LIPASE PRODUCTION BY MARINE FUNGUS ASPERGILLUS AWAMORI NAGAZAWA

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In
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by

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Lipase production by marine fungus *Aspergillus awamori* Nagazawa**" is based on the original research work carried out by Mr. Soorej M Basheer under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree.

M. CHANDRASEKARAN

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Lipase production by marine fungus *Aspergillus awamori* Nagazawa**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin under the guidance of Dr. M. Chandrasekaran, Professor, Cochin University of Science and Technology and the thesis or no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles or recognition.

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26-04-2007

Soorej M Basheer

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Soorej M Basheer

Dedicated to My Parents

ABBREVIATIONS

%	-	Percentage
°C	-	degree Celsius
A	-	Adenine
A°	-	Angstrom
A_{260}	-	Absorbance at 260 nm
A_{280}	-	Absorbance at 280 nm
ANOVA	-	Analysis of Variance
APS	-	Ammonium per sulphate
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pairs
BSA	-	Bovine serum albumin
C	-	Cytosine
cfu	-	colony forming units
Conc.	-	Concentration
DEAE Cellulose	-	Diethyl amino ethyl cellulose
DNA	-	Deoxy ribo nucleic acid
dNTP	-	deoxy nucleotide tri phosphate
dsDNA	-	double stranded DNA
DW	-	Distilled water
E. coli	-	*Escherichia coli*
EC	-	Enzyme classification
EDTA	-	Ethylene diamine tetra acetic acid
g	-	grams
G	-	Guanine
GC	-	Gas chromatography
h	-	Hours
HPLC	-	High performance liquid chromatography

kDa	-	Kilo Dalton
Km	-	Substrate concentration at which the reaction velocity is half maximum
kV	-	Kilo Volt
LB	-	Luria Bertani
M	-	Molar
ME	-	Malt extract
mg	-	milligram
ml	-	milliliter
mm	-	millimeter
MUF	-	4-Methylumbelliferyl
NJ	-	Neighbour Joining
nm	-	Nanometer
OD	-	Optical density
ORF	-	Open reading frame
PAGE	-	Polyacrylamide gel electrophoresis
P-B	-	Plackett Burman
PCR	-	Polymerase chain reaction
PEG	-	Polyethylene glycol
pNP	-	Para nitro phenol
PUFA	-	Poly unsaturated fatty acid
PVA	-	Poly vinyl alcohol
RNA	-	Ribonucleic acid
rpm	-	revolutions per minute
rRNA	-	ribosomal RNA
RSM	-	Response surface methodology
RT	-	Room temperature
SDS	-	Sodium dodecyl sulphate
SmF	-	Submerged fermentation

sp.	-	Species
SSF	-	Solid state fermentation
t pa	-	tonns per annum
T	-	Thymine
TAE	-	Tris acetic acid EDTA
Taq	-	*Thermus aquaticus*
TCA	-	Trichloro acetic acid
TE	-	Tris EDTA
TEMED	-	N-N-N'-N'-tetramethyl ethylene diamine
TLC	-	Thin Layer Chromatography
U/mg	-	Units/milligram
U/ml	-	Units/milliliter
UV	-	Ultra violet
V	-	volts
v/v	-	volume/volume
V_{max}	-	maximal velocity
µg	-	microgram
µl	-	microlitre
µM	-	micromole

CONTENTS

Chapter 1

INTRODUCTION

The use of enzyme-mediated processes can be traced to ancient civilizations. Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use. Until the 1960s, the total sale of enzymes were only a few million dollars annually, but the market has since grown spectacularly (Godfrey and West, 1996; Wilke, 1999). The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60 % of the total world supply of industrial enzymes is produced in Europe. At least 75 % of all industrial enzymes (including lipases) is hydrolytic in action. The global market for industrial enzymes is estimated at $2 billion in 2004 and is expected to rise at an average annual growth rate (AAGR) of 3.3 % to $2.4 billion in 2009. The Indian market for enzymes was estimated to be more than Rs 320 crores in the year 2004-05.

Lipases are considered to be the third largest group based on total sales volume after proteases and carbohydrases. The commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications (Jaeger *et al.*, 1999).

In addition to their biological significance, lipases hold tremendous potential for exploitation in biotechnology (Vakhlu and Kour, 2006). Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of biosurfactants, the oleochemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes

(Liese *et al.*, 2000). An increasing number of lipases with suitable properties are becoming available and efforts are underway to commercialize biotransformation and syntheses based on lipases (Liese *et al.*, 2000).

The major commercial application for hydrolytic lipases is their use in laundry detergents. Detergent enzymes make up nearly 32% of the total lipase sales. Lipase for use in detergents needs to be thermostable and remain active in the alkaline environment of a typical machine wash. An estimated 1000 tons of lipases are added to approximately 13 billion tons of detergents produced each year (Jaeger and Reetz, 1998). Lesser amounts of lipases are used in oleochemical transformations (Bornscheuer, 2000). Lipases play an important role in the processing of g-linolenic acid, polyunsaturated fatty acid (PUFA); astaxanthin, food colorant; methyl ketones, flavor molecules characteristic of blue cheese; 4-hydroxydecanoic acid used as a precursor of g-decalactone, fruit flavor; dicarboxylic acids for use as prepolymers; interesterification of cheaper glycerides to more valuable forms (e.g., cocoa butter replacements for use in chocolate manufacture) (Undurraga *et al.*, 2001); modification of vegetable oils at position 2 of the triglyceride to obtain fats similar to human milkfat for use in baby feeds; lipid esters including isopropyl myristate, cosmetics; and monoglycerides for use as emulsifiers in food and pharmaceutical applications.

Lipases (EC 3.1.1.3) (triacylglycerol acylhydrolases), which belong to the class of serine hydrolases, catalyze both the hydrolysis and the synthesis of esters from glycerol and long chain fatty acids.

A typical reaction catalyzed by lipases is shown in Fig. 1.1.

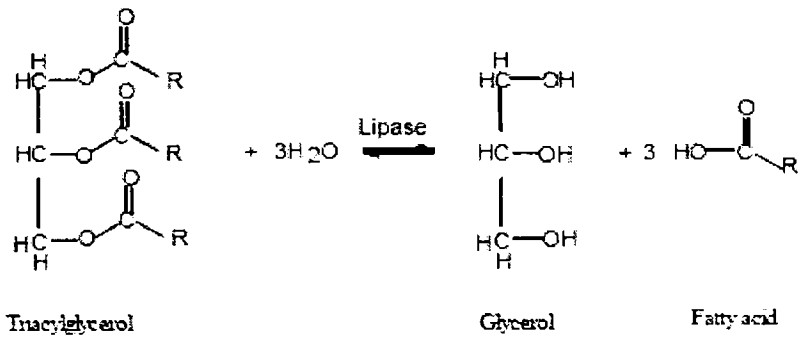


Fig. 1.1 Hydrolysis of triacylglycerol by lipase

Lipases are used in two distinct fashions. They are used as biological catalysts to manufacture products such as food ingredients, and by their application as such in making fine chemicals. Lipases are valued biocatalysts because they act under mild conditions, highly stable in organic solvents, show broad substrate specificity, and show high regio and/or stereoselectivity in catalysis (Snellman *et al.*, 2002). Lipases are active under ambient conditions and the energy expenditure required to conduct reactions at elevated temperatures and pressures is eliminated that reduces the destruction of labile reactants and products. Lipase catalysed processes offer cost-effectiveness too, in comparison with traditional downstream processing.

Lipases remain a subject of intensive study (Bornscheuer, 2000) because of their wide-ranging significance. Research on lipases is focussed particularly on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes, and general characterization of performance (Bornscheuer, 2000). It is expected that in the next few years lipases

will benefit from their versatility and continued penetration into the detergent and cosmetic markets (Hasan *et al.*, 2006).

Lipases from plants, animals and microbes, particularly bacterial and fungal sources were the subject of several earlier investigations (Yadav *et al.*, 1998a; Yamaguchi *et al.*, 1991). Although pancreatic lipases have been traditionally used for various purposes, it is now well established that microbial lipases are preferred for commercial applications due to their multifold properties, easy extraction procedures, and unlimited supply (Macrae and Hammond, 1985).

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. They are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications. Microbial lipases today occupy a place of prominence among biocatalysts owing to their ability to catalyze a wide variety of reactions in aqueous and non-aqueous media.

Lipase producing microorganisms include bacteria, fungi, yeasts and actinomycetes. Fungi are one of the most important lipase sources for industrial application because fungal enzymes are usually excreted extracellularly, facilitating extraction from the fermentation media. A large number of filamentous fungi have been studied for lipase production (Elibol and Ozer, 2002; Mahadik *et al.*, 2002; Maia *et al.*, 2001).

There are only few lipases used at an industrial scale in organic synthesis, presumably because of difficulties in establishing cost-effective scaling-up and downstream processing protocols. In near future, an important application area will

be the increasing use of lipases as biocatalysts for the preparation of chiral compounds in enantiomerically pure form (Jaeger and Reetz, 1998).

1.1 Enzymes from marine microbial sources

The marine environment, which encompasses about 71 percent of the Earth's surface, is potentially a vast resource for useful enzymes. Microbes live in various habitats in the marine environment, including neuston, plankton, nekton, seston and epibiotic, endobiotic, pelagic and benthic environments. These habitats harbor a diverse range of microbes including archaebacteria, cyanobacteria, actinomycetes, yeasts, filamentous fungi, microalgae, algae and protozoa. Almost all these groups are potential sources of useful enzymes. The ecological role of these microorganisms is in the mineralization and recycling of complex organic matter through degradative pathways and thus also contribute to the secondary production in the sea. Bacteria and fungi secrete different enzymes such as protease, amylase, lipase, chitinase, cellulase, ligninase, pectinase, xylanase, nucleases (DNases, RNases, restriction enzymes etc.) etc. based on their habitat and ecological functions. Harsh marine environments, such as deep ocean, hydrothermal vents, polar oceans and extremely saline bodies of water, have yielded valuable extremophilic microorganisms, which are the primary source of enzymes that are active at extreme conditions. An extensive review of marine microbial enzymes is presented by Chandrasekaran and Kumar (2002).

Despite the fact that lipases has been the subject of research at global and national level, marine microbial lipases remains unexploited and very little attention has been paid towards utilization of them. Hence, in the present study it was aimed to screen marine fungus isolated from seawater and sediments of South Indian Coastal environments for lipase production in order to select a potential strain that can produce novel lipase for further industrial application.

OBJECTIVES OF THE PRESENT STUDY

The primary objectives of this study include the evaluation of the potential of a marine fungus *Aspergillus awamori* Nagazawa, for lipase production through submerged fermentation, scale up studies using statistical optimization, purification, characterization of this enzyme and assessment of its potential applications.

Specific objectives of the present study include

1. Screening of potential fungus that produce lipase.
2. Optimization of bioprocess variables for the production of lipase employing statistical approach.
3. Purification and characterization of the lipase.
4. Isolation of gene encoding lipase.
5. Application studies with lipase.

Chapter 2

REVIEW OF LITERATURE

2.1 Lipases

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. They catalyse the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and fatty acids, and under certain conditions, the reverse reaction leads to esterification and formation of glycerides from glycerol and fatty acids. The hydrolysis is a reversible reaction and the potential of lipases for ester synthesis (Cao *et al.*, 1992; Chulalaksananukul *et al.*, 1990; Linko *et al.*, 1995a; Malcata *et al.*, 1990; Mustranta *et al.*, 1993) and for interesterification (Bloomer *et al.*, 1990; Lamboursain *et al.*, 1996; Safari and Kermasha, 1994) in organic media has been well documented. A true lipase will split emulsified esters of glycerine and long-chain fatty acids such as triolein and tripalmitin. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface (Martinelle *et al.*, 1995) and do not hydrolyze dissolved substrates in the bulk fluid. Lipases are serine hydrolases and they display little activity in aqueous solutions containing soluble substrates. In contrast, esterases show normal Michaelis–Menten kinetics in solution (Sharma *et al.*, 2001). Lipases may catalyze three types of reaction: hydrolysis, esterification, and transesterification. It is also well known that some lipases display position specificity (regiospecificity) toward fatty acids in triacylglycerols as well as fatty acid selectivity (Marangoni and Rousseau, 1995).

These reactions can be illustrated by Eqs. (1) and (2) as follows:

$$R_1COOH + R_2OH \leftrightarrow R_1COOR_2 + H_2O \quad (1)$$

Esterifcation (→) and hydrolysis (←)

$$R_1COOR_2 + R_3OH \leftrightarrow R_1COOR_3 + R_2OH \quad (2)$$

Transesterification

2.2 Action of lipase

Lipases belong to the class of serine hydrolases and therefore do not require any cofactor. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyse the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved (Fig. 2.1) (Saxena *et al.*, 1999). Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The occurrence of the lipase reaction at an interface between the substrate and the aqueous phase causes difficulties in the assay and kinetic analysis of the reaction (Macrae and Hammond, 1985). The usual industrial lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyse them initially into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids (Bjorkling *et al.*, 1991; Ghosh *et al.*, 1996).

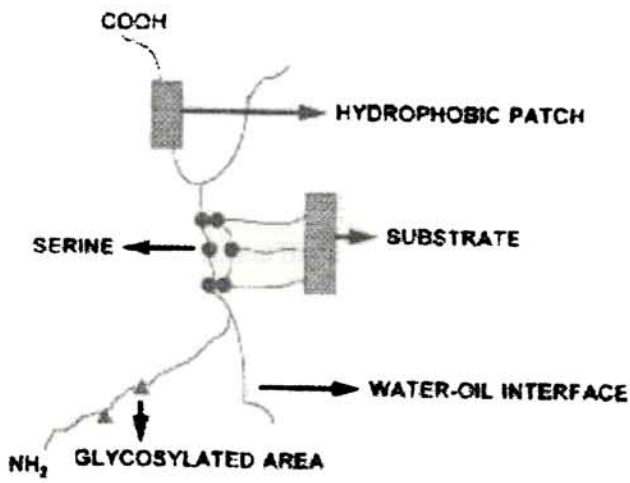


Fig. 2.1 Diagrammatic representation of a lipase molecule showing its main features. Substrate can be any triglyceride

In nature, the lipases available from various sources have considerable variation in their reaction specificities: this property is generally referred as enzyme specificity. Thus, from the fatty acid side, some lipases have affinity for short-chain fatty acids (acetic, butyric, capric, caproic, caprylic, etc.), some prefer unsaturated fatty acids (oleic, linoleic, linolenic, etc.), while many others are nonspecific and randomly split the fatty acids from the triglycerides. From the glycerol side of the triglycerides, the lipases often show positional specificity and attack the fatty acids at 1 or 3 carbon position of glycerol or at both the positions but not the fatty acid at the 2 position of the glycerol molecule (Saxena *et al.*, 1999). However, through random acyl migration, the 2-fatty acid monoglyceride undergoes rearrangement pushing the fatty acid to the 1 or 3 position of the glycerol molecule. As acyl migration is a slow process and the available lipases do not act on glycerol 2-mono fatty acid esters, the hydrolysis slows down and awaits the acyl migration to complete for enabling the lipase to attack the glyceride at the 1 and/or the 3 position. Interestingly, lipases function at the oil-water interface (Fig. 2.2). The amount of oil available at the interface determines the activity of the

lipases (Verger, 1997). This interface area can be increased substantially to its saturation limit by the use of emulsifier as well as by agitation. The saturation limit depends on the ingredients used as well as the physical conditions deployed. Thus, the activities of lipases can be pronouncedly increased by use of emulsifying agents as well as by methods that increase the size of the emulsion micelles (Borgstrom and Brockman, 1984; Brockerhoff and Jensen, 1974).

