixed with unwanted ingredients coming from solid substrate during extraction. Hence we intended to look for improved strains capable of producing higher amounts of lipase in SmF. We screened number of mutants of *A. niger* NCIM 1207 available in NCIM and optimized the lipase production using the promising mutant.

Chapter 3. Production of acidic lipase by mutants of *Aspergillus niger* NCIM 1207 in submerged fermentation

Mutants of *Aspergillus niger* NCIM 1207, isolated by subjecting conidia to UV-irradiation, were tested for the production of lipase (glycerol ester hydrolase EC 3.1.1.3). Mutants UV-10 and ANCR-1 showed seven fold and five fold enhanced productivity of enzyme, respectively, over the parent strain under submerged fermentation when grown in SOB medium containing 1% olive oil. Maximum lipase activity (41.0 IU/ml) was obtained in the culture broth when UV-10 was grown in medium supplemented with 0.5% Triton X-100. A higher concentration of oil in the medium did not help lipase production in the case of mutant UV-10. No increase in enzyme levels was observed when mutant UV-10 was grown in medium supplemented with glucose. However, the addition of glucose in the medium resulted in increased levels of lipase production by wild strain, *Aspergillus niger* NCIM 1207.

The lipase from *A. niger* NCIM 1207 was active at extremely acidic pH. Such lipases of microbial origin active at extremely acidic pH are not reported so far. Hence we carried out purification and characterization of this unique enzyme.

Chapter 4. Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207

An extracellular acidic lipase from *Aspergillus niger* NCIM 1207 has been purified to homogeneity with 149 fold purification and 54% final recovery. The molecular weight of the lipase was estimated to be 32.2 kDa using SDS-PAGE and MALDI-TOF suggesting that lipase is likely to function as a monomer. The isoelectric point (pI) of lipase was 8.5. The enzyme is rich in acidic and hydrophobic amino acids which is evident from amino acid analysis. The pH and temperature optima for purified enzyme were 2.5 and 50^{o}C respectively. The lipase was stable over a pH range between 8.0-11.0 and its half life of inactivation ($t^1/_2$) at 50^{o}C was 1h**.** The lipase from *A. niger* NCIM 1207 showed high stability in presence of majority organic solvents tested except 2-propanol, propan-1-ol, butan-1-ol, and tertiary-butanol. Ag^+ strongly inhibited the enzyme activity. Action on *p*-nitrophenyl esters of varying chain lengths suggested the high preference of the enzyme towards medium acyl chain length esters. The K_m and

V_{max} for *p*-nitrophenyl palmitate was 0.33 mM and 746 μmoles $min^{-1}mg^{-1}$ respectively. The K_m and V_{max} for *p*- nitrophenyl caprylate was 0.14 mM and 1066 μmoles $min^{-1}mg^{-1}$ respectively. The results analysis of hydrolysis product of triolein indicated that the enzyme cleaved only 3-position ester bond. Chemical modification studies revealed that His, Ser, Asp/Glu and Trp are involved in catalysis. The substrate protection studies suggested that Trp and Ser may also have a role in substrate binding.

We found that the lipase produced butyl esters from butter oil by trans-esterification. In addition, it is stable in majority of water miscible and water immiscible organic solvents which led us to look for its application in the formation of flavor esters.

Chapter 5. Enzymatic synthesis of isoamyl acetate by mycelium bound lipase of *Aspergillus niger* NCIM 1207

Commercial lipase preparations and mycelium bound lipase from *A. niger* NCIM 1207 were used for esterification of acetic acid with isoamyl alcohol to obtain isoamyl acetate. The esterification reaction was carried out at 30°C in *n*-hexane with shaking at 120 rpm. Initial reaction rates, conversion efficiency and isoamyl acetate concentration obtained using Novozyme 435 were the highest. The optimization studies were carried out using *Aspergillus niger* NCIM 1207 mycelium bound lipase. Isoamyl acetate production was maximal at an alcohol-acid ratio of 1.6. Acetic acid at higher concentrations than required for the critical alcohol-acid ratio lower than 1.3 and higher than 1.6 resulted in decreased yields of isoamyl acetate probably owing to lowering of micro-aqueous environmental pH around the enzyme leading to inhibition of enzyme activity. Mycelium bound *Aspergillus niger* lipase produced 80g/l of isoamyl acetate within 96 h even though extremely less amount of enzyme activity was used for esterification. The presence of sodium sulphate during esterification reaction at higher substrate concentration resulted in increased conversion efficiency when we used mycelium bound enzyme preparations of A*spergillus niger* NCIM 1207. This could be due to removal of excess water released during esterification reaction by sodium sulphate. High ester concentration (286.5g/l) and conversion (73.5%) were obtained within 24 h using Novozyme 435 under these conditions.

LIST OF ABBREVIATIONS

AMM	***Aspergillus* minimal medium**
DEPC	**Diethylpyrocarbonate**
DMSO	**Dimethyl sulfoxide**
DSS	**Dry solid substrate**
DORB	**Defatted rice bran**
DTNB	**2,2-Dithiobisnitrobenzoic acid**
EDAC	**1-ethyl-3-(3-dimethylaminopropyl) carbodiimide**
EDTA	**Ethylene diamine tetra acetic acid**
GRAS	**Generally Regarded As Safe**
HEPES	**2-(2-Hydroxyethyl) Piperazine-N-(4-Butanesulfonic acid).**
MES	**2-(N-Morpholinolethanesulfonic acid)**
NAI	**N-acetylimidazole**
NBS	**N-Bromosuccinimide**
NCIM	**National Collection of Industrial Microorganisms**
NEM	**N-ethylmaleimide**
PCMB	***p*-Chloromercurybenzoate**
PEG	**Polyethylene glycol**
PMSF	**Phenyl methyl sulfonyl fluoride**
***p*NPC**	***p*-nitrophenyl caprylate**
***p*NPP**	***p*-nitrophenyl palmitate**
SDS	**Sodium dodecyl sulphate**
SmF	**Submerged fermentation**
SOB	**Synthetic oil based medium**
SSF	**Solid state fermentation**
TFA	**Trifluoroacetic acid**
TNBS	**2,4,6-trinitrobenzenesulfonic acid**

CHAPTER - 1

General Introduction

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are most important kind of industrial enzymes and 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 hydrolysis of triacylglycerols to yield diacylglycerols, monoacylglycerols and free fatty acids under natural conditions. They also catalyze synthetic reactions such as esterifications, interesterifications, alcoholysis and acidolysis (Gandhi 1997). Because of their versatility, they find diverse industrial applications in food, chemical and pharmaceutical industries. Current applications of lipases in dairy industry include cheese flavor enhancement, acceleration of cheese ripening, the manufacture of cheese like products and the lipolysis of fats and cream (Vulfson 1994). They have also been found useful for resolution of racemic alcohols in the preparation of prostaglandins, steroids and carboxylic nucleoside analogues (Wooley & Petersen 1994). Research has been carried out on plant lipases, animal lipases and microbial lipases particularly bacterial and fungal. Mammalian lipases are most extensively studied during last two decades because of their medical importance. Even then, the first lipase whose X-ray structure was determined is from the fungus, *Geotrichum candidum*. It is also well established that microbial lipases are preferred for commecial applications due to their multifold properties, easy extraction procedures and unlimited supply. Additionally, development of new lipases with desired properties through continuous screening of microorganisms and improving lipase properties through protein engineering has

attracted much research interest in microbial lipases. Now most of the commercially available lipases are from fungal sources (Hou & Johnson 1992).

Definition of lipase

In 1856, Claud Bernard was the first to discover lipase in pancreatic juice as an enzyme that hydrolyzed insoluble oil droplets to soluble products. Though much information is available on lipases, it is very difficult to develop an appropriate definition for lipase. Two criteria have been used to classify lipolytic enzymes as true lipase 1) It should be activated by oil-water interphase and its activity increases as soon as water insoluble substrate forms an emulsion in the assay system.This phenomenon was termed "interfacial activation" (Sarda & Desnuelle 1958). 2) It should possess a "lid" acting as a surface loop in the protein structure that covers its active site. This "lid" structure moves away as soon as enzyme contacts the oil-water interphase to allow the substrate fit in to active site in the protein (Brzozowski et al. 1991; van Tilbeurgh et al. 1993). This criterium proved to be unsuitable for classification because some enzymes have a "lid" but do not exhibit interfacial activation (Verger 1997).

Therefore, lipases can be simply defined as carboxylesterases catalyzing hydrolysis and / or synthesis of long chain acylglycerols (Ferroto et al. 1997). Lipases resemble esterases in many aspects and hence it is difficult to differentiate lipases and esterases. However, lipases differ from esterases mainly on the basis of substrate specificity and interfacial activation (Long 1971). In contrast to esterases, lipases are activated only when adsorbed to oil-water interphase (Martinelle et al. 1995) and do not hydrolyze dissolved substrate in the bulk fluid. Lipases hydrolyze glycerolesters with acyl chain length ≥ 10 carbon atoms and esterases hydrolyze glycerol esters with acyl chain length ≤ 10 carbon atoms. Many lipases are active in organic solvents where they carry out number of useful synthetic reactions including esterification (Krishna et al. 2001; Rao & Diwakar 2001), transesterification, regioselective acylation of glycols and menthols, and synthesis of peptides (Ducret et al. 1998; Zhang et al. 2001) and other chemicals (Liese et al. 2000; Azim et al. 2001).

Different reactions catalyzed by lipases

Lipases catalyze the hydrolysis of esters, especially long chain triacylglycerols, to di- and mono-acylglycerols, glycerol and free fatty acids. They also catalyze the reverse reactions like esterification, transesterification (acidolysis, interesterification, alcoholysis), aminolysis, oximolysis and thiotransesterification in unhydrous organic solvents (Klibanov 1989; Gupta 1992; Gotor 2002).These reverse reactions are also carried out by lipases in biphasic systems (Brink et al. 1988) and in micellar solutions (Nagao & Keto 1990). Some of the reactions catalized by lipases are given in Figure 1. Lipases have gained immense importance in various industries because of such diverse reactions carried out by them in presence of water and organic solvents. The following basic reactions can be considered.

Hydrolysis

Fatty acids can be produced by ambient pressure saponification or chemically catalyzed hydrolysis. However, the use of lipases for hydrolysis of fats in the presence of excess water is more appealing since the reaction proceeds under mild conditions of pressure and temperature with the generation of reduced waste (Malcata et al. 1990). Currently, this technology is employed in the production of fatty acids, diglycerides, mono-glycerides. The lipases have also been used for the production of flavoring agents used in dairy products and detergents for laundry and household purposes.

Esterification

Synthesis of esters can be done chemically using acid or base catalysts. Recently, silica chloride proved to be efficient catalyst for esterification of carboxylic acids with alcohols as well as for transesterification of esters by both alcoholysis and acidolysis. (Srinivas et al. 2003). The use of lipases offers the advantages such as mild reaction conditions, reduced side reactions, and specificity. Examples of high-value chemicals obtained by lipase-catalyzed ester synthesis are numerous including the production of oleic acid esters of primary and secondary aliphatic and terpenic alcohols (Okamura et al. 1979), and the production of geranyl and menthyl esters from butyric acid and geranol, or lauric acid and menthol, respectively (Marlot et al. 1985). Enzyme mediated esterification

of acetic and butyric acids with four alcohols: n-butyl, isopentyl, 2 phenylethyl and geraniol has been reported (Larios et al. 2004).

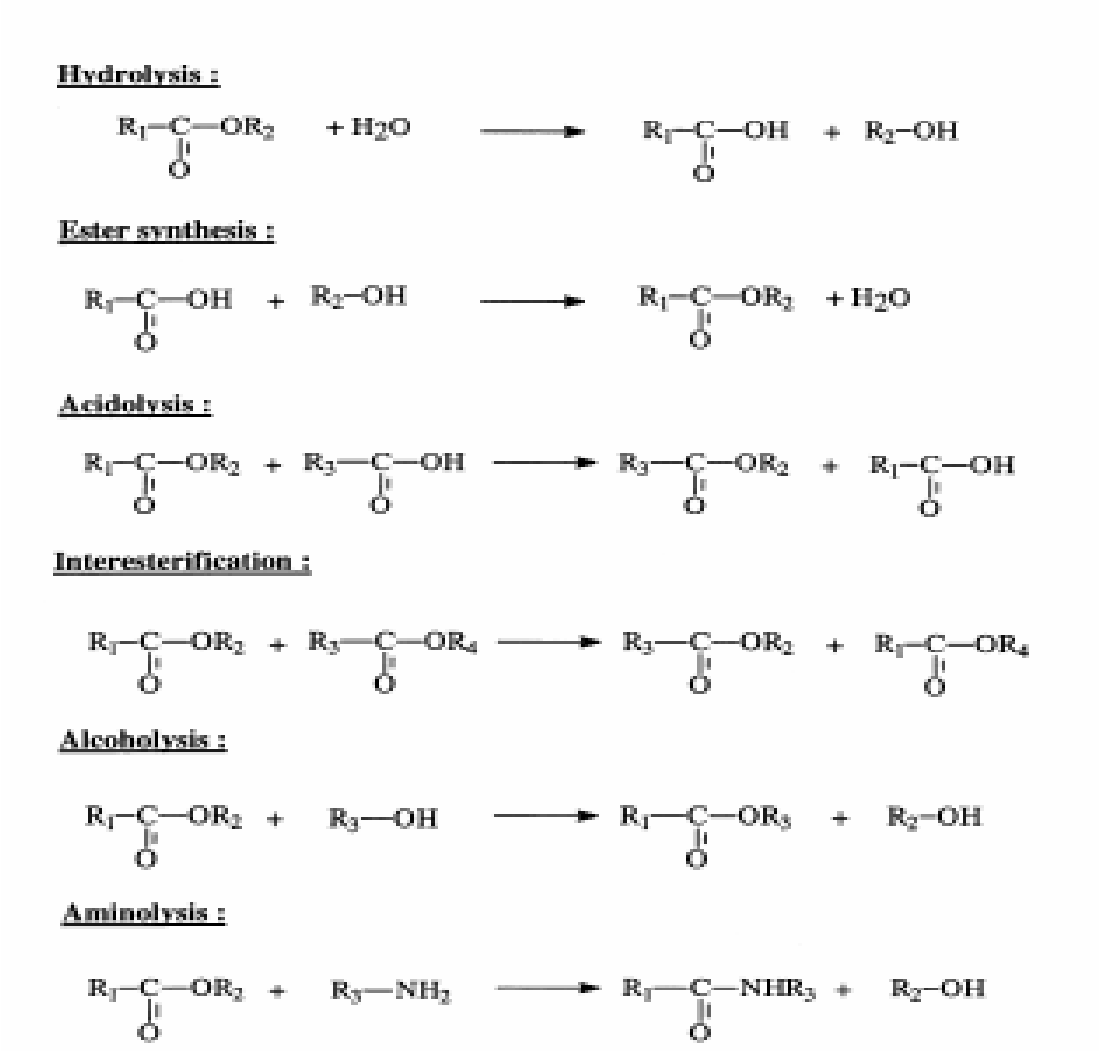

Figure 1. Different reactions catalyzed by lipase in aqueous and non-aqueous solutions.

Transesterification

The transesterification is the process of exchange of acyl radicals between an ester and an acid (acidolysis), an ester and another ester (interesterification), or an ester and an alcohol (alcoholysis). Generally, transesterification involves the acid- or base-catalyzed reaction of an ester and an alcohol that leads to double displacement of substituent groups. Transesterification/acylation reactions of secondary alcohols are efficiently

catalyzed by *N*-Heterocyclic carbenes (NHC) at room temperature. (Singh et al. 2004). Iodine also is known to catalyze a facile transesterification of β-ketoesters. (Chavan et al. 2003). The conversion of vegetable oils in to biodiesel is the best example of transesterification process using sodium or potassium hydroxide as catalyst. Alkali metals or alkali alkylates may be used for transesterification at lower temperatures. The application of lipases for the modification of fats and oils by transesterification offers great advantages of mild conditions, reduced sidereactions, and specificity. The cocoa butter analogues have been produced from cheaper feedstocks by lipase-catalyzed transesterification using palm oil mid fraction and steric acid (Macrae 1985) or tristearoylglycerol (Bloomer et al. 1990).

Catalysis on ''unnatural'' substrates

In addition to the synthesis and hydrolysis of carboxylic acid esters, lipases can utilize compounds other than water and alcohols as nucleophiles and thus catalyze different reactions such as aminolysis, thiotransesrification and oximolysis in organic solvents with selectivity (Klibanov 1989). The selectivity of lipase in the aminolysis of esters in anhydrous media has been successfully used for peptide and fatty amide synthesis (Montet et al. 1989). These results hold promise for using lipase technology in the synthesis of optically active peptides, polymers, surfactants and new detergents at low cost. Lipases show high catalytic efficiency in the resolution of chiral esters and amines via esterification and transestrification (Paiva et al. 2002), aminolysis, and ammonolysis reactions (Gotor 2002). Recently, *Pseudomonas cepacia* lipase was used to resolve 3-hydroxy-4-trityloxybutanenitrile giving (S) alcohol and (R) acetate with high enantioselectivity and yield (Kamal et al. 2006).

Structure of lipases and mechanism of lipolysis

The three dimentional structures of *Rhizomucor miehei* and human pancreatic lipases were determined in 1990 (Brady et al. 1990; Winkler et al. 1990, 1994). Since then, structures of many lipases especially from microbial origin have been solved (Cygler & 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). The lipase shares less than 20% amino acid sequence identity with other lipases. The structure contains a zinc binding site which is unique among all lipases with known structure which may be responsible for enhancing thermostability. The X-ray structure of *Pseudomonas aeruginosa* lipase is given in Figure 2. Very recently, the three dimentional structure of cold-adapted psychrotroph *Pseudomonas* sp. has been constructed by homology modeling based on crystal structure of acetyl esterase from *Rhodococcus* sp. and refined by molecular dynamics methods (Acharya 2007).

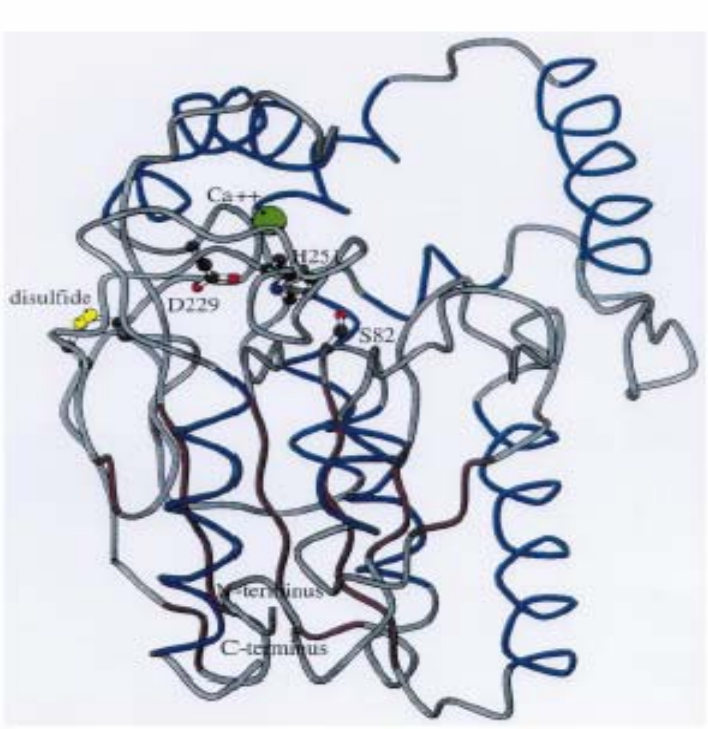

Figure 2 X-ray structure of Pseudomonas aeruginosa lipase. Indicated are the N and the C-terminus, and the catalytic triad formed by the nucleophile (S82), the acid (D229) and histidine residue (H251). In addition, the bound Ca^{++} ion and the disulfide bond formed between residues C183 and C235 are shown.

Although all lipases investigated so far are structrurally 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 secreated 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 unaccessible to solvent (Winkler & Gubernator 1994).The

binding of colipase to C-terminal domain through hydrophibic interaction keeps the lid in open conformation and allows the lipolysis to proceed at a high rate (Lowe 1997). Colipase 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).

Hydrolytic mechanism

As mentioned above, 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 & Gubernator 1994). The catalytic reaction involves many elementary steps: 1) The formation of non-covalent 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 that belong to so called "Oxanion hole"(Cygler et al 1995). 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 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 & Derewenda 1991; Derewenda & Sharp 1993). The catalytic mechanism of lipases based on catalytic triad is given in Figure 3.

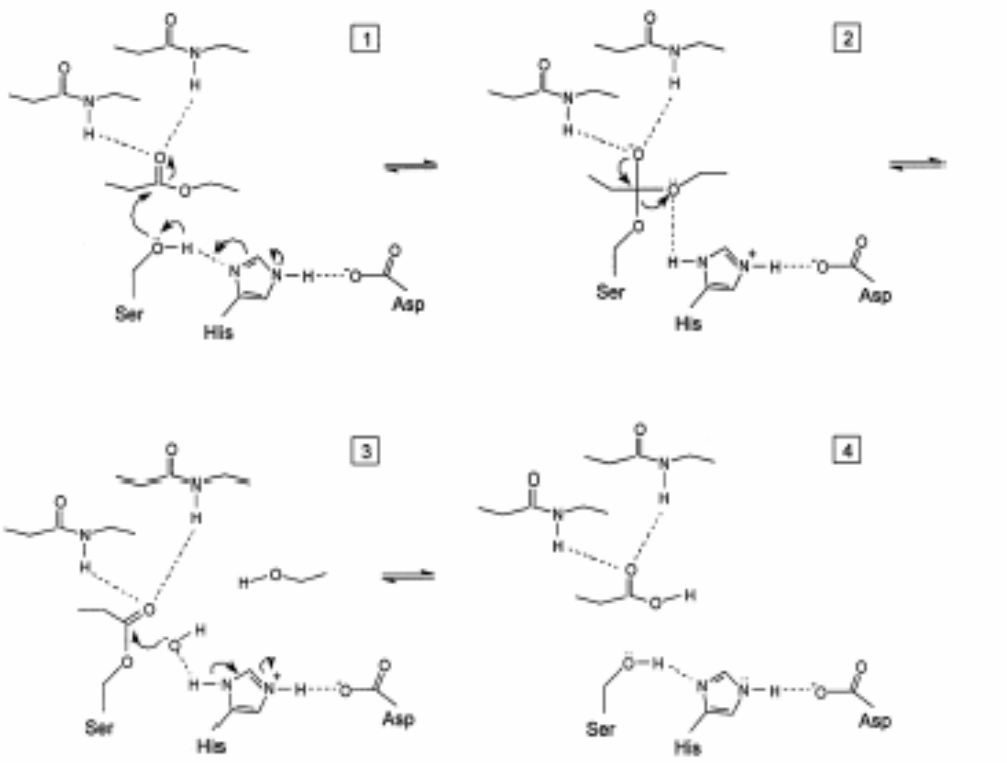

Figure 3. The catalytic mechanism of lipases based on a catalytic triad composed of serine (a nucleophile), histidine and aspartic or glutamic acid (connected through hydrogen bond). The tetrahedral intermediate is stabilized by an "oxyanion hole".

Interfacial activation

Lipase activity is greatly increased at the lipid-water interface of miceller or emulsified substrate (Sarda & Desnuelle 1958; Schmid & Verger 1998), 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 first absorbs to interface. This absorption leads to 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). 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) or a loop region in *Candida rugosa* lipase (Grochulski et al. 1994). 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). This region is also important for enantioselectivity (Liebeton et al. 2000) and activity in organic solvent (Mingarro et al. 1995; Fishman & Cogan 2003). In summary, lid like structural element constitutes the most important structural elements of lipases (Eggert et al. 2004).

Although all lipases show interfacial activation phenomenon, there are some exceptions. For example, Lipase from *Bacillus subtilis* (Lesuisse et al. 1993; van Pouderoyen 2001), guinea pig pancreatic lipase (Hjort et al. 1993) and *Staphylococus simulans* lipase (Sayari et al. 2001) do not show interfacial activation. Very recently, lipase from *Xanthomonas compestris* was reported which 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.

Sources of lipases

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. They are classified as mammalian, plant or microbial lipases based on their origin.

Mammalian lipases

Mammalian lipases are classified into four groups i.e the pancreatic, gastric, hepatic and lingual lipases according to their locations (Svendsen 1994). Among the mammalian lipases, human pancreatic lipase and animal pancreatic lipase are the most thoroughly studied lipases. Pancreatic lipases are secreated in to duodenum and act on dietary triacylglycerols hydrolyzing them in to free fatty acids and glycerol. Although they are active at oil-water interface, their activity is inhibited by physiological

concentrations of bile salts. This inhibition can be overcome by the addition of colipase, a small pancreatic protein which binds to both lipase and lipid micelles (Maylie et al. 1971). Human pancreatic lipase has a molecular weight of about 50 kDa and different mammalian lipases isolated from pig, rat, dog and mouse possess clear homology to human lipase (Winkler & Gubernator 1994).

All Gastric lipases are active and stable under acidic conditions. These lipases from different mammalian species have been found to be different in cellular distributions. Human gastric lipases secreted by cells located in the fundic region of the stomach are presumed to initiate the digestion of triacylglycerols (Moreau et al. 1989; Carriere et al. 1993). Rabbit gastric lipases are produced by the same cells which produce pepsinogen (Moreau et al. 1990), while dog gastric lipases are secreted by mucous pit cells (Carriere et al. 1993; Rousell et al. 2002). Unlike pancreatic lipases, gastric lipases do not require colipases as a cofactor for their activity (Roussel et al. 1999). Human gastric lipase, a 50 kDa glycoprotein, is directly secreted as active enzyme and this is major lipolytic enzyme involved in the digestion of dietary triglycerides (Miled et al. 2000). The role of lid and cap domains has been elucidated by site directed mutagenesis procedure. The lid and cap domains play an important role in catalytic reaction mechanism. It was also pointed out the cap and lid residues are involved in the binding with the lipidic substrate (Miled et al. 2003).

Lingual lipases secreted by serous glands of toung are accumulated in the stomach (Fink et al. 1984). They catalyze the hydrolysis of triacylglycerols in to fatty acids and di- or monoacylglycerols under acidic environment in the stomach (Hamosh et al. 1977, Fink et al. 1984). The activity of lingual lipases in low pH environment could be due to the prevention of inactivation by its substrates (Fink et al. 1984). Mouse and rats have high lingual lipase activities and only traces of gastric lipase activities. Guinea pig and rabbit have no lingual lipase activity but have some gastric lipase activities. By contrast, humans have predominantly gastric lipase but traces of lingual lipase activity (DeNigris et al. 1988). Like pancreatic lipases, lingual lipases are inhibited by bile salts but the inhibition cannot be prevented by colipase. However, this inhibition can be partially removed by lipids and proteins (Liao et al. 1984).

Hepatic lipase, a lipolytic enzyme and secreted glycoprotein, is made by hepatocytes and bound to heparin sulphate proteoglycans at the surface of liver sinusoidal capillaries (Perret et al. 2002). It has a number of structural and functional homologies with lipoprotein lipase (LPL). Unlike LPL, hepatic lipase has significant phospholipase as well as triacylglycerol lipase activity. It is more active than LPL in hydrolyzing the triglycerides and phospholipids of LDL and HDL (Peinado-Onsurbe et al. 1992). Hepatic lipases are divided in to an N-terminal domain and C-terminal domain. Both the domains are connected by a short domain-spanning region. The N-terminal domain harbours the active site which is covered by putative hinged loop and the C-terminal domain contains lipoprotein-binding site (Connelly 1999). Hepatic lipase activity is depressed by estrogens (Staels et al. 1990), corticotrophin (Schoonderwoerd et al. 1983), leptin (Liang & Tall 2001) and cholesterols (Benhizia et al. 1994). Androgens (Sorva et al. 1988), Triiodothyronine (Kihara et al. 1993) and heparin (Bush et al. 1989) enhance the activity of hepatic lipase. This lipase plays a key role in lipoprotein metabolism in two ways i.e a lipolytic mechanism and non-lipolytic mechanism (Brunzell and Deeb 2000). Lipolytic mechanisms involves the hydrolysis of triglycerols and phospholipids of intermediate density lipoproteins (IDL), large buoyany low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) producing more dense and smaller lipoprotein particles (Zambon et al. 2000). In non-lipolytic mechanism, hepatic lipase serves as a ligand to promote cellular uptake of apolipoproteins and HDL (Huff et al. 1997; Santamarina-Fojo et al. 1998). Both mechanisms are important for HDL metabolism (Dugi et al. 2000).

Plant Lipases

Plants lipases appear to be very attractive due to their low cost and ease of purification. They are known to be present in oil seeds and cereals. Lipase activity is generally present in post germinated seeds and is not found in non-germinated seeds. Oil seed plant lipases are present in low levels, which is the main drawback for their extensive use in plant scale operations (Villeneuve 2003). Many different oil plants contain lipases with various lipolytic activities especially from oat, rapeseed or castor bean. The castor bean lipase is present in the active form in dormant seeds and active under acidic condition (optimum pH 4.1). This lipase does not show regioselectivity but

hydrolyzes ricinolic acid which is the major constituent of castor bean oil (Lin et al. 1986). Other lipases were also found in various oil seeds such as peanut, linseed or soya.

Among the cereals, lipase from oat has been most extensively studied with respect to its positional and fatty acid selectivity (Piazza et al. 1992). Lipase from Oat showed fatty acid specificities similar to that of lipase from *Geotrichum candidum*. The rice bran and wheat germ are also good sources of lipases (Kumar & Prakash 2003). Wheat germ lipase is commercially available and active on short chain triacylglycerols such as triacetin or tributyrin. Among the other plant sources, lipase from cinnamon, white papper and potato tuber have also been reported (Haslbeck et al. 1985; Werman et al. 1995; Pinsirodom and parkin 1999).

Recently, the lipase activity of *Carica papaya* latex and its applications in oil and fat modifications has been reviewed (Villeneuve 2003). This lipase has a very strong activity towards short chain triglycerols. Very recently, acidic lipase from linseeds has been isolated and purified to homogeneity with the apparent molecular weight of 190 kDa and a subunit molecular weight of 42 kDa (Sammour 2005). The purified enzyme hydrolyzed wide range of triacylglycerols and active at pH 4.7.

Microbial lipases

Lipases widely occur in bacteria (Jaeger et al. 1994, 1999; Gao et al. 2000), yeasts (Rap & Backhaus 1992; Dalmau et al. 2000; Vakhlu & Kaur 2006) and fungi (Jaeger & Reetz 1998; Ferreira et al. 1999). Among the microbial lipases, lipases of bacterial and fungal origin have been most studied and also commercially exploited. Yeast lipases have received less attention in spite of the fact that *Candida rugosa* is the most frequently used organism for lipase synthesis (Benjamin & Pandey 1998; Dominguez de Maria et al. 2006).