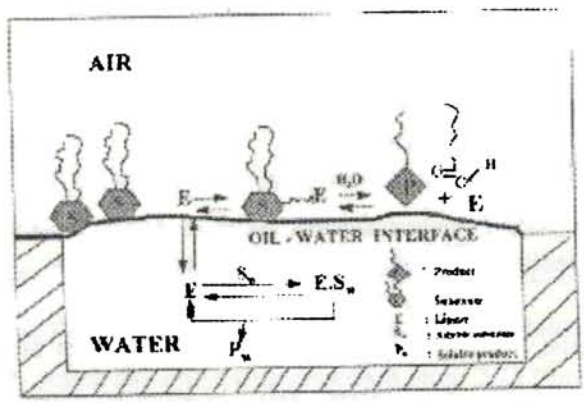


Fig.2.2. Lipolytic reaction at the oil–water interface

2.3 Microbes as source of lipase

Many microorganisms and higher eukaryotes produce lipases. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food (Sztajer *et al.*, 1988), compost heaps, coal tips, and hot springs (Wang *et al.*, 1995). Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomycetes. Some of the lipase-producing microorganisms are listed in Table 2.1.

Table 2.1 Some lipase-producing microorganisms

Source	Genus	Species	Reference(s)
Bacteria (Gram-positive)	Bacillus	*B. megaterium*	Godtfredsen, 1990
		B. cereus	El-Shafei and Rezkallah, 1997
		B. stearothermophilus	Gowland *et al.*, 1987
			Kim *et al.*, 1998
		B. subtilis	Kennedy and Rennarz, 1979
		Recombinant *B. subtilis* 168	Lesuisse *et al.*, 1993
		B. brevis	Hou, 1994
		B. thermocatenulatus	Rua *et al.*, 1998
		Bacillus sp. IHI-91	Becker *et al.*, 1997
		Bacillus strain WAI 28A5	Janssen *et al.*, 1994
		Bacillus sp.	Helisto and Korpela, 1998
		B. coagulans	El-Shafei and Rezkallah, 1997
		B. acidocaldarius	Manco *et al.*, 1998
		Bacillus sp. RS-12	Sidhu *et al.*, 1998a; Sidhu *et al.*, 1998b
		B. thermoleovorans ID-1	Lee *et al.*, 1999
		Bacillus sp. J 33	Nawani and Kaur, 2000
	Staphylococcus	*S. canosus*	Tahoun *et al.*, 1985
		S. aureus	Lee and Yandolo, 1986
		S. hyicus	Kampen *et al.*, 1998; Meens *et al.*, 1997; Oort *et al.*, 1989
		S. epidermidis	Farrell *et al.*, 1993; Simons *et al.*, 1998
		S. warneri	Talon *et al.*, 1995

	Lactobacillus	*Lactobacillus delbruckii* sub sp. bulgaricus	El-Sawah *et al.*, 1995
		Lactobacillus sp.	Meyers *et al.*, 1996
	Streptococcus	*Streptococcus lactis*	Sztajer *et al.*, 1988
	Micrococcus	*Micrococcus freudenreichii*	Hou, 1994
		M. luteus	Hou, 1994
	Propionibacterium	*Propionibacterium acne*	Sztajer *et al.*, 1988
		Pr. granulosum	Sztajer *et al.*, 1988
	Burkholderia	*Burkholderia* sp.	Yeo *et al.*, 1998
		Bu. glumae	El-Khattabi *et al.*, 2000
Bacteria (Gram-negative)	Pseudomonas	*P. aeruginosa*	Aoyama *et al.*, 1988; Hou, 1994; Ito *et al.*, 2001
		P. fragi	Mencher and Alford, 1967
		P. mendocina	Jaeger and Reetz, 1998
		P. putida 3SK	Lee and Rhee, 1993
		P. glumae	Frenken *et al.*, 1993; Noble *et al.*, 1994
		P. cepacia	Hsu *et al.*, 2000; Lang *et al.*, 1998; Pencreac'h and Baratti, 1996
		P. fluorescens	Lacointe *et al.*, 1996; Marangoni, 1994
		P. aeruginosa KKA-5	Sharon *et al.*, 1998
		P. pseudoalcaligenes F-111	Lin *et al.*, 1995; Lin *et al.*, 1996b
		Pseudomonas sp.	Sin *et al.*, 1998
			Dong *et al.*, 1999; Miyazawa *et al.*, 1998; Reetz and Jaeger, 1998
		P. fluorescens MF0	Guillou *et al.*, 1995

		Pseudomonas sp. KWI56	Yang *et al.*, 2000
	Chromobacterium	*Ch. viscosum*	Diogo *et al.*, 1999; Helisto and Korpela, 1998; Jaeger and Reetz, 1998; Rees and Robinson, 1995
	Acinetobacter	*Aci. pseudoalcaligenes*	Sztajer *et al.*, 1988
		Aci. radioresistens	Chen *et al.*, 1999
	Aeromonas	*Ae. hydrophila*	Anguita *et al.*, 1993
		Ae. sorbia LP004	Lotrakul and Dharmsthiti, 1997
Fungi	Rhizopus	*Rhizop. delemar*	Espinosa *et al.*, 1990; Haas *et al.*, 1992; Klein *et al.*, 1997; Lacointe *et al.*, 1996
		Rhizop. oryzae	Beer *et al.*, 1998; Coenen *et al.*, 1997; Essamri *et al.*, 1998; Hiol *et al.*, 2000; Salleh *et al.*, 1993
		Rhizop. arrhizus	Elibol and Ozer, 2001; Sztajer and Maliszewska, 1989
		Rhizop. nigricans	Ghosh *et al.*, 1996
		Rhizop. nodosus	Nakashima *et al.*, 1988
		Rhizop. microsporous	Ghosh *et al.*, 1996
		Rhizop. chinensis	Ghosh *et al.*, 1996
		Rhizop. japonicus	Nakashima *et al.*, 1988
		Rhizop. niveus	Kohno *et al.*, 1999; Kohno *et al.*, 1994
	Aspergillus	*A. flavus*	Long *et al.*, 1998; Long *et al.*, 1996
		A. niger	Chen *et al.*, 1995
		A. japonicus	Satayanarayan and Johri, 1981
		A. awamori	Satayanarayan and Johri, 1981
		A. fumigatus	Satayanarayan and Johri, 1981
		A. oryzae	Ohnishi *et al.*, 1994a; Ohnishi *et al.*, 1994b

	A. carneus	Helisto and Korpela, 1998
	A. repens	Kaminishi *et al.*, 1999
	A. nidulans	Mayordoma *et al.*, 2000
Penicillium	*Pe. cyclopium*	Chahinian *et al.*, 2000
	Pe. citrinum	Sztajer and Maliszewska, 1989
	Pe. roqueforti	Petrovic *et al.*, 1990
	Pe. fumiculosum	Hou, 1994
	Penicillium sp.	Ghosh *et al.*, 1996; Helisto and Korpela, 1998
	Pe. camambertii	Ghosh *et al.*, 1996
	Pe. wortmanii	Costa and Peralta, 1999
Mucor	*Mucor miehei*	Lacointe *et al.*, 1996; Plou *et al.*, 1998; Rantakyla *et al.*, 1996
	Mu. javanicus	Ishihara *et al.*, 1975
	Mu. circinelloides	Balcao *et al.*, 1998
	Mu. hiemalis	Ghosh *et al.*, 1996b
	Mu. racemosus	Ghosh *et al.*, 1996b
Ashbya	*Ashbya gossypii*	Stahmann *et al.*, 1997
Geotrichum	*G. candidum*	Ghosh *et al.*, 1996b; Sugihara *et al.*, 1991
	Geotrichum sp.	Macedo *et al.*, 1997
Beauveria	*Beauveria bassiana*	Hegedus and Khachatourians, 1988
Humicola	*H. lanuginosa*	Ghosh *et al.*, 1996b; Plou *et al.*, 1998; Takahashi *et al.*, 1998; Zhu *et al.*, 2001
Rhizomucor	*R. miehei*	Dellamora-Ortiz *et al.*, 1997; Jaeger and Reetz, 1998; Merek and Bednasski, 1996; Weber *et al.*, 1999
Fusarium	*Fusarium oxysporum*	Rapp, 1995

		F. heterosporum	Takahashi *et al.*, 1998
	Acremonium	*Ac. strictum*	Okeke and Okolo, 1990
	Alternaria	*Alternaria brassicicola*	Berto *et al.*, 1997
	Eurotrium	*Eu. herbanorium*	Kaminishi *et al.*, 1999
	Ophiostoma	*O. piliferum*	Brush *et al.*, 1999
Yeasts	Candida	*C. rugosa*	Brocca *et al.*, 1998; Frense *et al.*, 1996; Wang *et al.*, 1995; Xie *et al.*, 1998; Yee *et al.*, 1995
		C. tropicalis	Takahashi *et al.*, 1998
		C. antarctica	Arroyo *et al.*, 1999; Jaeger and Reetz, 1998; Weber *et al.*, 1999
		C. cylindracea	Helisto and Korpela, 1998; Kamiya and Gotto, 1998
		C. parapsilosis	Lacointe *et al.*, 1996
		C. deformans	Lacointe *et al.*, 1996
		C. curvata	Ghosh *et al.*, 1996b
		C. valida	Ghosh *et al.*, 1996b
	Yarrowia	*Y. lipolytica*	Merek and Bednasski, 1996; Pignede *et al.*, 2000
	Rhodotorula	*Rho. glutinis*	Papaparaskevas *et al.*, 1992
		Rho. pilimornae	Tahoun *et al.*, 1985
	Pichia	*Pi. bispora*	Hou, 1994
		Pi. maxicana	Hou, 1994
		Pi. sivicola	Sugihara *et al.*, 1995
		Pi. xylosa	Sugihara *et al.*, 1995
		Pi. burtonii	Sugihara *et al.*, 1995
	Saccharomyces	*Sa. lipolytica*	Tahoun *et al.*, 1985
		Sa. crataegenesis	Hou, 1994

	Torulospora	*Torulospora globora*	Hou, 1994
	Trichosporon	*Trichosporon asteroides*	Dharmsthiti and Ammaranond, 1997
Actinomycetes	Streptomyces	*Streptomyces fradiae* NCIB 8233	Sztajer *et al.*, 1988
		Streptomyces sp. PCB27	Sztajer *et al.*, 1988
		Streptomyces sp. CCM 33	Sztajer *et al.*, 1988
		Str. coelicolor	Hou, 1994
		Str. cinnamomeus	Sommer *et al.*, 1997

2.3.1 Bacterial lipases

A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal lipases (Brune and Gotz, 1992; Petersen and Drablos, 1994; Sugiura, 1984; Sztajer *et al.*, 1991). Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable (Macrae and Hammond, 1985). Staphylococcal lipases are lipoprotein in nature (Brune and Gotz, 1992).

2.3.2 Fungal lipases

Fungal lipases have been studied since 1950s and comprehensive reviews are available in the literature (Brockerhoff and Jensen, 1974; Lawrence, 1967; Sharma *et al.*, 2001). These lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents (Lawson *et al.*, 1994). The chief producers of commercial lipases are *Aspergillus niger*, *Candida cylindracea*, *Humicola lanuginosa*, *Mucor miehei*, *Rhizopus arrhizus*, *R. delemar*, *R. japonicus*, *R. niveus* and *R. oryzae* (Godfredson, 1990).

Among the different biological sources of the lipases studied, filamentous fungi are thought to be the best source for industrially useful lipases because these lipases are usually extracellular and soluble (Huang *et al.*, 2004). Fungal lipases from the genera *Geotrichum, Penicillium, Aspergillus, Rhizopus,* and *Rhizomucor* (Persson *et al.*, 2000) have been the most widely studied and consequently are the most widely used in industrial applications.

Among Mucorales, the lipolytic enzymes of the moulds *Mucor hiemalis*, *M. miehei*, *M. lipolyticus*, *M. pusillus*, *Rhizopus japonicus*, *R. arrhizus*, *R. delemar*.

R. nigricans, *R. nodosus*, *R. microsporus*, and *R. chinesis* have been studied in great detail (Lazar and Schroder, 1992). The thermophilic *M. pusillus* is well known as a producer of thermostable extracellular lipase. From a lipase-producing strain of *M. miehei*, two isoenzymes with slightly different isoelectric points but a high degree of antigenic identity were isolated (Huge-Jensen *et al.*, 1987). Novo Industries have commercialized a lipase of *M. miehei*, immobilized on a resin (Lipozyme TM).

Lipase producers within the order Entomophthorales include *Entomophthora apiculata*, *E. coronata*, *E. thaxteriana*, *E. virulenta*, *Basidiobolus* spp. and *Conidiobolus* spp. The genera *Pichia*, *Hansenula*, and *Saccharomyces* are also reported to produce lipase (Stead, 1986). Two kinds of cell-bound lipases were purified from *Saccharomyces lipolytica* (Lazar and Schroder, 1992). Lipases are reported from *Candida curvata*, *C. tropicalis*, *C. valida*, and *C. pellioculosa* (Lazar and Schroder, 1992) and are nonspecific towards the different ester bonds in triglycerides, with the exception of *C. deformans* (Lazar and Schroder, 1992).