Bacterial Lipases

The common bacterial lipase producing strains are presented in Table 1. Extracellular bacterial lipases are of immense commercial importance because of the ease of their production. Although there are number of bacterial strains which produce lipases, only few of them have been commercially exploited as wild or recombinant strains (Jaeger et al. 1994; Palekar et al. 2000; Gupta et al. 2004). Several bacterial lipases have

been launched in the market in past few years. However, lipases from the genus *Pseudomonas* are widely used for biotechnological applications (Pandey et al. 1999; Beisson et al. 2000). Most of the bacterial lipases have neutral (Dharmsthiti et al. 1998; Dharmsthiti & Luchai 1999) or alkaline pH optima (Kanwar and Goswami 2002; Sunna et al. 2002). There are very few lipases which exhibit acidic pH optima (Andersson et al. 1979) and some lipases possess broad pH optima (Bradoo et al. 1999). These lipases have temperature optima ranging between 30 – 70^{o}C (Dharmsthiti et al. 1998; Litthauer et al. 2002). Lipase from *Burkholderia* sp. is reported to have exceptionally high temperature optimum of 90-100^{o}C (Bradoo et al. 2002) and it is also reported to be active at extremely high alkaline pH of 11.0. Some lipases of *Pseudomonas* and *Bacillus stearothermophilus* showed thermostability up to 100-150^{o}C (Bradoo et al. 1999; Rathi et al. 2002).

Table 1. Lipase producing bacterial strains

Organisms	**References**
Gram positive bacteria	
Bacillus alcalophilus	Ghanem et al. 2000
Bacillus atrophaeus	Bradoo et al.1999
Bacillus cereus	El-Shafei and Rezkallah 1997
Bacillus coagulans	El-Shafei and Rezkallah 1997
Bacillus pumilus	Jaeger et al.1999
Bacillus stearothermophilus	Kim et al. 1998; Jaeger et al. 1999; Bradoo et al.1999
Bacillus subtilis	Jaeger et al. 1999
Bacillus thermocatenulatus	Rua et al. 1998; Jaeger et al. 1999; Pandey et al.1999;
Bacillus sp. HI-9	Becker et al., 1997
Bacillus thermoleovorans ID-1	Lee et al. 1999
Burkholderia glumae	Jaeger and Reetz 1998; Reetz and Jaeger 1998
Lactobacillus delbruckii	El-Sawah et al. 1995
Lactobacillus plantarum	Lopes Mde et al. 2002
Lactobacillus sp.	Meyers et al. 1996
Moraxella sp.	Jaeger et al. 1999

Mycobacterium chelonae	Pandey et al. 1999
Pasteurella multocida	Pratt et al. 2000
Propionibacterium acne	Jaeger et al. 1999
Proteus vulgaris	Jaeger et al. 1999
Serratia marcescens	Pandey et al.1999; Abdou 2003
Staphylococcus aureus	Jaeger et al. 1999
Staphylococcus epidermidis	Simons et al. 1998; Jaeger et al. 1999
Staphylococcus haemolyticus	Oh et al. 1999
Staphylococcus xylosus	Pandey et al.1999; van Kampen et al. 2001
Streptomyces exfoliates	Arpigny and Jaeger 1999
Sulpholobus acidocaldarius	Jaeger et al. 1999
Vibrio chloreae	Jaeger et al. 1999

Gram Negative Bacteria

Acinetobacter radioresistens	Chen et al., 1999; Liu and Tsai 2003
Acinetobacter calcoaceticus	Dharmsthiti et al.1998; Jaeger et al. 1999; Pandey et al. 1999; Pratuangdejkul & Dharmsthiti 2000
Acinetobacter sp	Barbaro et al.2001
Aeromonas sorbia LP004	Lotrakul and Dharmsthiti, 1997
Archaeglobus fulgidus	Jeager et al. 1999
Arthrobacter sp.	Pandey et al. 1999
Chromobacter viscosum	Jaeger et al. 1999
Pseudomonas aeruginosa	Sharon et al., 1998; Ito et al., 2001
Pseudomonas fragi	Schuepp et al. 1997; Ghanem et al. 2000
Pseudomonas fluorescens	Arpigny and Jaeger 1999; Pandey et al 1999
Pseudomonas luteola	Arpigny and Jaeger 1999; Littauer et al. 2002
Pseudomonas mendocina	Jaeger and Reetz, 1998; Jaeger et al. 1999; Surinenaite et al. 2002
Pseudomonas nitroreducens	Ghanem et al. 2000
Pseudomonas pseudomallei	Kanwar and Goswami 2002
Pseudomonas sp.	Reetz and Jaeger, 1998; Dong et al., 1999
Pseudomonas sp. KWI56	Yang et al. 2000
Pseudomonas wisconsinensis	Arpigny and Jaeger 1999
Psychrobacter immobilis	Jaeger et al. 1999

Yeast lipases

Yeast lipases have recently attracted special attention of biotechnological industries because yeast products have been considered to be safe and used by man since ages. These lipases are generally extracellular, monomeric glycoproteins although some lipases from *Yerrowia lipolytica* are reported to be intracellular. The list of lipases isolated from different yeasts are given in Table 2. Among the yeasts, *Candida rugosa* is most frequently used for lipase synthesis and extensive literature is available on *Candida rugosa* lipase (Benjamin & Pandey 1998; Dominguez de Maria et al. 2006). The molecular weight of yeast lipases vary between 33 to 65 kDa. Some yeast strains produce lipase isozymes as a result of post transcriptional and post translational modifications (Vakhlu & Kour 2006).

Table 2. Lipase producing yeast strains.

Organisms	**References**
Candida antarctica	Jaeger and Reetz 1998; Arroyo et al. 1999
Candida curvata	Ghosh et al. 1996
Candida cylindracea	Kamiya and Gotto 1998; Helisto and Korpela 1998
Candida parapsilosis	Lacointe et al. 1996
Candida rugosa	Yee et al. 1995; Brocca et al. 1998; Xie et al. 1998;
*Candida tropicali*s	Takahashi et al. 1998
Pichia bispora	Hou 1994
Pichia Burtonii	Sugihara et al. 1995
Pichia maxicana	Hou 1994
Pichia sivicola	Sugihara et al. 1995
Pichia xylosa	Sugihara et al. 1995
Rhodotorula glutinis	Papaparaskevas et al. 1992
Saccharomyces lipolytica	Tahoun et al. 1985
Saccharomyces crataegenesis	Hou 1994
Torulospora globora	Hou 1994
Trichosporon asteroides	Dharmsthiti and Ammaranond 1997
Yarrowia lipolytica	Merek and Bednasski 1996; Pignede et al. 2000

Fungal Lipases

Fungal lipases have been studied since 1950s and there are number of comprehensive reviews on fungal lipases. Fungi are well recognized as best lipase sources and are used preferably for industrial applications, especially in food industry. Among the fungi, mucorales have been studied in great details which include lipases from *Mucor hiemalis, Mucor miehei, Mucor pusillus, Rhizopus japonicus, Rhizopus arrhizus, Rhizopus delimar, Rhizopus nigricans* (Lazar & Schroder 1992). Lipase from *Mucor pusillus* is thermostable extracellular lipase. *Rhizopus* lipases are specifically suited for the conversion of triacylglycerols to their monoglycerides and for interesterification of fats and oils. These lipases have molecular weight of about 40-45 kDa and exhibit preference towards medium chain length (C_8 – C_{10}) fatty acids. The lipases from different fungal sources are given in Table 3.

Table 3. Lipase producing fungal strains

Organisms	**References**
Alternaria brassicicola	Berto et al. 1997
Ashbya gossypii	Stahmann et al. 1997
Aspergillus carneus	Helisto and Korpela 1998
Aspergillus flavus	Long et al. 1996; 1998
Aspergillus nidulans	Mayordomo et al. 2000
Aspergillus niger	Chen et al. 1995
Aspergillus oryzae	Ohnishi et al. 1994a,b
Aspergillus repens	Kaminishi et al. 1999
Eurotrium herbariorum	Kaminishi et al. 1999
Fusarium heterosporum	Takahashi et al. 1998
Fusarium oxysporum	Rapp 1995
Geotrichum candidum	Sugihara et al. 1991; Ghosh et al. 1996
Geotrichum sp.	Macedo et al. 1997
Humicola lanuginosa	Ghosh et al. 1996; Takahashi et al. 1998; Plou et al. 1998; Zhu et al. 2001
Mucor circinelloides	Balca˜o et al. 1998
Mucor hiemalis	Ghosh et al. 1996

Mucor miehei	Rantakyla et al. 1996; Lacointe et al. 1996; Plou et al. 1998
Mucor racemosus	Ghosh et al. 1996
Ophiostoma piliferum	Brush et al. 1999
Penicillium camambertii	Ghosh et al. 1996
Penicillium cyclopium	Chahinian et al. 2000
Penicillium funiculosum	Hou 1994
Penicillium roqueforti	Petrovic et al. 1990
Penicillium sp.	Helisto and Korpela 1998
Penicillium wortmanii	Costa and Peralta 1999
Rhizomucor miehei	Merek and Bednasski 1996; Jaeger and Reetz 1998; Dellamora-Ortiz et al. 1997
Rhizopus chinensis	Ghosh et al. 1996
Rhizopus delemar	Klein et al. 1997; Espinosa et al. 1990; Haas et al. 1992; Lacointe, et al. 1996
Rhizopus microsporous	Ghosh et al. 1996
Rhizopus nigricans	Ghosh et al. 1996
Rhizopus niveus	Kohno et al. 1994
Rhizopus oryzae	Salleh et al. 1993; Coenen et al. 1997; Takahashi et al. 1998; Hiol et al. 2000

Screening of lipase-producing microorganisms

Lipase producing organisms are conventionally screened using agar plates containing tributyrin or triolein. The zone of hydrolysis of these substrates around the colony is the indication of esterase or lipase activity. Recently, agar plates supplemented with olive oil have also been used for screening of lipase positive strains (Hube et al. 2000; Kim et al. 2001). The lipases in culture supernatants of various microbes can be detected by gel diffusion method which involves the appearance of clear hydrolysis zone due to lipolysis of triacylglycerol (Lawrence et al. 1967; Stead 1986). The indicators like Nile blue sulphate (Christen & Marshall 1984) and Victoria blue (Lawrence et al. 1967) were also added to agar plates to give colored hydrolysis zones. The use of these methods is limited due to generation of acidic metabolites other than fatty acids giving false positive results. To overcome these limitations, Rhodamin B was used in the agar

medium which imparts orange color to the hydrolysis zone under UV-light at 350 nm. This method is based on the formation of fluorescent Rhodamin B complex with liberated fatty acids released during hydrolysis of triolein. One can use olive oil in place of triolein in agar medium to observe the fluorescent zone of hydrolysis (Jette & Ziomark 1994; Jarvis & Thiele 1997). Approaches to rapid screening of especially acid lipase producing bacteria were developed and the feasibility assessment of the screening method was performed (Liu et al. 2007). This method is the combination of back titration and microwave treatment.

Lipase Assay

The conventional procedure for the lipase assay and also the procedure adopted for molecular screening of lipase have been discussed in many reviews (Beisson et al. 2000; Gupta et al. 2003). The lipase assay methods are based on the release of fatty acids or chromogenic or fluorescent products liberated after hydrolysis of lipid substrates. These products can be measured quantitatively by titrimetric methods, calorimetric and fluorescent assays, chromatographic procedures or immunological methods. Techniques to measure lipase and esterase activities in vitro have been reviewed recently (Gilham & Lehner 2005).

Titrimetry is most widely used and reliable assay procedure for characterizing lipase action, specificity and also interfacial activation phenomenon (Ferreto et al. 1997). In this method, triolein is used as substrate since lipases prefer triacylglycerols with larger chain length fatty acids. The pH stat method reported by Jaeger et al (1994) is highly sensitive that can measure the release of 1 μmol fatty acid / min. The method involves titration of assay mixtures to pH 9.0-10.0 after stopping the reaction with ethanol (Andersson 1980; Rathi et al. 2001; Bradoo et al. 2002). This method is very laborious and time consuming and hence cannot be used for rapid lipase assays. Until now, the conventional lipase assay of pH stat method with back titration is still used to determine gastric lipase activity (Chahinian et al. 2006).

Spectrophotometric assay methods use *p*-nitrophenylesters of various chain length fatty acids as substrates which release *p*-nitrophenol after hydrolysis. This product develops yellow color under alkaline condition which can be measured

spectrophotometrically at 410 nm (Winkler & Stuckman 1979; Pancreac'h & Baratti 1996). The use of short chain length fatty acid esters of *p*-nitrophenol provides the measure of esterase activity rather than lipase activity. The longer chain length fatty acid ester of *p*-nitrophenol, mainly *p*-nitrophenylpalmitate, is used to determine lipase activity under acidic, neutral or alkaline conditions. The calorimetric assays are performed using colorless substrate, β-naphthyl caprylate, which is hydrolyzed by lipase to liberate β-naphthol. This colored product is measured spectrophotometrically at 560 nm (Lanz & Williams 1973; Gandolfi et al. 2000). Sometimes, hydrolytic and synthetic activities of lipases cannot be correlated and hence specific screening methods for synthetic activities of lipases need to be developed. Very recently, Sandoval and Marty (2007) have reported sensitive and specific screening method to measure synthetic activities of lipases in vivo on an agar plate and in vitro using culture broth. These methods are sensitive enough to determine both intra- and extracellular lipases.

The fluorescent assay method involves the use of substrates, which after hydrolysis release fluorescent fatty acids that can be measured. The fluorescent triacylglycerol can be used as substrate in which one of the alkyl groups is substituted by fluorescent pyrenyl group (Thuren et al. 1987; Negre-Salvarye et al. 1991). Some times non-fluorescent substrate like 4-methylumbelliferyl oleate can also be used to release fluorescent product (in this case, 4-methylumbelliferone) after lipase hydrolysis (Jacks & Kircher 1967). A highly sensitive fluorescent based lipase assay was developed by Roberts (1985) using 4-methylumbelliferyl butyrate. This assay is more sensitive (6000 fold) than conventional titration. Jette and Ziomek (1994) described the fluorescent lipase assay based on the interaction of Rhodamin B with fatty acids released during lipase hydrolysis of triacylglycerols. The method is very rapid and thus allows the large number of samples to be processed for determination of lipase activity.

The chromatographic techniques, such as TLC, GC and HPLC are considered as conclusive methods for determining fatty acids liberated by lipase hydrolysis of lipid substrates. Immunological methods are highly specific and can be employed easily. However, these methods cannot be used routinely due to the stringent requirement of purified enzymes and poly- or monoclonal antibodies.

Lipase production

Microorganisms produce both intracellular and extracellular lipases. Extracellular lipases have attracted great attention because of their ease of isolation and purification since they are produced in high amounts in fermented broth. Lipase production is carried out in liquid media containing proper carbon, nitrogen and other nutrients. Cells are grown in either shake flasks or fermenter with constant stirring by maintaining proper temperature and pH. By and large, these are inducible enzymes which require the presence of lipid source in the medium for their induction. The lipid source could be an oil or any other inducer such as triacylglycerols, fatty acids, hydrolyzable esters, tweens or glycerols (Ghosh et al. 1996; Shirazi et al. 1998; Rathi et al. 2001). The nonlipid carbon source could also be employed for lipase production in bacteria. Kanwar et al (2002) reported the lipase production from *Pseudomonas* sp. in presence of n-alkane as substrates. Maximum production of about 25 units / ml was achieved when the organism was grown in medium containing n-hexadecane as sole carbon source. The combination of olive oil and n-hexadecane were employed as carbon source for alkaline lipase production from *Achromobacter radioresistens* (Liu and Tsai 2003).

Carbon Sources

Vegetable oils such as olive, sunflower, sesame, cotton seed, corn, peanut were used as lipid source in the medium resulting in the production of high levels of lipase activity (Gao & Breuil 1995). Rapeseed oil and corn oil were most suitable substrates for cell growth and lipase production from *Rhizopus oryzae* (Essamri et al. 1998). Other oils such as palm oil (Rathi et al. 2002), mustard oil (Kumar et al. 2005) and babusa oil (Castilho et al. 2000) have been used for lipase production. Lipase was also produced when medium was supplemented with non-conventional carbon sources like potato starch and starch (Bapiraju et al. 2004). Groundnut oil refinery residues (Miranda et al. 1999) and sugarcane bagasse (Cordova et al. 1998) were also used for lipase production.

Certain long chain fatty acids are known to support lipase production (Ghosh et al. 1996). For instance, high lipase yields were obtained when *Candida rugosa* was grown in medium containing fatty acids or lipid, but non-fat related acids did not induce lipase production (Dalmou et al. 2000). Long chain saturated fatty acids such as stearic

acid or arachidonic acid was found to be effective lipase inducers (Ito et al. 2001). Unsaturated fatty acids such oleic acid also served as good inducers of lipase from *Candida rugosa* and it was observed that lipase production increased with increase in chain length of fatty acid (Wei et al. 2004). Oleic acid esters were shown to be the best carbon source for both cell growth and lipase production by *Candida rugosa*. The combination of dodecane and glycerol trioleate resulted in the highest activity in batch fermentation. This was due to improved solubility of solid substrates in the medium (Wei et al. 2004).

The addition of surfactants (Triton, Tween, SDS, PEG, Gum Arabic, AOT, SPAN) to the culture medium has often been shown to enhance the secretion of lipase activity in to the medium (Odibo et al. 1995; Berto et al. 1997; Abdel-Fattah 2002). This may be due to the alteration of cell wall permeability leading to increased protein secretion or to surfactants effect on cell bound enzymes (Costas et al. 2004). Lin et al (1996) reported that the addition of Triton X-100 enhanced the alkaline lipase production from *Pseudomonas pseudoalkaligenes* F-111 by 50 fold compared to the value obtained in olive oil based medium without Triton-X-100. No such enhancement in lipase production from *Yerrowia lipolytica* was observed in surfactant supplemented medium (Dominguez et al. 2003). On the contrary, lipase production was lowest in Tween 80 supplemented medium.

Recent papers show that it is possible to get extracellular lipases with different compositions of isozymes (Dalmou et al. 2000; de la Casa et al. 2006; Dominguez de maria et al. 2006). *Geotrichum* sp. FO40 B produced lipase A, B and C. All A,B and C lipases are produced when plant oils were used as carbon source but only lipase C is produced when tributyrin is used in the medium (Ota et al. 2000).

Nitrogen Sources

Besides carbon source, different organic and inorganic nitrogen sources have been used for lipase production. Generally, organic nitrogen sources such as peptone, tryptone or yeast extract is preferred for lipase production in bacterial systems (Oh et al. 1999; Ghanam et al. 2000; Lanser et al. 2002). Inorganic nitrogen sources such as NH_4Cl,

NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ have been proven to be effective for lipase production especially from fungal sources (Rathi et al. 2001).

The use of yeast extract resulted in high lipase production from thermophilic fungi such as *Emericella regulate, Humicola sp., T. lanuginosus, P. purpurogenum* and *Chysosporium sulfureum* (Venkateshwarlu & Reddy 1993). There have been several reports that some protein complexes can stimulate lipase production (Nahas 1988; Thomas et al. 2003; Cihangir & Sarikaya 2004). Combination of yeast extract, polypeptone and soybean meal was found to be most suitable for maximum lipase production from *A. oryzae* (Ohnishi et al. 1994a). Lipase production and biomass from *Rhizopus sp*. were found to be the best with corn steep liquor followed by peptone and meat extract (Bapiraju et al. 2005). *Candida cylindraceae* produced significant levels of lipase when it was grown in synthetic medium containing both peptone (3.4g/L) and yeast extract (4.9g/L) as nitrogen source. Fickers et al (2004) observed marked increase in lipase yields when *Y. lipolytica* mutant was grown in medium supplemented with casein hydrolysate and more specifically tryptone N1. Various fish protein hydrolysates were used as nitrogen source for the production of lipase from *R. oryzae* (Ghorbel et al. 2005). Defatted meat fish protein hydrolysates were the best indicating the presence of some constituent in the lipid fraction which may suppress the lipase synthesis in *R. oryzae*. Inorganic nitrogen sources such as $(NH_4)_2SO_4$, NH_4Cl significantly favored the lipase production by *Candida cylindraceae* (D'Annibale et al. 2006). Lipase production by *F. oxysporum* was investigated in which sodium nitrate was found to be most suitable (Camargo-de-Morias et al. 2003). Urea as a nitrogen source gave maximum lipase production from *Geotrichum* strain (Ginalska et al. 2004).

Thus, the microbial lipases are produced in presence of lipid substrates (oils, fatty acids, fatty acid esters) as carbon source in combination with organic or inorganic nitrogen sources. The requirement of metal ions varies with the organisms used for lipase production. Physical parameters such as pH, temperature, agitation and aeration influence the growth of the organism and also lipase production. Lipases are produced either by submerged fermentations (SmF) or by solid state fermentations (SSF) although the former is preferred under certain situations.

Multiple forms of lipases

There are several reports on the multiple forms of lipases produced by microorganisms. These multiple forms could be the result of post transcriptional processing, presence of different genes or deglycosylations. *Geotrichum candidum* produces two extracellular lipases, lipase I & II which are encoded by two homologous genes (Shimada et al. 1990). Chang et al (1994) identified multiple forms of lipases in *Candida rugosa*. The relative abundance of various forms of lipases is altered due to the presence of Tween 80 or Tween 20 in the medium (Chang et al. 1994). *Rhizopus niveus* produced two lipases Lipase I & II which differ in their molecular weight. Lipase I is presumed to be converted to Lipase II by limited proteolysis (Kohno et al. 1994). Benjamin and Pandey (2001) characterized three distinct forms of lipases from *Candida rugosa* produced in SSF. All these forms existed in three iso-forms possessing different molecular weights. Lopez et al (2004) reported three forms in *Candida rugosa* with different selectivity and reactivity in aqueous and organic media.

Some microorganisms produce multiple forms of lipases due to post-translational modifications such as glycosylations. *Geotrichum candidum* NRRL Y-553 produces multiple glycosylated lipases with slight variations in pI values (Baillargeon & McCarthy 1991). Four different lipases are produced by *Geotrichum candidum* ATCC 34614 (Sugihara et al. 1994). Lipase I is non-specific in positional specificity whereas Lipase IV has unusual positional specificity. *Geotrichum candidum* ATCC 66592 produces two lipases with molecular weights of 61 and 57 kDa. The 61 kDa lipase was separated in to two lipases with pI 4.5 and 4.7 using crossed electrofocussing immunoelectrophoresis. However, when deglycosylated with endoglycosidase H, the two forms showed an identical pI of 4.6 (Jacobsen and Poulsen 1992).

Purification of the lipase

Microbial lipases have been isolated and purified to homogeneity. Most of the commercial lipase are crude preparations since commercial applications do not require purified enzymes. 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 purified enzyme preparations are necessary for determining primary

amino acid sequences, understanding of 3-D structure and structure and function relationship of the proteins (Aires-Barros et al. 1994; Saxena et al. 2003).

The general purification steps involve removal of cells or mycelium from the fermented broth, concentration, ammonium sulphate or organic solvent precipitation followed by a combination of several chromatographic methods. In case of intracellular lipases, an additional step of cell lysis is required to extract the enzyme. The chromatographic methods include ion-exchange, affinity and size exclusion chromatography. However, the specific procedure and its efficiency differ from case to case. Ammonium sulphate or organic solvent precipitated enzyme preparations give a high average yields albeit with limited purification (Aires-Barros et al. 1994). Such precipitated preparations are suitable for use in detergent formulations. Lipases are hydrophobic in nature and hence the purification could be achieved employing affinity chromatography techniques such as hydrophobic interaction chromatography (Imamura & Kitaura 2000; Queiroz et al. 2001).

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 specer arm as a ligand and aqueous two phase systems (Saxena et al. 2003). The novel methods of lipase purification have been well described in special purification reviews (Gupta et al. 2004). Techniques employed for purification of some of the microbial lipases are given in Table 4.

Table 4: Purification strategies employed for some microbial lipases.

Organism name	Purification scheme	Fold purifi-cation	% Reco very	Mol. Wt kDa	References
Acinetobacter calcoaceticus	Aqueous two phase system	41	68	-	Bompensieri et al.1998
Anthrodia cinnamomea	Ammonium sulfate precipitation, Phenyl sepharose	17.2	33.6	60	Shu et al. 2006
Aspergillus carneus	Hydrophobic interaction chromatography	24	38	27	Saxena et al. 2003
Aspergillus Niger	P^H precipitation and series of chromatographic steps	50	10	35.5	Namboodiri et al. 2000
Aspergillus nidulans	Phenyl sepharose, affinity binding on linolenic acid-agarose	1186	10	29	Mayordomo et al. 2000
Aspergillus oryzae	Ammonium sulfate, Acetone precipitation, Anion exchange chromatography, Gel filteration	792	11	25	Toida et al. 1998
Bacillus sp.J33	Ammonium sulfate precipitation, phenyl sepharose	175	15.6	45	Nawani et al. 2000
Bacillus sp. RSJ-1	Ultrafiltration, Ammonium sulfate precipitation, dialysis, ion exchange, gel filtration	201.45	19.7	-	Sharma et al. 2002
Burkholderia sp. YY62	Ammonium sulfate precipitation, DEAE anion exchange, sepharose CL-4B gel filteration, DEAE anion exchange, HPLC	74	9.0	40	Yeo et al. 1998
Burkholderia multivorans	Adsorbing on polypropylene matrix Accurel MP-1000	3.0	66	-	Gupta et al. 2005
Geotrichum marianum	Octyl sepharose 4 fast-flow chromatography, Bio gel	76	46	62	Huang et al. 2004

Mucor hiemalis	Ultrafiltration, Ammonium sulfate filteration, Sephadex G-75,Q-sepharose,Sephacryl S-200	2200	18.1	49	Hiol et al. 1999
Penicillium citrinum	Ammonium sulfate precipitation, gel filteration on Superose 6 column, hydrophobic interaction chromato-graphy on Phenyl superose column	379	15.2	63	Krieger et al. 1999
Penicillium Citrinum	Ultrafiltration, Hydrophobic interaction chromatography	810	68	33	Krieger et al.1997
Penicillium chrysogenum	Ultrafiltration, Phenyl sepharose, Mono Q-Hr 5/5,PD-10	30.3	44	40	Ferrer et al. 2000
Pseudomonas aeruginosa KKA-5	Ammonium sulfate, Hydroxyl-apatite column chromatography	518	2.6	30	Sharon et al.1998
Pseudomonas sp. LP7315	Ammonium sulfate precipitation, anion exchange chromatography, preparative electrophoresis	64.1	12	59	Sakiyama et al. 2001
Pseudomonas sp. strain S5	Affinity chromato-graphy, anion exchange	387	52	60	Zaliha et al. 2005
Rhizomucor Miehei	Ion exchange, Octyl sepharose affinity chromatography	135	12	-	Uvarani et al. 1998
Rhizopus oryzae	Ammonium sulfate precipitation, sulfopropyl-sepharose chromatography Sephadex G-75	1200	22	32	Hiol et al. 2000
Serratia marcescens	Ion exchange, gel filteration	45	42	52	Abdou et al. 2003

(-) indicates no data available

Application of lipases

Lipases are considered to be the third largest group of enzymes based on total volume of sales. Lipases have been exploited for wide variety of different applications which make them commercially important enzymes leading to billion dollar business (Jaeger et al. 1999). Majority of lipases in current industrial use are of microbial origin and are produced by submerged fermentation. In addition to natural function of hydrolyzing ester bonds, lipases can also catalyze esterification, interesterification and transesterification reactions in nonaqueous media. Apart from their preference to their natural substrates, lipases also perform the enantioselective and regioselective hydrolysis and synthesis of wide variety of nonnatural esters (Boland et al. 1991). This versatality of lipases makes them ideal biocatalysts of choice for potential applications in food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries (Houde et al. 2004). There are excellent review articles published recently on various industrial applications of lipases (Schmid & Verger 1998; Cammarota & Freire 2006; Hasan et al. 2006).

Lipases in fat and oleochemical industry

There are some fats that are more valuable because of their structure. Chemical methods are reported for converting less valuable fats in to more useful species. However, these chemical methods tend to release random products. Cocoa butter is used in the production of chocolates. The preparation of transesterified triglycerides is based on the regioselectivity of certain lipases. Many lipases have sn-1,3-specificity which is used to regioselectively interesterify positions 1 ans 3 of natural triglycerides. The cocoa butter constitutes triglycerides with oleic acid in the *sn*-2-position and stearic and palmitic acid at sn-1- and sn-3-positions respectively. These cocoa butter equivalents can be prepared by lipase mediated interesterification of natural triglycerides such as middle fraction of palm oil or sunflower oil (Bloomer et al. 1990). Fugi oil company of Japan is producing several thousand tons of chocolate fats using lipase (*Rhizopus*) mediated transesterification. The other company, Unichema, also produces chocolate fat by transesterification of high oleic sunflower oil using *Rhizomucor miehei* lipase. Betapol, a highly digestive triglyceride with palmitic acid at *sn*-2-position is prepared by inter-

esterification of tripalmitin with oleic acid using *Rhizomucor miehei* lipase. The inter-esterification of suitable triglycerides mixtures with the use of sn-1,3-specific lipases in combination with catalytic hydrogenation of double bonds led to preparation of margarines and shortening from plant oils (Holemans et al. 1987; Moore 1987).

The scope for the application of lipases in the oleochemical industry is enormous. More than 60 million tons of fats and oils are produced world wide and approximately 2 million tons per annum are utilized in high energy consuming processes such as hydrolysis, glycerolysis and alcoholysis. The conventional processes for steam fat splitting and glycerolysis of oils involve high temperatures of 240–260^{o}C and high pressures. These processes result in products that are unstable and also require re-distillation to remove impurities and products of degradation. The enzymatic methods have not yet been exploited at commercial level. There are relatively small-scale enzymatic fat splitting processes are reported for the production of high value polyunsaturated fatty acids. *Candida cylindracea* lipase has been used commercially for the production of soaps (McNeil et al. 1991) and one of the Japanese companies, Miyoshi Oil and Fat claimed that the enzymatic method yielded a superior product and it was cheaper than the conventional chemical process.

Use of lipases in textile industry

In textile industry, lipases are used for removing size lubricants which provides a fabric with greater absorbancy needed for improved levelness in dying. Commercial preparation used for the desizing of denim and other cotton fabrics consists of amylases and lipases. Enzymes used in textile industries are supplied by Rakuto Kasai Israel Limited and are used for desizing, stone washing of Denim and Jeans, enzymatic wash, bio-polishing etc. Synthetic fibers have been enzymatically modified which can be used in the production of several consumers items such as fabrics, yarns etc. Polyesterase, related to lipase, has been used for the treatment of polyester fabric which improves its ability to uptake the chemical compounds such as dyes, antistaining compounds, antimicrobial compounds etc. Bayer AG described the enzymatic degradation of polyesteramide by treatment with esterase, lipase or protease solutions has been reported (PCT Publication No.WO 97/43014). Degradation of polymers of aliphatic polyethylenes

by lipase from *Pseudomonas* has been reported by Amano Pharmaceuticals KK (JP 5344897). Genecore International, Inc also discloses a method of improving the wettability and absorbance of a polyester fabric by treatment with a lipase (Hasan et al. 2006).

Lipases in detergent industry

There is a great commercial success of proteases to be used in detergents. After the proteases, major efforts are being made to introduce lipases as a second group of detergent enzymes since the biggest market of their use is in detergent formulations (Jaeger & Reetz 1998). Standard wash liquids contain ionic and non-ionic surfactants, oxidants at alkaline pH (pH 10.0) and temperatures about 50^{o}C. These conditions are rather hostile to enzymes. Therefore, major screening programs were initiated to look for the lipases that are stable and active under alkaline conditions and also at higher temperatures. Novo-Nordisk made several detergent formulations containing lipases which were used successfully for the removal of died soils from textiles and also for elimination of stains. The major commercial lipases used in the detergent formulation are given in Table 5. Other lipases used as detergents include those from *Candida* (Nishioka et al. 1990), *Chromobacterium* (Minoguchi & Muneyuki 1989).