The imperfect fungus *Geotrichum candidum* is responsible for acid formation in dairy products by lipolyzing fat. The *G. candidum* lipase features specificity towards fatty acids with a *cis* double bond at C_9, hence is applied for the structural analysis of triglycerides (Litchfield, 1972).

The intracellular and extracellular lipases of *Aspergillus niger* are 1,3-(regio)-specific (Okumura *et al.*, 1976). *A. oryzae* was reported to be an efficient host for the heterologous expression of the lipase from *Rhizopus miehei* and *Humicola lanuginose* (Lazar and Schroder, 1992). The lipase of *Penicillium roqueforti* is responsible for the flavour of Blue cheese (Eitenmiller *et al.*, 1970). Lipolytic activity has also been detected in *P. camemberti*, the white surface mould of Brie and Camembert cheese. Lipases with specificity for butyric acid have been

isolated from strains of the *Penicillium* species such as *P. cyclopium* (Iwai *et al.*, 1975), *P. verrucosum* var. *cyclopium*, and *P. crustosum* (Lazar and Schroder, 1992). The *P. cyclopium* lipase has a much higher activity towards di- and monoglycerides than triglycerides. *H. lanuginosa* lipases show a high degree of hydrolytic activity with coconut oil and oils having a high content of lauric acid. The two lipases differ in their positional specificity (Ibrahim *et al.*, 1987).

2.3.3 Metagenomics and Lipase

Metagenomics, an approach to access global microbial genetic diversity, has been used to discover novel, potentially important enzymes, including lipases (Daniel, 2005; Lorenz and Eck, 2005; Streit and Schmitz, 2004). Several genes encoding metagenomic lipases have been identified in metagenomic libraries prepared from various environmental samples, including soils (Henne *et al.*, 2000; Lee *et al.*, 2004), pond and lake water (Ranjan *et al.*, 2005; Rees *et al.*, 2003), and a solfataric field (Rhee *et al.*, 2005). A novel lipase-encoding gene, *lipG*, was isolated from a tidal flat-derived metagenomic library and sequenced (Lee *et al.*, 2006).

2.3.4 Lipase from marine sources

Marine organisms like fishes are found to be rich sources of lipase. But there are not many reports on lipase production by marine microorganisms. Marine *Vibrio* sp. VB-5 produces a lipase that hydrolyzes n-3 polyunsaturated fatty acid (PUFA)-containing fish oil. Saturated and monoenoic fatty acids were liberated easily from fish oil by lipase. It is hoped that the lipase from VB-5 is capable of catalyzing the esterification reaction with n-3 PUFA, since the reversible reaction is known for lipase (Chandrasekaran and Rajeev Kumar, 2002).

2.4 Fermentation production of lipase

A number of reports exist on influences of various environmental factors such as temperature, pH, nitrogen, carbon and lipid sources, agitation, and dissolved oxygen concentration on lipase production (Nahas, 1988; Watanabe *et al.*, 1977). Lipase production is generally stimulated by lipids (Omar *et al.*, 1987; Suzuki *et al.*, 1988). The lipase activity steadily increases to a peak and declines. Lipase production is usually coordinated with, and dependant on the availability of triglycerides. Besides this, free fatty acids, hydrolysable esters, bile salts, and glycerol also stimulate lipase production. High production of lipase in case of *P. fragi* occurs in peptone-supplemented medium, although different peptones vary in their effectiveness (Lawrence *et al.*, 1967a; Nashif and Nelson, 1953). Though *Pseudomonas* sp. grow in a basal medium with ammonium sulphate, glucose, citrate or pyruvate, it required an organic nitrogen source for lipase production (Alford and Pierce, 1963). A mixture of arginine, lysine and glutamic acid in medium was observed to be effective for lipase production (Alford and Pierce, 1963). A strain of *Penicillium roqueforti* produced maximum amount of lipase when grown in 0.5% casitone and 1% proflobroth (Eitenmiller *et al.*, 1970). Growth and lipase production by *Micrococcus* sp. were unaffected by peptone of 0.5% to 2%, but lipase production by *Pseudomonas* sp, *A. wentii*, *M. hiemalis*, *R. nigricans*, and *M. racemosus* were stimulated by peptone (Akhtar *et al.*, 1980; Chander *et al.*, 1981; Chander *et al.*, 1980). Soybean meal extract in *Rhizopus oligosporus* culture medium supported good growth and lipase production (Nahas, 1988). Physiological regulation of lipase activity by thermotolerant strain of *P. aeruginosa* EF_2 under various conditions in batch, fed-batch, and continuous cultures support the contention that nitrate generally stimulates production of lipase (Gilbert *et al.*, 1991).

Milk is a good medium for growth of psychrotrophic bacteria and for lipase production which was found to be susceptible to catabolite repression by

glucose (Akhtar *et al.*, 1980; Chander *et al.*, 1981; Chander *et al.*, 1980; Gilbert *et al.*, 1991). While glucose is essential for production of lipase by *P. fragi* (Alford and Pierce, 1963), *A. wentii* (Chander *et al.*, 1980), *M. hiemalis* (Akhtar *et al.*, 1980), *R. nigricans*, and *M. racemosus* (Chander *et al.*, 1981). *P. aeruginosa* EF_2 (Gilbert *et al.*, 1991) showed no such requirement (Nadkarni, 1971). Lipase activity per milligram dry weight of mycelium was much higher on lactose, mannose, xylose, fructose, dextrin, and rhamnose in case of *Talaromyces emersonii* (Oso, 1978). Mannitol, galactose, sucrose (Chander *et al.*, 1980), fructose, lactose, maltose, raffinose or ribose produced less amount of lipase (Chander *et al.*, 1981) and caused decreased growth with corresponding reduction in lipase activity in *M. racemosus* (Chopra *et al.*, 1981). Polysaccharides such as glycogen, hyaluronate, laminarin, gum arabic, and pectin stimulated production of lipase in *Serratia marcescens* (Nishio *et al.*, 1987) and *Saccharomycopsis lipolytica* (Ruschen and Winkler, 1982).

Triglyceride is important for lipase production as it can act both as an inducer and as inhibitor. Among the triglycerides, olive oil was observed to be effective in inducing lipase (Akhtar *et al.*, 1980). Salts of unsaturated fatty acids inhibited lipase production by *P. fragi* (Smith and Alford, 1966), whereas tributyrin and trioctanoin had no effect on lipase production by *P. fragi* and *M. freudenreichii* (Lawrence *et al.*, 1967a). Butter oil, corn oil or olive oil inhibited lipase production by *P. roqueforti* (Eitenmiller *et al.*, 1970), *Saccharomycopsis* sp., *B. licheniformis*, *M. caseolyticus* and *Staphylococcus* sp. (Saxena *et al.*, 1999). Triglycerides such as olive oil, groundnut oil and cotton seed oil, and fatty acids such as oleic acid, linoleic acid and linolenic acid stimulated lipase production by *P. mephitica* (Saxena *et al.*, 1999). Lipids are considered not to be true inducers (Lawrence *et al.*, 1967a; Nashif and Nelson, 1953). *A. wentii* showed reduced growth and lipase production when the synthetic and natural lipids were added to the growth medium (Chander *et al.*, 1980). Emulsification of culture media

containing oil by gum acacia supported good growth and lipase production in *R. oligosporus* (Nahas, 1988). Triolein, olive oil, tributyrin, and oleic acid butylester were able to induce lipase in immobilized protoplasts, whereas Tween 80 enhanced lipase activity (Johri *et al.*, 1990).

The initial pH of the growth medium is also important for lipase production. Maximum activity was observed at pH > 7.0 for *P. fragi* (Nashif and Nelson, 1953) and at pH 9.0 for *P. aeruginosa* (Nadkarni, 1971) wherein development of acidity in media reduced lipase activity (Nashif and Nelson, 1953). In contrast, maximum growth at acidic pH (4.0–7.0) was reported for *S. lipolytica* (Jonsson and Snygg, 1974), *M. caseolyticus* (Jonsson and Snygg, 1974), *B. licheniformis*, *A. wentiib* (Chander *et al.*, 1980), *M. hiemalis* (Akhtar *et al.*, 1980), *R. nigricans.*, *Mucor racemosus* (Chander *et al.*, 1981), *R. oligosporus* (Nahas, 1988) and *P. aeruginosa* EF_2 (Gilbert *et al.*, 1991).

The best temperature for lipase production by *T. emersonii* was determined to be 45°C (Oso, 1978). Temperatures in the range of 22–35°C were however observed to be optimum for maximum lipase production for *A. wentii* (Chander *et al.*, 1980), *M. heimalis* (Akhtar *et al.*, 1980), *R. nigricans* (Chander *et al.*, 1981), *M. Racemosus* (Chopra *et al.*, 1981) *R. oligosporus* (Nahas, 1988), and *P. aeruginosa* (Gilbert *et al.*, 1991).

Aeration has variable effect on lipase production by different organisms. The degree of aeration appears to be critical in some cases since shallow layer cultures (moderate aeration) produced much more lipase than shake cultures (high aeration) (Nashif and Nelson, 1953). Vigorous aeration greatly reduced lipase production by *R. oligosporus* (Nahas, 1988), *P. fragi* (Lu and Liska, 1969), *P. aeruginosa* (Nadkarni, 1971), and *M. racemosus* resulted in increased lipase production in static culture conditions (Chopra *et al.*, 1981). However, high

aeration was needed for high lipase activity by *A. wentii* (Chander *et al.*, 1980) and *M. hiemalis* (Akhtar *et al.*, 1980). Changing the ratio of surface area to volume and hence, aeration of cultures of *P. fragi* had no effect on the quantity of lipase produced per cell; but increasing aeration by shaking resulted in both increased growth and lipase production, followed by a rapid decrease of lipase activity as shaking continued (Lawson *et al.*, 1994). The stationary conditions favoured maximum lipase production in *T. emersonii* (Oso, 1978). Lipase synthesis by two strains of *P. fluorescens* (psychrotroph), stimulated in milk medium at 7°C, was immediately preceded by a decrease in O_2 tension which resulted in earlier production of lipase (Rowe and Gilmour, 1982). Oxygen is the limiting factor in shake-flask culturesLow oxygen concentration negatively affects the metabolism of *R. delemar*, which explains that low oxygen concentration is a useful tool to scale down fermentation processes in cases where a transient or local oxygen limitation occurs (Giuseppin, 1984). The different fermentation systems of a few reported lipases are listed in Table 2.2.

2.5 Lipase assay

Lipase hydrolyse triglycerides and give rise to free fatty acids and glycerol. Numerous methods are available, in literature, for measuring the hydrolytic activity as well as for the detection of lipases (Brockerhoff and Jensen, 1974; Jensen, 1983; Tietz and Shuey, 1993). These methods can be classified as follows: 1. titrimetry, 2. spectroscopy (photometry, fluorimetry, infrared), 3. chromatography, 4. radioactivity, 5. interfacial tensiometry, 6. turbidimetry, 7. conductimetry, 8. immunochemistry, 9. microscopy (Beisson *et al.*, 2000).

Tributyrin plate assay and titrimetry are the most commonly used methods for screening of lipase producers and estimation of lipase activity, respectively (Akhtar *et al.*, 1983; Linfield *et al.*, 1984).

A simple and reliable method for detecting lipase activity in microorganisms uses the surfactant Tween 80 in a solid medium to identify a lipolytic activity (Sierra, 1957). The formation of opaque zones around the colonies is an indication of lipase production by the organisms. Modifications of this assay use various Tween surfactants in combination with Nile blue or neet's foot oil and Cu^{2+} salts. Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate and clear zones around the colonies indicate production of lipase (Cardenas *et al.*, 2001). Screening systems making use of chromogenic substrates have also been described (Yeoh *et al.*, 1986). Plates of a modified Rhodamine B agar is used to screen lipase activity in a large number of microorganisms (Wang *et al.*, 1995). There are several methods available in literature for lipase activity and they are summarized in Table 2.3.

Table 2.2: Fermentation systems reported for lipase production in microbes

Organism	Fermentation	Media	Special Features	Reference
Fungus				
Metarhizium anisopliae	SmF	Basal Medium + 2% olive oil	-	Silva *et al.*, 2005
Aspergillus sp.	SmF	Mineral Growth Medium		Cihangir and Sarakaya, 2004
Aspergillus carneus	SmF	Modified medium of Aisaka and Terada	Alkaline Thermostable	Saxena *et al.*, 2003a
Fusarium oxysporum	SmF	Nitrate medium		Camargo-de-Morais *et al.*, 2003
Rhizopus oligosporous	SSF	Almond meal		Ikram-ul-Haq *et al.*, 2002
Mucor sp.	SmF	Basal medium		Abbas *et al.*, 2002
Rhizopus oryzae	SmF			Hiol *et al.*, 1999
Aspergillus nidulans	SmF	2% Olive oil as substrate	Cold active lipase	Mayordomo *et al.*, 2000
Candida rugosa	SSF	Coconut oil cake and wheat bran		Benjamin and Pandey, 2000
Aspergillus oryzae	SmF	Basal medium + 2% Olive oil		Toida *et al.*, 1998
Bacteria				
Burkkolderia cepacia	SmF	Basal medium + emulsified coconut oil	Alkaline lipase	Rathi *et al.*, 2001
Pseudomonas sp.	SmF	GYP Medium + Olive oil		Sarkar *et al.*, 1998
Aeromonas sobria	SmF	Whey and Soyabean meal		Lotrakul and Dharmsthiti, 1997b
Pseudomonas pseudoalcaligens	SmF	Nutrient broth + Olive Oil		Lin *et al.*, 1996a

Table 2.3: Assays for the determination of lipase activity

Assay and substrate	Product analysed	Principle involved	Remarks	Reference(s)
Plate assays				
Tributyrin, acylglycerols and esters of long-chain fatty acids	Short-chain fatty acids	Halo-based or colour change of Phenol Red/Victoria Blue/Nile Blue Sulphate, or measurement of fluorescence after complexation of fatty acid with fluorescent dye Rhodamine B	Convenient for rapid screening	Hube *et al.*, 2000 Kim *et al.*, 2001 Martínez and Soberón-Chávez, 2001
Titrimetry				
Fats and oils, triacyl-glycerols,methyl esters	Fatty acids	Neutralization reaction either directly by pH-stat or by pH indicator	Most reliable and commonly used procedure	Ferrato *et al.*, 1997 Ghosh *et al.*, 1996a
Spectrophotometry				
Fatty acid conjugates of β-naphthol	β-Naphthol	Estimation of β-naphthol by complexation with Fast Blue BB	The ester is not stable at extreme pH	Degrassi *et al.*, 1999 Nachlas and Blackburn, 1958
p-Nitrophenyl esters	*p*-Nitrophenol	Coloured product measured at 410 nm	Convenient method. Preferred during purification procedures. Disadvantage of undergoing spontaneous hydrolysis	Winkler and Stuckmann,1979
Tweens	Fatty acid	Precipitation of fatty acid with calcium or copper and measurement of turbidity	Simple, reproducible and sensitive; can be used for quantitative assays but often used for plate assays	von-Tigerstrom and Stelmaschuk, 1989