Table 5: Some of the commercial lipases used in detergents

Brand name	Company	Producing organisms	Properties
Lipolase	Novo-Nordisk	Recombinant fungal lipase from *Humicola lanuginosa* expressed in host *Aspergillus oryzae*	Active at pH 10.0 and temperature 40^{o}C
Lipomax	Gist-Brocades	Recombinant *Pseudomonas pseudoalkaligenes*. It was recloned and expressed in the same organism	Active at pH 11.0 and temperature 45^{o}C
Lumafast	Genencore	Recombinant lipase from *Pseudomonas mendocina* cloned and expressed in *Bacillus* species	Active at pH 10.5 and temperature 40^{o}C

Laundering is generally performed under alkaline conditions and hence lipases active under such conditions are preferred (Gerhartz 1990; Satsuki & Watanabe 1990). It is estimated roughly that 1000 tons of lipases are sold every year in the area of detergents (Godfrey & West 1996). However, compared to other enzymes, the use of lipases in detergents is still limited mainly due to shortage of enzymes with suitable properties. The functional importance of lipases in detergent industry is related to the removal of fatty residues in laundry, dishwashers as well as for cleaning of clogged drains. Continuous efforts in screening for improved lipases with suitable properties could be the potential solution to these problems (Jaeger & Reetz 1998). Enhancement in the desirable lipase properties by protein engineering also can also be solution to the problems faced in the use of lipases in detergents. Recently, an alkaline lipase from *Fusarium oxysporum* was isolated and found to be active at pH 8.0 and temperature of 50^{o}C. (dos Prazeres et al. 2006). This lipase is compatible with various ionic and non-ionic surfactants as well as commercial detergents which makes this lipase a potential additive for detergent formulations.

Lipases in bakery and dairy products

Lipases have been extensively used for the hydrolysis of milk fat. Current applications include the flavour enhancement of cheeses, the acceleration of cheese ripening, the manufacturing of cheese like products, and the lipolysis of butterfat and cream (Falch 1991). The action of lipases on milk fat gives many dairy products such as soft cheeses with specific flavor characteristics. The cheese is prepared by the action of Rennet paste which contains the active component, chymosin, involved in clotting of milk through hydrolysis of k-casein (Vulfson 1994). Rennet also contains lipases and esterases which contribute to cheese ripening. Apart from Rennet, proteases and lipases from other sources have increasingly been used in cheese making. Lipases also play an important role in the preparation of enzyme modified cheeses (EMC). EMC is prepared by incubating cheese curd with lipases at elevated temperatures in order to produce free fatty acids. The EMC, an important commercial flavor, is used for the manufacture of dips, sauces, soups, snacks, dressings and crackers (http://www.au-kbc/frameresearch.html).

The release of free fatty acids from milk fat caused by the action of lipases contributes many flavor characteristics to dairy products. Liberation of short chain fatty acids ($C_4 - C_6$) lead to the development of a sharp, tangy flavor while the release of medium chain length fatty acids ($C_{12} - C_{14}$) imparts a soapy taste to the product (Vulfson 1994). In addition, these fatty acids are readily metabolized by the microbial consortia found in cheese to produce other flavor ingredients such as acetoacetate, β-keto acids, methyl ketones, and flavor esters and lactones (Kingsella & Hwang 1976). Larios et al (2004) synthesized short chain flavor esters with high conversions in organic solvents using *Candida antarctica* lipase (CAL-B). Flavors similar to goat, sheep or raw milk can be generated by the addition of lipases to pasteurized milk.

Microbial lipases have been used to extend the shelf life of breads, increase the loaf volume and improve the crump structure. They are also used to enhance and control the nonenzymatic browning of bakery products. Generally, lipases from *A. niger, R. oryzae* and *C. cylindracea* are used in bakery products.

Resolution of racemic mixtures

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 2003).

Pseudomonas AK lipase was used to prepare chiral intermediate to be used in the synthesis of epithilone, potent antitumor agent (Zhu & Panek 2001). *Candida rugosa* lipase was used for resolution of antimicrobial compounds in to their (S) and (R) enantiomers (Ono et al. 2001). Novozyme 435 (lipase from *C. antarctica*) was used to resolve racemic flurbiprofen by enantioselective esterification with alcohols (Zhang et al. 2005). Lipase mediated resolution of racemic Baclofen was reported which is used in the therapy of pain and as a muscle relaxant (Murlidhar et al. 2001). Enzymatic resolution of

racemic 2-pentanol and 2-heptanol was reported using CAL B (*C. antarctica* lipase) in which S-(+)-2-pentanol is formed that is required for the synthesis of anti-Alzheimer drugs. We have reported the enantioselective hydrolysis of *meso*-cyclopent-2-en-1,4-diacetate to obtain 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate using lipase from *Trichosporon beigelli* as catalyst through medium-engineering approach (Kalkote et al. 2000). This intermediate is used in the synthesis of cyclopentanoid natural products such as prostaglandins, prostacyclins, thromboxanes and also in the synthesis of of some anti-HIV drugs (Harre et al. 1982). Mannich bases are anticancer agents (Dimmock et al. 1997; Gul et al. 2000) and their anticancer properties are based on a deamination process which afford α, β-unsaturated ketones responsible for selective alkylation of thiol groups in the biological systems (Gul et al. 2005). Mannich base derivatives undergo interesting sequential reactions in presence of whole microbial cells leading to (S)-phenylpropanol or *para*-methoxyphenol (Raminelli et al. 2007). Thus preparation of enantiomerically enriched secondary alcohols was described using whole cells.

Lipases are also used in the production of enantiopure novel herbiside, (S)-indanofan which is active against grass weeds (Tanaka et al. 2002). This (S)-enantiomer is obtained by lipase mediated enzymatic resolution followed by chemical inversion technique. The distereomers of 4-hydroxyproline is an important starting material for many agrochemicals and pharmaceuticals. The enantioselective hydrolysis of racemic 4-oxo-1,2-pyrrolidinedicarboxylic acid dimethyl ester using CAL B lipase led to production of *cis*-4-hydroxy-D-proline or *trans*-4-hydroxy-D-proline with 99.5% diastereomeric excess (Sigmund et al. 2001).

Lipase mediated synthesis of flavors and fragrance compounds has been reported with (-) menthol being the most prominent. Pure (-) menthol esters have been isolated from racemic menthol via transesterification reaction using *Burkholderia capacia* lipase (Athawale et al. 2001). The final product, menthyl methacrylate was further polymerized to get sustained release perfume. The another perfumery constituent, (-) methyl jasmonate is synthesized by using commercially available lipase P (Amano) to get chiral intermediate (+)-(6S)-methyl-7-epicucurbate (Kiyota et al. 2001).

Lipases in biodiesel production

Shortages of fossil fuel reserves, hike in price of crude oil and environmental concern have compelled us to think of alternative fuels. Incresed efforts in R & D are required to develop alternative methods of producing fuels from renewable resources. One of the approaches is to produce alkyl esters (Biodiesel) from vegetable oils which can be carried out using different catalytic processes (Fukuda et al. 2001). Biodiesel is methyl or ethyl ester of fatty acids made from virgin or used vegetable oils (edible or non-edible). Currently, biodiesel is produced mainly from field crop oils such as rapeseed, sunflower and soybean oil. The main commodity source for India can be non-edible oil obtained from plant species like Jatropha, Pongamia etc.

The chemical catalyzed processes pose pollution and bye product separation problems. Therefore, in last few years, enzymatic synthesis of biodiesel has shown significant progress (Shimada et al. 2002; Zhang et al. 2003). Biodiesel can be a substitute for conventional diesel fuel because it does not produce sulphur dioxide and much soot particulate which are the main causes for environmental pollution. Immobilized *Pseudomonas* lipase was used to produce both methyl and ethyl esters by transesterification of soybean oil with methanol and ethanol (Noureddini et al. 2005). de Oliveira et al (2004) produced ethyl esters of fatty acids from castor oil in *n*-hexane using commercial lipases, Novozyme 435 and Lipozyme IM. Crude soybean oil is also used for biodiesel production using Novozyme 435 in solvent-free medium (Du et al. 2004). Palm kernel oil was used to prepare ethyl esters (biodiesel) with highest conversion. This biodiesel has the properties comparable to international biodiesel specifications (Abigor et al. 2000). Recently, methanolysis of cottonseed oil for biodiesel production was studied using *C. antarctica* lipase in tertiary butanol as a solvent. In this process it was found that tertiary butanol improves the enzymatic process because it dissolves both methanol and the glycerol (Royon et al. 2006).

Lipases in leather industry

Hides and skins of the animals contain proteins and fat in between collagen fibres which should be partially or fully removed before tanning. The proteins can be removed by proteases and fats can be removed with the help of lipases. Recently both proteases and lipases have been used for soaking, bating and un-hairing of the hides and skins.

Traditional methods used for bating involve the use of manure of dog, pigeon or hen which are very unpleasant, unreliable and very slow in action. Biotechnological developments have completely replaced these methods with enzymatic methods. Enzymatic bating process is carried out under acid or alkaline conditions using acidic or alkaline lipases. Soaking process removes dirt and other contaminants present on the skin. The enzymatic soaking process is performed using mixture of protease and lipase under alkaline or acidic conditions. Un-hairing is performed using conventional and most wide spread method in which lime and sodium sulphide are used. These chemicals dissolve the hair and open up the fibre structure. However, protease-assisted technology of un-hairing results in cleaner grain surface and improved area yield and softness. The proteases used are active at high alkaline pH conditions.

Degreasing is an essential step in the processing of animal skins and hides. Conventional methods make use of organic solvents and surfactants, which create environmental problems such as emissions of volatile organic compounds. Lipases degrade fat specifically without changing leather itself. Additionally, lipases not only hydrolyze fat on the outside of the skins and hides but also the fat inside the skin structure. This makes the enzymatic treatment method environmentally sound for removal of fat (Hassan et al. 2006). The amount of surfactant can be reduced if lipases are used for degreasing of skins and hides. Degreasing can be done under alkaline and acidic condition using lipases active at both alkaline and acidic pH. Acid active lipases are used to treat the skins stored under pickled state. The combination of acid lipase and acid protease (NovoCor ABL and NovoCor ADL) used for acid bating of fur and wool are marketed by Novo Industries, Denmark. NovoCor AD, a lipase active at acid pH is used for degreasing of hides and skins. Lipase from *Rhizopus nodosus* was used for degreasing of leathers from wooled sheep skins (Muthukumaran & Dhar 1982).

Lipases in waste/effluent treatment

Waste waters from dairies (Jung et al. 2002; Omil et al. 2003) and slaughter houses (Masse et al. 2001; 2003) are rich in biodegradable organic molecules especially fats and proteins. These organic molecules cause land and water pollution due to high BOD and COD. Hence the effluent treatment is very much necessary in industrial processing units such as food processing, leather processing and dairy processing (Godfrey & reichelt 1983). The large number of pretreatment systems is employed to remove fatty substances prior to main biological treatment. However, these pretreatments are not cost effective due to high cost of reagents used. In addition, they are operating with low removal efficiency of dissolve or emulsified fats leading to production of problematic sludge (Willey 2001).

Pretreatments involving dissolution and hydrolysis of fats may improve the biological degradation of waste water/effluents containing high fats. Hydrolysis pretreatments have been mostly tested on waste activated sludge or municipal waste (Knezevic et al. 1995; Lin et al. 1997) which contain higher amounts (10 times) of solids than waste water from dairy or slaughter house. Little information is available on the use of alkaline / acid / enzymatic hydrolysis for the hydrolysis of fats and oils. The use of lipases in the treatment of such effluents from several origins could be a new promising area for lipase applications. Commercial lipases have been used in the pretreatment of slaughter house effluents (Masse et al. 2001). Both cells and lipases from *Pseudomonas aeruginosa* were used for the treatment of lipid rich waste waters (Dharmsthiti & Kuhasuntisuk 1998). Immobilized lipase has also been used to hydrolyze triglycerides in waste water treatment plants (Tschocke 1990). *Candida rugosa* lipases were employed in the treatment of domestic waste water and also in the cleaning of sewer systems and sinkholes (Jaeger & Reetz 1998).

Solid state fermentation (SSF) is the promising approach to produce low costs lipases to be used in effluent treatments. Enzymatic preparations obtained by SSF have been used in the pretreatment of dairy industry and slaughter house effluents with high fat content (Cammarota et al. 2001; 2003; Jung et al. 2002). Enzyme production can be carried out in situ with the use of industrial waste as a source of nutrient for the fermentative process. This enzymatic prehydrolysis stage obtained through SSF results in

considerable increase in efficiency of organic matter removal leading to generation of acceptable quality of effluent for further biological treatment. *Penicillium restrictum* is the most promising organism producing a mixture of lipase, protease and amylase when cultivated on babusu cake solid medium (Gombert et al. 1999; Palma 2000). More scientific studies are being carried out to investigate the possibility of enzymatic hydrolysis processes competing with traditional biological treatments with a view to improving the quality of pretreated effluent. The development of such processes is necessary for the pretreatment of waste effluents with high fat content. Cammarota & Freire (2006) have recently published a good review article on the use of hydrolytic enzymes for the treatment of waste water with high oil and grease content.

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CHAPTER – 2

Production of acidic lipase by *Aspergillus niger* NCIM 1207 in solid state fermentation

Abstract

This chapter deals with the production of the lipase in submerged and solid state fermentation using *Aspergillus niger* NCIM 1207. Fifty strains of species of *Rhizopus, Mucor, Geotrichum, Penicillium* and *Aspergillus* were screened for their ability to form butyl esters from butter oil in presence of butanol. *Rhizopus* strains showed major intracellular activity while *A. niger* NCIM 1207 produced mainly extracellular activity. We selected *A. niger* strain for lipase production using both submerged fermentation (SmF) and solid state fermentation (SSF). The major optimization studies on lipase production were carried out using SSF. Denovo biosynthesis of lipase occurred only in the presence of lipid substrate and was completely repressed by glucose. Addition of Triton X-100 (0.5%, w/v) in fermentation medium resulted in enhanced lipase activity (18 IU/ml). The pH and temperature optima for lipase were 2.5 and 50^{o}C, respectively. The enzyme also exhibited high activity (75%) at extremely acidic pH of 1.5. The crude enzyme is also stable over a wide pH range (pH 2.5 to 9.0) for 24 h at room temperature. The enzyme retained 63% of its original activity on incubation at 70^{o}C for 5 h. Lipase production was studied in SSF and the highest yields of enzyme were obtained using wheat bran as solid substrate in combination with olive oil as lipid substrate. Maximum lipase activity (630 IU/g dry solid substrate) was recovered when fermented wheat bran was extracted with NaCl (1%) supplemented with Triton X-100 (0.5%). The crude enzyme was stable in various organic solvents. It retained 90 % of its original activity when it was incubated in 25 % Hexane, chloroform, acetonitrile, isooctane, ethylene glycol, propylene glycol and in n-hexadecane for 24 h. This organism, being GRAS cleared, can be used for large-scale production of enzyme for commercial purpose.

Introduction

Lipases (glycerol ester hydrolyses EC 3.1.1.3) catalyze the hydrolysis of acyl glycerols to fatty acids, di-acyl glycerols, mono-acyl glycerols and glycerol. Under certain conditions, they also catalyze the synthesis of esters by transesterification, thioesterification and aminolysis. Lipases occur widely in bacteria (Jaeger et al. 1999; Gao et al. 2000), yeasts (Rapp & Backhaus 1992; Dalmou et al. 2000) and fungi (Jaeger & Reetz 1998; Ferreira et al. 1999). In recent years, research in microbial lipases has

increased because of their practical applications in industry, in the hydrolysis of fats, production of fatty acids and food additives, synthesis of esters and peptides, resolution of racemic mixtures or addition in detergent (Maleata 1996).

Fungi are widely recognized as the best lipase sources and are used preferably for industrial application, especially in the food industry. *Aspergillus niger* is among the most well known lipase producer and its enzyme is suitable for use in many industrial applications (Fu et al. 1995; Macris et al. 1996) Most of the research concentrates on extracellular lipases that are produced by variety of organisms (Rapp & Backhaus 1992). Studies on conditions for the production of extracellular lipases by *A. niger* show variations among different strains but the requirement of lipid carbon source is essential for enzyme production (Ohnishi et al.1994; Macris et al.1996; Cihnagir et al. 2004; Falony et al. 2006).

The technique of solid state fermentation (SSF) involves the growth and metabolism of microorganisms on moist solids without any free flowing water. According to Pandey et al (2001) there are two separate fermentation processes:

1) The solid substrate fermentation in which solid substrate itself acts as a carbon source and the fermentation is carried out nearly in absence of water. This is the most commonly used method of fermentation which involves cultivation of microorganisms on natural material.

2) The solid state fermentation which is carried out employing a natural inert solid support (which is not a substrate for the organism) in absence or near-absence of water. This system is less frequently used which involves cultivation of microbes on inert support impregnated with a liquid medium.

SSF processes have been extensively used in Asian and African countries but these processes were neglected by European countries. The keen evaluation of SSF and SmF has revealed that there are enormous and practical advantages of SSF over SmF. The advantages include non-aseptic conditions, use of raw materials as substrates, use of variety of inert solid supports, low capital and energy expenditure, less expensive downstream processing (since we deal with low volume extractions), less water consumption and low wastewater generation, higher volumetric productivity, higher product concentration, easier control of contamination and simpler fermentation media

(Durand & Chereau 1988; Raimbault 1998; Gowthaman et al. 2001). However, SSF has some limitations such as limited choice of microorganisms capable of growth under reduced moisture conditions, controlling and monitoring of parameters such as temperature, pH, humidity, air flow, difficulty in rapid determination microbial growth. In recent years, SSF has been receiving much importance even in Western countries since they have cheap and abundant agro-industrial waste to be used as substrate.

Application of SSF for production of antibiotics, secondary metabolites, bio-fuels, organic acids, aromatic compounds and industrial enzymes has been extensively studied (Pandey et al. 2003; Krishna 2005; Rodriguez et al. 2006). In early days, SmF was the preferred technology for enzyme production using genetically modified strains. However, there is significant interest in using SSF technology for production of industrial enzymes (Krishna 2005). Enzyme titers obtained in SSF processes are higher than in SmF processes. Thermo-stability of excreted enzymes and the low levels of catabolite repression are the added advantages of SSF technology (Acuna-Arguellel et al. 1995; Minjares Carranco 1997; Favela-Torres 1998; Diaz-Godinez 2001; Mateos Diaz et al. 2006). Cultures show tolerance to high concentration of metal ions yielding increased production of citric acid (Shankaranand & Lonsane 1994). Almost all the industrial enzymes can be produced using SSF technology. Much work has been carried out on the production of industrial enzymes such as amylase, glucoamylase (Selvakumar et al. 1996; Anto et al. 2006,), cellulases (Krishna 1999; Macris et al. 1999; Litifian et al. 2007) xylanases (Deschamps & Huet 1985; Wiacek-Zychlinska et al. 1994; Puchart et al. 1999), pectinases (Taragono & Pilosof 1999; Bottella et al. 2007) through SSF. Attempts are being made also to produce other enzymes such as phytase (Krishna et al. 2004) and tannase (Sabu et al. 2006) using SSF technology. Almost all commercial enzymes except alkaline proteases are derived from fungi. Few reports are available on production of bacterial enzymes in SSF (Archana & Satyanarayana 1997).

Lipases are currently produced through SmF as well as SSF, although SmF is preferred for certain situations. However, several reports on lipase production demonstrated that SSF gives highest titers and stable production of lipase. A number of solid substrates have been employed for the cultivation of microorganisms to produce lipases. Some of the substrates are sugar cane bagasse (Cordova et al. 1998), gingly oil

cake (Kamini et al. 1998), vegetable oil refinery residue (Miranda et al. 1999), Babassue oil cake (Gombert et al. 1999), cotton cake (Emtiazi et al. 2003), barley bran and triturated nut (Dominguez et al. 2003), soy cake (Di Luccio et al. 2004). Use of synthetic polymers like Amberlite IRA 900 (Christen et al.1997) and nylon sponge (Dominguez et al. 2003) have been reported.

Although, almost all the literature on SSF refers to fungal systems, there are very few reports on lipase production in SSF by *A. niger* to date (Olama & EI-Sabaeny 1993; Falony et al. 2006). Lipases active at highly acidic pH have not been reported so far from microbial sources. The objective of this study was production of acidic lipase by *A. niger* NCIM 1207 employing SSF as a major route of fermentation. The characterization of the enzyme with regard to thermo-stability, pH stability and optimum temperature and pH conditions for reaction are also described.

Materials and methods

Materials

Malt extract, yeast extract, bacto-peptone were obtained from Difco Chemical Co. Detroit, USA. Celite 545 was obtained from Fluka Chemie, AG Switzerland. n-Butanol (water content < 0.1%), laboratory grade reagent, was procured from May and Baker Ltd., UK. Butter oil, wheat bran, rice polish, DORB, soybean, maize, olive oil, sunflower oil, coconut oil, til oil, have been purchased from local supplier. All other chemicals were of analytical grade and procured from B.D.H., UK.

Methods

Microorganisms and growth media

The cultures were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India and were maintained on MYG medium (malt extract 0.5%, yeast extract 0.25%, glucose 1.0% and agar 2.0%). The rich MGYP medium contained (w/v) malt extract 0.3%, yeast extract 0.3%, bacto-peptone 0.5% and glucose 2.0%. *Aspergillus* minimal medium (AMM) contained (w/v) $NaNO_3$ 0.05%, $MgSO_4.7H_2O$ 0.05%, KCl 0.05%, KH_2PO_4 0.2%, yeast extract 0.1%, bacto-peptone 0.5%. Synthetic oil based (SOB) medium is AMM with 1% olive oil. The pH of the medium was adjusted to 5.5 with NaOH prior to sterilization.

Lipase production in SmF

The culture was grown in 500 ml Erlenmeyer flasks containing 100 ml MGYP or SOB medium. The flasks were inoculated with spores (approximately 10^6/ml) from a 7 days old culture on MYG slope and incubated at 30^{o}C on a rotary shaker (150 - 180 rpm) for 72 h. The mycelium was harvested by filtration and the culture filtrate was used as a source of extracellular enzyme.

Preparation of dry mycelium

The mycelium harvested by filtration was washed with distilled water twice to remove traces of medium contents and finally washed with chilled acetone. The acetone treated mycelium was vacuum dried for 6 h to remove acetone and water. This vacuum dried mycelium was used as a source of cell bound (intracellular) lipase enzyme in trans-esterification experiments.

Celite adsorption of extracellular enzyme

A standard procedure based on Colman and Macrae (1973) was used to immobilize the extracellular lipase. Celite 545 (1.0 g) was added to 20 ml of the culture filtrate with mixing. Ice-cold acetone (25 ml) was then added over a period of 5 min while stirring with magnetic stirrer and the suspension was stirred for an additional 30 min at 0^{o}C, then filtered and air dried. The celite adsorbed preparation (1.2 g) contained approximately 200 mg (±25) of water. This celite-adsorbed enzyme preparation was used as a source of extracellular enzyme in trans-esterification experiments.

Lipase catalyzed transesterification of butter oil as a measure of lipase activity

Screening of microorganisms for lipase activity was based on the ability to form butyl esters from butter oil in presence of butanol. Determination of lipase activity was carried out by transesterification of butter oil with butanol. The transesterification reaction was carried out in a 25 ml stoppered conical flask, which was shaken at 100 strokes per minute in a controlled temperature water bath at 35^{o}C. The reaction mixture contained 50 mg vacuum dried mycelium or 500 mg celite adsorbed enzyme preparation, 250 mg butter oil and 5.5 g of water saturated butanol. Fifty μl of water / buffer was added to the reaction mixture when the vacuum dried mycelium was used.

Lipase production in SSF

Erlenmeyer flasks (500 ml) containing 10g of wheat bran (or any other agricultural residue) moistened with 24 ml SOB medium with 1 ml of oil were sterilized at 121^{o}C for 30 min. After cooling, the flasks were inoculated with spore suspension (1 ml) containing 10^{6} spores from 7 days old culture grown on MYG slope. The contents of each flask were mixed thoroughly with inoculating needle for uniform distribution of fungal spores in the medium. The flasks were incubated at 30^{o}C. A flask was harvested, its contents were extracted for enzyme and lipase activity was assayed every 24 h for the period of 6 days.

Enzyme extraction

After SSF 100 ml of aqueous solution of NaCl (1%) was added to each flask and the mixture shaken on a rotary shaker (180 rpm) for 2 h at room temperature for extraction of enzyme from the fermented Koji (Al-Asheh & Duvnjak 1994; Ebune et al. 1995) At the end of extraction, the suspension was squeezed through a double layer muslin cloth and it was centrifuged at 5000 rpm for 20 min at 4^{o}C. The clear supernatant obtained was used as the extracellular enzyme.

Enzyme assay using pNPP substrate

The spectrophotometric method (Vonderwulbeche et al. 1992) with slight modifications was used for rapid and routine measurement of lipase activity using *p*-nitrophenylpalmitate (*p*NPP) as the substrate. The substrate solution was prepared by adding solution A (40 mg of *p*NPP in 12 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 emulsion obtained remained stable for at least 2 h. The assay mixture consisted of 0.9 ml of substrate solution, 0.1 ml of suitable buffer (0.5 M) and 0.1 ml of suitably diluted enzyme. The assay mixture was incubated at 50^{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.

Effect of pH on enzyme activity and stability

Lipase assay was performed from pH 1.5 to 4.5 using various buffer systems at 50 mM concentration using *p*NPP as substrate. Buffer systems used were KCl-HCl buffer (pH 1.0-2.0), citrate-phosphate buffer (pH 2.5-5.0). The effect of pH on lipase stability was determined by incubating the enzyme sample in 50 mM buffer systems at room temperature and the residual activity was assayed after 24 h under standard assay conditions. The buffer systems used were KCl-HCl buffer (pH 1.5-2.0), citrate-phosphate buffer (pH 2.5-6.0), phosphate buffer (pH 7.0), borate buffer (pH 8.0-9.0) and bicarbonate buffer (9.0-11.0).

Effect of temperature on enzyme activity and stability

Lipase activity was determined at different temperature under standard assay conditions. For temperature stability studies, the enzyme solution was incubated at different temperatures (50^oC – 75^oC) and the residual activity was assayed every hour of incubation up to 5 h under standard assay conditions.

Effect of organic solvent on enzyme stability

The effect of different organic solvents on enzyme stability was determined by pre-incubating 3 ml of crude enzyme in 1ml of organic solvent for 24 h at 30^oC with shaking at 160 rpm. Residual activity of suitably diluted enzyme was measured by spectrophotometric assay performed under standard assay conditions.

GLC analysis

Analysis of esters was carried out by GLC using capillary column (Phillips, 0.25 um film of silicon OV1, 3.8 m × 0.22 mm; injector and FI detector at 300^oC). For samples, which contained incompletely solvolyzed or unchanged triglycerides, the temperature was set at 40^oC for 3 min then rising at 3^oC/min up to 320^oC to elute unchanged triglycerides. Esters were identified by interpolation from standards. Analysis was normally carried out on 1 ml samples using added undecane (0.15 mg/ml) as an internal standard, which was prepared in n-hexane.

Results

Screening of lipase producing microorganisms in SmF

Fifty strains of species of *Rhizopus, Mucor, Geotrichum, Penicillium and Aspergillus* were screened for their ability to form butyl esters from butter oil in the presence of butanol. Lipase activity was based on the formation of butyl esters formed. Strains of two species *R. arrhizus* NCIM 877, 878, 879 and *A. niger* NCIM 1207 were found to be promising lipase producers. Data on the formation of one of the key esters (butyl oleate) are presented since it is not practical to show all the different esters formed. When grown on MGYP medium, *Rhizopus* strains produced both intra- and extracellular lipases while *A. niger* NCIM 1207 showed neither mycelial bound nor extracellular lipase (Table 1). Growth of strains in SOB medium demonstrated that *Rhizopus arrhizus* strains produced highest levels of intracellular lipase and *A. niger* produced maximum extracellular lipase (Table 1). In general, higher biomass was obtained in SOB medium. The transestrification reaction was carried out at different pH using mycelium bound lipases. It was observed that *A. niger* NCIM 1207 lipase was active at extremely acidic pH of 2.5 and lipase from *R. arrhizus* NCIM 877 was active at pH 5.5 - 6.0 (Figure 1).

Table 1. Lipase activity of cultures grown in MGYP medium and SOB medium.

Cultures	#Biomass (mg dry weight)		Ester formed (mg/ml)			
			Intracellular activity		Extracellular activity	
	MGYP	SOB	MGYP	SOB	MGYP	SOB
R. arrhizus NCIM 877	285	1085	0.56	5.10	0.35	0.23
R. arrhizus NCIM 877	225	990	0.34	3.04	0.13	0.30
R. arrhizus NCIM 877	275	1190	0.45	3.62	0.24	0.45
A. oryzae NCIM 643	465	1206	Trace	0.57	Trace	Trace
A. oryzae NCIM 1031	310	1050	0.10	0.51	Trace	Trace
A. niger NCIM 1207	450	1350	ND	0.58	ND	2.25

The biomass was obtained from 100 ml of the medium
Extracellular activity - Celite bound activity ND – Not detected
Intracellular activity - Mycelium bound activity Trace - < 0.05 mg ester formed

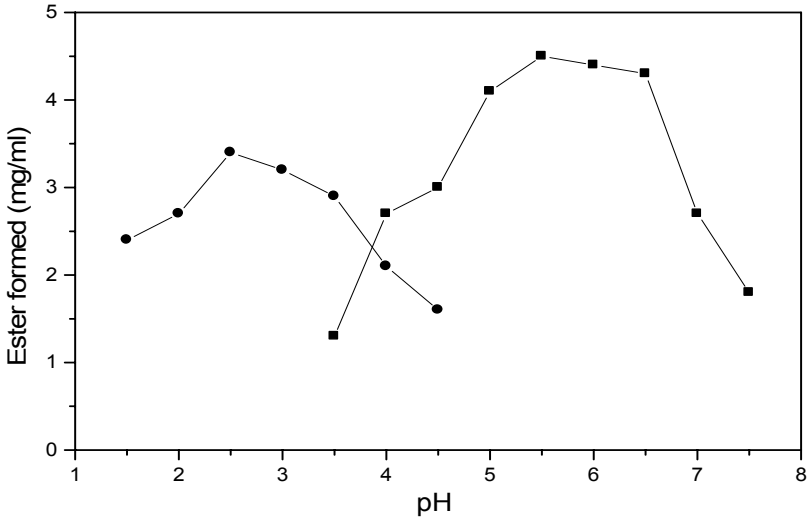

Figure 1. Effect of pH on trans-esterification activity of *A. niger* NCIM 1207 (●)and *R. arrhizus* (■) mycelium bound lipases.

Lipases that are active at extremely acidic pH have not been reported so far and hence we selected *Aspergillus niger* NCIM 1207 for further studies. In future experiments, spectrophotometric assay method was used for rapid and routine measurement of lipase activity using *p*NPP as the substrate.

Lipase production in submerged fermentation

Submerged fermentation was carried out in SOB medium in presence and absence of glucose (1%) and Triton X-100 (0.5%). Lipase activity was measured 2 days after the addition of Triton X-100 in the medium. The use of carbohydrates such as glucose in SOB medium appeared to be beneficial for enzyme production in case of *A. niger* NCIM 1207 (Table 2). This could be attributed to rapid assimilation of easily metabolizable carbon source compared to lipid producing more biomass and lipase activity. The addition of Triton X-100 to the fermentation medium resulted in enhanced levels (18 IU/ml) of enzyme production. Since *A. niger* NCIM 1207 produced high levels of extracellular lipase active at pH 2.0, further studies on optimization of enzyme production in SSF and its characterization were carried out.

Table 2. Effect of glucose on extracellular lipase production by *A. niger* NCIM 1207.

Carbon source	**Biomass[a] (mg dry weight)**	**Lipase activity[b] (IU/ml)**
AMM+glucose(1%)	400±40	0.02
AMM+olive oil(1%)	845±45	3.5
AMM+glucose(1%)+olive oil(1%)	1100±95	6.8
AMM +glucose(1%)+olive oil(1%)+ [c]Triton X-100 (0.5%)	1060±75	18.0

[a] The biomass was derived from 100 ml of the medium.

[b] The culture was grown in SmF and the activities were calculated after 5 days of incubation using *p*NPP as substrate.

[c] Triton X-100 (0.5%) was added on 3rd day of fermentation and the activity was determined after 5th day of fermentation.

Optimization of lipase production in SSF

Effect of moisture on lipase production

To check the influence of moisture on lipase activity during SSF, wheat bran (10.0 g) was moistened with different amounts of SOB medium and the results are shown in Figure. 2. Maximum enzyme yield was obtained when wheat bran was moistened with SOB medium in a 1:2.5 ratio. There was a decline in enzyme production above this ratio as the porosity of the medium is decreased.

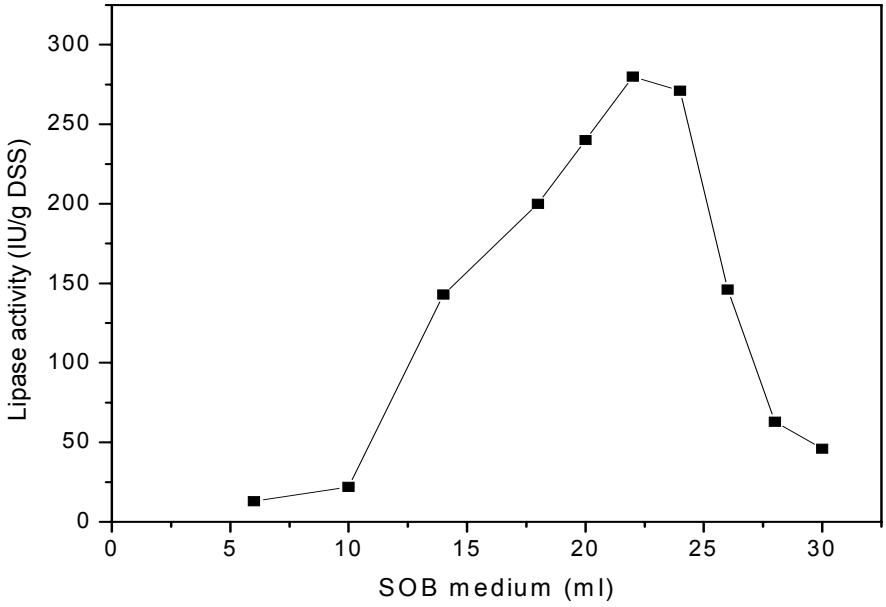

Figure 2. Effect of moisture level on lipase production in SSF.

Effect of different agricultural residues and oils on lipase production

Among the different agricultural residues tested for lipase production by *A. niger* NCIM 1207, wheat bran supported enzyme production far better than any other natural substrate (Table 3). Further optimization studies were carried out using wheat bran as the substrate.

The effect of different oils on lipase production was studied. From the results (Table 3) it could be concluded that *A. niger* NCIM 1207 lipase required a lipid based carbon source for their production, although their role in lipase synthesis is poorly understood. All oils tested supported enzyme production however, til oil and olive oil gave maximum yields of lipase activity.

Table 3. Production of lipase by *A. niger* NCIM 1207 in SSF using various solid substrates and oils.

Solid substrates and oil	Lipase production (IU/g DSS)
Wheat bran + olive oil	305 ± 27
Rice polish + olive oil	80 ± 5.0
DORB + olive oil	135 ± 20
Maize grains + olive oil	48 ± 1.2
Soybean seeds + olive oil	64 ± 1.5
Wheat bran + sarsonka oil	290 ± 5.0
Wheat bran + til oil	340 ± 24
Wheat bran + sunflower oil	125 ± 4.0
Wheat bran + castor oil	130 ± 4.0
Wheat bran + coconut oil	180 ± 5.0

Time course of enzyme production

SSF was carried out using wheat bran (10.0g) as solid substrate moistened with SOB medium (25 ml). The time course of lipase production using wheat bran as a substrate is given in Figure 3. Maximum enzyme activity was found on the 5th day of fermentation. A prolonged incubation time beyond this period did not help to further increase the enzyme yield.

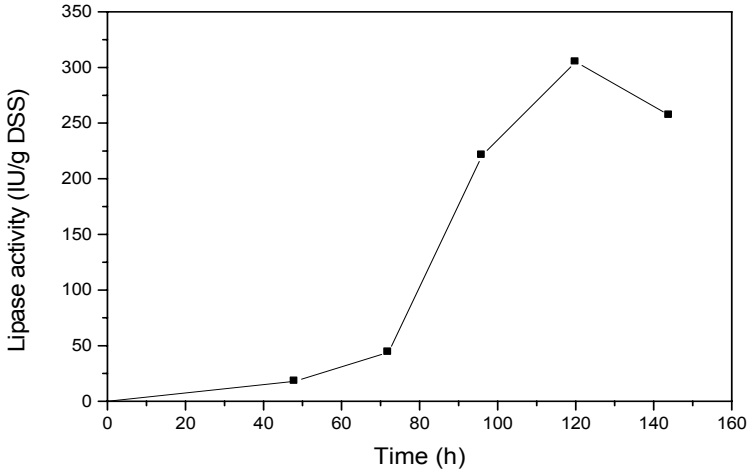

Figure 3. Time course of lipase production in SSF using wheat bran.

Extraction of enzyme using different salt solutions with and without surfactants

Extraction of enzyme from fermented wheat bran was carried out with tap water, distilled water, 1% NaCl or 1% $(NH4)_2SO_4$. There was no marked difference in the recovery of enzyme but NaCl (1%) was found to be effective for extraction of enzyme (Table 4). The supplementation of NaCl with Triton X-100 as surfactant (0.5%) helped in increased recovery (twofold) of enzyme from the fermented wheat bran. The yield of lipase was as high as 630 IU/g of DSS.

Table 4. Effect of different salt solution on lipase extraction from fermented wheat bran.

Salt solution	**Lipase production(IU/g DSS)**
Tap water	265 ± 24
Distilled water	230 ± 16
NaCl (1%)	310 ± 24
NaCl (2%)	260 ±14
$(NH_4)SO_4$ (1%)	270 ± 18
NaCl (1%) + Tween 80 (0.5%	320 ± 20
NaCl (1%) + Triton X-100 (0.5)	630 ± 24
NaCl (1%) + Triton X-100 (1.0 %)	625 ± 27

Characterization of enzyme in aqueous solutions

Effect of pH on enzyme activity and stability

Lipase assay was performed from pH 1.5 to 5.0 using variety of buffers at 50 mM in reaction mixture at 50°C for 20 min using *p*NPP as substrate. The optimum activity of lipase was observed between 2.5 and 3.0 with sharp decline in activity above the pH 3.5. However, the enzyme was very active even at pH 1.5 with 75% residual activity (Figure 4). The lipase was stable at a broad pH range from 2.5 (70% residual activity) to 9.0 (50% residual activity) after 24 h incubation at room temperature (Figure 5).

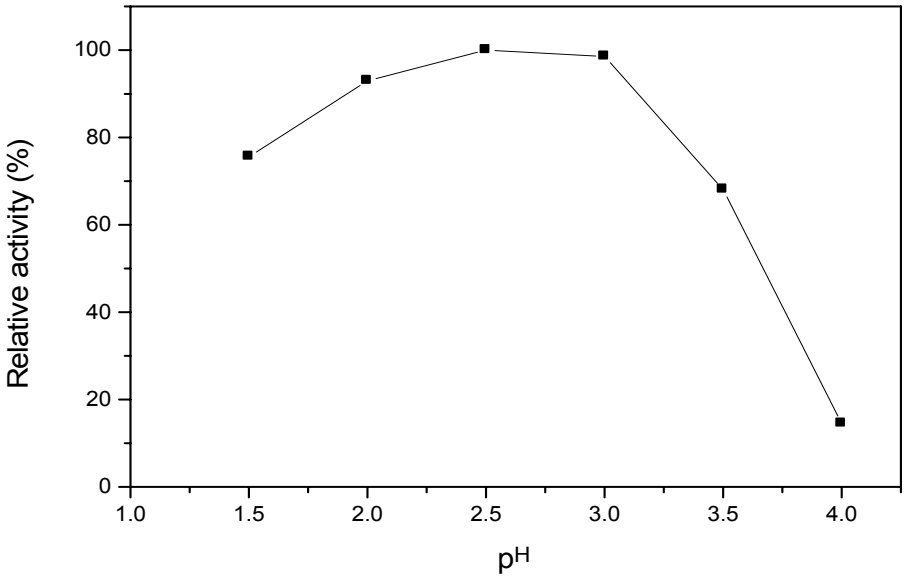

Figure 4. Effect of pH on lipase activity. Lipase assay was performed from pH 1.5 – 4.5 using various buffers at 50 mM at 50°C.

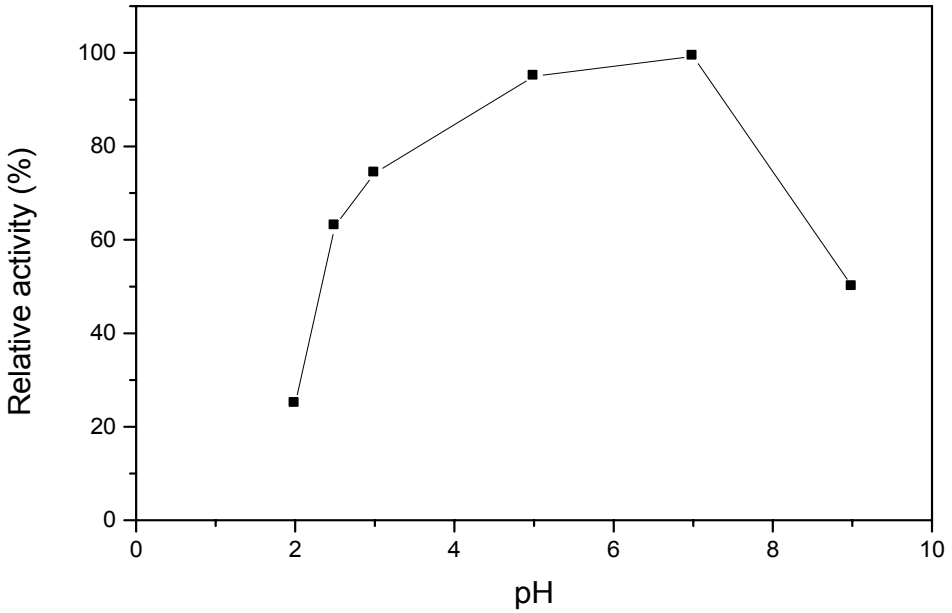

Figure 5. Effect of pH on lipase stability. The enzyme was incubated in 50 mM buffer systems. The buffer systems used were KCl-HCl buffer (pH 1.5-2.0), citrate-phosphate buffer (pH 2.5-6.0), phosphate buffer (pH 7.0), borate buffer (pH 8.0-9.0) and bicarbonate buffer (9.0-11.0). The residual activity was assayed after 24 h.

Effect of temperature on lipase activity and stability

Lipase activity was determined at different temperatures under standard assay conditions. The enzyme exhibited maximum activity at 50^{o}C with substantial activity between 30 and 55^{o}C (Figure 6). Studies on lipase stability at different temperatures revealed no loss of activity after 5 h incubation at 60^{o}C. The enzyme showed 40% of its original activity when incubated at 75^{o}C for 4 h (Figure 7).

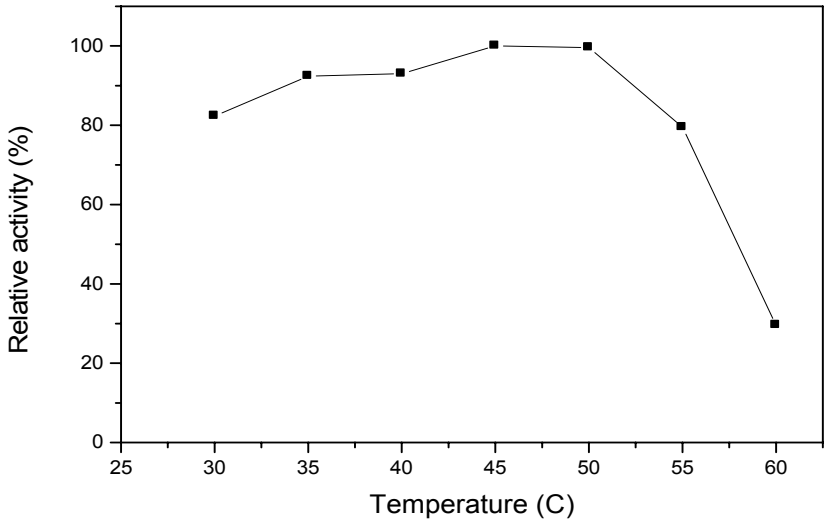

Figure 6. Effect of temperature on lipase activity.

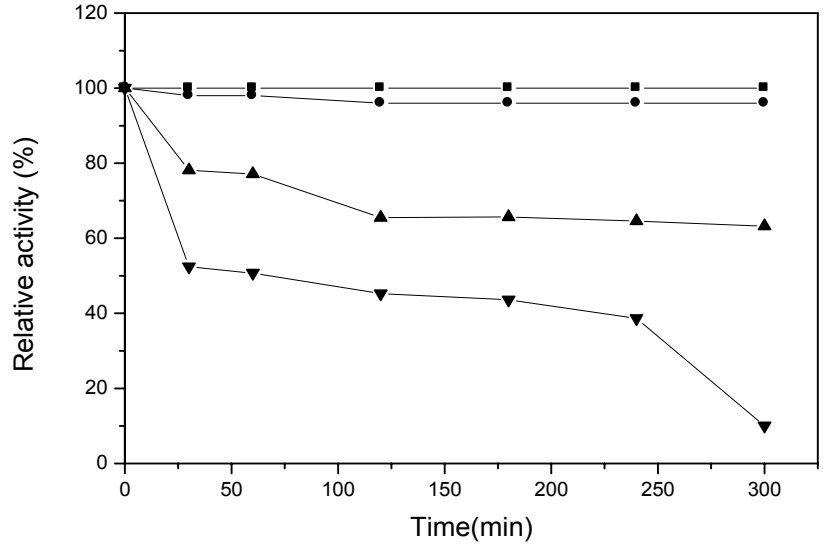

Figure 7. Effect of temperature on lipase stability. The enzyme solution was incubated at different temperatures and residual activity was assayed every hour up to 5 h.

50^{o}C (■---■), 60^{o}C (●---●), 70^{o}C (▲---▲), 75^{o}C (▼---▼)

Characterization of enzyme in organic solvent

Effect of different organic solvents on stability of crude enzyme

Attempts were made to check effect of water miscible and water immiscible solvents at 25% concentration on stability of lipase and the results are shown in Table 5. Crude enzyme showed more than 90% of its original activity even after 24 h of incubation in hexane, chloroform, acetonitrile, DMSO, ethyl acetate, isooctane, toluene, and n-hexadecane. Lipase was not stable in propan-1-ol and 1,4 dioxane loosing 97% and 89% of its original activity respectively.

Table 5. Effect of organic solvent on enzyme stability

Organic Solvent	**Relative Activity (%) after 24 h**	**Relative Activity (%) after 48 h**
Hexane	97.76	97.6
Chloroform	98.4	79.84
2-Propranol	74.24	72.0
Methanol	71.2	69.0
Isoamyl alcohol	38.88	22.8
Acetonitrile	95.84	85.0
DMSO	91.72	77.0
1,4 dioxane	11.2	10.1
Ethyl acetate	90.4	73.44
Isooctane	96.0	95.4
Toluene	91.0	80.16
Propan-1-ol	2.88	0
Butan-1-ol	37.6	25.6
Tertiary-butyl alcohol	43.52	37.0
n-hexadecane	91.52	91.0
Control	100.0	100.00

Discussion

It was observed that lipase activity in *Rhizopus* strains is associated with the cell. *A. niger* NCIM 1207 produced mainly high levels of extracellular lipase based on the formation of butyl esters. We also found out that lipase of *A. niger* NCIM 1207 was active at extremely acidic pH suggesting the uniqueness and novelty of the enzyme. This was the main reason why we selected this fungal strain for future studies. We were able to increase the activity in both SmF (18.0 IU/ml) and SSF (630.0 IU/g dry solid substrate) under the optimized conditions. Previous reports on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms. The present studies reported that *A. niger* NCIM 1207 produced mainly extracellular lipase in SmF conditions only in the presence of oil suggesting that lipase activity is induced by the presence of lipid substrates in the medium. Although lipid substrates generally act as inducers of lipases in many fungi (Large et al.1999; Wei et al. 2004), in some cases, such as *Aspergillus* (Pokorny et al. 1994; Long et al. 1996) and *Rhizopus* (Nahas 1988; Salleh et al. 1993), lipases are produced constitutively. On the contrary, lipid substrates inhibit the lipase production in some fungi (Lin et al. 2006). It is also worth noting that lipase production increased (two fold) upon addition of glucose in SOB medium. Such increase in lipase activity was also observed in case of *Aspergillus wentii* (Chander et al. 1980), *Mucor hiemalis* (Akhtar et al. 1980; Lin & Ko 2005) and *Geotrichum* (Ginalska et al. 2004). Rodriguez et al (2006) observed no differences in the activities when oil based medium was supplemented with sugars such as xylose, glucose, saccharose. Probably, this could be due to lower concentrations of sugars which are utilized before the oil and consequently, before lipase production.

The use of surfactants like Triton X-100 during SSF fermentation helps to increase enzyme production (Al-Asheh & Duvnjak 1994, Ebune et al. 1995; Mandviwala & Khire 2000). We also found that the incorporation of Triton X-100 in fermentation medium resulted in a threefold increase in enzyme production in SmF. This effect could be attributed to increased permeability of cells. Increase in enzyme levels in SSF was observed when enzyme from koji was extracted with NaCl (1%) supplemented with 0.5% Triton X-100. Similar enhanced recovery of lipase (1062 IU/g DM) was observed when

Rhizopus homothalicus fermented Koji was extracted with Triton X-100 at 0.5% (Rodriguez et al. 2006).

In the light of several advantages of SSF, lipase production from *A. niger* NCIM 1207 was attempted on various natural substrates among which wheat bran emerged as the most suitable with olive oil as lipid carbon source. Maximum enzyme activity (9.14/g of DDS) was obtained when *A. niger* was grown on wheat bran and olive oil (Falony et al. 2006). ul-Haq et al (2002) reported that *Rhizopus oligosporus* produced high levels of extracellular lipase by SSF using almond meal as the substrate. Bhushan et al (1994) reported lipase production from alkalophilic yeast species by SSF using rice bran and wheat bran. The universal suitability of wheat bran may be attributed to the presence of sufficient nutrients in it. In addition, it remains loose even in moist conditions, there by providing a large surface area (Feniksova et al. 1960).

Various oils such as olive, sunflower, corn, peanut, castor, coconut oils were used as energy and carbon sources for lipase production. Our fungal strain produced similar high lipase activity when grown on til (sesame) oil, olive oil, sarsonka oil suggesting that the cheapest and available oils could be used as carbon sources for industrial production of lipase. Generally, olive oil has been used for lipase production in both SmF and SSF (Pokorny et al. 1997; Gombert et al.1999; Lima et al. 2003; Falony et al. 2006; Rodriguez et al. 2006). We also obtained higher lipase activity when olive oil or til oil was used as a lipid substrate in SSF. Fatty acids present in the greatest proportions in these oils are oleic and linoleic acids. Triglycerides of olive oil and til oil contain 28% and 39% of oleic acid respectively. Better production appears therefore to be correlated with high content of oleic acid in the olive oil (Iwai & Tsujisaka 1984; Lima et al. 2003). Highest lipase activities were also obtained when sesame and corn oil were used in the medium which could be due to high oleic acid content in these oils (Hatzinkolaou et al. 1996; Maia et al. 2001). Rodriguez et al (2006) reported that lipase production was 40 times lower in the medium without oil as compared to medium with oil. Olive oil cake and olive mill water, the byproducts generated by olive oil industries, have also been used for lipase production as it contains variable quantities of residual oil (Cordova et al.1998).

The moisture content in SSF is a crucial factor that determines the success of the process (Feniksova et al.1960). The moisture level in SSF has a great impact on the physical properties of the solid substrate (Feniksova et al.1960; Pokorny et al. 1997). Moisture levels higher than optimum causes decreased porosity, lower oxygen transfer and alteration in wheat bran particle structure. Likewise, lower moisture than optimum decreases the solubility of the solid substrate, lowers the degree of swelling and produces a higher water tension. In the present work, maximum lipase production was obtained with wheat bran to SOB medium ratio of 1:2.5.

Lipases are diverse in their sensitivity to solvents. There is general observation that the water miscible solvents are more detrimental than water immiscible solvents (Nawani et al. 1998). In this work, the stability of *A. niger* NCIM 1207 lipase was investigated in various water miscible and water-immiscible solvents. Crude lipase from *A. niger* NCIM 1207 showed high stability in majority of both water miscible as well as water immiscible solvents even after exposure for 24 h at room temperature. Similar stability was found for lipase produced by *A. niger* MYA 135 in water miscible and water immiscible organic solvents (Pera et al. 2006). Crude lipase of *Penicillium crysogenum* is stable in hexane and 1,4 Dioxane at concentration of 50% whereas it is unstable in xylene (Bancerz et al. 2005). Activity of *Fusarium solani* lipase was increased by incubation in 50% n-hexane and toluene after 1 h (Maia et al. 2001). Lipase from *A. niger* was stable in 50% propan-2-ol or butanol up to 24 h (Macris et al.1996). The lipase from *A. niger* NCIM 1207 was stable in 25% hexane, chloroform, 2-propanol, methanol, acetonitrile, DMSO, dichloromethane, ethyl acetate, isooctane, toluene, ethylene glycol, propylene glycol, n-hexadecane. It was not stable in propan-1 ol and 1,4 dioxane. The results demonstrated that this lipase could become the potential candidate for use in organic synthesis and related application.

Generally, bacterial lipases have neutral or alkaline pH optima (Kanwar & Goswami 2002; Sunna et al. 2002) with the exception of lipase from *P. fluorescens* which exhibited acidic pH optimum at pH 4.8 (Anderson et al. 1979). Lipases from yeast have pH optima ranging between pH 5.0 - 7.5. Lipases from *Aspergillus* strain reported till today are active at pH 4.0 to 7.0 range and temperature between 40 and 55^{o}C (Pabai et al. 1995; Kamini et al. 1998; Namboodiri et al. 2000). Lipase from *A. niger* NCIM 1207 was

active at pH 2.5 with 75 and 90% residual activity at extreme acidic pHs 1.5 and 2.0, respectively. This enzyme is also stable over a broad pH range from 2.5 to 9.0 up to 24 h at room temperature. The optimum temperature of *A. niger* NCIM 1207 lipase is 50^{o}C and it is stable at 60^{o}C for 5 h. Lipase from *Aspergillus terreus* has excellent temperature tolerance (15 to 90^{o}C) and is also highly thermostable, retaining 100% activity at 60^{o}C for 24 h (Yadav et al.1998).While lipase from *Rhizomucor miehei* retains 90 % of its activity upon exposure to 65^{o}C for 30 min. (Uvarani et al. 1998). Very recently, it was observed that strains from the genus *Thermus* were able to grow even at 80^{o}C producing extremely thermostable lipases which are stable at 80^{o}C for 30 min (Dominguez et al. 2007).

In conclusion, a lipase producing strain of *A. niger* NCIM 1207 has been selected, which produced mainly extracellular enzyme. Elevated enzyme levels (630 IU/g dry solid substrate) were obtained in SSF using wheat bran which, to the best of our knowledge, were among the highest reported in the literature from *Aspergillus* sources. This lipase has some properties in common with lipases from other *A. niger* strains except the optimum pH (2.5) and the stability at highly acidic pH (2.5). In organic solvent, the lipase exhibited highest activity at pH 2.5, which is evident from transesterification activity. Such unique lipase, active and also stable at extremely acidic pH (2.5), has not been reported so far. This lipase was stable in majority of organic solvents such as hexane, chloroform, 2-propanol, methanol, acetonitrile, DMSO, dichloromethane, ethyl acetate, isooctane, toluene, ethylene glycol, propylene glycol, n-hexadecane. These results, together with the fact that this fungus is generally recognized as a safe (GRAS) microorganism in the food, beverage and pharmaceutical industry, make this process worthy of future investigation.

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CHAPTER – 3

Production of acidic lipase by mutants of *Aspergillus niger* NCIM 1207 in submerged fermentation

Abstract

Mutants of *Aspergillus niger* NCIM 1207, isolated by subjecting conidia to UV-irradiation, were tested for the production of lipase (glycerol ester hydrolase EC 3.1.1.3). Mutants UV-10 and ANCR-1 showed seven fold and five fold enhanced productivity of enzyme, respectively, over the parent strain under submerged fermentation when grown in SOB medium containing 1% olive oil. Maximum lipase activity (41.0 IU/ml) was obtained in the culture broth when UV-10 was grown in medium supplemented with 0.5% Triton X-100. A higher concentration of oil in the medium did not help lipase production in the case of mutant UV-10. No increase in enzyme levels was observed when mutant UV-10 was grown in medium supplemented with glucose. However, the addition of glucose in the medium resulted in increased levels of lipase production by wild strain, *Aspergillus niger* NCIM 1207.

Introduction

Microorganisms can generate new genotypes by two means: mutation and genetic recombination. In mutation, gene is modified either by spontaneous mutation or by induced mutation. Although some mutations are detrimental which can be eliminated by selection, some are beneficial to either microorganisms or humans. The mutation can be detected and preserved indefinitely. This is exactly what has been done in strain improvement program that led to the great expansion of the fermentation industry in the second half of the twentieth century. Mutations have improved the productivity of industrial cultures (Parekh et al. 2000). The mutation involves the treatment of a population of cells with mutagenic agent to obtain high yielding mutants. Treatment of cells with mutagenic agents continues untill desired killing (99.99% for some industrial programs) is obtained and survivors are selected randomly for product formation in flasks. The most useful mutagens are nitrosoguanidine (NTG), methylmethane sulfonate (MMS), ethylmethane sulfonate (EMS), ultraviolet light (UV).

Recombinant DNA technology approaches have been used for genetic modification of bacterial, yeast and fungal strains to promote expression of desirable genes, to hinder the expression of others, to alter the specific genes, or to inactivate genes for blocking specific pathways (Lemaux 2000). Recombinant DNA technology has been

used: 1) to increase enzyme productivities be use of multiple gene copies 2) to produce in industrial organisms enzymes from microbes that are difficult to grow or handle genetically 3) to produce useful enzymes in safe host that are not pathogenic or toxin producing 4) to improve stability, activity or specificity of an enzyme by protein engineering (Falch 1991). Recombinant DNA method has been adopted by industries to increase production levels and to produce enzymes from industrially unknown organisms in industrial organisms (Cowan 1996). Many enzymes currently used in food processing are derived from recombinant microorganisms. Virtually all laundry detergents use genetically-engineered enzymes (Cowan 1996). Most of the host strains used to develop production strains for food processing have been derived from relatively small number of bacterial, yeast and fungal species primarily *B. subtilis*, *B. licheniformis*, *S. cerevisiae*, *A. niger, A. oryzae*. These organisms are generally safe sources on native enzymes and are proven for their efficient growth under industrial production conditions (Olempska-Beer et al. 2006). Some organisms such as *E. coli K-12, Fusarium venenatum* and *Pseudomonas fluorescens*, with no history of use in industrial production of enzymes, have also been successfully used as hosts for expression of food processing enzymes. These organisms are recognized as non-pathogenic and GRAS cleared based on FDA regulations. More information can be found in several reviews on applications of mutation, genetic engineering and metabolic engineering to obtain improved microbial strains and processes (Bigelas 1989; Khetan & Hu 1999; Khosla & Keasling 2003; Adrio & Demain 2006).

Many microbial lipase genes have been cloned including some important commercial lipases like *Pseudomonas cepacia, Pseudomonas pseudoalcaligenes, Pseudomonas putida, Candida rugosa, Candida antarctica, Humicola lanuginosa, Rhizomucor miehei, Rhizopus delimar, Geotrichum candidum*. However, recombinant production has been limited to few microbial lipases, such as *C. antarctica* (Hoegh et al. 1995), *R. miehei* (Huge-Jensen et al. 1989), *Thermomyces lanuginosa* (Boel and Huge-Jensen 1989), *Pseudomonas pseudoalkaligenes* (Cox et al.1994). Bacterial lipases from various *Bacillus* species can be over-expressed in *E. coli* using conventional over-expression systems (Eggert et al. 2000; Kim et al. 2000; Nthangeni et al. 2001). Scientists at Novo Nordisk isolated desirable lipase for use in detergents from *Humicola*. For

production, the gene was cloned in to *Aspergillus oryzae,* which resulted in production of 1000 fold more enzyme (Carlsen 1990). The synthetic gene of *Rhizopus oryzea* was functionally overexpressed in *Pichia. pastoris*, to get high amount of *Rhizopus oryzae* lipase in *Pichia pastoris*.(Minning et al. 2001).

Among the microorganisms, fungi are widely recognized as the best lipase sources and are used preferably for industrial applications, especially in the food industry. *Aspergillus niger* is superior to several other fungal organisms with respect to lipase production and its enzyme is suitable for use in many industrial applications (Fu et al. 1995, Macris et al. 1996). Most research has been concentrated on extra-cellular lipases produced by a wide variety of organisms (Rapp & Backhaus 1992). In previous chapter, *A. niger* NCIM 1207 was grown in SSF to obtain enhanced production of acidic lipase. However, SSF has some limitations such as the limited choice of microorganisms capable of growth under reduced moisture conditions, controlling and monitoring of parameters such as temperature, pH, humidity and air flow (Nahara et al. 1982; Lonsane et al. 1985). In addition, the broth obtained from SSF is colored probably due to black spores of *Aspergillus* strains and it is also mixed with a number of unwanted ingredients coming from solid substrates during extraction.

The objective of the present studies was to identify mutants of *A. niger* capable of producing enhanced levels of acidic lipase under submerged conditions (SmF). Several mutants of *A. niger* NCIM 1207 were isolated and some exhibited enhanced levels of extra-cellular β-glucosidase activity (Gokhale et al. 1988). We also described the optimization studies on lipase production by one of the mutants capable of producing enhanced levels of acidic lipase in submerged fermentation (SmF).

Materials and Methods

Materials

Malt extract, yeast extract, bacto-peptone were obtained from Hi-Media Laboratories Limited, Mumbai, India. Olive oil was purchased from local supplier. All other chemicals were of analytical grade and procured from B.D.H. UK.