Fluorescence assay				
Triacylglycerols with alkyl group substituted with a fluorescent group, e.g. conjugated pyrenyl group	Flourescent free prenyl groups	Shift in fluorescence wavelength after triacylglycerol hydrolysis	Rapid assay, but expensive substrate limits its usage	Negre-Salvayre *et al.*, 1991
Non-fluorescent 4-methyl umbelliferyl oleate	Fluorescent 4-methyl umbelliferone	Product is analysed, as it is fluorescent		Roberts, 1985
Chromatographic procedures (TLC/GC/HPLC)				
Triacylglycerols, fats and oils	Fatty acids	Analysis and quantification of the product or residual substrate through specific columns	Use depends upon availability of the instrument. Time-consuming for routine analysis, but often recommended for substrate-specificity determination	Bereuter and Lorbeer, 1995
Interfacial pressure : monolayer method				
Lipid	Fatty acids	Change in surface pressure due to breakdown of triacylglycerol	Highly sensitive. Elaborate and extensive set-ups required for accurate estimation	Jaeger *et al.*, 1994 Verger, 1980
Interfacial pressure : oil-drop method				
Lipid	Fatty acids	Oil-drop shape is monitored; changes from apple to pear shape upon hydrolysis	Extensive set-ups are required	Nury *et al.*, 1991

Interfacial pressure : atomic force microscopy				
Lipid bilayers	Fatty acids	Regions of bilayers hydrolysed by lipases showing deep defects are detected by the atomic-force microscopy tip	Provided the first nanoscale picture of kinetics of lipid degradation by lipases. Sophisticated instruments involved	Beisson *et al.*, 2000
IR spectroscopy				
Vegetable oils, trioctanoylglycerol	Fatty acid esters and free fatty acids	Lipolysis monitored by recording the Fourier-transform IR spectrum of the entire reaction mixture	Expensive and sophisticated instruments involved	Walde and Luisi, 1989

2.6 Lipase purification

Most of the microbial lipases are extra cellular in nature. Usually the fermentation process is followed by the removal of cells from the culture broth, either by centrifugation or by filtration and the cell-free culture broth is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents (Saxena *et al.*, 2003b). About 80 % of the purification schemes attempted thus far have used a precipitation step, with 60 % of these using ammonium sulphate and 35 % using ethanol, acetone or an acid (usually hydrochloric) followed by a combination of several chromatographic methods such as gel filtration and affinity chromatography. Precipitation is used as a crude separation step, often during the early stages of a purification procedure, and is followed by chromatographic separation. Increase in lipase activity depends on the concentration of ammonium sulfate solution used (Pabai *et al.*, 1995b). In comparison to other techniques, which give lower yields (60–70 %), precipitation methods often have high average yield (87 %) (Aires-Barros *et al.*, 1994).

This is followed by gel filtration, and ion exchange chromatography. In recent years, affinity chromatographic techniques have come into use as this technique decreases the number of steps necessary for lipase purification as well as increases specificity. Currently, reversed-micellar (Dekker *et al.*, 1986; Yadav *et al.*, 1998b) and two-phase systems (Dunhaupt *et al.*, 1991; Hustedt *et al.*, 1985), membrane processes, and immunopurification (Harlow and Lane, 1988; Hill *et al.*, 1989) are being used for purification of lipases.

2.7 Lipase properties

Lipases are active in organic solvents, and they catalyze a number of useful reactions including esterification (Chowdary *et al.*, 2001; Hamsaveni *et al.*, 2001; Krishna and Karanth, 2001; Rao and Divakar, 2001); transesterification,

regioselective acylation of glycols and menthols; synthesis of peptides (Ducret *et al.*, 1998; Zhang *et al.*, 2001), and other chemicals (Azim *et al.*, 2001; Berglund and Hutt, 2000; Bornscheuer, 2000).

Lipases purified from *S. aureus* and *S. hyicus* show molecular weights ranging between 34-46 kDa. They are stimulated by Ca^{++} and inhibited by EDTA. The optimum pH varies between 7.5 and 9.0. The purified lipase of *P. fragi*, *P. fluorescens*, and *P. aeruginosa* were monomeric with molecular weight of 33 kDa, 45 kDa, and 29 kDa, respectively (Brune and Gotz, 1992; Nishio *et al.*, 1987; Sztajer *et al.*, 1991). The lipase was inhibited by Zn^{++}, Fe^{++}, and Al^{+++} and activated by Ca^{++} (Kugimiya *et al.*, 1986). The lipase gene of *P. fragi* has been cloned and sequenced (Lawrence, 1967).

Lipases from different species of *Psuedonomas* were purified by acidification of the culture supernatant, ammonium sulphate precipitation (Nishio *et al.*, 1987), sepharose CL-6B chromatography (Sztajer *et al.*, 1991), and isoelectric focussing using CHAPS (Nishio *et al.*, 1987).

2.7.1 pH optima

Extracellular lipase of *A. niger*, *Chromobacterium viscosum* and *Rhizopus* sp. are active at acidic pH (Fukumoto *et al.*, 1963; Laboureur and Labrousse, 1966). An alkaline lipase active at pH 11.0 has been isolated from *P. nitroreducens* (Watanabe *et al.*, 1977).

2.7.2 Temperature optima and thermal stability

Lipases of *A. niger* (Fukumoto *et al.*, 1963), *R. japonicus* (Aisaka and Terada, 1980), and *C. viscosum* (Yamaguchi *et al.*, 1973) are stable at 50°C, while lipases of thermotolerant *H. lanuginosa* and *P. nitroreducens* are stable at 60°C

and 70°C (Liu *et al.*, 1973) respectively. *C. gigantea* lipase had half life for inactivation of 35.7, 46.4 and 22.9 min respectively at 45°C, 50°C and 55°C (Tombs, 1991), similar to lipases of *R. japonicus* (Suzuki *et al.*, 1986). Purified lipase from *A. terreus* (Yadav *et al.*, 1998a) retained 100% of its activity at 60°C after 24 h. But, the maximum activities of *C. gigantea* and other lipases from mesophiles were at 30–35°C. Thermophilic bacterial lipases obtained from Icelandic hot spring showed higher lipase activity at 40 to 60°C (Sigurgisladottir *et al.*, 1993).

2.7.3 Activation and inactivation of the enzyme

Cofactors are not required for the expression of lipase activity. Divalent cations, such as calcium, generally stimulate the activity. It has been postulated that this is based on the formulation of calcium salts of long-chain fatty acids (Godfredson, 1990; Macrae and Hammond, 1985). The lipase activity is inhibited drastically by Co^{++}, Ni^{++}, Hg^{++}, and Sn^{++}; and is slightly inhibited by Zn^{++}, Mg^{++}, EDTA, and SDS (Patkar and Bjorkling, 1994). In *H. lanuginosa* S-38, sulphhydryl-reducing agents, like dithiothreitol, did not alter the enzyme activity, but did render it more susceptible to heat inactivation. Inactivation is accelerated by the addition of urea. Reducing compounds (cysteine, 2-mercaptoethanol), chelating agents, (EDTA, *o*-phenanthroline), and thiol group inhibitors (*p*-chloro mercuric benzoate, monoiodoacetate) did not show a detectable effect on lipase in *M. pusillus*, suggesting that lipase is not a metallo-enzyme and it does not require either free -SH group or an intact S–S bridge for its activity. Spontaneous and cyclic AMP-induced lipase formation is greatly enhanced in *Serratia marcescens* SM-6 on exposure to glycogen, hyaluronate, pectin B, and gum Arabic (Nishio *et al.*, 1987).

2.7.4 Substrate specificity

Specificity of lipases is controlled by the molecular properties of the enzyme, structure of the substrate, and factors affecting binding of the enzyme to the substrate (Jensen, 1983). Substrate specificity of lipases is often crucial to their application for analytical and industrial purposes. Specificity is shown both with respect to either fatty acyl or alcohol parts of their substrates (Jensen, 1983). Many microbes produce two or more extracellular lipases with different fatty acid specificities. Tributyrin is hydrolysed slowly by some microbial lipases (Patkar and Bjorkling, 1994). In contrast, *M. miehei* lipase preferentially releases butyric acid from milk fat especially at low pH (Moskowitz *et al.*, 1977). *Geotrichum candidum* produces a lipase, which shows pronounced specificity for the hydrolysis of esters of a particular type of long-chain fatty acid. Lipases show both regio- and stereospecificity with respect to the alcohol moeity of their substrates (Chapman, 1969).

Lipases can be divided into two groups on the basis of the regiospecificity exhibited acylglycerol substrates (Macrae and Hammond, 1985). Lipases in the first group catalyse the complete breakdown of triacylglycerol to glycerol and free fatty acids together with diacylglycerols and monoacylglycerol as intermediates in the reaction. These intermediates do not accumulate since they are hydrolysed more rapidly than the triacylglycerol. Examples of the first group of lipases include lipase from *C. cylindracea* (Benzonana and Desnuelle, 1965). The second group of lipases release fatty acids regiospecifically from the outer 1 and 3 positions of acylglycerols. These lipases hydrolyse triacylglycerol to give free fatty acids, 1,2-diacylglycerols (Macrae and Hammond, 1985; Priest, 1992), and 2-mono acylglycerol. Many extracellular microbial lipases, such as those from *A. niger* (Okumura *et al.*, 1976) and *R. arrhizus* (Saxena *et al.*, 1999), show 1,3-(regio)-specificity. Lipases excreted by *R. japonicus*, *M. miehei*, *H. lanuginosa*, *C. viscosum*, and *P. fluorescens* are also 1,3-(regio)-specific (Macrae and

Hammond, 1985). Till date, there are no authentic reports of lipases which catalyse the release of fatty acids selectively from the central 2-position of acylglycerols (Macrae and Hammond, 1985), except for a report of Asahara *et al.*, (1993). Partial stereospecificity in the hydrolysis of triacyl glycerols has been observed in *R. arrhizus*, *R. delemar*, *C. cylindracea*, and *P. aeruginosa*. Owing to this property, these enzymes can be used to isolate optically pure esters and alcohols (Lavayre *et al.*, 1982).

2.8 Sequencing and cloning of lipase gene

Lipase gene from many microorganism and higher animals has been cloned. The lipase genes from *S. hyicus* and *S. aureus* have been cloned, sequenced, and compared with other lipases. This revealed two conserved domains separated by 100 amino acids, which are likely to form active site (Brune and Gotz, 1992). Putative active site residues around His 269 and Ser 369 of the *S. hyicus* lipase are highly conserved in the two *S. aureus* lipases and in several eukaryotic lipases. Some reports on cloning of lipase gene are summarized in Table 2.4.

2.9 Applications of lipase

The industrial applications of lipases have grown rapidly in recent years and are likely to markedly expand further in the coming years. Lipases may be used to produce fatty acids (Linko *et al.*, 1990; Marangoni, 1994), biosurfactants (Chopineau *et al.*, 1988), aroma and flavor compounds (Gandhi *et al.*, 1995), lubricant and solvent esters (Linko *et al.*, 1994), polyesters (Linko *et al.*, 1995b), and biomodified fats (Marangoni, 1994; West, 1988). Lipases are also widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, and production of cosmetics, and pharmaceuticals (Kazlauskas and Bornscheuer, 1998). Lipase can be used to accelerate the degradation of fatty waste

(Masse *et al.*, 2001a; Masse *et al.*, 2001b) and polyurethane (Takamoto *et al.*, 2001). Major applications of lipases are summarized in Table 2.5. Most of the industrial microbial lipases are derived from fungi and bacteria (Table 2.6).

Table 2.4: Works already reported at genetic level

Microbial Source of gene	Work reported	Significance	Reference
Aci. calcoaceticus BD413	Cloned in *E. coli* Phage M13	Sequence of 21kb revealed one complete ORF,Lip A	Kok *et al.*, 1995
P. aeruginosa 14BS3	A recombinant plasmid and a lac promoter constructed, cloned in *Xanthomonas campestris*	Expression of alkaline lipase 12 fold increase in lipase production	Leza *et al.*, 1996
Rhizopus oryzae DSM 853	Cloning of lipase gene		Beer *et al.*, 1998
Candida rugosa	Lip I gene (1647) Synthesized	Over expressed in *P. pastoris*	Brocca *et al.*, 1998
B. thermoleovorans ID-1	Thermophilic lipase cloned and sequenced		Cho *et al.*, 2000
Y. lipolytica	Isolated Lip 2 gene	Gene encoded A 334 amino acid precursor protein.	Pignede *et al.*, 2000
A. oryzae	L3 Lipase gene cloned , Gene expressed in *E. coli*	L3 gene had an ORF of 954 nucleotides including 3 introns Encoded a functional tri acyl glycerol lipase	Toida *et al.*, 1998
Pseudomonas sp..,KB 700A	Cloned in *E. coli* TG1 and expressed	Encoded a protein of 474 amino acid	Rashid *et al.*, 2001
Bacillus sphaericus 205y	Gene isolated and sequenced	Expressed in *E. coli*	Rahman et al., 2003
Geobacillus sp. strain T1	A thermoalkaliphilic T1 lipase gene isolated	overexpressed in pGEX vector in the prokaryotic system	Leow et al., 2007
Yarrowia lipolytica	Extracellular lipase gene was cloned into the pPICZalphaA	The lipase was successfully expressed and secreted in *Pichia pastoris*	Yu et al., 2007
Candida thermophila SRY-09	Cloned and expressed im *Pichia pastoris*	Heterologously expressed lipase	Thongekkaew and Boonchird, 2007

Table 2.5 Industrial applications of microbial lipases (Vulfson, 1994)

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

Table 2.6 Some commercially available microbial lipases (Krishna and Karanth, 2002)

Supplier	Sources of Lipases
Amano Pharmaceutical Co., Nagoya, Japan (www.amano-enzyme.co.jp)	*Aspergillus niger, C. rugosa, C. lipolytica, G. candidum, H. lanuginosa, M. javanicus, Penicillium* sp., *P. roquefortii, Pig liver esterase, P. fluorescens, R. delemar, R. javanicus, R. niveus*
Biocatalysts, Pontypridd, Wales, UK (www.biocatalysts.com)	Lipomod AC, Lipomod RD, Animal, *C. lipolytica, Ch. viscosum, G. candidum, H. lanuginosa, M. javanicus, M. miehei, P. cyclopium, P. roquefortii, P. fluorescens, R. delemar, R. javanicus, R. japonicus*
Biocon India, Bangalore, India (www.biocon.com)	Lipases for leather and detergent industries, Biolipase conc., Lipase FAP, Pregastric esterase, Lipoprotein lipase, Cholesterol esterase, Biolipase A
Boehringer Mannheim, Penzberg, Germany (Now Roche Diagnostics, merged with Roche group) (www.roche.com/diagnostics)	*C. rugosa, C. antarctica, Ch viscosum, G. candidum, H. lanuginosa, M. javanicus, M. miehei, P. cyclopium, P. roquefortii*, Porcine pancreas, *Pseudomonas, P. fluorescens, R. delemar, R. javanicus, R. japonicus*, Chirazyme screening set
Novozymes A/S, Novo Nordisk, Bagsvaerd, Denmark (www.novozymes.com)	Lipolase (detergent lipase), and lipases from several sources
Diversa Corp., San Diego, CA (www.diversa.com) (Innovase LLC, a joint venture of Diversa with Dow Chemical Co, (www.dow.com)	Detergent lipases, and other lipases
Thermogen Inc., Woodridge, IL (www.thermogen.com)	ThermoCat, Quick Screen, Esterase kits
Biocatalytics Inc., Pasadena, CA (www.biocatalytics.com)	Various lipases, custom made enzymes, salt-immobilized enzymes
Juelich Enzyme Products GmbH, Wiesbaden, Germany (www.juelichenzyme.com)	Various lipases and esterase, *Rhodotorula pilimanae, R. arrhizus*, Pig liver esterase, *R. niveus, C. rugosa*

Sigma–Aldrich–Fluka (www.sigma-aldrich.com)	Porcine pancreas, *C. lipolytica, M. javanicus, P. roquefortii, R. arrhizus, R. niveus, C. rugosa*, Wheat germ
Genzyme Biochemicals, Spingfield Mill, Kent, UK (www.genzyme.com)	*Ch. viscosum*, Lipoprotein lipase, C. *rugosa, Pseudomonas* sp.
Gist-Brocades, Delft, Holland (www.gist-brocades.nl) (Now part of DSM group: www.dsm.com) Enzyme business of GB is acquired by Genencor (www.genencor.com)	*M. miehei, R. arrhizus*, Piccantase (for dairy) Lipomax, Lumafast (Detergent lipases)
Hoechst, Frankfurt, Germany (Now Aventis, after merger with Rhone-Poulenc SA) (www.aventis.com)	Pancreatin
Nagase & Co Ltd., Ohama, Japan (www.nagase.co.jp)	*Rhizopus*
Merck, Darmstadt, Germany (www.merck.com)	Porcine pancreas
Roehm, Darmstadt, Germany (www.roehm.de)	*Aspergillus oryzae, A. niger, B. subtilis*, Pancreas
Seppim, 61500, Sees, France (www.sfrl.fr)	Lipase
Serva, Heidelberg, Germany (Invitrogen group) (www.serva.de)	Porcine pancreas, *Rhizopus* sp., Wheat germ

There is a renewed interest in the development of more industrial applications of lipases. For each application, the lipase selection is based on specific characteristics including substrate, positional and stereoisomer specificity as well as temperature and pH stability (Yamaguchi and Mase, 1991). The use of lipases for the modification of the positional distribution of fatty acids in butter fat triacylglycerols was reported (Safari and Kermasha, 1994). Indeed, it was shown that the interchange of palmitic or myristic acid with oleic acid at the sn-2-position of the glycerol backbone can suppress the cholesterol-raising potential of milk fat (Hayes *et al.*, 1991). Lipases from *Pseudomonas fluorescens* (Kalo *et al.*, 1989), *Aspergillus niger* (Kalo *et al.*, 1988b), *Candida cylindracea* (Kalo *et al.*, 1988a), and *Mucor miehei* (Kalo *et al.*, 1988b; Safari *et al.*, 1993) have been investigated for the interesterification of butter fat. Among several commercial enzymes, lipase from *Rhizopus niveus* showed an interesting potential for the production of interesterified butter fat with an increased proportion of oleic acid at the sn-2 position (Safari and Kermasha, 1994). *R. japonicus* lipase has been used to produce hard butter suitable for chocolate manufacture by interesterification of palm oil with methyl stearate (Matsuo *et al.*, 1981).

The importance of thermostable lipases for different applications has been growing rapidly. Most of the studies realized so far have been carried out with mesophilic producers. Many lipases from mesophiles are stable at elevated temperatures (Sugihara *et al.*, 1991). Thermostable lipolytic enzyme has been applied to the synthesis of biopolymers and biodiesel and used for the production of pharmaceuticals, agrochemicals, cosmetics, and flavours (Haki and Rakshit, 2003).

2.9.1 Lipases in fat and oleochemical industry

The lipase catalysed transesterification in organic solvents is an emerging industrial application such as production of cocoa butter equivalent, human milk

fat substitute "Betapol", pharmaceutically important polyunsaturated fatty acids (PUFA) rich/low calorie lipids, "designers fats or structured lipid" and production of biodiesel from vegetable oils (Jaeger and Reetz, 1998; Nakajima *et al.*, 2000). *Mucor miehei* (IM 20) and *Candida antarctica* (SP 382) lipases were used for esterification of free fatty acids in the absence of organic solvent or transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols (Akoh, 1993).

Immobilized *M. miehei* lipase in organic solvent catalysed the reactions of enzymatic interesterification for production of vegetable oils such as; corn oil, sunflower oil, peanut oil, olive oil and soybean oil containing omega-3 polyunsaturated fatty acids (Li and Ward, 1993).

The scope for the application of lipases in the oleochemical industry is enormous. Fats and oils are produced world wide at a level of approximately 60 million t pa and a substantial part of this (more than 2 million t pa) is utilized in high energy consuming processes such as hydrolysis, glycerolysis and alcoholysis. The saving of energy and minimization of thermal degradation are probably the major attractions in replacing the current chemical technologies with biological ones. Miyoshi Oil & Fat Co., Japan, reported the commercial use of *Candida cylindracea* lipase in the production of soaps (McNeill *et al.*, 1991). The company claimed that the enzymic method yielded a superior product and was cheaper overall than the conventional Colgate–Emery process.

2.9.2 Use of lipase in textile industry

Lipases are used in the textile industry to assist in the removal of size lubricants, in order to provide a fabric with greater absorbency for improved levelness in dyeing. Its use also reduces the frequency of streaks and cracks in the denim abrasion systems. Commercial preparations used for the desizing of denim

and other cotton fabrics, contains both alpha amylase and lipase enzymes (Cortez, 2000).

PCT Publication No. WO 97/43014 (Bayer AG) describes the enzymatic degradation of polyesteramide by treatment with an aqueous solution comprising an esterase, lipase or protease. JP 5344897 A (Amano Pharmaceutical KK) describes a commercial lipase composition, which dissolves in solution with an aliphatic polyester with the result that the fiber texture is improved without losing strength. Polymers of aliphatic polyethylene are also disclosed which can be degraded by lipase from *Pseudomonas* spp. PCT Publication No. 97/33001 (Genencor International, Inc.) discloses a method for improving the wettability and absorbance of a polyester fabric by treating with a lipase (http://www.wipo.int).

2.9.3 Lipases in detergent industry

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. The cleaning power of detergents seems to have peaked; all detergents contain similar ingredients and are based on similar detergency mechanisms. To improve detergency, modern types of heavy duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes, such as protease, amylase, cellulase and lipase (Ito *et al.*, 1998). The lipase of *H. lanuginosa* DSM 3819 is suitable as a detergent additive because of its thermostability, high activity at alkaline pH, and stability towards anionic surfactants (Huge and Gormsen, 1987).

In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase, which originated from the fungus *T. lanuginosus* and was expressed in *A. oryzae*. Lipases used as detergents also include those from *Candida* (Nishioka *et al.*, 1990) and *Chromobacterium* (Minoguchi and Muneyuki, 1989). Laundering is generally

carried out in alkaline media, lipases active under such conditions are preferred (Gerhartz, 1990; Satsuki and Watanabe, 1990; Umehara *et al.*, 1990), for example, the *A. oryzae* derived lipase. Alkaline lipase produced by *Acinetobacter radioresistens* had an optimum pH of 10.0 and was stable over a pH range of 6.0 - 10.0; therefore have great potential for application in the detergent industry (Chen *et al.*, 1998).

2.9.4 Lipases in food processing, flavour development and improving quality

Lipases have also been used for addition in food to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known flavour and fragrance compounds (Macedo *et al.*, 2003). Lipases are used in production of leaner meat such as in fish. The fat is removed during the processing of the fish meat by adding lipases and this procedure is called biolipolysis. The lipases also play an important role in the fermentative steps of sausage manufacture and to determine changes in long-chain fatty acid liberated during ripening. Earlier, lipases of different microbial origin have been used for refining rice flavour, modifying soybean milk and for improving the aroma and accelerating the fermentation of apple wine (Seitz, 1974).

2.9.5 Diagnostic tool

Lipases are also important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or increasing levels can indicate certain infection or disease. Lipases are used in the enzymatic determination of serum triglycerides to generate glycerol, which is subsequently determined by enzyme linked colorimetric reactions. The level of lipases in blood serum can be used as a diagnostic tool for detecting conditions such as acute pancreatitis and pancreatic injury (Lott and Lu, 1991).

2.9.6 Bakery products, confectionery and cheese flavouring

Lipases are extensively used in the dairy industry 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 (http://www.au-kbc.org/frameresearch.html).

A whole range of microbial lipase preparations has been developed for the cheese manufacturing industry: *Mucor meihei* (Piccnate, Gist-Brocades; Palatase M, Novo Nordisk), *A. niger* and *A. oryzae* (Palatase A, Novo Nordisk; Lipase AP, Amano; Flavour AGE, Chr. Hansen) and several others (http://www.au-kbc.org/frameresearch.html). Lipases also play a crucial role in the preparation of so called enzyme modified cheeses (EMC). EMC is a cheese that is incubated in the presence of enzymes at elevated temperature in order to produce a concentrated flavour for use as an ingredient in other products (dips, sauces, dressings, soups, snacks, etc.) (http://www.au-kbc.org/frameresearch.html).

2.9.7 Cosmetics

Unichem International (Spain) has launched the production of isopropyl myristate, isopropyl palmitate and 2- ethylhexylpalmitate for use as an emollient in personal care products such as skin and sun-tan creams, bath oils etc. Immobilized *Rhizomucor meihei* lipase was used as a biocatalyst. The company claims that the use of the enzyme in place of the conventional acid catalyst gives products of much higher quality, requiring minimum downstream refining (http://www.au-kbc.org/beta/bioproj2/uses.html).

Retinoids (Vitamin A and derivatives) are of great commercial potential in cosmetics and pharmaceuticals such as skin care products. Water-soluble retinol derivatives were prepared by catalytic reaction of immobilized lipase (Maugard

et al., 2002). Lipases have been used in hair waving preparation (Saphir, 1967), as a component of topical antiobese creams (August, 1972) or in oral administration (Smythe, 1951).

2.9.8 Lipases in tea processing

The quality of black tea is dependent largely on the dehydration, mechanical breaking, and enzymatic fermentation to which tea shoots are subjected. During manufacture of black tea, enzymatic breakdown of membrane lipids initiate the formation of volatile products with characteristic flavour properties, emphasize the importance of lipid in flavour development. Lipase produced by *Rhizomucor miehei* enhanced the level of polyunsaturated fatty acid observed by reduction in total lipid content (Latha and Ramarethinam, 1999).

2.9.9 Medical applications

Lipases isolated from the wax moth (*Galleria mellonella*) were found to have a bacteriocidal action on *Mycobacterium tuberculosis* (MBT) H37Rv. This preliminary study may be regarded as part of global unselected screening of biological and other materials for detecting new promising sources of drugs (Annenkov *et al.*, 2004).

Lipases may be used as digestive aids (Gerhartz, 1990). Lipases are the activators of Tumor Necrosis Factor and therefore can be used in the treatment of malignant tumors (Kato *et al.*, 1989). Although human gastric lipase (HGL) is the most stable acid lipase and constitutes a good candidate tool for enzyme substitution therapy (Ville *et al.*, 2002). Lipases have earlier been used as therapeutics in the treatment of gastrointestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies, etc. (Mauvernay *et al.*, 1970).

Lipase from *Candida rugosa* has been used to synthesize lovastatin, a drug that lower serum cholesterol level. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride, a widely used coronary vasodilator, was carried out with *S. marcescens* lipase (Matsumae *et al.*, 1993).

2.9.10 Lipases as biosensors

A biosensor based on the enzyme-catalysed dissolution of biodegradable polymer films has been developed. The polymerenzyme system; poly (trimethylene) succinate, was investigated for use in the sensor, which is degraded by a lipase. Potential fields of application of such a sensor system are the detection of enzyme concentrations and the construction of disposable enzyme based immunosensors, which employ the polymer degrading enzyme as an enzyme label (Sumner *et al.*, 2001).

Lipases may be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, and these function as lipid biosensors (Karube and Sode, 1988) and may be used in triglycerides (Iwai, 1990) and blood cholesterol determinations (Imamura *et al.*, 1989).

2.9.11 Degreasing of leather

Lipases represent a more environmentally sound method of removing fat. For bovine hides, lipases allow tensides to be replaced completely. For sheepskins, which contain up to 40 % fat, the use of solvents is very common and these are replaced with lipases and surfactants. If surfactants are used for sheepskins, they are usually not as effective and may be harmful to the environment. Maps (India) offers a range of lipases for degreasing which work in different pH conditions; Palkodegrease, lipase for degreasing in neutral to alkaline pH conditions and

Palkodegrease AL, Lipase for degreasing in acidic pH conditions (http://www.mapsenzymes.com/Enzymes Leather.asp).