Methods

Microorganisms and growth media

Aspergillus niger NCIM 1207 and its mutants were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India, and were maintained on MYG medium as described in Chapter 2. Synthetic oil based medium (SOB) as described in Chapter 2 was used for production of enzyme.

Screening of mutants for lipase production in submerged fermentation

Cultures were grown in 250 ml Erlenmeyer flasks containing 70 ml of SOB medium. Flasks were inoculated with spores (approximately 10^6/ml) from a 7-day-old culture on MYG slope and incubated at 30^{o}C on a rotary shaker (150–180 rpm) for different period of time. The mycelium was separated by filtration and the culture filtrate was used as a source of extra-cellular enzyme.

Determination enzyme activity and biomass

Lipase activity was estimated by spectrophotometric method using *p*NPP substrate as described in chapter 2. The biomass was determined by washing the separated mycelium with water several times and kept for drying at 80^{o}C till we obtained constant weight.

Results

Screening of mutants for lipase production

Mutants of *A. niger* were isolated by exposing conidia to ultraviolet irradiation and plating the irradiated conidia on MGYP agar plates (Gokhale et al. 1988). Some of the mutants were tested for lipase production and comparative enzymic activities are given in Table 1. Auxotrophic mutants exhibited comparatively low levels of lipase production. Introduction of methionine auxotrophy resulted in approximately 80% decrease in enzyme activity compared to the wild strain. Among the mutants tested, UV-10 and carbendazim resistant mutants produced higher amounts of lipase in the medium. UV-10 mutant was selected for further optimization studies on lipase production in submerged fermentation.

Table 1. Production of lipase by mutants of *Aspergillus niger* NCIM 1207.

Strain	**Genotype / Phenotype**	**Lipase activity (IU/ml)**
Aspergillus niger NCIM 1207	Wild	3.2
UV-4	met, cyst	0.58
UV-7	met, cyst	1.09
UV-10	Prototroph, compact colony Morphology	25.12
UV-12	aspn	0.33
UV-22	Leu	1.59
UV-61	leu, met	0.57
UV-101	Prototroph, big spore head	2.52
ANCR-1	Carbandazim resistant	15.05
UV-III-39	Prototroph, fawn color spores	2.83

met – methionine, cyst – cystein, aspn – asparagines, leu – leucine,

Time course of lipase production

The profile of lipase production by UV-10 mutant and the wild strain is shown in Figure 1. Compared to the wild strain, mutant UV-10 produced more lipase activity at all intervals of time with highest activity detected on 7th day of incubation. This activity was eight times more than that secreted by wild type strain.

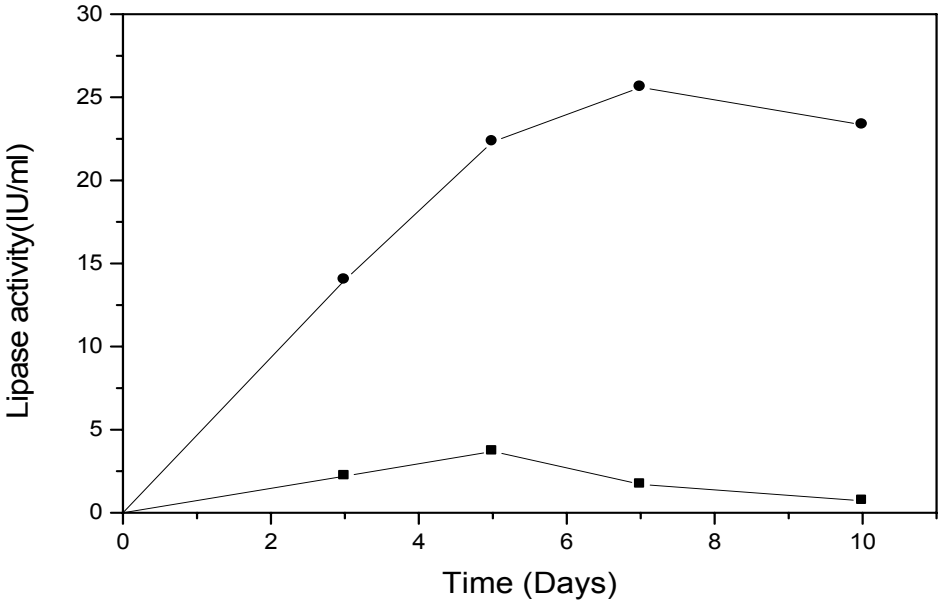

Figure 1. Time course of lipase production in submerged fermentation. (■) *Aspergillus niger* NCIM 1207; (●) Mutant UV-10.

Effect of different amounts of oil in SOB medium on lipase production

The effect of different amounts of oil in the medium on lipase production demonstrated that *A. niger* NCIM 1207 yielded both more biomass and lipase activity (9.6 IU/ml) with increase in oil concentration. However, no increase in lipase production by UV-10 was observed even though there was an increase in biomass with increase in oil concentration (Table 2).

Table 2. Effect of oil concentration in the medium on lipase production

Strain	Concentration Of oil (%)	Lipase activity after			
		5th Day		7th day	
		Biomass (mg dry weight)	Lipase activity (IU/ml)	Biomass (mg dry weight)	Lipase activity (IU/ml)
A. niger NCIM 1207	1.0	535	2.2	622	1.7
	2.5	1447	7.4	1305	3.1
	5.0	2584	6.8	2459	9.6
Mutant UV-10	1.0	405	22.3	677	25.6
	2.5	1127	23.6	1825	25.6
	5.0	2568	21.0	2860	25.8

Effect of glucose concentration on lipase production

To study the effects of glucose on lipase production, the organisms were grown in SOB medium containing different concentrations of glucose. Data indicating the enzyme activity and biomass are given in Table 3. The observations indicate that no repression of enzyme production was observed up to 2.5% glucose for parent strain. However, the repression was observed even at 1% glucose for mutant strain. Higher concentrations of glucose did effect pronounced repression in case of both wild as well as mutant strain. While there was 5.5 fold increase in lipase at 2.5% glucose in case of wild strain, lipase production by mutant strain was drastically decreased at this concentration of glucose. Maximum repression of lipase was caused by 5% glucose. The addition of glucose always resulted in a sudden drop in the pH of the medium. This drop in pH was more pronounced when mutant was grown in glucose supplemented media. The biomass of both strains increased with concomitant increase in glucose concentration in the SOB medium. It also appeared from the results that the lipase production by both the strains was severely affected when the pH of the medium falls below 3.0.

Table 3. Effect of various concentration of glucose on lipase production.

Strain	Glucose (%)	5th day			7th day		
		Biomass (mg dry weight)	pH	Lipase activity (IU/ml)	Biomass (mg dry weight)	pH	Lipase activity (IU/ml)
A. niger NCIM 1207	-	593	3.1	3.2	570	3.3	1.7
	1	923	3.2	6.1	737	3.8	5.6
	2.5	1250	3.0	7.2	1028	4.0	9.0
	5.0	1609	2.0	0.3	1722	2.0	0.9
Mutant UV-10	-	697	3.2	13.1	614	4.1	25.5
	1	1152	2.7	16.3	1015	3.0	23.6
	2.5	1456	2.6	11.6	1450	2.8	12.7
	5.0	1820	1.9	4.3	1950	2.0	5.0

Effect of glucose and Triton X-100 on lipase production

Submerged fermentation was carried out in the presence and absence of glucose (1%) and Triton X-100 (0.5%). Lipase activity was measured 2 days after the addition of Triton X-100 in the medium (Table 4). There was a three fold increase in lipase production in case of parent strain grown in presence of Triton X-100. However, mutant UV-10 produced 1.6 times more lipase in presence of Triton X-100. The addition of glucose in the SOB medium did increase lipase secretion in the case of the mutant strain.

Table 4. Effect of glucose and Triton X-100 on lipase production.

Medium used	Lipase activity (IU/ml)	
	A. niger NCIM 1207[a]	Mutant UV-10[b]
SOB	3.2	25.5
SOB + Triton X-100 (0.5%)	11.8	40.2
SOB + Glucose (1%)	6.1	24.0
SOB + Glucose (1%) + Triton X-100 (0.5%)	18.0	41.0

[a] Triton X-100 was added on 3rd day of fermentation and activity was determined after 5th day.

[b] Triton X-100 was added on 5th day of fermentation and activity was determined after 7th day.

Discussion

Some mutants of *A. niger* NCIM 1207 were tested for lipase production under submerged condition. In general, introduction of auxotrophy resulted in decreased enzyme production. Methionine auxotrophs produced low levels of lipase. At present we cannot correlate between auxotrophy and lipase production. This is possible only when a large number of auxotrophs are screened for lipase production. One of the mutants, designated as UV-10, was found to be the best lipase producer (25.0 IU/ml) compared to the parent strain (3.2 IU/ml) under submerged conditions. Tan et al (2003) isolated mutant of *Candida* sp. by a series of mutation methods including UV, NTG treatment and quick neutron mutation. The lipase produced by the mutant increased ten times compared to the parent strain. Mutants of *Yarrowia lipolytica* with enhanced lipase productivity were obtained either by chemical mutagenesis or genetic engineering (Fickers et al. 2003; 2005). New strains were developed using one of these mutants by gene amplification of *LIP2* gene which produced 1,58,246 U/ml of lipase after feeding a combination of tryptone and olive oil in batch culture. Electro-fusion of protoplasts derived from *Rhizopus cohnii* mutants produced 19 fusion products showing higher lipase biosynthesis capacity than that of parent strain (Sawicka-Zukowska et al. 2004). One of the fusants produced 3.5 times higher lipase activity than that produced by parent strain

The effect of different amounts of olive oil in the medium demonstrated that *A. niger* NCIM 1207 yielded more biomass and lipase activity with increase in oil concentration up to 2.5%. Higher concentration of oil (5%) led to repression of enzyme synthesis. Production of lipase decreased with increase in glucose concentration in case of mutant strain suggesting the repression of enzyme synthesis. These results indicate the differences in pattern of lipase biosynthesis in mutant strain in response to olive oil concentration. Lipase synthesis has been reported to be repressed by high concentration of olive oil (Kok et al.1996; Lin et al. 1996; Chen et al.1998; Lima et al. 2003). The repression of lipase could be attributed to oxygen transfer limitations (Lima et al. 2003) or due to the cell lysis (Chen et al.1998).

It is well known that the addition of glucose in the medium stimulates the lipase production (Kamini et al. 1998; Rathi et al.2001; Bapiraju et al. 2005; Costas et al. 2004; Lin et al. 2005). Costas et al (2004) have achieved maximum lipolytic activities of

Issatchenkia orientalis in a medium with glucose and fructose as carbon sources. Kamini et al (1998) have indicated that the addition of glucose and sucrose increased lipase production by *A. niger*. Sucrose was the best for high lipase production by *P. chrysogenum* but with low biomass. However the biomass was significantly increased when glucose was added in the medium (Bancerz et al. 2005). We observed significant increase in lipase production by wild strain in glucose supplemented media. However, no increment in lipase production was found in case of mutant strain. Higher concentrations (5% and above) of glucose caused sudden drop in pH of the fermentation medium below 3.0. This drop in pH could be responsible for inactivation of lipase. Such phenomenon was reported earlier when *A. niger* NCIM 1207 was grown in presence of high concentration of glucose for cellulase production (Gokhale et al. 1991). Parent strain *Aspergillus niger* NCIM 1207 and its Mutant UV-10 showed totally different pattern of utilization of carbon source. Inhibitory effect of glucose was reported by (Nahas 1988; Macris et al. 1996; Dalmau et al. 2000; Wei et al. 2004). Fadiloglu & Erkmen (2002) has reported inhibitory effect of glucose and lactose on *Rhizopus oryzae*. He also reported that addition of glucose and lactose to the medium with oil show negative effect on both biomass and activity.

It is well documented that surfactants can increase the cell permeability facilitating the export of several molecules across the cell membrane. Our optimization studies revealed that the mutant exhibited maximum lipase activity (41.0 IU/ml) when grown in Triton X-100 supplemented SOB medium. However, the effect of Triton X-100 on the mutant was not as significant as in case of wild type indicating the altered cell wall/cell membrane permeability in case of mutant strain. Such permeability changes may sometimes lead to increased productivity presumably through an increased rate of export from the cell (Li et al. 2001). The effect of addition of surfactants like Tween20, Tween80, Triton-X-100, SDS, PEG and gum arabic on lipase production was investigated (Silva et al. 2005). It was found that highest lipase activity was obtained when SDS, Tween 20 and Tween 80 were added after 50 h of *Metarhizium anisopliae* growth. Li et al (2001) obtained good recovery of lipase from *Acinetobacter radioresistens* using Tween 80 as carbon source. Tween 20 and Tween 80 were also used in the media as sole carbon source which stimulated lipase production since these

compounds have been placed in the category of synthetic lipids (Thomson et al. 1999). In present study, Triton-X 100 was added after 72 h and 120 h of growth of *A. niger* NCIM 1207 and mutant UV-10 respectively. Lipase activity was measured 2 days after the addition of Triton X-100 in the medium. There was a three fold increase in lipase production in the case of the parent strain grown in the presence of Triton X-100. However, mutant UV-10 produced 1.6 times more lipase in the presence of Triton X-100.

Mala et al (2001) isolated UV and nitrous acid derived mutants of *A. niger* selected on media containing bile salts. Nitrous acid mutants exhibited increased efficiency of lipase production compared with UV mutants. However, this lipase was active at pH 6.0 and was not active at extremely acidic pH. Increase in lipase yield 200% was reported by Ellaiah et al (2002) in case *A. niger* mutant derived from UV and NTG mutagenesis.

Visual selection for altered colony morphology on the surface culture has been proposed as a means of identifying improved strains (Rawland & Normansell 1983). A mutant of *Penicillium funiculosum* characterized by compact colony morphology showed enhanced productivity of both endoglucanase and xylanase enzymes (Lachke et al. 1986) Different morphological mutants of *A. niger* NCIM 1207 produced two fold increase in β-glucosidase production over the wild strain (Gokhale et al. 1988). Present studies revealed that the mutant UV-10 exhibiting compact colony morphology produced enhanced levels of lipase under submerged conditions. This mutant could be an excellent source of lipase.

Alteration in cell wall/cell membrane permeability is a common mechanism of resistance to toxic chemicals. These permeability changes may result in increased productivity presumably due to an increased rate of export from the cell. Any resistance mutation causing alteration in cell wall/cell membrane structure is potentially capable of allowing increased export of metabolites. Schimenti et al. (1983) have isolated nystatin resistant mutants of *T. reesei* that showed increased production of cellulase. Carbendazim resistant mutant (ANCR-1) of *A. niger*, isolated in this study, also showed five fold increase in lipase production compared to wild strain.

In conclusion, the mutants of *A. niger* NCIM 1207 were screened for lipase production in submerged fermentation (SmF). Mutant UV-10 produced enhanced levels (seven fold) of lipase when grown in SOB medium containing 1% olive oil. Maximum lipase activity was obtained when UV-10 was grown in medium supplemented with 0.5% Triton X-100. A higher concentration of oil in the medium did not help lipase production in the case of mutant UV-10. No increase in enzyme production was observed when mutant UV-10 was grown in medium supplemented with glucose. However, the addition of glucose in the medium resulted in increased levels of lipase production by wild strain, *Aspergillus niger* NCIM 1207.

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CHAPTER – 4

Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207

Abstract

An extracellular acidic lipase (3.1.1.3) from *Aspergillus niger* NCIM 1207 has been purified to homogeneity using ammonium sulfate precipitation followed by phenyl sepharose and sephacryl-100 gel chromatography. This protocol resulted in 149 fold purification of lipase with 54% final recovery. The purified enzyme showed a prominent single band on SDS-PAGE. The molecular weight of the lipase was estimated to be 32.2 kDa using SDS-PAGE and MALDI-TOF suggesting that lipase likely to function as a monomer. The isoelectric point (pI) of lipase was 8.5. The enzyme is rich in acidic and hydrophobic amino acids. The pH and temperature optima for purified enzyme were 2.5 and 50^{o}C respectively. The lipase was stable over a pH range between 8.0-11.0 and its half life of inactivation ($t^1/_2$) at 50^{o}C was 1h**.** The lipase from *A. niger* NCIM 1207 showed high stability in presence of various organic solvents since it retained more than 90% of its original activity after exposure for 24 h at 30^{o}C in 25% hexane, methanol, DMSO, isooctane, toluene, n-hexadecane, acetone and ethanol. There was total inactivation of enzyme activity in presence of 2-propanol, propan-1-ol, butan-1-ol, and tertiary-butanol. Ag^{+} strongly inhibited the enzyme activity. Action on *p*-nitrophenyl esters of varying chain lengths suggested the high preference of the enzyme towards medium acyl chain length esters. The K_m and V_{max} for *p*-nitrophenyl palmitate was 0.33 mM and 746 μmoles $min^{-1}mg^{-1}$ respectively. The K_m and V_{max} for *p*- nitrophenyl caprylate was 0.14 mM and 1066 μmoles $min^{-1}mg^{-1}$ respectively. The results analysis of hydrolysis product of triolein indicated that the enzyme cleaved only 3-position ester bond. Chemical modification studies revealed that His, Ser, Carboxylate and Trp are involved in catalysis. The substrate protection studies suggest that Trp may have a role in substrate binding.

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 (Vulfson 1994). 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.

Extracellular lipases have been proven to be efficient and selective biocatalysts in many industrial applications such as biosensors, pharmaceuticals, foods, cosmetics, detergents (Pandey et al. 1999). Filamentous fungi are widely recognized as preferred sources of extracellular lipases, which facilitate the enzyme recovery from the broth. Most of the fungal lipases reported so far are from *Geotrichum, Rhizopus, Mucor, Rhizomucor, Aspergillus and Penicillium. Aspergillus niger* is one of the most important microorganisms used in biotechnology which produces many extracellular enzymes that are recognized as GRAS (Generally Regarded As Safe) by the FDA (Schuster et al. 2002). *A. niger* is also known to produce several lipases and esterases (Pokorny et al. 1997) and some of them are well characterized.

Lipases have been purified from animal, plant, fungal 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 (Vicente et al. 1990; Yadav et al. 1998) and aqueous two-phase systems (Queiroz et al. 1995; Bradoo et al. 1999), membrane processes (Sztajer & 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).

Active site directed chemical modification is an important tool for identifying the residues at the active site of an enzyme responsible for catalytic activity. Presently, site directed mutagenesis is widely employed for this purpose which requires the knowledge of three dimensional structure of protein. In contrast, the active-site directed chemical modification is simpler because it does not require the information of three dimensional structure of an enzyme.

During screening of lipase producing organisms, we found that *A. niger* NCIM 1207 produced high levels of extracellular lipase active at pH 2.5. Although many *Aspergillus* lipases have been reported, none of them was found to be active at such acidic pH. In this chapter, we describe the separation, purification and characterization including active site determination of unique lipase produced by *A. niger* NCIM 1207 under submerged fermentation.

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 and Bromophenol Blue, 1(3) monoolein, 2-monoolein, 1,2(2,3) diolein, 1,2 diolein , triolein, oleic acid, *N*-ethylmaleimide (NEM), iodoacetate, phenylmethyl-sulfonylfluoride (PMSF), diethylpyrocarbonate (DEPC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *N*-bromosuccinimide (NBS), *N*-acetylimidazole (NAI), citraconic anhydride, phynylglyoxal HEPES, and MES were purchased from Sigma Chemical Company, U.S.A. Sephacryl-100, Phenyl Sepharose CL-4B were obtained from Pharmacia, Sweden. All other chemicals used were of analytical grade and of the highest purity available locally.

Methods

Enzyme assay

Enzyme activity was assayed as described in Chapter 2. The amount of *p*-nitrophenol liberated following the hydrolysis of *p*-nitrophenylpalmitate , at pH 2.5 and 50^{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. However, during enzyme purification steps, protein concentrations were determined by using the formula 1.7 A_{280} = 1mg/ml of protein.

Enzyme purification

Cultivation of *Aspergillus niger* NCIM 1207 and enzyme production were carried out as described in Chapter 2. Unless otherwise stated, all operations were carried out at 6 ± 1^{o}C.

Ammonium sulfate precipitation

Ammonium sulfate was added to culture filtrate (500 ml) to get 90% saturation at 4^{o}C, with constant stirring and left overnight. The precipitated protein was collected by filtration, dissolved in minimum volume of 10mM Glycine-NaOH buffer pH 9.0. The protein solution was extensively dialyzed against the same buffer, lyophilized and subjected to Phenyl-Sepharose chromatography.

Phenyl-Sepharose chromatography

The enzyme sample obtained from the above step was loaded onto a Phenyl-Sepharose column (2.5 x 16.5 cm) pre-equilibrated with 10 mM Glycine-NaOH buffer, pH 9.0, containing 20%(w/v) ammonium sulfate at a flow rate of 60 ml/h. The column was then washed with the same buffer till the flow-through fractions showed no lipase activity. The bound enzyme was then eluted with decreasing concentrations of ammonium sulfate. Fractions of 5ml were collected and those exhibiting specific activity more than 4.5 IU/mg were pooled, dialyzed extensively against the 10 mM Glycine-NaOH buffer, pH 9.0. The dialyzed fraction was concentrated by lyophilization and used for the next step.

Sepacryl S-100 chromatography

The concentrated enzyme obtained from the above step was chromatographed on a Sephacryl S-100 column (1.5 x110cm) pre-equilibrated with 10mM Glycine-NaOH buffer, pH

9.0, at a flow rate of 12 ml/h. Fractions of 1.8 ml were collected and those having specific activity more than 28.0 IU/ml were pooled and concentrated by lyophilization. This concentrated fraction was stored at –20^{o}C till further use.

Molecular mass determination (Mr)

The relative molecular mass (Mr) of the purified lipase was determined by Matrix Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) mass spectrophotometry, using Voyager DE-STR (Applied Biosystems, USA) equipped with a 337nm nitrogen laser. The matrix was prepared in deionized water containing sinapinic acid (10 mg/ml), 50% acetonitrile and 0.1% TFA. Lipase was mixed with matrix (1:1) and 2μl of the sample was spotted on plate, dried at room temperature and analyzed.

Molecular weight was also determined by using 12% (w/v) SDS-polyacrylamide gel electrophoresis at pH 8.8 according to Laemmli (1970) with different molecular weight markers. After elecrophoresis, the bands were visualized by silver staining according to Deutscher (1990).

Isoelectric focusing

a) The Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE) was performed (7.5% concentration) in a vertical gel apparatus using wide range ampholyte pH (3-10). Approximately 20 µg of purified protein was applied to the gel and focused at 200–400 V for a period of 4-5 h. Protein band was visualized with Coomassie Brillient Blue R-250 stain (Deutscher 1990).
b) Isoelectric point (pI): The Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE) was performed in a vertical gel apparatus using wide range Ampholytes pH (3-10). Approximately 20 µg of purified protein was applied to the gel and focused at 200 –400 V for a period of 4-5 h. After running the gel, gel has cut into 0.5 cm pieces and immersed in 1ml of KCl (10mM), soaked for 30 min and this fractions were checked for its pH.

Amino acid analysis

Salt-free lyophilized enzyme sample (100 µg) was hydrolyzed in gas phase 6N HCl for 20h at 110^{o}C. Following hydrolysis, the sample was again lyophilized, dissolved in 200µl of sample loading buffer (0.4M sodium borate, pH 10.2) and 1µl of the sample was subjected to analysis on a Hewlett-Packard Amino Quant with a diode array detector. Primary amino acids were detected as orthophthaladehyde derivatives.

Positional specificity

The total reaction mixture of 2 ml containing 20mM triolein in 50mM citrate phosphate buffer (pH 2.5) was incubated with 10 IU of the enzyme activity at 30^{o}C, under shaking (200rpm). Aliquots of 200ul were removed at fixed time intervals and the products were extracted with 0.8 ml of diethyl ether. The extracted samples were applied to silica gel-60 TLC plates (Merck, Darmstadt, Germany) for product analysis. The plates were developed in a chamber with a solvent mixture of chloroform and acetic acid (96:4). The hydrolysis products were visualized using saturated iodine chamber and compared with standards from Sigma.

Circular dichroism (CD) and analysis of secondary structure

The secondary structure of native lipase at 25^{o}C was determined by CD spectroscopy. Spectra were recorded on a Jasco-710 Spectropolarimeter in the far UV region (190 to 250 nm) using a 250 µl sample holder and 0.1 cm path length cell. The concentration of enzyme used was 125µg/ml. The temperature of the sample was controlled at 25^{o}C. Results are expressed as [θ] (mdeg cm^{2} dmol^{-1}).

Lipase characterization

Effect of pH on enzyme activity and stability

The effect of pH on enzyme activity was determined by assaying lipase activity at 50^{o}C in a pH range of 2.0-11.0 using following 50 mM buffer systems: KCl-HCl buffer (pH - 2.0), citrate phosphate buffer (pH 2.5 – 6.0), Phosphate buffer (pH 7.0), and Glycine-NaOH buffer (pH 8.0 – 11.0).

Stability assay was performed by incubating the purified enzyme at 30^{o}C for 24 h in 50 mM buffers of different pH values (KCl-HCl buffer, pH - 2.0; citrate phosphate buffer, pH 2.5 – 6.0; Phosphate buffer, pH 7.0; and Glycine-NaOH buffer, pH 8.0 – 11.0). The residual activity was then assayed using under standard assay conditions considering the enzyme activity at zero time as 100%.

Effect of temperature on enzyme activity and stability

Measurements of enzyme activity were carried out at pH 2.5 under standard assay conditions. The reaction mixture was incubated at different temperatures covering the range of 30 – 70^{o}C.

For determining the temperature stability, enzyme solution was incubated in 50 mM Glycine- NaOH buffer (pH 9.0) for 3 h at different temperatures covering the range of 30-70^{o}C. The residual enzyme activity was determined using standard assay conditions and compared with the control without incubation.

Effect of organic solvents on enzyme stability

Lipase stability in water-miscible organic solvents was determined by incubating the enzyme solution prepared in 10 mM Glycine-NaOH buffer (pH 9.0) with 25% (v/v) organic solvent for 24 h at 30^{o}C. The residual activity was estimated under standard assay conditions.

Lipase stability in water-immiscible organic solvents was determined by incubating the enzyme solution prepared in 10 mM Glycine-NaOH buffer (pH 9.0) with 25% (v/v) organic solvent for 24 h at 30^{o}C with shaking at 100 rpm on rotary shaker. The residual activity was estimated under standard assay conditions.

Effect of metal ions and EDTA on enzyme activity

For determining effect of metal ion and EDTA on lipase activity, enzyme assays were performed in presence of various metal ions and EDTA at a final concentration of 0.1 and 1 mM using *p*NPP as substrate.

Determination of Michalis-Menten constant

Values for the K_m and V_{max} were determined from double reciprocal plots of substrate concentration versus initial reaction rates (Lineweaver and Burk 1934). Enzyme assay with appropriately diluted lipase were performed using either *p*-nitrophenyl caprylate or *p*-nitrophenylpalmitate as a substrate under standard assay conditions. The concentrations 0.066 mM to 0.8 mM were chosen to run the assay.

Chemical modification studies

Effect of group specific reagents on lipase activity

Purified lipase (5μg)) was incubated with chemical reagents specific to different amino acid functional groups with specific concentration. Reaction conditions were given in Table 1. After 30 min incubation at 25^{o}C, residual activity of enzyme samples was determined by standard assay method.

Chemical modification of histidine residues

Purified lipase (5 μg) in 1 ml 50mM sodium phosphate buffer, pH 6.0, was treated with DEPC (100 mM, freshly diluted in absolute ethanol) at 25^{o}C. The reagent was added in reaction mixture to get final concentration of 0.5, 1.0, 1.5, and 2.0 mM. The reagent was allowed to react with enzyme for the period of 30 min. The aliquots were removed and assayed for enzyme activity under standard assay conditions. Control sample was treated similarly as above; only DEPC in the reaction mixture was replaced by absolute ethanol.

For the determination of number of histidine residues involved in active site, the lipase(100μg) was incubated with different concentration of DEPC (0, 15, 20, 25, 30 mM).The aliquot was removed from each concentration after 5, 10, 15, 20 min for analysis of residual lipase activity. The pseudo-first order rate constant was calculated from the slope of the logarithm plots of the residual activity against the reaction time. The order of the reaction was estimated from the slopes of the plots of log (pseudo-first order rate constant) against log (inhibitor concentration). The K_m and K_{cat} values of partially DEPC modified enzymes were also determined under standard assay conditions.

Chemical Modification of serine

Purified lipase (5 μg) in 1 ml 50mM sodium phosphate buffer, pH 7.5, was treated with PMSF (100 mM, freshly diluted in absolute ethanol) at 25^{o}C. The reagent was added in reaction mixture to get final concentration of 0.5, 1.0, 1.5, and 2.0 mM. The reagent was allowed to react with enzyme for the period of 30 min. The aliquots were removed and assayed for enzyme activity under standard assay conditions. Control sample was treated similarly with the addition of ethanol instead of PMSF in the reaction mixture.

The number of serine residues involved at active site was determined by incubating lipase(100μg) with different concentrations of PMSF (0, 2, 4, 6, 8mM). The aliquots were removed from each concentration after 5, 10, 15, 20 min for analysis of residual lipase activity. The pseudo-first order rate constant was calculated from the slope of logarithmic plots of residual lipase activity against the reaction time. The order of the reaction was estimated from the slopes of the plots of log (pseudo-first order rate constant) against log (inhibitor concentration). The K_m and K_{cat} values of partially PMSF modified enzymes were also determined under standard assay conditions

Chemical modification of carboxylate residues

Purified lipase (5 μg) in 1 ml 50mM MES/HEPES buffer (75:25), pH 6.0, was treated with EDAC (100 mM) at 25°C. The reagent was added in reaction mixture to obtain an effective concentration of 50 mM, 100 mM, 150 mM, 200 mM respectively. The reagent was allowed to react with enzyme for a period of 30 min. The aliquots were removed and assayed for enzyme activity under standard assay conditions.

To determine the number of essential carboxyl groups involved in active site, the lipase (100 μg) was incubated with different concentrations of EDAC (0, 10, 20, 30,40, 50mM). The aliquots were removed from each reaction mixture after 10, 20, 30, 40 and 50 min for analysis of residual lipase activity. The pseudo-first order rate constants were calculated from the slope of logarithmic plots of residual activity against the time of reaction. The order of the reaction was estimated from the slopes of the plots of log (pseudo-first order rate constant) against log (inhibitor concentration). The K_m and K_{cat} values of partially EDAC modified enzymes were also determined under standard assay conditions.

Chemical modification of tryptophan residues

Aliquots of purified lipase (5μg) in 1ml 50mM sodium acetate buffer, pH 3.0–5.0 were titrated with freshly prepared *N*-bromosuccinimide (NBS, 1 mM) at 25^{o}C. The reagent was added in reaction mixture to obtain an effective concentration of 1, 10, 50, 100, 200 μM, respectively. The mixture was kept for the period of 30 min and the aliquots were removed and assayed for enzyme activity under standard assay conditions.

Number of tryptophan residues was determined by incubating 100 µg of purified lipase with 5 µl of NBS (1mM) at 25°C for 10 min. The addition was continued till no further decrease in optical density at 280 nm was observed. The decrease in absorbance and corresponding residual activity was determined by regular lipase assay. After each addition of NBS, the number of tryptophan residues oxidized (Δn) per mole of enzyme was calculated from the equation (Spande and Witkop 1967).

$$\Delta n = \frac{1.31 \times \Delta A_{280} \times M.W \times V}{5500 \times W}$$

Where ΔA_{280} is the decrease in absorbance at 280 nm, 1.31 is an empirical factor based upon oxidation of model tryptophan peptides by NBS (Patchornik et al. 1958), 5500 is the molar absorption extinction coefficient for tryptophan at 280 nm, M.W- Molecular weight of protein, V- Initial volume of titrated solution(ml), W- Weight of protein titrated (mg). The K_m and K_{cat} values of partially NBS modified enzymes were also determined under standard assay conditions.

Substrate protection and kinetic studies

In all chemical modification reactions the protective effect of substrate on modification was studied by incubating enzyme (10µg) with excess amount of substrate; *p*NPP (0.2mM-0.53mM) followed by treatment with various modifying reagents. The residual activity of enzyme was assayed periodically by standard assay method.

Results

Purification of lipase

The protein in the broth was precipitated by addition of ammonium sulfate (90% saturation). The precipitated enzyme was dissolved in minimal volume of Glycine-NaOH buffer (50mM, pH 9.0) followed by dialysis. The results of a typical procedure for the purification of *A. niger* NCIM 1207 lipase to homogeneity are summarized in Table 1. The enzyme was purified approximately 150 fold with an overall yield of 54% and specific activity of 1373 U/mg. The purified enzyme could be stored in Glycine-NaOH buffer pH 9.0 and -20^{o}C for one year without any apparent loss in its initial activity. The elution profile of lipase from Phenyl Sepharose column is shown in Figure 1. Final purification was achieved by gel filtration chromatography using Sephadex S-100. Elution pattern of lipase by Sephadex S-100 chromatography is shown in Figure 2. The pooled fraction was analyzed by SDS-PAGE, which revealed a single band of a protein (Figure 3).

Table 1. Summary of steps of purification of lipase from *Aspergillus niger* NCIM 1207.

Purification Steps	**Total Activity (IU)**	**Total Protein (mg)**	**Specific Activity (IU/mg)**	**Fold Purification**	**Yield (%)**
Culture filtrate	1700	185.10	9.18	1	100.00
Ammonium Sulfate Precipitation	1684	42.66	39.47	4.29	99.05
Phenyl Sepharose CL 4B Chromatography	1246	5.47	227.78	24.78	73.29
Gel Filtration Chromatography (S-100)	920	0.67	1373.13	149.56	54.11

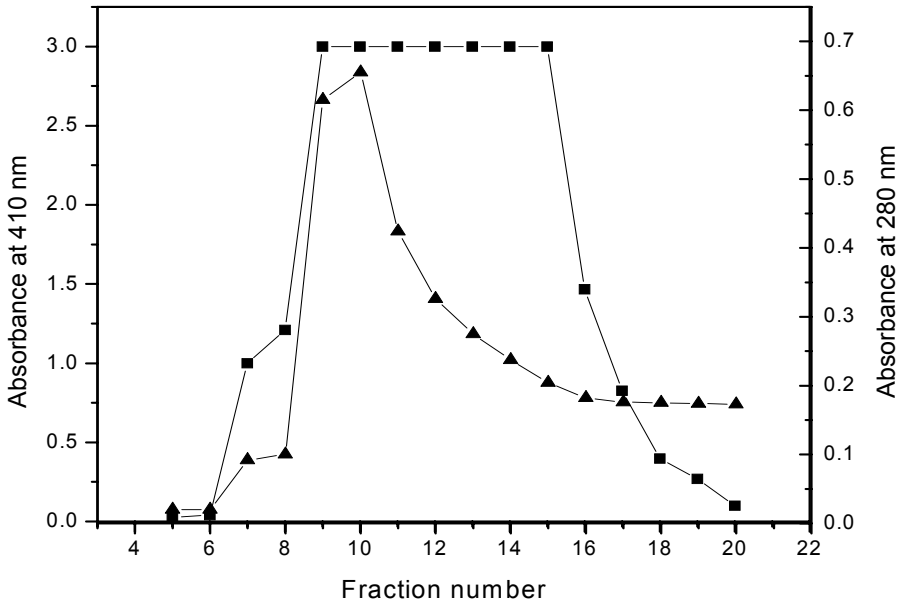

Figure 1. Elution profile of lipase from Phenyl Sepharose CL 4B column.
Protein (--▲--), Activity (--■--).

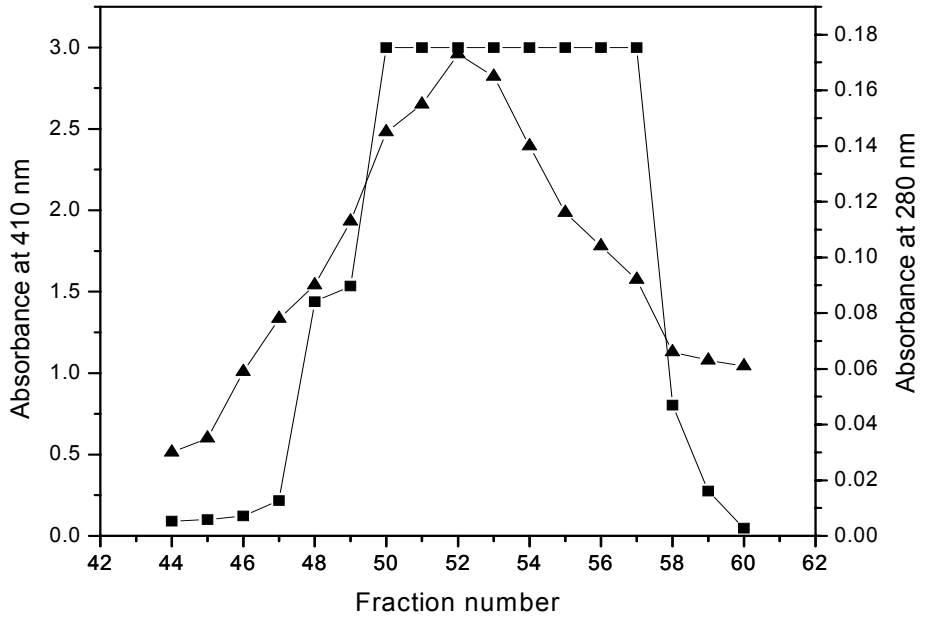

Figure 2. Elution profile of lipase on Sephacryl S-100 column.
Protein(--▲--), Activity (--■--).

Molecular properties of lipase

The apparent molecular weight of the purified enzyme determined by SDS-PAGE (Figure 3) and MALDI-TOF (Figure 4) was 32.2 kDa suggesting that it is a monomer. Molecular weight based on the amino acid composition (Table 2) was 33.34 kDa which is comparable to that obtained by the above methods. The enzyme is a basic protein with a pI of 8.5 (Figure 5).

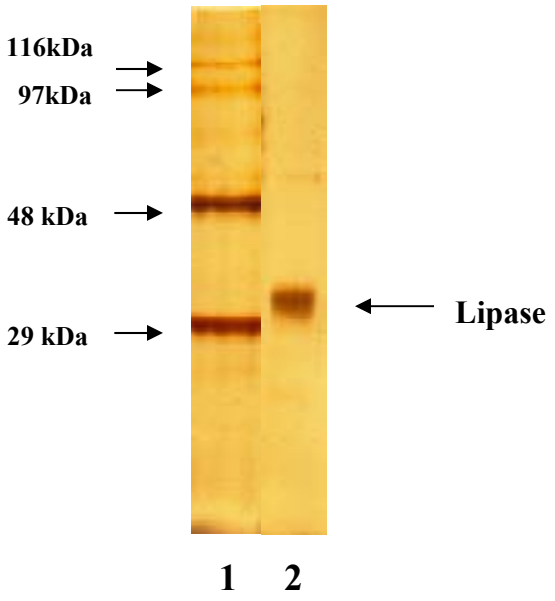

Figure 3. SDS-PAGE of purified acidic lipase. The purified protein was electrophoresed on 12% (w/v) of SDS-PAGE and stained with silver stain. The markers are β-galactosidase (116.0 kDa), Phosphorylase b (97.4 kDa), Fumarase (48.0 kDa) and Carbonic anhydrase (29.0 kDa).

Lane 1: Marker, **Lane 2**: Sephacryl S-100 eluted purified enzyme

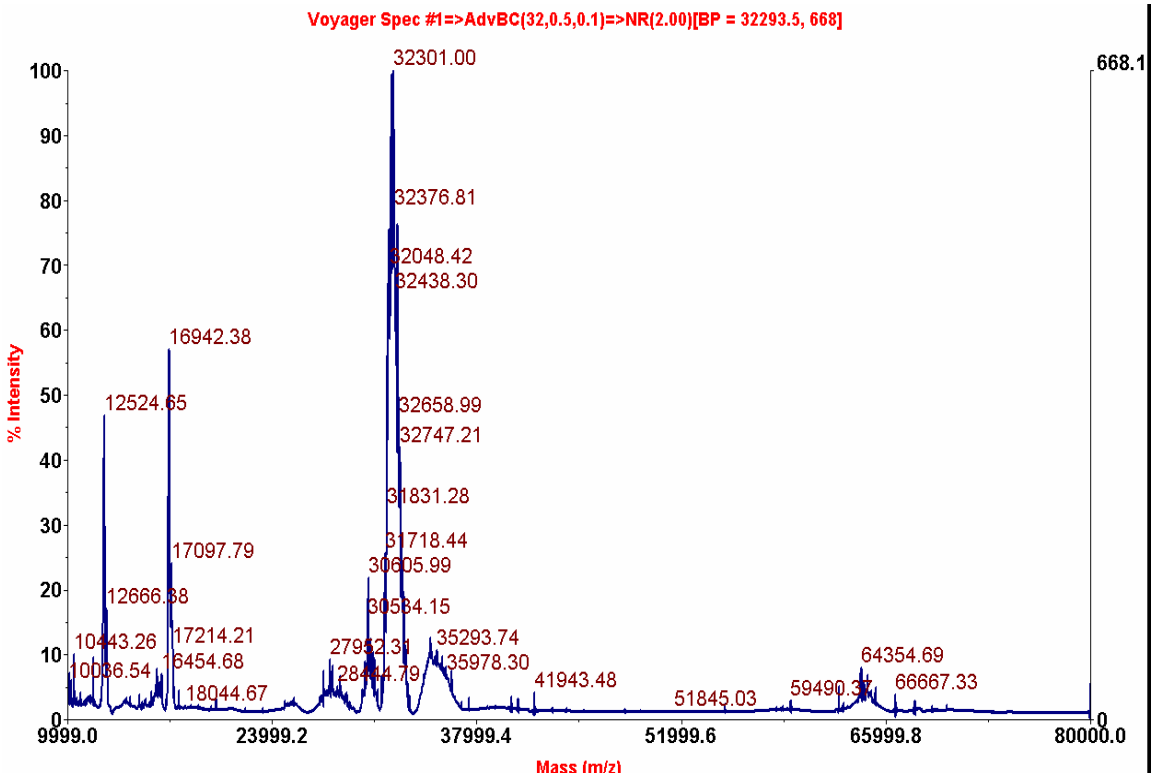

Figure 4. MALDI-TOF analysis of purified lipase of *A. niger* NCIM 1207.

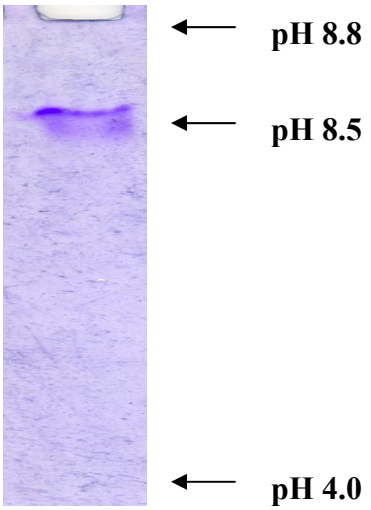

Figure 5. IEF-PAGE of purified enzyme from *Aspergillus niger* NCIM 1207.

Purified enzyme (20 μg) was elecrophoresed in a vertical gel apparatus using wide range Ampholyte pH (3-10) as described in methods. After electropheresis, gel was stained with Coomassie Brillient Blue R-250 stain (Deutcher 1990).

Amino acid analysis

The purified lipase consists of 333 amino acid residues (Table 2). The content of hydrophobic amino acids (L, V, I, M, P, A, F, W) is about 37.0 % (residues/mole). The content of acidic (Asx and Glx) and the basic (Lys, Arg, His) amino acids is 18.9 % and 6.3 % respectively. The enzyme has high glycine content (19.2%) but extremely low amounts of sulfur containing amino acids.

Table 2. Amino acid composition of purified lipase from *Aspergillus niger* NCIM 1207.

Amino acid	Residues / mole of enzyme
Ala	35
Val	17
Leu	25
Ile	13
Pro	11
Phe	12
Trp	7* [a]
Met	3
Gly	64
Ser	31
Thr	27
Cys	0 * [b]
Tyr	5
Lys	9
Arg	7
His	5
Asx	36
Glx	26
Total	333

Determined spectrophotometrically [a] Spande T.F and Witkop B (1967), [b]Habeeb A.F.S.A. (1972).

Circular dichroism (CD)

The secondary structure of native lipase at 25°C was determined by CD spectroscopy. Figure 6 shows the far UV spectra (290-200 nm) of lipase indicating two elipticity minima's at 222 and 208 nm which suggested the predominance of α-helical structure.

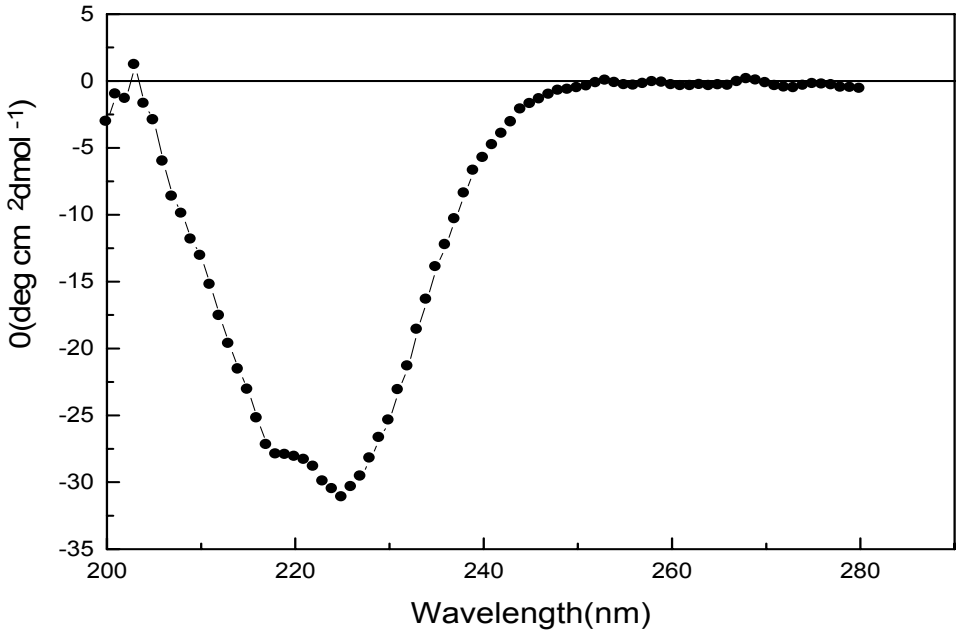

Figure 6. Far UV CD spectra of *A. niger* NCIM 1207 lipase at 25°C in 50 mM phosphate buffer, pH 7.0.

Biochemical properties of lipase

pH optimum and stability

Maximum lipase activity was obtained at pH 2.5 at 50^{o}C and decreased significantly from 100% to 50% when the pH was increased from 1.5 to 4.0 (Figure 7). The activity was not much affected at pH 1.5 where it showed 70% of the original activity. The data suggested that the lipase is active at extremely acidic pH.

Lipase from *A. niger* NCIM 1207 showed stability at alkaline pH range (pH 8.0 – 11) as it retained 100% of its original activity after incubation for 24 h (Figure 8). However the enzyme was not stable in the acidic pH ranging from 2.0 to 7.0. It was not stable at acidic pH range.

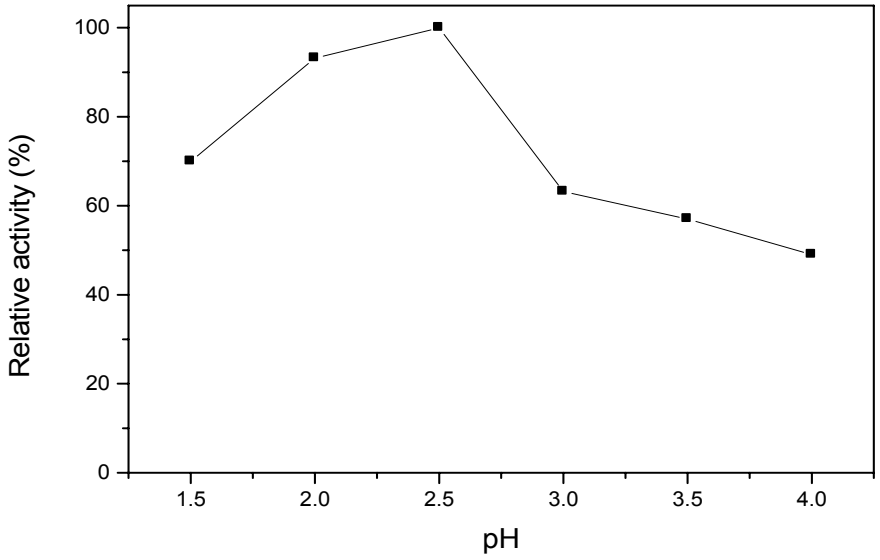

Figure7. Effect of pH on activity of purified lipase from *Aspergillus niger* NCIM 1207. Enzyme activity was assayed in a series of pH (1.5–4.0) at 50^{o}C as described in methods. Maximum enzyme activity obtained was taken as 100%.

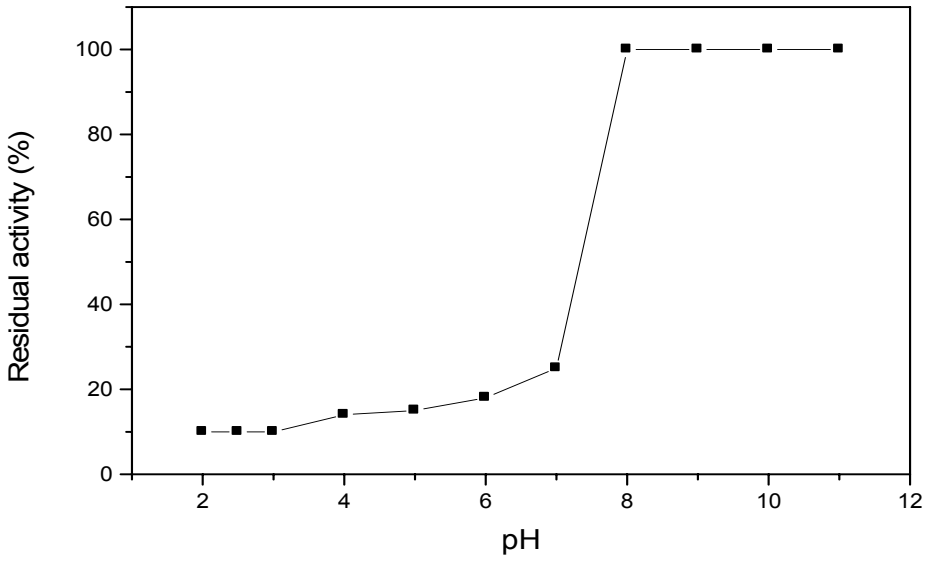

Figure 8. Effect of pH on stability of purified enzyme from *Aspergillus niger* NCIM 1207. A suitably diluted purified enzyme was incubated in a series of pH (2.0-11.0) at 30^{o}C for period of 24 h and the residual activity was determined under standard assay conditions as described in methods. Maximum activity obtained was taken as 100%.

Temperature optimum and temperature stability

The purified enzyme exhibited maximum activity at a temperature of 50^{o}C. The activity decreased sharply above the optimum temperature with almost 95% loss of its original activity at 70^{o}C (Figure 9). The lipase was stable at 40^{o}C for 3 h. However, lipase activity was significantly affected at higher temperatures since incubation at 50^{o}C for 1 h resulted in 52% loss of lipase activity (Figure 10).

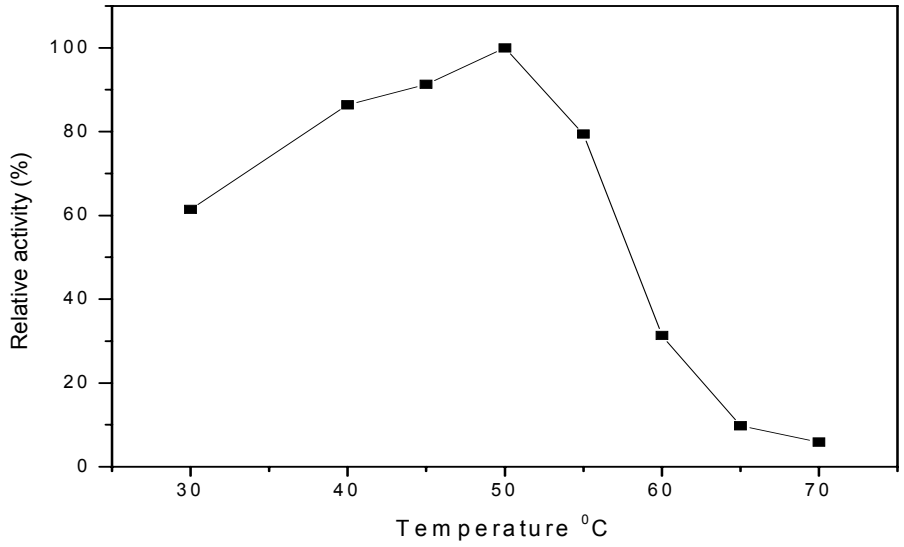

Figure 9. Effect of temperature of purified lipase. Lipase activity was assayed at different temperatures (30-70^{o}C) at optimum pH as described in methods. Maximum enzyme activity obtained was considered as 100%.

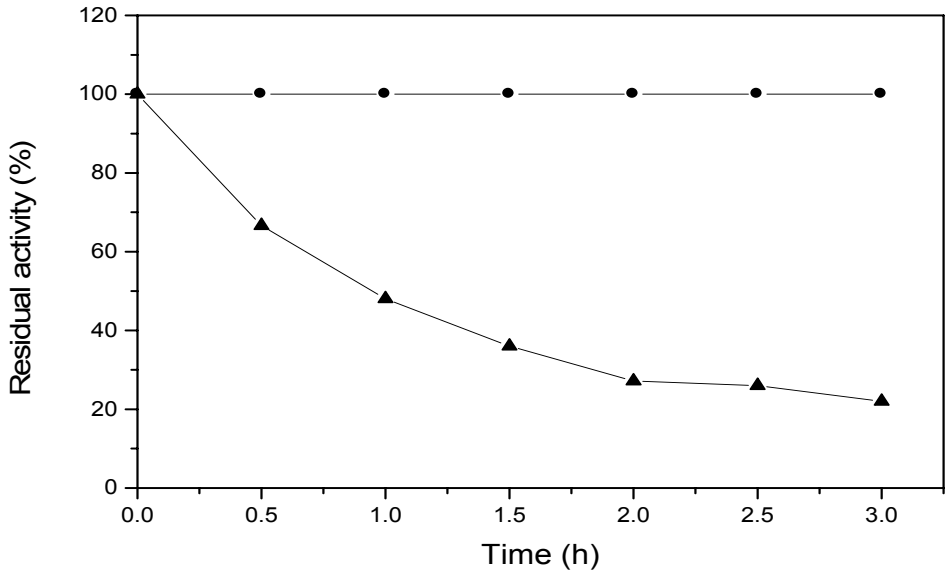

Figure 10. The effect of Temperature on stability of purified lipase. Purified enzyme samples were incubated at 40^{o}C & 50^{o}C. Aliquots were removed at suitable time interval and assayed for residual activity. 40^{o}C (●), 50^{o}C (▲).

Effect of organic solvents on enzyme stability

The results of effect of water miscible and water immiscible solvents (25%) on lipase stability are shown in Table 3. The enzyme was stable in all water miscible solvents and in some of the water immiscible solvents even after 24 h. Incubation of enzyme with chloroform, 2-propanol, and tertiary butyl alcohol, led to immediate loss (Within 5 min) of 50% of its initial activity. Total inactivation was observed within 4 h when enzyme was incubated with xylene, acetonitrile, propan-1-ol and butan-1-ol.

Table 3. Effect of organic solvents on stability of enzyme.

Organic Solvent	**Residual activity (%) (0 h)**	**Residual activity (%) (4 h)**	**Residual activity (%) (24 h)**
Hexane	99.43	105.08	111.1
Chloroform	53.14	34.69	32.59
2-Propranol	43.53	17.52	-
Methanol	86.97	82.21	110.0
Isoamyl alcohol	96.34	87.65	33.00
Xylene	-	-	-
Acetonitrile	-	-	-
DMSO	100.00	99.88	92.30
1,4 dioxane	76.25	29.85	10.06
Ethyl acetate	91.32	83.31	89.63
Isooctane	99.78	99.54	99.47
Toluene	100.00	99.85	99.77
Propan-1-ol	-	-	-
Butan-1-ol	23.74	-	-
Tertiary-butyl alcohol	49.07	13.90	-
n-hexadecane	99.88	99.14	100.00
Acetone	100.00	98.18	93.61
Ethanol	92.46	91.16	93.09
Control	100.00	100.00	100.00

Effect of metal ion on lipase activity

A. niger NCIM 1207 lipase did not show an obligate requirement of metal ions for its activity and EDTA did not affect the activity suggesting that it is neither a metal requiring enzyme nor a metalloenzyme. However the enzyme was inhibited by low concentrations (0.1mM) Ag^{2+} which appeared to be the most potent inhibitor. There is no significant inhibition observed by Hg^{2+} even at 1 mM concentration (Table 4).

Table 4. Effect of metal ions on *Aspergillus niger* NCIM 1207 lipase activity.

Metal ion	**Relative activity (%) in presence of metal ions**	
	0.1 mM	**1.0 mM**
Control	100.00	100.00
$AgNO_3$	11.0	3.36
$BaCl_2$	110.11	107.47
$CaCl_2$	108.21	101.89
$CuSO_4$	108.68	92.37
$CoSO_4$	100.00	93.10
$FeCl_3$	110.26	103.25
$FeSO_4$	107.03	102.80
$HgCl_2$	98.73	79.47
$MgSO_4$	107.47	99.12
$MnCl_2$	113.20	109.38
$NiCl_2$	111.24	97.65
$ZnCl_2$	105.60	103.95
NaCl	111.24	106.74
EDTA	109.82	103.55

Substrate specificity

Fatty acid specificity was studied with *p*-nitrophenyl esters of fatty acids with varying alkyl chain length. The highest hydrolytic activity was obtained with *p*-nitrophenyl caprylate (C_8) indicating a clear preference of the enzyme for medium alkyl chain length fatty acids (Table 5).

Table 5. Substrate specificity of *A. niger* NCIM 1207 lipase.

Substrate	**Relative activity (%)**
p-nitrophenyl caprylate	100.00
p-nitrophenyl decanoate	57.35
p-nitropheny laurate	52.35
p-nitropheny myristate	42.81
p- nitropheny palmitate	33.01
p -nitropheny stearate	21.40

The substrate specificity was determined by estimating the lipase activity using *p*-nitrophenyl esters of fatty acids (C_8 – C_{18}) under standard assay conditions. Activity towards *p*-nitrophenyl caprylate was taken as 100%.

Positional Specificity

We have investigated the positional specificity of purified lipase using triolein as substrate. The result of the thin-layer chromatography analysis of the hydrolysis products of triolein is shown in (Figure 11). Lipase from *A. niger* NCIM 1207 hydrolyzed triolein producing only 1, 2 diolein as end product after 4 h indicating that the enzyme has 3-position specificity.

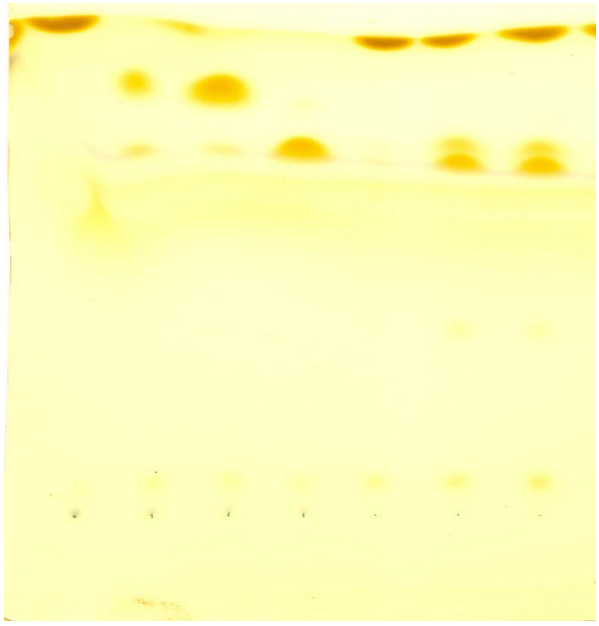

Figure 11. Thin-layer chromatograph of the hydrolysis products of triolein catalyzed by *A. niger* NCIM 1207 lipase. Reaction mixture containing 20mM triolein, enzyme solution (10 IU) in 2ml of citrate phosphate buffer (50 mM, pH 2.5) was shaken at 200 rpm at 30^{o}C. At 0h, 2h and 4h, the reaction products were extracted with 0.8 ml diethyl ether and analyzed by thin layer chromatography. Lane 1:triolein; Lane 2:diolein; Lane 3:1,3 diolein; Lane 4:1,2 diolein; Lane 5: 0h; Lane 6:2h; Lane 7: 4h.

Kinetic studies of lipase

Kinetic constants for the purified lipase from *A. niger* NCIM 1207 were obtained using standard assay conditions. The effect of varying substrate concentrations on the reaction rate was studied and Michaelis constant (K_m) and maximum velocity (V_{max}), were calculated for *p*-nitrophenyl palmitate (Figure 12) and *p*-nitrophenyl caprylate from Lineweaver-Burk plots (Figure 13). Results of kinetic studies (Table 6) indicated that enzyme shows higher affinity for *p*-nitrophenyl caprylate (lower K_m) and higher rate of hydrolysis (higher V_{max}) than *p*-nitrophenyl palmitate (*p*NPP).

Table 6. Kinetic parameters of hydrolysis of different *p*-nitrophenyl esters by lipase.