Lipase enzymes can remove fats and grease from skins and hides, particularly those with a moderate fat content. Both alkaline stable and acid active lipases are used in skin and hide degreasing. Deliming and bating are the most suitable processing stages for using lipases. The enzyme loosens and removes the hair on the skins, which can then be filtered off. The end product is of a higher quality when compared to leather manufactured using traditional methods. *Rhizopus nodosus* lipase was used for the degreasing of suede clothing leathers from wooled sheep skins (Muthukumaran and Dhar, 1982). Acid active lipases can be used to treat skins that have been stored in a pickled state (http://www.biowise.org.uk).

2.9.12 Waste/effluent/sewage treatment

Lipases are utilized in activated sludge and other aerobic waste processes, where thin layers of fats must be continuously removed from the surface of aerated tanks to permit oxygen transport (to maintain living conditions for the biomass). This skimmed fat-rich liquid is digested with lipases (Bailey and Ollis, 1986) such as that from *C. rugosa*. Effective breakdown of solids and the clearing and prevention of fat blockage or filming in waste systems are important in many industrial operations. Examples include: (i) degradation of organic debris-a commercial mixture of lipase, cellulase, protease, amylase, inorganic nutrients, wheat bran, etc. is employed for this purpose; (ii) sewage treatment, cleaning of holding tanks, septic tanks, grease traps, etc. Effluent treatment is also necessary in industrial processing units, such as abattoirs, the food processing industry, the leather industry, the poultry waste processing (Godfrey and Reichelt, 1983). Both *P. aeruginosa* LP602 cells and the lipase were shown to be usable for lipid-rich wastewater treatment (Dharmsthiti and Kuhasuntisuk, 1998). Fats in wastewater

treatment plants that contains mainly triglycerides is hydrolysed by immobilized lipase (Tschocke, 1990).

Bacterial lipases are involved in solution of such environmental problems as the breakdown of fats in domestic sewage and anaerobic digesters (Godfrey and Reichelt, 1983).

Simple alkyl ester derivatives of restaurant grease were prepared using immobilized lipases as biocatalysts. The lipase from *Pseudomonas cepacia* was found to be the most effective in catalysing the methanolysis and ethanolysis of grease (Hsu *et al.*, 2002). A mixture of industrial cellulase, protease, and lipase, in equal proportion by weight, reduced total suspended solids (TSS) by 30-50% and improved settling of solids in sludge. An increase in solid reduction was observed with increasing enzyme concentration.

2.9.13 Oil biodegradation

Monitoring of soil microbial lipase activity is a valuable indicator of diesel oil biodegradation in freshly contaminated, unfertilized and fertilized soils (Margesin *et al.*, 1999). Fungal species can be used to degrade oil spills in the coastal environment, which may enhance ecorestoration as well as in the enzymatic oil processing in industries (Gopinath *et al.*, 1998).

2.9.14 Pulp and paper industry

The pulp and paper industry processes huge quantities of lignocellulosic biomass every year. The technology for pulp manufacture is highly diverse, and numerous opportunities exist for the application of microbial enzymes. Historically, enzymes have found some uses in the paper industry, but these have been mainly confined to areas such as modifications of raw starch. The enzymatic

pitch control method using lipase have been in use in a large-scale paper-making process as a routine operation since early 1990s (Bajpai, 1999). Lipase for wastepaper deinking can increase the pulping rate of pulp, increase whiteness and intensity, decrease chemical usage, prolong equipment life, reduce pollution level of waste water, save energy and time and reduce composite cost. The addition of lipase from *Pseudomonas* species (KWI-56) to a deinking composition for ethylene oxide–propylene oxide adduct stearate improved whiteness of paper and reduced residual ink spots (Fukuda *et al.*, 1990).

2.9.15 Use of lipases in production of biodiesel

The limited (and fast diminishing) resources of fossil fuels, increasing prices of crude oil, and environmental concerns have been the diverse reasons for exploring the use of vegetable oils as alternative fuels (Shah *et al.*, 2004). The biodiesel fuel from vegetable oil does not produce sulphur oxide and minimize the soot particulate one third times in comparison with the existing one from petroleum. Because of these environmental advantages, biodiesel fuel can be expected as a substitute for conventional diesel fuel (Iso *et al.*, 2001). Immobilized *P. cepacia* lipase was used for the transesterification of soybean oil with methanol and ethanol (Noureddini *et al.*, 2005). Fatty acid ethyl esters have also been prepared from castor oil using *n*-hexane as solvent and two commercial lipases, Novozym 435 and Lipozyme IM, as catalysts (Oliveira *et al.*, 2004). Novozyme 435 have also been used to catalyse the transesterification of crude soybean oils for biodiesel production in a solvent-free medium (Du *et al.*, 2004).

Simple alkyl ester derivatives of restaurant grease were prepared using immobilized lipases from *Thermomyces lanuginose* and *C. antarctica*, as biocatalysts (Hsu *et al.*, 2002). Fatty acids esters were produced from two Nigerian lauric oils, palm kernel oil and coconut oil, by transesterification of the oils with

different alcohols using PS30 lipase as a catalyst. In the conversion of palm kernel oil to alkyl esters (biodiesel), ethanol gave the highest conversion of 72 %. Some of the fuel properties compared favourably with international biodiesel specifications (Abigor *et al.*, 2000).

2.10 Current status of lipase research in India

Research on microbial lipases in India date back to late seventies when a few reports on screening and production of lipase from a few fungi and bacteria appeared. The initial emphasis on screening exercises was followed by process optimization for maximum lipase production. Physico-chemical conditions of lipase production by *M. racemosus*, *A. wentii*, and *P. chrysogenum* was reported (Akhtar *et al.*, 1980; Chander *et al.*, 1980). Lipolytic activity of thermophilic fungi of paddy straw compost was reported (Satayanarayan and Johri, 1981). *A. niger*, *A. flavus*, *A. fumigatus* and *Penicillium glaucum* were reported as the potential lipase producers isolated from the kernels of chironji and walnut (Saxena *et al.*, 1999).

Large-scale process optimization for lipase production was reported for *A. terreus*, *A. carneus* and *B. stearothermophilus* (Yadav *et al.*, 1998a). Extracellular microbial lipases was utilized for transesterification reactions for producing valuable transformed edible oils which cannot be obtained by chemical interesterification methods (Chakrabarty *et al.*, 1987). Lipases from *H. lanuginosa* and *Y. lipolytica* have also been reported for the synthesis of geranyl esters (Chand and Kaur, 1998). An extracellular lipase isolated from the conidia of *N. crassa*, had an apparent molecular weight of 54 kDa and 27 kDa, suggesting thereby the presence of two identical subunits (Kundu *et al.*, 1987).

Extensive work on various aspects of lipase from production and purification to characterization and industrial applications has been carried out on various fungi and bacteria (Ghosh *et al.*, 1996; Yadav *et al.*, 1998a). Novel thermostable and alkaline lipases from *A. terreus* and *A. carneus* are being developed for the production of biosurfactants, glycerides, and pharmaceutically important compounds. These lipases show regio- and chemoselective cleavage of polyphenolic compounds. Lipase from a strain of *B. stearothermophilus* shows remarkable activity even at 100°C. Besides this, a rapid zymogram for lipase activity in polyacrylamide gels was developed (Yadav *et al.*, 1998b).

The ability of lipases to show increased stability and selectivity in organic solvents has been exploited (Parmar *et al.*, 1992). Biotransformations on polyacetoxy arylmethyl ketones, benzylphenylketone peracetates, esters of polyacetoxy aromatic acids, and peracetylated benzopyranones, using commercial lipases are reported (Parmar *et al.*, 1992). The enantioselective behaviour of microbial lipases for the resolution of racemic drugs (Qazi, 1997), lipase-catalysed ester interchanges for the modification of selected Indian vegetable oils into cocoa butter substitutes and high oleic oils (Sridhar *et al.*, 1991), and enhancement of enzyme activity in aqueous-organic solvent mixtures (Gupta, 1992) were reported.

Chapter 3

MATERIALS AND METHODS

3.1 SCREENING & SELECTION OF POTENTIAL STRAIN FOR LIPASE PRODUCTION

3.1.1 Source of fungal isolates

In the present study fungal isolates were screened for lipase production using the isolates obtained during an earlier investigation as part of the 'Drug Discovery' project sponsored by CSIR during 2001-2004 and available as stock culture at Microbial Technology Laboratory, DBT, CUSAT as well as other isolates collected from marine environments of Kerala coast.

3.1.2 Strategy for screening and selection of potential strain that produce lipase

1. Screened all fungal isolates for lipase production in Malt Extract broth (ME broth) (composition given under section 3.1.3) supplemented with Tween 80 as lipase inducer and selected those which produced lipase on Rhodamine plate using olive oil as substrate.
2. In the second phase, all those isolates which showed positive for lipase production were subjected to screening using different oils as substrates and assayed for lipase production using para-nitrophenyl acetate as assay substrate.
3. The strain, which showed maximal lipase production, was selected and considered as potential strain, and used.

3.1.3 Medium

Malt Extract medium (ME) with the composition given below was used for the screening studies. Tween 80 was used as lipase inducer in the medium.

Composition of Malt Extract medium (ME medium)

Tween 80	2.0 % (v/v)
Malt Extract	1.0 % (w/v)
Ammonium nitrate	0.1 % (w/v)
KH_2PO_4	0.1 % (w/v)
$MgSO_4.H_2O$	0.05%(w/v)
Yeast Extract	0.1 % (w/v)
NaCl	1.0 % (w/v)
pH	5.5

When used as solid agar medium 2.0 % agar (w/v) was added to the ME broth and was referred to as Malt Extract Agar (ME Agar).

3.1.4 Screening of Lipase producing fungi

3.1.4.1 Phase I- Screening

Primary screening of lipase producing fungal isolates were performed using 100 cultures in ME broth added with Tween 80-2.0 % (v/v) (HiMedia, Mumbai). A loopful of 5 day old agar slope culture was transferred into 100 ml of ME broth prepared in 250 ml conical flask and incubated for a period of 3-7 days at room temperature (28±2°C) at 150 rpm. After incubation, the culture broth was filtered to remove the fungal mat, and the supernatant obtained was treated as the crude enzyme for lipase assay. The lipolytic activity of the culture supernatant was observed, using Rhodamine plate assay (Kouker and Jaeger, 1987) as detailed below.

3.1.4.1.2 Rhodamine B-Olive oil plate assay

A rapid assay of lipase activity was done using Rhodamine B-Olive oil plates, with the composition as given below, to identify specific lipase producers (Kouker and Jaeger, 1987). Lipase activity was detected as orange-red fluorescent halos upon UV irradiation.

Composition of Rhodamine B- Olive oil Agar plates

Olive oil	3 % (v/v)
Agar	2 % (w/v)
Rhodamine B	1 % (v/v)
Tris HCl buffer (pH 7)	50 mM
$CaCl_2$	1 mM

The Rhodamine agar medium was prepared in distilled water, autoclaved, and cooled to 60°C. The cooled medium was added with 3 % of olive oil previously sterilized at 160°C for 2 h in hot air oven and 1 % filter sterilized Rhodamine B (1mg/ml). The contents were mixed well to dissolve and the medium was poured into Petri dishes.

Circular wells of 3 mm were punched in the agar plates and 10 µl of the culture supernatant was dispensed into each well. Lipase activity was identified as an orange fluorescent halo under UV light at 350 nm after 24 h of incubation at 37°C.

3.1.4.2 Phase II- Screening

Phase II screening of potential strain that produces maximum lipase was done using different substrates that included Olive oil, Coconut oil, Gingelley oil, Palm oil, Sun flower oil and Tributyrin. The enzyme was assayed using pNP acetate as substrate and protein content determined as mentioned under sections

3.1.5.1 and 3.1.5.2 respectively. The strain that produced maximum lipase was selected based on the results.

3.1.5 ANALYTICAL METHODS

All the analytical procedures were performed as detailed below and the experimental data were statistically analysed using Microsoft Excel.

3.1.5.1 Lipase assay using p-Nitrophenyl acetate

Lipase activity was estimated according to the method of Huggins and Lapides (1947), using pNP acetate as substrate. Lipase assay was done by incubating 0.2 ml of the enzyme sample for 30 min at 37°C using 2.3 ml of 50mM buffered substrate (pNPA in Tris buffer pH 7.0). OD was measured at 415 nm. One unit of enzyme activity was defined as the amount of enzyme that released one µmol of pNP per minute under the assay conditions described.

3.1.5.2 Protein estimation

Protein content was estimated according to the method of Lowry *et al.*, (1951), using Bovine Serum Albumin (BSA) as the standard. OD was measured at 750 nm and expressed in milligram per milliliter (mg/ml).

Reagent

(a) Sodium carbonate in 0.1 N sodium hydroxide 2.0 % (w/v)
(b) Cupric sulphate in distilled water 0.5 % (w/v)
(c) Solution of sodium potassium tartrate in distilled water 1.0 % (w/v)
(d) *Working reagent: 100 ml of reagent (a) added with 1ml each of reagent (b) and reagent (c)
(e) *1:1 Folin and Ciocalteau's phenol reagent diluted with distilled water

*Prepared fresh before use

Estimation

An aliquot of 200 µl of the sample was made upto 2 ml with distilled water and added to 5 ml of freshly prepared working reagent (d), mixed thoroughly, and incubated for 10 min. 0.5ml of reagent (e) was added and incubated for 30 min followed by measuring the absorbance at 750 nm in a UV-Visible spectrophotometer (Shimadzu, Japan).

3.1.6 Maintanence of lipase producing cultures

All the short listed cultures were maintained in ME agar slants supplemented with 2.0 % (w/v) NaCl and stored at 4°C. They were subcultured periodically once in a month. They were also stocked under paraffin oil at room temperature.

3.2 CULTURE IDENTIFICATION

The selected fungal strain was identified as *Aspergillus awamori* Nagazawa at MTCC, IMTECH, Chandigarh. The identity was also checked using 28S rDNA technique.

3.2.1 Ribotyping using partial 28S rRNA gene

PCR-based detection of fungal DNA sequences is a rapid, sensitive, and specific method used in the identification of organism. Coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes which evolve slowly, and are relatively conserved among fungi, provide a molecular basis of establishing phylogenetic relationships (White *et al.*, 1990). Identity of the selected strain was further confirmed by ribotyping using a primer pair (NL1F-NL4R) for 28S rDNA as given in Table 3.1. A portion of the 28S rRNA gene was amplified from the genomic

DNA (O'Donnell, 1993) using these specific primers. Product of PCR amplification was subjected to nucleotide sequencing, followed by homology analysis.