Substrate	K_m **(mM)**	V_{max} **(µmoles min^{-1}mg^{-1})**	K_{cat} **(min^{-1})**	K_{cat}/K_m **(mM^{-1}min^{-1})**
p-nitrophenyl palmitate	0.33	746	1.8×10^9	5.45×10^9
p-nitrophenyl caprylate	0.14	1066	2.5×10^9	1.7×10^{10}

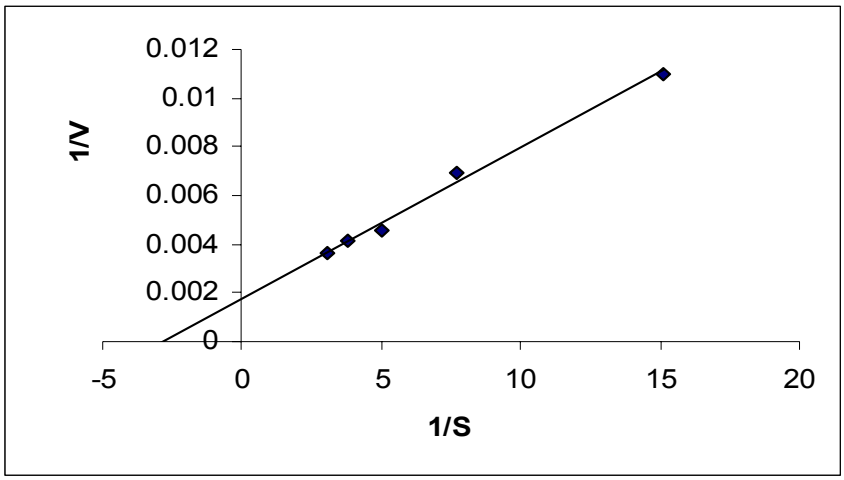

Figure 12. Lineweaver-Burk plot for the hydrolysis of *p*-nitrophenyl palmitate.

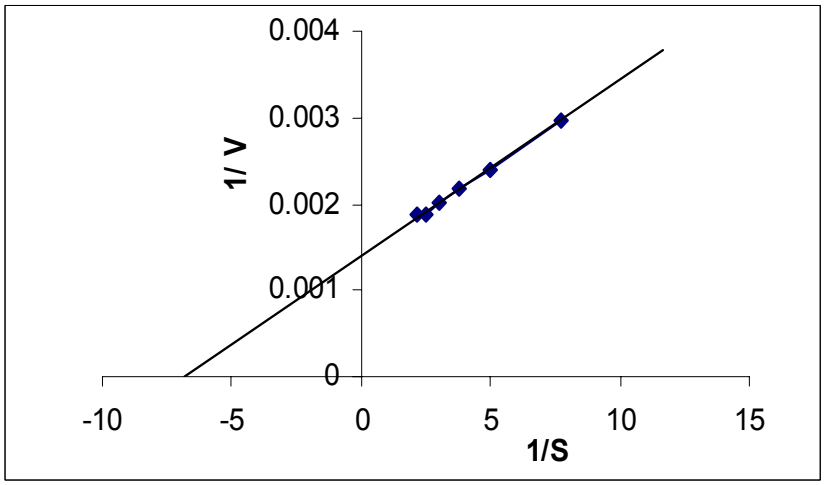

Figure 13. Lineweaver-Burk plot for the hydrolysis of *p*-nitrophenyl caprylate.

Chemical modification studies

The importance of amino acid functional groups for the activity of lipase was determined by chemical modification studies using chemical agents with restricted amino acid specificity. The results of the inactivation studies are given in Table 7. The enzyme was not inhibited by NEM, NAI, citraconic unhydride and phenylglyoxal suggesting the non-involvement of cystein, tyrosine, lysine and arginine residues in catalysis. Strong inhibition of enzyme by DEPC, PMSF, EDAC and NBS was observed which indicated the involvement of histidine, serine, carboxylate and tryptophan for its catalytic activity In view of these observations, the role of above-mentioned amino acid residues for catalysis was further investigated.

Table7. Effect of group specific chemical modifiers on lipase activity.

Chemical Reagent	**Conc^{n}**	**Possible Reaction site**	**Buffer**	**Residual activity (%)**
DEPC	2 mM	His	Sodium phosphate,50 mM pH 6.0	00
TNBS	2 mM	Lys	Sodium bicarbonate,4%,pH 8.4	55
Citraconic unhydride	5 mM	Lys	Sodium bicarbonate,50 mM, pH 7.8	100
NBS	1 μM	Try	Sodium acetate, 50 mM, pH 4.5	00
PMSF	5 mM	Ser	Sodium phosphate ,50 mM, pH 7.5	18
EDAC	50 mM	Asx/Glx	MES/HEPES, 75:25 mM , pH 6.0	00
NEM	10 mM	Cys	Sodium phosphate,50 mM,pH 7.5	100
Iodoacetate	10 mM	Cys	Sodium phosphate,50 mM,pH 8.0	100
NAI	10 mM	Tyr	Sodium borate,50 mM,pH 7.5	100
Phenylglyoxal	10 mM	Arg	Sodium bicarbonate,50 mM, pH 8.0	100

Modification of Histidine

Carbethoxylation of purified lipase at pH 6.0 resulted in total loss of its initial activity. No loss was observed in control samples. The inactivation was found to concentration dependent and it followed pseudo-first-order kinetics yielding a slope of 2.3 (Figure 14 & 15). These observations suggested that the enzyme inactivation was the

result of modification of two histidine residues per mole of enzyme. The DEPC-inactivation could not be prevented by pre-incubating the enzyme with excess of substrate prior to modification (Table 8). The kinetic analysis of DEPC modified enzyme showed decrease in K_{cat} values and no change in K_m (Table 9) suggesting its involvement in catalysis.

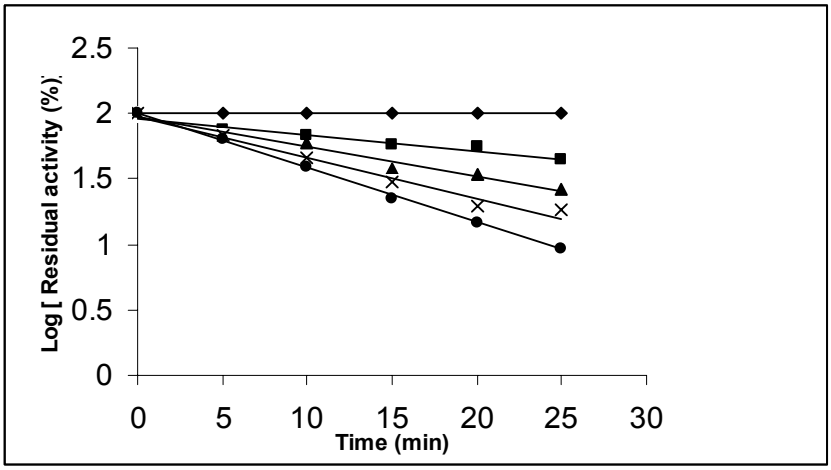

Figure14. Effect of DEPC on lipase activity. Enzyme (100 µg) in 50mM sodium phosphate buffer, pH 6.0,was treated with DEPC (♦, 0 mM; ■,15 mM; ▲,20 mM; ×, 25mM; ●,30mM). Samples (20µl) were removed from the reaction mixture at different time interval and assayed for residual activity.

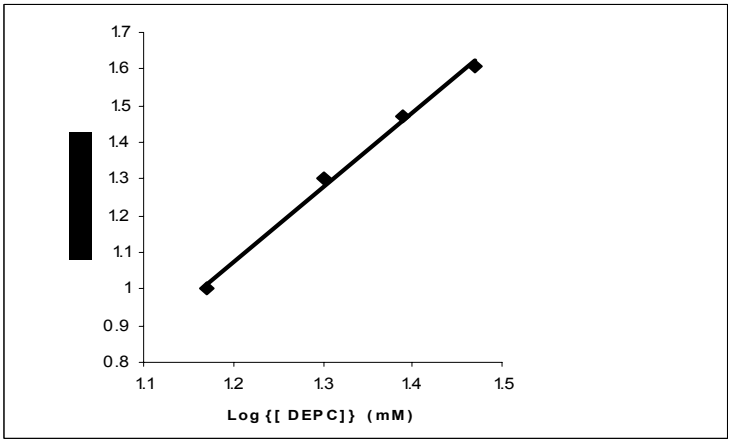

Figure 15. The linear relationship of log (*k*) and log (DEPC concentration). The pseudo-first order rate constant *k* was calculated from the slope of data given in Figure14.

Table 8. Effect of histidine modification on lipase activity and substrate protection study.

Reaction mixture	R.A. (%)	Inhibition (%)	Protection (%)
Enzyme (Control)	100	0	100
Enzyme + DEPC (50 mM)	00	100	00
Enzyme + pNPP(0.2 mM) + DEPC(50 mM)	1.27	98.73	1.27
Enzyme + pNPP(0.4 mM) + DEPC(50 mM)	1.64	98.36	1.64
Enzyme + pNPP(0.53 mM) + DEPC(50 mM)	0.91	99.09	0.91

Table 9. Effect of histidine modification on the K_m and K_{cat} values of lipase.

Percentage activity	Residue modified	Reagent used	K_m	K_{cat}
100	None	----------	0.33	1.8×10^{9}
36	Histidine	DEPC	0.33	5.2×10^{8}
33			0.33	4.9×10^{8}
27			0.33	4.7×10^{8}

Modification of serine

The incubation of purified enzyme with 5 mM PMSF resulted in significant loss (82%) of its initial activity within 30 min indicating the important role of serine in catalytic activity of the enzyme. The PMSF-inactivation was found to be concentration dependent. No loss of activity was observed in control samples. The logarithm of residual activity was plotted as a function of time at various PMSF concentrations which followed pseudo-first-order kinetics for every fixed PMSF concentration (Figure 16). After calculating pseudo-first-order rate constants (k) from the slope of the plots, log k x 10^3 was plotted against log of PMSF concentration which yielded a slope of 0.67 (Figure 17). These results indicated that the loss of activity was due to modification of a single serine residue per mole of the enzyme. The PMSF-mediated inactivation was prevented by pre-incubating the enzyme with excess of substrate prior to modification (Table 10) which suggests that it could also have a possible role in substrate binding.

The kinetic analysis of PMSF modified enzyme showed decrease in K_{cat} values and no change in K_m (Table 11) suggesting its involvement in catalysis.

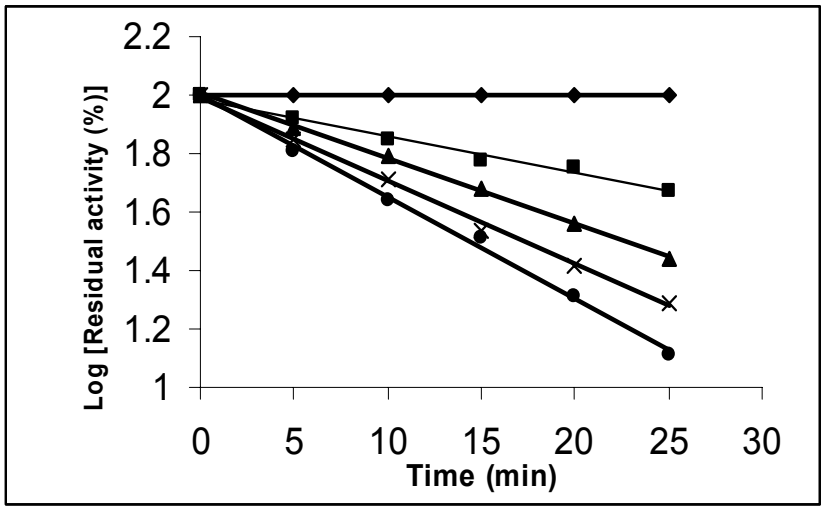

Figure 16. Effect of PMSF on lipase activity. Enzyme (100 µg) in 50mM sodium phosphate buffer, pH 7.5, was treated with PMSF (♦, 0 mM; ■, 2.0 mM; ▲, 4.0 mM; ×, 6.0mM; ●, 8.0 mM). Samples (20µl) were removed from the reaction mixture at different time interval and assayed for residual activity.

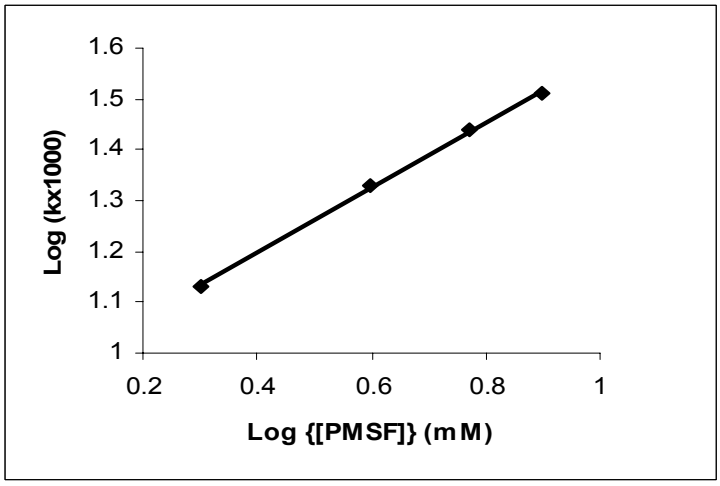

Figure 17. The linear relationship of log (k) and log (PMSF concentration). The pseudo-first order rate constant (k) was calculated from the slope of data given in Figure 16.

Table 10. Effect of serine modification on activity of lipase: substrate protection study..

Reaction mixture	**R.A (%)**	**Inhibition (%)**	**Protection (%)**
Enzyme (Control)	100	0	100
Enzyme + PMSF(10 mM)	18.75	81.25	00
Enzyme + pNPP(0.2 mM) + PMSF (10 mM)	80.56	19.44	62.00
Enzyme + pNPP(0.4 mM) + PMSF (10 mM)	80.56	19.44	62.00
Enzyme + pNPP(0.53 mM) + PMSF (10 mM)	75.24	24.76	56.49

Table 11. Effect of serine modification on the K_m and K_{cat} values of lipase.

Percentage activity	**Residue modified**	**Reagent used**	K_m	K_{cat}
100	None	---------	0.33	1.8×10^{9}
64	serine	PMSF	0.33	1.59×10^{8}
59			0.33	1.43×10^{8}
48			0.33	0.95×10^{8}

Modification of carboxylate residues:

EDAC-modification of purified lipase at pH 6.0 resulted in 78% loss of its initial activity. No loss of activity was observed in control samples. EDAC-mediated inactivation is dependent on concentration of the reagent. The plots of residual activity versus time of inactivation for various EDAC concentrations were linear (Figure18). The plot of pseudo-first-order constants (k x 10^3) as a function of log of EDAC concentration gave a slope of 0.60 indicating the involvement of single carboxylic residue per mole of enzyme (Figure 19). The substrate protection studies revealed only 13% protection (Table 12) suggesting its main role in catalysis. The kinetic parameters of the partially inactivated enzyme sample showed slight increase in K_m and decrease in K_{cat} values (Table13).

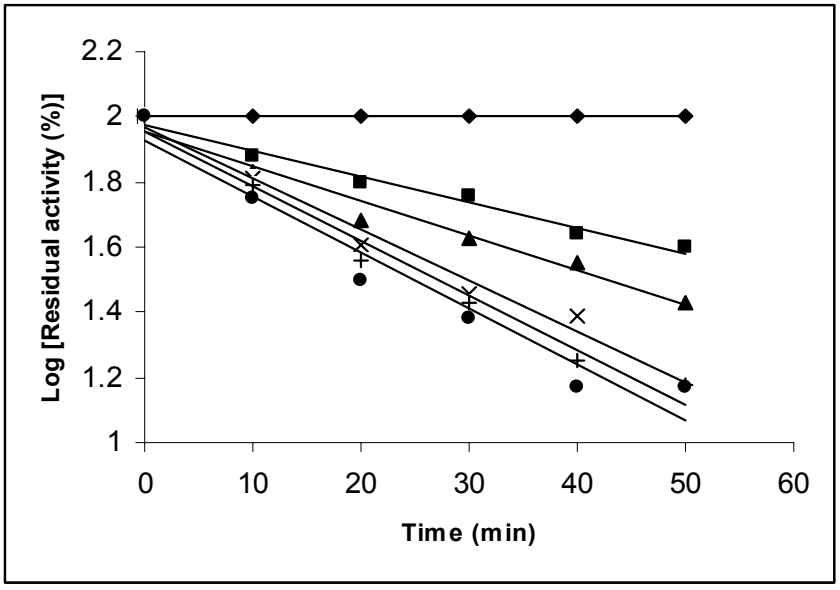

Figure 18. Effect of EDAC on lipase activity. Enzyme (100 µg) in 50mM MES/HEPES, 75:25 mM , pH 6.0,was treated with EDAC). (♦,0mM; ■,10 mM; ▲,20 mM; ×, 30 mM; +, 40 mM; ●,50 mM). Samples (20µl) were removed from the reaction mixture at different time interval and assayed for residual activity.

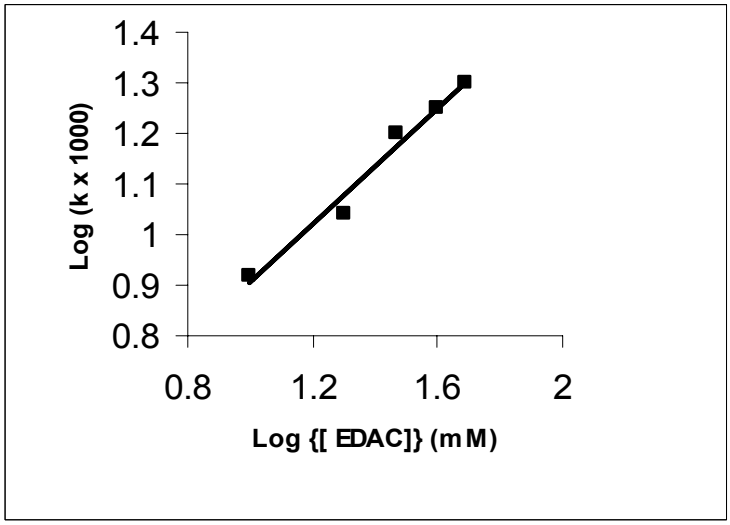

Figure 19. The linear relationship of log (*k*) and log(EDAC concentration).The pseudo-first order rateconstant *k* was calculated from the slope of data Figure 19.

Table 12. Effect of carboxylate modification on activity of lipase: substrate protection study.

Reaction mixture	**R.A (%)**	**Inhibition (%)**	**Protection (%)**
Enzyme (Control)	100	0	100
Enzyme + EDAC (150 uM)	22.19	77.81	00
Enzyme + pNPP (0.2 mM)+EDAC(150 uM)	35.89	64.11	13.7
Enzyme + pNPP (0.4 mM)+ EDAC(150 uM)	32.75	67.25	10.56
Enzyme + pNPP(0.53 mM)+EDAC(150 uM)	31.10	68.9	8.91

Table 13. Effect of carboxylate modification on the K_m and K_{cat} values of lipase

Percentage activity	**Residue modified**	**Reagent used**	K_m	K_{cat}
100	None	--------	0.33	1.8×10^{9}
50	Corboxylate	EDAC	0.4	7.28×10^{8}
35			0.5	6.96×10^{8}

Modification of tryptophan

Lipase modification by NBS led to complete loss of its initial activity. Loss in enzyme activity was due to tryptophan modification. A plot of percent residual activity versus the number of tryptophan residues modified showed that four tryptophan residues are involved in enzyme catalysis (Figure 20). The NBS mediated inactivation of lipase was partially prevented by pre-incubating the enzyme with excess amount of substrate (*p*NPP), prior to modification reaction (Table 14) suggesting that it may also have a role in substrate binding. The K_m of partially modified enzyme remained unchanged with decrease in K_{cat} (Table 15).

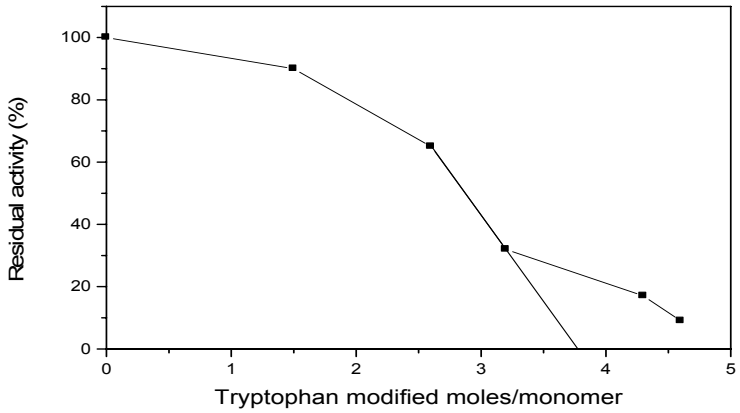

Figure 20. Oxidation of lipase tryptophan residues was carried out with stepwise addition of NBS to the enzyme. The number of tryptophan residue oxidized was determined as described in materials and methods section.

Table 14. Effect of tryptophan modification on lipase activity: substrate protection study.

Reaction mixture	**R.A. (%)**	**Inhibition (%)**	**Protection (%)**
Enzyme (Control)	100	0	100
Enzyme + NBS (50μM)	0	100	0
Enzyme + pNPP (0.2 mM) + NBS(50μM)	64.25	35.75	64.25
Enzyme + pNPP (0.4 mM) + NBS(50μM)	73.65	26.35	73.65
Enzyme + pNPP (0.53 mM) + NBS(50μM)	68.10	31.9	68.10

Table 15. Effect of tryptophan modification on K_m and K_{cat} values of lipase.

Percentage activity	**Residue modified**	**Reagent used**	K_m	K_{cat}
100	none	--------	0.33	1.8×10^{9}
64	Tryptophan	NBS	0.33	2.6×10^{6}
44			0.33	2.4×10^{6}
38			0.33	2.0×10^{6}

Discussion

Most of the fungal lipases are extracellular and produced under submerged fermentation. The general purification procedure involves the separation of mycelium or cells from the culture broth, precipitation of proteins by ammonium sulfate or organic solvents, followed by ion exchange, affinity or hydrophobic chromatography and gel filtration. In most of the cases precipitation by ammonium sulfate has been attempted which often gives high (85-90%) average yields (Aires-Barros et al. 1994).

The purification steps involved ammonium sulphate precipitation followed by hydrophobic interaction chromatography using Phenyl Sepharose column in presence of 20% ammonium sulphate. The partially purified lipase was found to be unstable at acidic or neutral buffer systems but it was stable at pH values above 8.0. Hence we opted to use alkaline buffer system (Glycine-NaOH, pH 9.0) during all purification steps. The hydrophobic interaction chromatography has been used for most of the lipases since these enzymes are hydrophobic showing interaction with hydrophobic supports (Nawani et al. 2000; Saxena et al. 2003). The retention of lipase on hydrophobic support is dependent on the salt used and it increases with the ionic strength. Queiroz et al (1995) used 20% of the ammonium sulfate in the eluent and observed total retention of lipase on the hydrophobic column. By washing the column with 10 mM phosphate buffer, maximum recovery (89%) was obtained. We also observed 95% retention of *A. niger* lipase on phenyl sepharose column in presence of 20% ammonium sulphate and it was eluted with distilled water with 73% recovery.

New techniques have been developed for purification and a combination of these new methods with the traditional chromatographic techniques led to rapid and high recovery of lipases. Employing multi-step strategies or combination of novel methods with the traditional procedures, the recovery of purified fungal lipases varied between as low as % 0.02% in case of *R. niveus* (Kermasha et al.1998) and as high as 68 % in case of *P. citrinum* (Krieger et al.1997). We were able to purify *A. niger* lipase to homogeneity with 54% yield which is supposed to be the highest reported so far for *Aspergillus* lipases.

Aspergillus niger NCIM 1207 produced a single extracellular lipase with a molecular weight of 32.2 kDa as determined by SDS-PAGE and MALDI TOF procedures. The molecular weight of *A. niger* lipase is comparable to lipases from *Aspergillus carneus* (27.0 kDa, Saxena et al. 2003), *Aspergillus nidulans* (29.0 kDa, Mayordoma et al. 2000), *Aspergillus niger* (35.5 kDa, Namboodiri et al. 2000). Some bacterial lipases such as from *Pseudomonas* sp (30.0 kDa, Dong et al. 1999), *Pseudomonas aeruginosa* MB 5001 (29 kDa, Chartrain et al 1993), *Pseudomonas aeruginosa* KKA-5 (30.0 kDa, Sharon et al. 1998), *Flavobacterium odoratum* (32.5 kDa, Labuschangne et al. 1997), *Bacillus coagulans* BTS-3 (31 kDa, Kumar et al. 2005), *Acinetobacter* sp. ES-1 (32.0 kDa, Lee et al. 2006) also showed comparable molecular weights. The molecular masses of most of the fungal lipases have been reported to be in the range of 11-67 kDa (Saxena et al. 2003). The lipase from *Pythium ultimum* exists as a tetramer with a molecular weight of 270 kDa with a monomeric molecular weight of 68 kDa (Mozaffar and Weete 1993). Lipase purified from *A. niger* NCIM 1207 was shown to be homogeneous monomer with alkaline pI (8.5). Most of the lipases purified from *Aspergillus* strains exhibit acidic pI (Mayordomo et al. 2000; Namboodiri et al.2000; Saxena et al. 2003).

The amino acid analysis data revealed that the acidic lipase consisted of 333 amino acid residues corresponding to molecular weight of 33.34 kDa which is in good agreement with molecular weight obtained by SDS-PAGE and MALDI-TOF methods. The higher content of hydrophobic amino acids (37.0%) indicated the hydrophobic nature of the enzyme. The purified lipase contained highest amount of glycine followed by serine and threonine. It also has high amount of acidic amino acids (19.2%). Amino acid data of *Mucor hiemalis* lipase also shows high content (43 mol/mol) of glycine (Hiol et al.1999).

Lipase from *A. niger* NCIM 1207 showed pH optimum of 2.5 at 50^{o}C. This appears to be a unique enzyme having extremely acidic pH optimum. Majority of lipases of *Aspergillus* origin showed acidic pH optima (5.0-6.0) except lipase from *A. carneus* which exhibited alkaline pH optimum of 9.0 (Saxena et al. 2003). Lipase from *Penicillium* strains showed pH optima ranging from 7.0-10.0 (Krieger et al.1997; Bian et al. 2005). Fungal lipases from *Rhizopus miehei, Geotrichum candidum and Mucor*

hiemalis were active at neutral pH (Uvarani et al. 1998; Hiol et al. 1999; Gopinath et al. 2003). Purified enzyme from our strain was extremely stable even after 5 days of incubation at pH 8.0 – 11.0 showing 100 % residual activity whereas it showed only 10% residual activity at acidic pH. Lipase from *A .carneus* (Saxena et al. 2003) also showed alkaline pH stability. Lipase from *M. hiemalis* (Hiol et al. 1999) and *A. terreus* (Yadav et al. 1998) showed stability at broad pH range between 4.0 – 10.0.

Bacterial lipases are active at high temperatures (Lee et al. 2001; Kambourova et al. 2003; Kumar et al. 2005; Karadzic et al. 2006). The fungal lipases are generally active and stable at 40-50^{o}C (Namboodiri et al. 2000; Hiol et al. 2000). Acidic lipase of *A. niger* NCIM 1207 was found to be active at 50^{o}C and its half life was found to be 1h at 50^{o}C. The thermo-tolerant lipase isolated from *A. terreus* (Yadav et al. 1998) showed high thermal tolerance (15^{o}C - 90^{o}C). Lipase from *Aspergillus carneus* and from *A. niger* retained 100% and 40 % residual activity at 70^{o}C for 5 min respectively (van Heerden et al. 2002; Saxena et al. 2003) while lipase from *Rhizopus oryzae* showed total loss in activity after 30 min at 50^{o}C (Hiol et al. 2000).

Lipases are known to catalyze the reactions in both aqueous as well as organic solvents. They show remarkable stability in organic solvents which helps to carry out synthetic reactions leading to production of pharmaceutically important products. The acidic lipase of *A. niger* 1207 was stable in majority of polar and non-polar organic solvents for 24 h but was inactivated in presence of xylene, acetonitrile, and propane-1 ol immediately. The lipase from *A. carneus* was stable in presence of isooctane and toluene (Saxena et al. 2003). Lipase from *A. oryzae* was stable in isooctane, hexane and cyclohexane (Toida et al. 1998). Acidic lipase from *A. niger* NCIM 1207 was not inhibited by ethanol, methanol, and strong dehydrating solvents like acetone. However, some fungal and yeast lipases were inhibited by these solvents (Rapp 1995; Hiol et al. 1999; Toida et al.1998; Saxena et al. 2003). Majority of bacterial 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 solvent, since it retained almost 100% activity after exposure by 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. 2007).

Heavy metal ions like Ag^{+} (Toida et al.1998; Gopinath 2002, 2003), Hg^{2+} (Kamini et al. 2000; Sharma et al. 2001; Saxena et al. 2003) inhibit many microbial lipases. The enzyme from *A. niger* NCIM 1207 was not inhibited by majority of the metal ions tested except Ag^{+} which totally inactivated the lipase. Not much inhibition (20%) was observed in presence of Hg^{2+}. The activity of lipase from *B. coagulans* MTCC 6375 was enhanced in presence of Hg^{2+} (Kanwar et al. 2006). Lipase from *Aspergillus carneus* was inhibited by Cu^{2+},Cd^{2+},Hg^{2+}, Zn^{2+} and Pb^{2+} and stimulated by Mg^{2+} and Na^{2+} (Saxena et al. 2003) . Most of the lipases are not inhibited by metal ions such as Na^{+}, K^{+}, NH^{4+},Ca^{2+},Co^{2+},Mn^{2+},Ba^{2+},Mg^{2+},Fe^{2+}(Hiol et al. 1999; Kamini et al. 2000; Gopinath et al. 2002, 2003; Kanvar et al. 2006; Saxena et al. 2003). Metal ions like Ca^{2+}, Mg^{2+} are well known activators of lipases (Labuschagne et al.1997; Kim et al. 2000; Gopinath 2002, 2003; Saxena et al. 2003; Yu et al. 2007) because Ca^{2+} or Mg^{2+} form complexes with ionized fatty acids, changing their stability and behaviors at the interfaces (Gulmova 1993). Activity of lipase from *A. niger* NCIM 1207 was neither inhibited nor enhanced in presence of Ca^{2+} and Mg^{2+}. EDTA did not affect the enzyme activity suggesting that this lipase is neither metal requiring nor metallo-enzyme.

The substrate specificity studies demonstrated that the highest hydrolytic activity was obtained with *p*-nitrophenyl caprylate (C_8) indicating a clear preference of the enzyme for medium alkyl chain length fatty acids. Such substrate specificity towards medium alkyl chain length fatty acids were reported for many lipases (Lee et al. 2001; Lescic et al. 2001; Kanwar et al. 2006; Lee et al. 2006).

Lipase from *A. niger* NCIM 1207 hydrolyzed triolein resulting in the formation of 1,2-diolein as a main final product indicating that the enzyme cleaved 3-position bond of esters. Most of the microbial lipases such as lipases from *Aspergillus niger, Rhizopus delimar, Rhizopus miehei, Mucor javanicus* and *Yerrowia lipolytica* show 1, 3-positional specificity (Weete 1998; Saxena et al. 2003; Aloulou et al. 2007) releasing 2-monoacylglycerol and 1,2- and 2,3-diacylglycerol as products from the substrate. The

lipases with preference at 2-position are very rare in nature. Lipase C produced by *Geotrichum* sp. FO401B (Ota et al. 2000) and lipase form III and IV from *Geotrichum Candidum* ATCC 34614 (Sugihara et al. 1994) are good examples of this group of lipases. Lipases possessing no strict preference for position and fatty acid features hydrolyze all the ester bonds in various substrates to give glycerol and fatty acids as final products (Hiol et al. 1999; Lescic et al.2001; Kamini et al. 2000). Lipase from *A. niger* NCIM 1207 appears to be unique in relation to positional specificity because it cleaved triolein at only 3-position releasing 1,2-diolein as main product. Such lipases have not been reported so far in the literature.