Table 3.1: NL1F & NL4R primer sequences, used for amplification of 28S rDNA of the fungus

Sequence	Reference
NL1F- 5'-GCATATCAATAAGCGGAGGAAAAG-3'	O'Donnell, 1993
NL4R- 5'-GGTCCGTGTTTCAAGACGG-3'	O'Donnell, 1993

3.2.1.1 Polymerase chain reaction (PCR)

A PCR was performed in the Thermal cycler (Eppendorf, Germany) using the genomic DNA from *A. awamori* and 28S rDNA specific primers.

The PCR mixture contained 1 µl of genomic DNA (360 ng), 2 µl of each primer (100 pmol/µl), 2 µl 10 mM deoxyribonucleoside-triphosphate, 2.5 µl 10X PCR buffer containing $MgCl_2$, 2 µl of (3U/µl) *Taq* DNA Polymerase, and 13.5 µl of deionized water to make up the total volume to 25 µl. The PCR reaction was conducted with the initial denaturation of the template DNA at 94°C for 2 min followed by denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec and elongation at 72°C for 1 min. This cycle was then followed by 29 cycles of denaturation, annealing and elongation, followed by an extended final elongation step at 72°C for 7 min.

All PCR products and fragments were electrophoresed in a 1 % (w/v) agarose gel prepared in TAE-buffer (0.1 M Tris, 0.05 M Na_2EDTA (pH 8.0) and 0.1 mM glacial acetic acid). The gel electrophoresis was conducted in TAE-buffer

for 1 h at 80V. DNA bands in the gel were observed on a UV transilluminator after staining with ethidium bromide solution.

3.2.1.2 DNA sequencing

Nucleotide sequences of the PCR amplicon was determined using the ABI Prism 310 genetic analyzer, with the big dye Terminator kit (Applied Biosystems), at BioServe India Ltd., Hyderabad. The identity of the sequence obtained was established by comparing with the gene sequences in the database using BLAST software (Altschul *et al.*, 1980).

3.2.1.3 Phylogenetic tree construction

Phylogenetic tree was constructed using the neighbour joining method implemented in Multiple Sequence Alignment-CLUSTAL W (Thompson *et al.*, 1994) (http://align.genome.jp). Tree was constructed using nucleotide evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Tree was visualized using the CLUSTAL W N-J tree. The sequence was submitted to GenBank using the submission tool BankIt (http://www.ncbi.nlm.nih.gov/ BankIt).

3.3 SUBMERGED FERMENTATION (SmF) FOR PRODUCTION OF LIPASE BY ASPERGILLUS AWAMORI NAGAZAWA

Bioprocess for the production of lipase by *Aspergillus awamori* Nagazawa under submerged fermentation was optimized by studying the effect of various parameters using Malt Extract broth (section 3.1.3).

3.3.1 Medium preparation

ME medium was prepared as 50 ml broth in 250 ml Erlenmeyer flask. All the components, except oil, were dissolved in distilled water, pH adjusted to 5.5 and autoclaved. Oil was sterilized separately in hot air oven for 2 h at 160°C, cooled to room temperature, and then added (2 % v/v) to the autoclaved medium.

3.3.2 Inoculum preparation

The spore inoculum was prepared from a freshly raised 10 days old ME agar slant by dispersing the spores in 0.1% Tween 80 (v/v) prepared in saline water. 2 ml of this inoculum, containing 7 x 10^7 cfu/ml, was used for inoculating each flask unless otherwise mentioned.

3.3.3 Inoculation and Incubation

The prepared ME medium with Oil was inoculated with 2 ml (4 % v/v) of the prepared spore suspension. The inoculated medium was incubated at 28±2°C and 150 rpm in an environmental shaker (Orbitek, Scigenics India).

3.3.4 Recovery of enzyme

After incubation for the desired period (4 days arbitrarily selected) the fungal mycelia was removed by filtration through Whatman No. 1 filter paper and the culture supernatant was treated as the crude enzyme for all assays. This was the general procedure followed for all the studies conducted with respect to submerged fermentation, unless otherwise mentioned.

3.3.5 ANALYTICAL METHODS

3.3.5.1 Lipase assay in microtitre plate

Lipase assay was done using different pNP derivatives according to modified method of Nuria Prim *et al.*, (2003) in microtitre plate. PNP Acetate-pNPC2 (Sigma-Aldrich, USA), pNP Butyrate-pNPC4 (Sigma-Aldrich, USA), pNP Caprylate-pNPC8 (Fluka-Chemie, Germany), pNP Laurate-pNPC12 (Fluka-Chemie, Germany) and pNP Palmitate -pNPC16 (Sigma-Aldrich, USA) were used.

Microtitre plate lipase assay is preferred for the following advantages. It is easy to perform, requires small quantity of sample, accuracy of assay, and easy to assay large number of samples.

3.3.5.1.1 Substrate preparation

Solution A (Stock substrate solution)

Substrates described in section 3.3.5.1 were dissolved in isopropanol and sonicated for 6 min in a continuous mode for proper emulsification. A concentration of 0.15% (w/v) or 0.15 % (v/v) of substrate was prepared in isopropanol.

Solution B

50 mM Tris buffer (pH 7.0) containing 0.1 % gum arabic (Sigma-Aldrich, USA) and 0.4 % Triton X-100 (SRL, Mumbai).

Buffered substrate (1:10 dilution of the substrate stock solution A in Solution B)

To 9 ml of continuously stirred solution of **B** 1 ml of solution **A** was added dropwise.

3.3.5.1.2 Procedure

(a) An aliquot of 230 µl of buffered substrate was taken in a flat bottom microtitre plate and incubated at 37°C for 10 min in the microtitre plate reader (BIO-RAD, Model 680 series Microplate reader, USA).

(b) To the preincubated buffered substrate, 20 µl of appropriately diluted enzyme solution was added.

(c) Incubated at 37°C for 30 min and the released pNP was determined by immediate measurement of the absorbance at 415 nm against suitable blanks.

One unit of enzyme activity was defined as the amount of enzyme that released one µmol of pNP per minute under the assay conditions described.

All the assays were conducted in Microtitre plates unless otherwise mentioned.

3.3.5.2 Protein estimation

Protein content of the sample was estimated according to the method of Lowry *et al.*, (1951), using Bovine Serum Albumin (BSA) as the standard as described under section 3.1.5.2.

3.3.5.3 Specific Activity

Specific activity of the sample was calculated by dividing the enzyme units with the protein content and was expressed as U/mg protein.

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme activity (U/ ml)}}{\text{Protein (mg/ml)}}$$

3.3.5.4 Protease assay

Protease activity was determined by caseinolytic method (Kunitz, 1947) with minor modification. Hammerstein casein, used as substrate for the assay, was measured by the increase in absorbance at 280 nm.

The method is as described below:

1) 2 ml of 1 % (w/v) Hammerstein casein prepared in 0.05 M phosphate buffer (pH 7.0) and 0.5 ml of the same buffer were preincubated at 40°C for 10 min.
2) To the above solution, 0.5 ml of diluted enzyme solution was added and incubated at 40°C for 30 min. Appropriate control/blanks were also incorporated.
3) The reaction was arrested with 2.5 ml of 0.44 M trichloroacetic acid (TCA) solution. To the control, TCA was added before the addition of enzyme sample.
4) The reaction mixture was transferred to centrifuge tubes and the precipitated protein was removed by centrifugation at 10,000 rpm for 15 min (Sigma-laboratory Centrifuge, Germany).
5) The absorbance of the clear supernatant was measured at 280 nm in UV-Visible spectrophotometer (Shimadzu, Japan) against suitable blanks. The TCA soluble fractions of protein formed were quantified by comparison with a standard graph plotted with tyrosine as standard.
6) One unit of protease activity was defined as the amount of enzyme that liberated 1μg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions.
7) Enzyme activity was expressed as Units per ml.

3.3.5.5 Biomass

The fungal mycelia in the culture broth was separated by filtration through Whatman filter No.1 and dried at 80°C until a constant weight is attained (Silva *et al.*, 2005) and estimated as fungal biomass. Dry weight of the biomass was calculated and expressed as mg/ml. Values were the mean of three sets of experiments run simultaneously.

3.3.5.6 Lipase assay using olive oil as enzyme substrate (Titration method)

Lipase assay was performed using olive oil as a substrate. The enzyme activity was determined by titration of the free fatty acids liberated from olive oil against standard alkali solution (Ota and Yamada, 1966).

3.3.5.6.1 Reagents:

(i) Olive oil
(ii) Polyvinyl alcohol (PVA) (mol.wt 14,000)
(iii) 0.05 N NaOH
(iv) Phenolphthalein indicator
(v) Acetone
(vi) 0.05 M Phosphate buffer

3.3.5.6.2 Preparation of emulsion:

1. 2 % (w/v) of Polyvinyl alcohol (PVA) was prepared in 100 ml distilled water.
2. To 75 ml of 2 % PVA, 25 ml of olive oil was added.
3. Homogenized using a homogenizer for 10 min.
4. Added 2.5 ml of emulsion and 2 ml of 0.05 M PO_4 buffer to a clean conical flask. Maintained one control and one test for each reaction.
5. After 10 min of pre-incubation, added 0.5 ml of enzyme to the test.

6. Reaction was allowed to proceed for 20 min at 37°C.
7. After 20 min, 10 ml of acetone was added to both the test and control flasks.
8. 0.5 ml of heat-inactivated enzyme was added to the control.
9. Few drops of phenolphthalein was added onto the test and control.
10. Reaction mixture was titrated against 0.05 N NaOH.
11. Lipase activity was calculated using the following formula:

    Difference in titer value between test and control = (T-C)

    $$\text{Lipase activity (U/ml/min)} = \frac{(T-C) \times \text{Normality of NaOH} \times 100}{\text{Time of incubation}}$$

One unit of lipase activity is defined as the amount of enzyme that releases one micromole of free fatty acids per ml per minute under assay conditions.

3.3.6 Optimization of Bioprocess variables for lipase production by *Aspergillus awamori* Nagazawa

Various physico-chemical and bioprocess parameters affecting lipase production by fungus under submerged fermentation were optimized towards maximal enzyme production using ME medium. Strategy adopted for the optimization was to evaluate individually the effect of different parameters ('one-variable-at-a-time' method) on lipase production under SmF, conduct statistical optimization, and perform a time course experiment under optimized conditions.

The parameters optimized included incubation time, oil as substrate, incubation temperature, initial pH of the medium, agitation, additional carbon and nitrogen sources, sodium chloride concentration, percentage of sea water, inorganic salts, surfactants and inoculum concentration. Medium preparation, inoculum preparation, inoculation and incubation, and recovery of enzyme were done as

described under sections 3.3.1, 3.3.2, 3.3.3 and 3.3.4 respectively unless otherwise mentioned. In each case, samples were assayed for lipase activity, and protein content, and specific activity calculated as detailed under sections 3.3.5.1, 3.3.5.2 and 3.3.5.3, respectively, unless otherwise described.

3.3.6.1 Incubation time

Optimal incubation time for maximal enzyme production was determined by incubating the inoculated media for a total period of 144 h and analysing the samples at a regular interval of 12 h for enzyme activity. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.2 Substrate

Ideal substrate that induces maximal lipase production was studied using different oil substrates (2 % conc. arbitrarily selected). Twelve different oils, viz., groundnut oil, vegetable oil, palmolein oil, sunflower oil, mustard oil, castor oil, rice bran oil, gingelley oil, coconut oil, neem oil, and Tween 80 were used for the study. After 4 days of incubation, enzyme activity was estimated. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively. The selected substrate was used in the subsequent optimization studies unless otherwise mentioned.

3.3.6.3 Incubation temperature

Optimal incubation temperature for maximal enzyme production was evaluated by incubating the inoculated media with rice bran oil (selected as optimal substrate based on the results of the previous experiment) at the following

temperatures 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C and determining the enzyme activity after 4 days of incubation. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.4 Initial pH of the medium for enzyme production

Initial pH of the medium that could support maximal enzyme production was determined by adjusting the pH of the medium to various levels i.e., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 with either 1 N HCl or 1 N NaOH and determining the enzyme activity after 4 days of incubation. Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.5 Agitation

Effect of agitation on lipase production was studied by incubating the inoculated media taken in the conical flasks both at static and shake flask conditions at different rpm (100, 150, 200 and 250 rpm) and enzyme was assayed after 4 days of incubation. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.6 Inoculum concentration

Optimal inoculum concentration that supports maximal enzyme production was evaluated using different concentrations of conidial inoculum (1-15 %) prepared as mentioned under section 3.3.2. One ml spore inoculum contained 7×10^7 cfu/ml. After 4 days of incubation, enzyme activity was estimated. Medium

preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.7 Additional carbon sources

Need for additional carbon sources, other than the oil substrate, for the maximal enzyme production, was evaluated using various sugars viz., arabinose, dextrose, fructose, galactose, lactose, maltose, mannose, mannitol, sucrose, sorbitol and xylose. The sugars were added into the medium, so that the final concentration of the sugar was 0.1 M. After 4 days of incubation, enzyme activity was estimated. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.8 Effect of nitrogen sources

Effect of different nitrogen sources on lipase production was evaluated using different amino acids, complex organic sources, and inorganic nitrogen sources as detailed below. After 4 days of incubation, enzyme activity was estimated. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.8.1 Amino acids

Requirement for addition of amino acids for maximal enzyme production was determined by preparing the media supplemented with each of the amino acids viz., alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine at 0.1 M level, independently, after replacing the nitrogen sources of the original medium with the desired aminoacid.