The lipase of *A. niger* NCIM 1207 showed high affinity for medium chain length fatty acid esters (*p*NPC). The K_m and V_{max} for *p*-nitrophenyl palmitate were 0.33 mM and 746μmol min^{-1} mg^{-1} respectively and for *p*-nitrophenyl caprylate 0.14 mM and 1066.08 μmol min^{-1} mg^{-1} respectively. For *P. cepacia* lipase, Pencreac'h and Baratti (1996) reported K_m and V_{max} values of 12 mM and 30 mmol/min respectively, using *p*NPP as substrate. Kambourova et al. (2003) reported that the K_m and V_{max} for lipase of *B. stearothermophilus* are 0.33 mM and 188 μmol min^{-1} ml^{-1} respectively for the substrate, *p*NPP. *B. coagulans* lipase possess K_m and V_{max} 0.44 $mmol^{-1}$ min^{-1} and 28 mM respectively for hydrolysis of *p*NPP and 0.7 $mmol^{-1}$ min^{-1} and 32 mM for *p*NPC (Kanwar et al. 2006).

The Ser-His-Asp triad is well known structural feature of the serine proteases (Schrag et al. 1991; Brumlik & Buckley 1996). It is also observed in catalytic sites of several lipases with carboxylate residue of either aspartic acid or glutamic acid. These amino acids in the catalytic triad of lipases have been determined by either chemical modification studies (Boots et al. 1995; Ruiz et al. 2007) or by site directed mutagenesis (Frenken et al. 1992; Hyun-Ju et al. 2000). We employed chemical modification method to determine the amino acids responsible for catalysis. We found that the catalytic triad of *Aspergillus niger* NCIM 1207 lipase comprised of two His, one Ser and one carboxylate residues. In addition, we found that Trp plays an important role in catalytic activity since there was decrease in K_{cat} value without change in K_m. However, substrate protection studies revealed that the tryptophan inactivation was prevented by the excess of substrate. Hence, we feel that it could be also involved in substrate binding. Many reports are

available in which Trp is shown to be responsible for interfacial activation (Lookene et al. 1997; Feng et al. 2002). We also feel that it may serve the same function in case of *A. niger* NCIM 1207 lipase.

In conclusion, an extracellular acidic lipase from *Aspergillus niger* NCIM 1207 was purified approximately 150 fold with an overall yield of 54% and specific activity of 1373 U/mg. It is a single polypeptide chain with a molecular weight of 32.2 kDa and a pI of 8.5. It is neither metal requiring nor metallo-enzyme. The present studies showed that *A. niger* NCIM 1207 lipase is a unique acidic lipase because it is active at high acidic pH and is specific for 3-position in the ester bond. Moreover, the high stability of the enzyme in presence of organic solvents suggests that it has the potential for industrial applications such as flavor and bio-diesel production.

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CHAPTER – 5

Enzymatic synthesis of isoamyl acetate by mycelium bound lipase of *Aspergillus niger* NCIM 1207

Abstract

Commercial lipase preparations and mycelium bound lipase from *A. niger* NCIM 1207 were used for esterification of acetic acid with isoamyl alcohol to obtain isoamyl acetate. The esterification reaction was carried out at 30^{o}C in *n*-hexane with shaking at 120 rpm. Initial reaction rates, conversion efficiency and isoamyl acetate concentration obtained using Novozyme 435 were the highest. The optimization studies were carried out using *Aspergillus niger* NCIM 1207 mycelium bound lipase. Isoamyl acetate production was maximal at an alcohol-acid ratio of 1.6. Acetic acid at higher concentrations than required for the critical alcohol-acid ratio lower than 1.3 and higher than 1.6 resulted in decreased yields of isoamyl acetate probably owing to lowering of micro-aqueous environmental pH around the enzyme leading to inhibition of enzyme activity. Mycelium bound *Aspergillus niger* lipase produced 80g/l of isoamyl acetate within 96 h even though extremely less amount of enzyme activity was used for esterification. The presence of sodium sulphate during esterification reaction at higher substrate concentration resulted in increased conversion efficiency when we used mycelium bound enzyme preparations of A*spergillus niger* NCIM 1207. This could be due to removal of excess water released during esterification reaction by sodium sulphate. High ester concentration (286.5g/l) and conversion (73.5%) were obtained within 24 h using Novozyme 435 under these conditions.

Introduction

Fusel oil is a by-product generated during alcohol production by fermentation and its distillation. Generally the industrial alcohol produced by fermentation contains 0.1-0.2% of fusel oil which is recovered during the process of rectification of alcohol. With 300 distilleries in India and annual ethanol production more than 14000 million liters, 68 million liters of fusel oil was anticipated. The current selling price of fusel oil is Rs. 30-35/liter which is mainly used in paint, shoe polish industries and also as fuel for energy source. The fusel oil contains mainly isoamyl alcohol (55-60%) followed by n-propyl alcohol (15-20%), isobutyl alcohol (6-8%) and traces of n-butyl alcohol and ethanol. Esters obtained from alcohols of fusel oil can be used as solvents, flavoring agents, extractants, and plasticizers (Patil et al. 2002).

Esters of short chain acids and alcohols are compounds with great application in food, beverage, cosmetic and pharmaceuticals industries due to their characteristic flavor and fragrance (Leblanc *et a*l., 1998). Esters resulting from long chain fatty acids (12 – 20 carbon atoms) with short chain alcohols (3 –8 carbon atoms) are used as additives in food and detergent industries. The acetates of most alcohols are also commercially available. Ethyl, isopropyl, butyl, isobutyl, amyl and isoamyl acetates are used in cellulose nitrate and other lacquer type coatings due to their high solvent power. Butyl and hexyl acetates have excellent solvent properties useful for polyurethane coating systems. Ethyl, isobutyl, amyl and isoamyl acetates are frequently used as flavors. Among all these esters, isoamyl acetate is of commercial importance in the food industry (75 tons per year) because of its strong banana flavor (Welsh et al. 1990; Guvenc et al. 2002; 2003).

Most of the simple esters used commercially are produced synthetically by chemical reaction of an alcohol with an acid in presence of acid catalysts (Kirk & Othmer 1991) or from extraction from natural sources (Welsh et al. 1990). Natural flavor esters extracted from plant materials are often in short supply and are most expensive for commercial exploitation (Prapulla et al. 1992). The chemically synthesized product is cheap but not natural. Hence biotechnological production of flavor esters through enzymatic synthesis can be attractive / alternative route to the traditional chemical synthetic methods, particularly in the production of natural flavor and fragrances. This is due to selectivity of the enzymes, milder operation conditions, the degree of purity of the product and their acceptability in the food industry (Rocha et al. 1999). Esters obtained through bio-catalytic route are considered to be “Natural” satisfying the consumers demand (Gillies et al. 1987)

The lipases (triacylglycerol acylhydrolases E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. Many lipases are active in organic solvents and catalyze number of useful reactions such as esterification (Choudhary et al. 2001; Hamsaveni et al. 2001), trans-esterification, regio-selective acylation of glycols and menthols (Liese et al. 2000; Azim et al. 2001). Flavor esters are generally produced by free and immobilized lipases in organic solvents (Rizzi et al. 1992; Gubicza et al. 2000; Krishna et al. 2001). Recently, lipase catalyzed production of various esters such as isoamyl butyrate (Macedo et al. 2004), isoamyl propionate

(Krishna & Karanth 2002), hexyl butyrate and laurate (Chang et al. 2003; 2005), citronellyl flavor ester (Melo et al. 2005) has been investigated.

Many acetate esters are compounds of natural flavors, which can be synthesized by lipase-catalyzed esterification in organic solvents. However, there is very little information available on production of acetate esters in organic solvents. The use of acetic acid as acyl donor in trans-esterification or direct esterification was previously attempted with little success due to the toxicity of acetate on lipase activity causing dead end inhibition (Segel 1975). Acetic acid lowers the pH of the environment and interferes with their aqueous layers (Gillies et al. 1987; Claon and Akoh 1994). This aqueous acidic environment is responsible for the loss of lipase activity.

The literature on the production of isoamyl acetate using various sources of lipases can be summarized as following: Isoamyl acetate (26g/l) in 24 h in heptane was obtained with 80% conversion efficiency using 50g/l *Mucor miehei* lipase (Langrand et al. 1988). *Candida cylindraceae* lipase (20g/l) was used to obtain isoamyl acetate (17g/l) with 100% conversion in hexane in 48 h. (Welsh et al. 1990). Razafindralambo et al (1994) reported the production of 30g/l of isoamyl acetate with 80% conversion in heptane in 24 h using *Rhizomucor miehei* free lipase at 50g/l concentration. Krishna et al. (2000) have reached an isoamyl acetate (7g/l) with conversion of 95% using 3g/l Lipozyme IM-20 in heptane with equimolar substrate concentration (60mM) in 72 h. They also have obtained 150g/l of isoamyl acetate in heptane in 72 h using same enzyme at 10g/l concentration (Krishna et al. 2001).

The purpose of this study is to look for the possibility of using *A. niger* NCIM 1207 lipase (mycelium bound) for bioconversion of isoamyl alcohol to isoamyl acetate. For comparison, the commercial lipase enzyme preparations were also tested for isoamyl acetate formation. The optimization studies on the effect of various reaction parameters alcohol-acid molar ratio, substrate concentration, reaction time on isoamyl acetate formation were carried out using mycelium bound lipase.

Materials and Methods

Materials

Following commercial enzyme preparations were used.

1. Novozyme 435, Lipase from *Candida antarctica* (Sigma), 10,000 U/g
2. Lipase from *Candida rugosa* (Sigma), 1, 140 U/mg
3. Lipase from *Rhizomucor miehei* (Sigma), 20,000 U/g
4. Lipolase Ultra 50 T , 50 KULU/g, (kilo ultra lipase units), is a protein engineered lipase produced by genetically modified *Aspergillus*..
5. Lipase from *Mucor miehei* (Sigma) with 5770 U/mg solid
6. Lipase from *Aspergillus niger* (Fluka) with 194 U/g solid

The mycelium bound and celite bound lipases of *A. niger* NCIM 1207 were prepared as described in Chapter 2. All the other chemicals used were of analytical grade.

Methods

Esterification reaction

Esterification reaction was carried out in *n*-hexane with a working volume of 10 ml in 25 ml stoppered flask. An appropriate amount of enzyme was added in to a reaction mixture containing a solution of isoamyl alcohol and acetic acid and sodium sulphate pre-equilibrated at the conditions of experiment. The reaction mixture was incubated at 30^{o}C with shaking at 120 rpm and the samples were withdrawn at different time intervals to analyze the isoamyl acetate content in the reaction mixture.

Analysis

The samples (for isoamyl acetate) were analyzed on Varian (Varian Analytical Instruments, USA) gas chromatograph (Model CP-3800 GC) equipped with CP Wax 52 CB column (30 m length, 0.32 mm internal diameter) and a flame-ionization detector. Nitrogen gas was used as a carrier gas with a flow rate of 30 ml/min. Column oven, injection port and detector temperatures were 220, 230 & 240^{o}C respectively. The ester formed was calculated as being equivalent to acid consumed. The conversion of acetic acid to isoamyl acetate was calculated as the ratio of ester concentration to initial acid concentration [(mmol of ester formed / mmol of ester calculated for initial acid concentration) x 100].

Results

Effect of enzyme source

The effect of lipase source on isoamyl production was investigated by comparing different commercial lipases and the results are given in Table 1. Mycelium bound lipase of *A. niger* NCIM 1207 was also tested for isoamyl acetate formation. It was observed that extent of esterification was higher for Novozyme 435 lipase yielding 100% in 4 h. Mycelium bound lipase of *Aspergillus niger* NCIM 1207 also gave high esterification but the rate of reaction was slower. No other commercial preparations produced isoamyl acetate except *C. rugosa* lipase which gave only 2.5g/l of isoamyl acetate with 3.1% esterification. The degree of esterification obtained with three enzyme preparations at different reaction time is shown in Figure 1. Although the conversion was almost 100% using both the enzymes for esterification, the reaction reached equilibrium for Novozyme 435 in 4 h where as lipase from *A. niger* took almost 96 hrs to reach equilibrium. Therefore most of the optimization experiments were carried out using Novozyme 435. Some optimization studies were also carried out using mycelium bound lipase of *A. niger* NCIM 1207.

Table 1. Bioconversion using different commercial enzyme sources. The esterification reaction was carried out using acid: alcohol molar ratio of 1.6 with 37.7g/l acetic acid.

Enzyme source	Isoamyl acetate (g/l)	Conversion (%)
Novozyme 435 (Sigma) (10g/l)	81.0	100.0 (4 h)
Aspergillus niger NCIM 1207 mycelium bound lipase (100g/l).	80.0	99.0 (96 h)
Celite bound enzyme (10g/l)	-	-
Lipolase Ultra 50 T (10g/l)	-	-
Candida rugosa lipase(Sigma) (10g/l)	2.5	3.1 (96 h)
Rhizomucor miehei lipase (Sigma) (10g/l)	-	-
Mucor miehei lipase (Sigma) (10g/l)	-	-
Aspergillus niger lipase (Fluka) (10g/l)	-	-

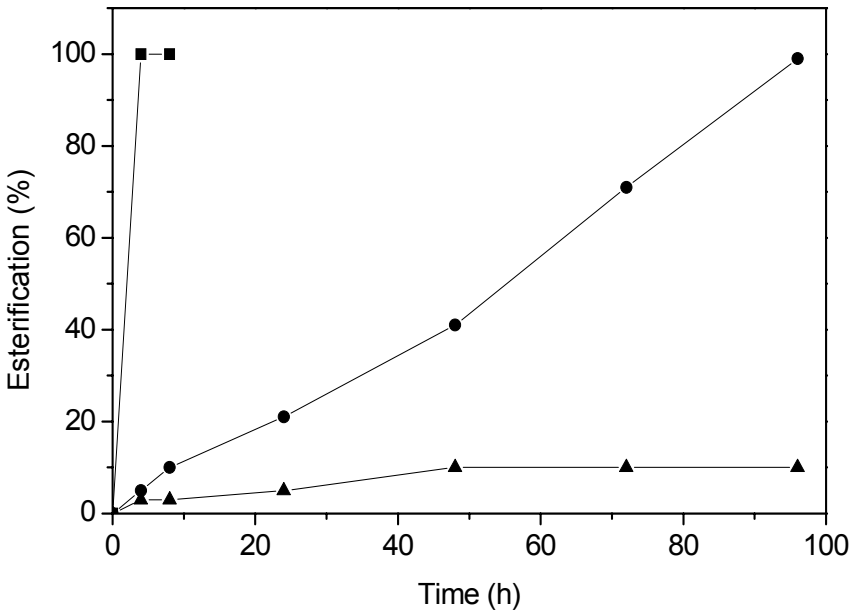

Figure 1. Effect of enzyme source on isoamyl acetate production. The esterification reaction was carried out in *n*-hexane containing 1M isoamyl alcohol, 0.6M acetic acid, 500mg of sodium sulphate and Novozyme 435 (■), *C. rugosa lipase* (▲), Mycelium bound lipase of *A. niger* NCIM 1207 (●).

The effect of alcohol to acid molar ratio on the esterification yield was investigated by fixing alcohol concentration at 0.8M and by varying acid concentration (0.32M – 1.3M). All the experiments were performed at mycelium bound *A. niger* NCIM 1207 lipase 100g/l. Reaction mixtures were incubated at 30^{o}C with shaking at 120 rpm for 96h. The rate of reaction increased with increase in alcohol to acid ratio, because the equilibrium of the reaction was pushed toward the product formation as the alcohol concentration increased. At isoamyl alcohol to acetic acid molar ratios more than 1.6 did not change the reaction rate significantly. A maximum yield of isoamyl acetate (100% in 96 h) was achieved when alcohol to acid ratio of 1.3-1.6 was employed (Figure 2).

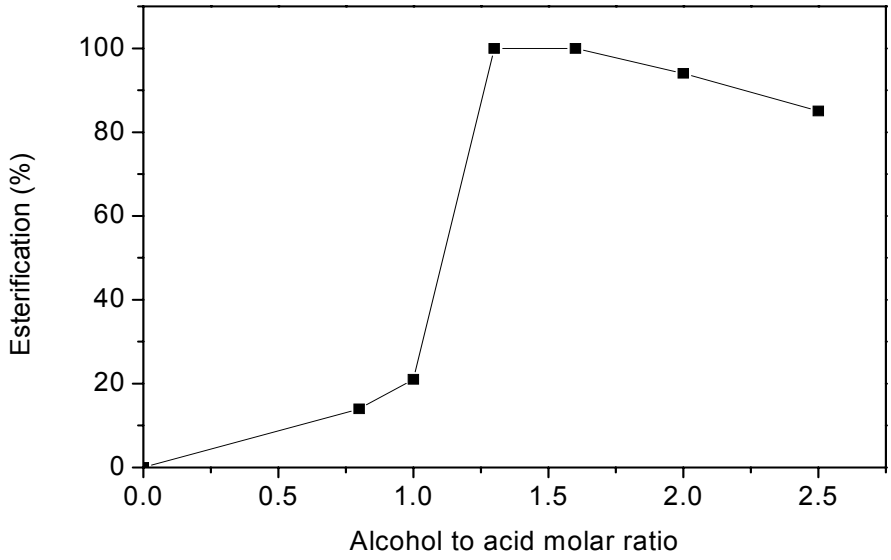

Figure 2. Effect of alcohol to acid molar ratio on synthesis of isoamyl acetate. The esterification reaction was carried out in n-hexane with different alcohol to acid molar ratios at fixed concentration (0.8M) of isoamyl alcohol using *A. niger* NCIM 1207 lipase (100g/l).

Effect of substrate (acetic acid) concentration on activity of mycelium bound lipase of *A. niger* NCIM 1207

We also evaluated the effect of different substrate (acetic acid) concentration (0.15M – 0.62M) on esterification using mycelium bound lipase of *A. niger* NCIM 1207 with fixed alcohol to acid ratio at 1.6. We found that the conversion was 98% up to 0.3M acetic acid concentration followed by sudden drop to 60% with higher substrate level of 0.6M (Table 2). This reduction in the conversion could be due to the acid inactivation of the enzyme or to the excess water generated during the esterification reaction, which might shift the equilibrium of the reaction towards hydrolysis. The first possibility could be ruled out since the mycelium bound lipase was found to be active and stable even at pH 2.0 for 24 h. The second possibility was confirmed by carrying out the esterification reactions in presence of sodium sulphate, which removes water generated during esterification reaction. The presence of sodium sulphate (5%) resulted in 99% conversion of acetic acid (0.62M) yielding 80g/l of isoamyl acetate. The results showed that the drop in conversion was due to excess water formed during esterification (Table 2, Figure 3).

Table 2. Effect of substrate (acetic acid) concentration on isoamyl acetate formation using mycelium bound lipase of *Aspergillus niger* NCIM 1207.

Substrate (M)	Isoamyl acetate (g/l)		Conversion (%)	
	Without sodium sulphate	With sodium sulphate 5%)	Without sodium sulphate	With sodium sulphate (5%)
0.15	20	20.4	98	100
0.31	40	40.9	98	100
0.47	52	54.5	85	89
0.62	50	80.0	61	99

Esterification reaction was carried out in 25 ml stopper flask containing different concentration of acetic acid (0.15 – 0.62M) at alcohol to acid molar ratio of 1.6 and 1 g mycelium bound enzyme in 10 ml n-hexane. The reaction mixture was incubated at 30^{o}C with shaking at 120 rpm. Samples were taken after every 24 hrs till 96 hrs.

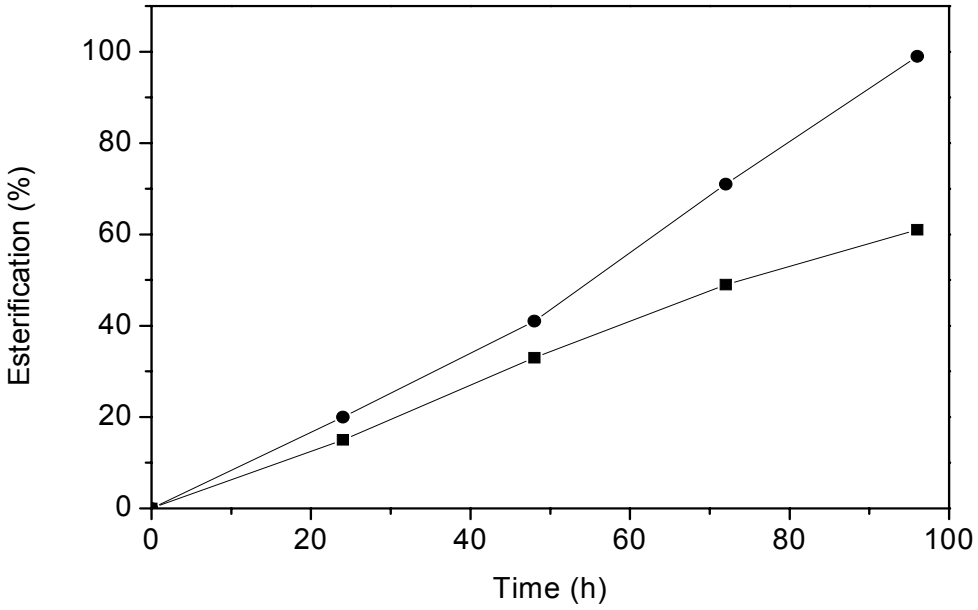

Figure 3. Formation of isoamyl acetate by mycelium bound lipase of *A. niger* NCIM 1207 in presence (●) and in absence (■) of sodium sulphate (5%). The esterification reaction was carried out in *n*-hexane containing isoamyl alcohol (1.0M) and acetic acid (0.62M) and enzyme (100g/l).

Effect of substrate concentration on Novozyme 435.

The effect of varying concentrations of acetic acid (0.8M – 3.0 M) on extent of esterification is presented in Table 3. The esterification of about 100% within 8 h was observed up to 1.0 M acid concentration and it was reduced to 90% at 1.25 M acid concentration. The presence of sodium sulphate in the reaction mixture did not help to enhance the conversion indicating water generated has no effect on synthetic reaction probably at these concentrations of the substrate. The further increase in concentration of acetic acid resulted in less conversion possibly due to water produced during esterification. Therefore the experiment was planned to see the effect of addition of sodium sulphate during esterification at higher (>1.25M) acetic acid concentration. It was observed that the addition of sodium sulphate helped to increase the esterification of isoamyl alcohol to isoamyl acetate (Table 3). Maximum isoamyl acetate concentration of 286 g/l was obtained using 20 g/l of Novozyme 435 (Figure 4) with 73% esterification

Table 3. Effect of sodium sulphate on isoamyl alcohol formation by Novozyme 435.

Enzyme (g/l)	Acetic acid (g/l)	Sodium sulphate (g/l)	Product (g/l)	Esterification (%)
10	48.0 (0.8M)	Nil	103.0	99.3
10	60.0 (1.0M)	Nil	128.0	98.4
10	75.5 (1.25M)	Nil	148.0	90.7
10	75.5 (1.25M)	50	152.0 (8 hrs)	93.2
10	105.0 (1.74M)	50	187.5 (8 hrs)	83.0
10	143.7 (2.37M)	50	219.0 (24 hrs)	70.3
20	143.7 (2.37M)	50	220.5 (24 hrs)	70.5
20	143.7 (2.37M)	100	265.5 (8 hrs)	85.2
20	180.0 (3.0M)	100	259.5 (8 hrs)	66.5
20	180.0 (3.0M)	150	286.5 (24 hrs)	73.5

Esterification reaction was carried out in 25 ml stopper flask containing different concentration of acetic acid (0.8M – 3.0M) at alcohol to acid molar ratio of 1.6 and 100 or 200 mg of Novozyme 435 in 10 ml n-hexane. The reaction mixture was incubated at 30^{o}C with shaking at 120 rpm. Samples were taken after 8 h and 24 h.

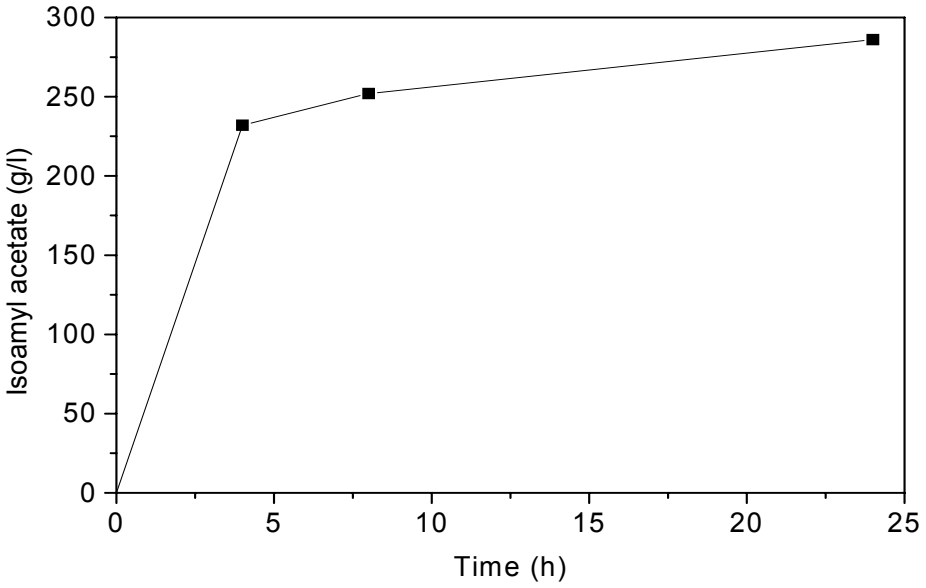

Figure 4. Profile of isoamyl acetate formation using Novozyme 435 (20 g/l) at higher concentration of acetic acid (180g/l) in presence of sodium sulphate (150 g/l).

Discussion

Various enzyme sources either in the form of free lipases (Langrand et al. 1988; Razafindralambo et al. 1994) or immobilized lipases (Gubicza et al. 2000; Krishna et al. 2000) have been used for production of flavor acetates in organic solvents. The effect of various commercial lipases and also mycelium bound lipase on isoamyl acetate production was investigated. We found that the reaction rate and esterification were higher using Novozyme 435 yielding 100% esterification in 4h. Novozyme 435 was also found suitable in the synthesis of other acetate esters in hexane (Bourg-Garros et al. 1998), in heptane (Yadav & Trivedi 2003) and also in solvent-free systems (Guvenc et al. 2002). *A. niger* NCIM 1207 produces lipase active at acidic pH and it was also found to produce isoamyl acetate (80g/l) indicating the new potential candidate for production of flavor esters in organic solvents.

Guvenc et al (2007) carried out optimization studies on lipase-catalyzed production of industrially important isoamyl acetate by response surface methodology using Novozyme 435. They found that acid/alcohol molar ratio was the most effective

parameter in the production of isoamyl acetate. The maximum esterification was obtained with acid /alcohol ratio of 0.8 with 12% enzyme concentration. In our studies, we found that alcohol / acid molar ratio of 1.3 – 1.6 was optimum for getting maximum esterification. The extent of esterification increased with increase in alcohol to acetic acid molar ratio because the equilibrium of the reaction was pushed toward the product formation as the nucleophile (alcohol) concentration is raised. The esterification leveled off at the alcohol to acid molar ratios higher than 1.6. The strong effect of acid inhibition on enzyme activity was observed at alcohol to acid ratios below 1.3 leading to decreased esterification. Such strong effect of acid inhibition was also reported by Guvenc et al (2002; 2007) and Romeo et al (2005; 2007). The acid inhibition of lipase could be due to either accumulation of water during reaction which favors hydrolysis or due to substrate (acid) inhibition. It was also asserted that alcohols are terminal inhibitors of lipases and acids may cause acidification of micro-aqueous interface leading to enzyme inactivation.

The isoamyl acetate concentration obtained so far in literature using Novozyme 435 are 150g/l in 72 h in heptane (Krishna et al. 2001) and 381g/l in 6 hrs in solvent-free system (Guvenc et al. 2002), both with 80% conversion. Very recently, Novozyme 435-mediated synthesis of isoamyl acetate was carried out using isoamyl alcohol obtained from fusel oil and acetic acid in solvent free system by RSM technique (Guvenc et al. 2007). They obtained 490g/l of isoamyl acetate with 75% esterification efficiency using 12% enzyme. In our study, we were able to produce 281g/l of isoamyl acetate with 73.5% esterification using Novozyme 435 under optimized conditions. The amount of enzyme used was only 2% which is much less compared to others. We also carried out experiments using mycelium bound *A. niger* NCIM 1207 lipase which gave 80g/l of isoamyl acetate with 99% esterification indicating that this could be potential candidate for production of isoamyl acetate. It is noteworthy to mention that the amount of *A. niger* enzyme used in terms of enzyme activity was approximately 70 times less (1400 IU/l) as compared to Novozyme 435 (100,000 PLU/l).

In conclusion, mycelium bound lipase of *A. niger* NCIM 1207 was able to produce isoamyl acetate indicating its potential use in bioconversion of fusel oil to isoamyl acetate.

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Conclusions

- After screening of fungal strains available in NCIM, *Aspergillus niger* NCIM 1207 was found to produce both intra- and extracellular lipase that are active at extremely acidic pH of 2.5. **Lipases active at such acidic pH are not reported so far in the literature from microbial sources.**

- Solid state fermentation studies revealed that the *A. niger* NCIM 1207 produces very high levels of lipase activity (630 IU/g DSS). Such high levels are not reported so far in the literature for any *Aspergillus* strain.

- Mutant of *A. niger* NCIM 1207 designated as UV-10, possessing compact colony morphology, was identified which produces seven fold higher lipase under submerged fermentation than the parent strain. This increased lipase production could be attributed to alteration in cell wall leading to permeability changes in the mutant strain.

- The purified enzyme is monomer with molecular weight of 32.2 kDa and pI of 8.5. The enzyme possess 3-positional specificity cleaving triolein in to 1,2-diolein. **Lipases with such positional specificity are not reported so far and hence it appears to be unique.**

- This acidic lipase was found to be stable in majority of organic solvents. We were able to employ this enzyme for production of isoamyl acetate which is used in flavor and food industry.

List of Publications

Nutan D. Mahadik, Ulka S. Puntambekar, Kulbhushan B. Bastawde, Jayant M. Khire, Digambar V. Gokhale (2002). Production of acidic lipase by *Aspergillus niger* in solid state Fermentation. Process biochemistry **38**:715-721

Nutan D. Mahadik, Ulka S. Puntambekar, Kulbhushan B. Bastawde, Jayant M. Khire, Digambar V. Gokhale (2004). Production of acidic lipase by a mutant of *Aspergillus niger* NCIM 1207 in submerged fermentation. Process biochemistry **39**:2031-2034

Nutan D. Mahadik, Ulka S. Puntambekar, Kulbhushan B. Bastawde, Jayant M. Khire, Digambar V. Gokhale **(2003).** Process for preparation of acidic lipase. **US Patent 6,534,303**

K. D. Trimukhe, **Nutan D. Mahadik**, Digambar V. Gokhale, Anjani J. Varma (2007). Environment friendly cross-linked chitosan as a matrix for selective adsorption and purification of lipase of *Aspergillus niger* NCIM 1207. Green Chemistry (Communicated).

Nutan D. Mahadik, Kulbhushan B. Bastawde, Digambar V. Gokhale. Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207. Manuscript in preparation.

Nutan D. Mahadik, Kulbhushan B. Bastawde, Digambar V. Gokhale. Enzymatic synthesis of isoamyl acetate by *Aspergillus niger* lipase. Manuscript in preparation.