ME medium without any nitrogen source was used as control. After 4 days of incubation, enzyme activity was estimated. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.8.2 Organic nitrogen sources

Effect of complex organic nitrogen sources on enzyme production was studied using yeast extract, beef extract, malt extract, casein, gelatin, peptone, soyabean meal, tryptone and urea individually at 1 % (w/v) level, replacing the nitrogen sources of the ME medium with the desired complex organic nitrogen source. ME medium, without any nitrogen source, was used as control. After 4 days of incubation, enzyme activity was estimated. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.8.3 Inorganic nitrogen sources

Effect of inorganic nitrogen sources on enzyme production was studied by the addition of ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium hydrogen carbonate, ammonium acetate, ammonium oxalate, ammonium dihydrogen orthophosphate, ammonium iron sulphate, sodium nitrate, and potassium nitrate at 0.1 M level, replacing the nitrogen sources of the ME medium with the desired inorganic nitrogen source. ME medium, without any nitrogen source, was used as control. After 4 days of incubation, enzyme activity was estimated. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.9 Sodium chloride concentration

Effect of sodium chloride concentration on enzyme production was determined by the addition of sodium chloride to the medium with varying concentrations viz: 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 7.5%, 10%, 12.5% and 15% NaCl. Medium without sodium chloride was used as control. After 4 days of incubation, enzyme activity was estimated. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.10 Seawater

Requirement for inclusion of sea water in ME medium for maximal enzyme production was evaluated by preparing the ME medium with various levels of seawater (25%, 50%, 75% and 100%) and assaying the enzyme production after 4 days of incubation. ME media prepared with 1% NaCl in distilled water and ME media without any seawater or NaCl were used as controls for comparison purpose. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.11 Inorganic salts

Effect of inorganic salts on enzyme production was studied by the addition of calcium chloride, magnesium sulphate, zinc sulphate, potassium chloride, copper sulphate, ferrous sulphate, manganese chloride, and cobalt chloride at 0.1 M level, after removing the inorganic salts of the original medium. ME media without any inorganic salts was used as control. Enzyme was assayed after 4 days of incubation. Medium preparation, inoculation and incubation, and enzyme

extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.6.12 Surfactants

Impact of surfactants on lipase production was evaluated using various surfactants viz: Triton X 100, SDS, PEG 6000, Tween 80, Tween 20, Gum Arabic, Sodium deoxycholic acid, and brij 35 at 0.25 % (w/v). Medium without any surfactants was used as control. Enzyme was assayed after 4 days of incubation. Medium preparation, inoculation and incubation, and enzyme extraction were performed as detailed under sections 3.3.1, 3.3.3 and 3.3.4 respectively.

3.3.7 Optimization of bioprocess variables for lipase production by Statistical approach

Optimization of medium for maximal enzyme production by fungus through statistical approach was carried out employing Plackett-Burman (PB) Design and Response Surface Methodology (RSM) as detailed below. The statistical software package used was Design-Expert® 6.0 (Stat Ease Inc., Minneapolis, U.S.A).

3.3.7.1 Plackett-Burman Design (PB Design)

After studying the effect of different parameters by 'one-variable-at-a-time' method eleven factors were selected for further optimization using statistical approach. The effect of eleven factors *viz.*; incubation days, pH, soyabean meal, ammonium sulphate, sodium chloride, rice bran oil, calcium chloride, potassium dihydrogen orthophosphate, PEG 6000, temperature, and inoculum conc. on lipase production was evaluated with Plackett-Burman Design (Haaland, 1989). The

parameters were varied over two levels and the minimum and maximum ranges selected for the parameters are given in Table 3.2.

Table 3.2: Minimum and maximum ranges for the parameters selected in Plackett-Burman Design for optimization of lipase production by *A. awamori*

S. No.	Factors	Level	
		Minimum (-)	Maximum (+)
1.	Incubation period (days)	3	5
2.	pH	3.0	5.0
3.	Soyabean (%)	0.5	1
4.	Ammonium Sulphate (M)	0.05	0.1
5.	NaCl (%)	1	2
6.	Ricebran oil (%)	2	4
7.	$CaCl_2$ (M)	0.05	0.1
8.	KH_2PO_4 (M)	0.05	0.1
9.	PEG 6000 (%)	0.05	0.1
10.	Inoculum (%)	1	4
11.	Temperature (°C)	30	40

The statistical software package Design-Expert® 6.0 (Stat Ease Inc., Minneapolis, U.S.A) was used to generate a set of 12 experimental designs. Production was set up by inoculating the media with respective inoculum percentages as suggested by the model and incubated for specified incubation period (3-5 days), at specified temperature (30-40°C), at 150 rpm. For each experiment, the lipase production was calculated in terms of U/ml. The experiments were done in triplicate. Regression analysis of the experimental data obtained was conducted using statistical software.

Based on the results obtained from the Plackett–Burman design, the fitted first-order model is:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i$$

Y is the predicted response, β_0, β_i, are constant coefficients, and x_i is the coded independent variables or factors.

Effect of each variable on the production was determined by calculating their respective E-values (Gupta *et al.*, 2004).

$$E = \frac{\text{(total of responses at high level)} - \text{(total of responses at low level)}}{\text{Number of trials}}$$

3.3.7.2 Response Surface Methodology (RSM)

The important parameters affecting enzyme production by *A. awamori* were optimized using a response surface type Box-Behnken (Box and Behnken, 1960) model experimental design. The treatments considered in the design were concentration of organic nitrogen source (Soyabean meal), inorganic nitrogen source (Ammonium sulphate), inorganic salt (KH_2PO_4) in the medium, incubation period, and incubation temperature.

Based on the results of the one-variable-at-a-time experiments and PB Design, the effect of five factors *viz.* concentration of soyabean meal (A), ammonium sulphate (B), potassium dihydrogen orthophosphate (C) in the medium, incubation period (D), and incubation temperature (E) were studied on lipase production using Response Surface Methodology. Other components of the medium were: Rice bran oil 2 % (v/v); $CaCl_2$ 0.05 M; PEG 6000 0.05 % (w/v); NaCl 1 % (w/v); inoculum 1 % (v/v) and pH 3.0.

3.3.7.2.1 Box-Behnken Design

Box-Behnken design model is a second-order design that allows estimation of quadratic effects, and is based on combining a two-level factorial design with an incomplete block design. This design was used for creating the quadratic response model.

Each factor in the design was studied at three different levels. All the variables were taken at a central coded value, considered as zero. A design model with 46 runs in 2 blocks of 23 cases was used as exhibited in Table 3.3, and each independent variable was tested at three levels. The levels were coded in standardised units with the values -1, 0 and +1 representing the lower, middle and higher levels respectively.

Design-Expert® 6.0 (Stat-Ease, Inc., Minneapolis, USA) was used to analyze the experimental design. The average maximum lipase activity was taken as the dependent variable or response (Y). Regression analysis was performed on the data obtained. The results of the Box-Behnken design were then used to fit a quadratic equation by multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

The following quadratic model was chosen to represent the relationship fitted between the above five variables.

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i x_i + \sum_{i=1}^{5} \beta_{ii} x_i^2 + \sum_{i=1}^{5} \sum_{j=1}^{5} \beta_{ij} x_i x_j$$

Or in the expanded form,

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{55} X_5^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5 + \beta_{34} X_3 X_4 + \beta_{35} X_3 X_5 + \beta_{45} X_4 X_5$$

In this model, Y represents the dependent variable-enzyme yield; X_1, X_2, X_3, X_4, and X_5 are the independent variables denoting soyabean meal concentration, ammonium sulphate concentration, potassium dihydrogen orthophosphate concentration, incubation period and incubation temperature respectively. β_1, β_2, β_3, β_4 and β_5 are linear coefficients, β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35} and β_{45} are second order interaction coefficients, and β_{11}, β_{22}, β_{33}, β_{44} and β_{55} are the quadratic coefficients. The design of experiments in terms of actual factors are given in Table 3.4.

The levels tested were soyabean meal- 0.5, 0.75 and 1% w/v; ammonium sulphate-0.05, 0.08 and 0.1 M; potassium dihydrogen orthophosphate-0.05, 0.08 and 0.1 M; incubation period-3, 4 and 5 days; and incubation temperature 30, 35 and 40°C. Analysis of Variance (ANOVA) was performed and 3-dimensional response surface curves were plotted by Design Expert software to study the interaction among various physico-chemical factors.

Table-3.3: Box-Behnken design for 5 variables at 3 levels- 2 blocks and 46 runs for optimization of lipase production by *A. awamori*

BLOCK	RUN	Incubation period (A) X_1 (days)	Soyabean meal (B) X_2 (%w/v)	Ammonium Sulphate (C) X_3 (M)	KH_2PO_4 (D) X_4 (M)	Temperature (E) X_5 (°C)
1	1	+1	-1	0	0	0
1	2	0	+1	0	0	-1
1	3	0	0	-1	-1	0
1	4	-1	-1	0	0	0
1	5	+1	0	+1	0	0
1	6	0	+1	0	0	+1
1	7	0	0	0	0	0
1	8	-1	0	-1	0	0
1	9	0	-1	0	0	+1
1	10	0	0	0	+1	+1
1	11	+1	0	-1	0	0
1	12	-1	+1	0	0	0
1	13	0	0	0	0	0
1	14	0	0	0	-1	+1
1	15	0	-1	0	0	-1
1	16	+1	+1	0	0	0
1	17	-1	0	+1	0	0
1	18	0	0	-1	+1	0
1	19	0	0	0	-1	-1
1	20	0	0	0	0	0
1	21	0	0	0	+1	-1
1	22	0	0	+1	+1	0
1	23	0	0	+1	-1	0
2	1	0	-1	-1	0	0
2	2	+1	0	0	-1	0
2	3	0	+1	0	+1	0
2	4	-1	0	0	0	+1
2	5	-1	0	0	-1	0
2	6	0	0	-1	0	+1
2	7	+1	0	0	0	-1
2	8	+1	0	0	0	+1
2	9	+1	0	0	+1	0
2	10	0	-1	0	-1	0
2	11	-1	0	0	+1	0
2	12	0	0	0	0	0
2	13	0	0	0	0	0
2	14	0	0	-1	0	-1
2	15	0	+1	-1	0	0
2	16	0	-1	0	+1	0
2	17	-1	0	0	0	-1
2	18	0	0	+1	0	-1
2	19	0	-1	+1	0	0
2	20	0	0	0	0	0
2	21	0	0	+1	0	+1
2	22	0	+1	+1	0	0
2	23	0	+1	0	-1	0

Table-3.4: Box-Behnken design for optimization of lipase production by *A. awamori*

BLOCK	RUN	Incubation period (A) X_1 (days)	Soyabean meal (B) X_2 (%w/v)	Ammonium Sulphate (C) X_3 (M)	KH_2PO_4 (D) X_4 (M)	Temperature (E) X_5 (°C)
1	1	5	0.50	0.08	0.08	35
1	2	4	1.00	0.08	0.08	30
1	3	4	0.75	0.05	0.05	35
1	4	3	0.50	0.08	0.08	35
1	5	5	0.75	0.10	0.08	35
1	6	4	1.00	0.08	0.08	40
1	7	4	0.75	0.08	0.08	35
1	8	3	0.75	0.05	0.08	35
1	9	4	0.50	0.08	0.08	40
1	10	4	0.75	0.08	0.10	40
1	11	5	0.75	0.05	0.08	35
1	12	3	1.00	0.08	0.08	35
1	13	4	0.75	0.08	0.08	35
1	14	4	0.75	0.08	0.05	40
1	15	4	0.50	0.08	0.08	30
1	16	5	1.00	0.08	0.08	35
1	17	3	0.75	0.10	0.08	35
1	18	4	0.75	0.05	0.10	35
1	19	4	0.75	0.08	0.05	30
1	20	4	0.75	0.08	0.08	35
1	21	4	0.75	0.08	0.10	30
1	22	4	0.75	0.10	0.10	35
1	23	4	0.75	0.10	0.05	35
2	1	4	0.50	0.05	0.08	35
2	2	5	0.75	0.08	0.05	35
2	3	4	1.00	0.08	0.10	35
2	4	3	0.75	0.08	0.08	40
2	5	3	0.75	0.08	0.05	35
2	6	4	0.75	0.05	0.08	40
2	7	5	0.75	0.08	0.08	30
2	8	5	0.75	0.08	0.08	40
2	9	5	0.75	0.08	0.10	35
2	10	4	0.50	0.08	0.05	35
2	11	3	0.75	0.08	0.10	35
2	12	4	0.75	0.08	0.08	35
2	13	4	0.75	0.08	0.08	35
2	14	4	0.75	0.05	0.08	30
2	15	4	1.00	0.05	0.08	35
2	16	4	0.50	0.08	0.10	35
2	17	3	0.75	0.08	0.08	30
2	18	4	0.75	0.10	0.08	30
2	19	4	0.50	0.10	0.08	35
2	20	4	0.75	0.08	0.08	35
2	21	4	0.75	0.10	0.08	40
2	22	4	1.00	0.10	0.08	35
2	23	4	1.00	0.08	0.05	35

3.3.7.2.2 Validation of the model

In order to validate the response surface model, a random set of experiments was set up according to the conditions predicted by the model. The responses obtained from the trials conducted as above following the Box-Behnken design model for five variables, was used to estimate the coefficients of the polynomial model using standard regression techniques. The estimate of "Y" was used to generate an optimal combination of factors that can support maximal enzyme production using predictive models from response surface methodology. The software Design-Expert® 6.0 (Stat Ease Inc., Minneapolis, U.S.A) was used to fit the response surface-Box Behnken model to the experimental data. All the experiments were carried out independently in triplicates.

3.3.8 Time course study under optimal condition

Time course experiment was conducted with the optimized conditions determined after statistical optimization of various variables. The conditions selected include the following:

- Rice bran oil (2 %)
- Inoculum (1 %-7 x 10^7 cfu/ml)
- Calcium chloride (0.05 M)
- PEG 6000 (0.05 % w/v)
- pH 3.0
- Sodium chloride (1 % w/v)
- Soyabean meal (0.77 % w/v)
- Ammonium sulphate (0.1 M)
- Potassium dihydrogen orthophosphate (0.05 M)
- Incubation temperature of 35°C

Medium preparation, inoculation and incubation, and enzyme recovery were performed as detailed under 3.3.1, 3.3.3 and 3.3.4 respectively.

3.4 ENZYME PURIFICATION

Lipase produced by *Aspergillus awamori* under SmF was purified (Fig. 3.1) employing standard protein purification procedures which included ammonium sulphate precipitation, followed by dialysis, and ion exchange chromatography as detailed below. All the operations were done at 4°C unless otherwise specified.

3.4.1 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was done according to Englard and Seifter (1990). Ammonium sulphate (Sisco Research Laboratories Pvt. Ltd., India) required to precipitate lipase enzyme was optimized by its addition at varying levels of concentrations (20 %, 40 %, 60 %, 80 % and 90 % saturation) to the crude extract. The weight of ammonium sulphate to be added was calculated using the chart given as Appendix-1.

(i) To precipitate the protein, ammonium sulphate was slowly added initially at 20 % saturation to the crude extract while keeping in ice with gentle stirring.

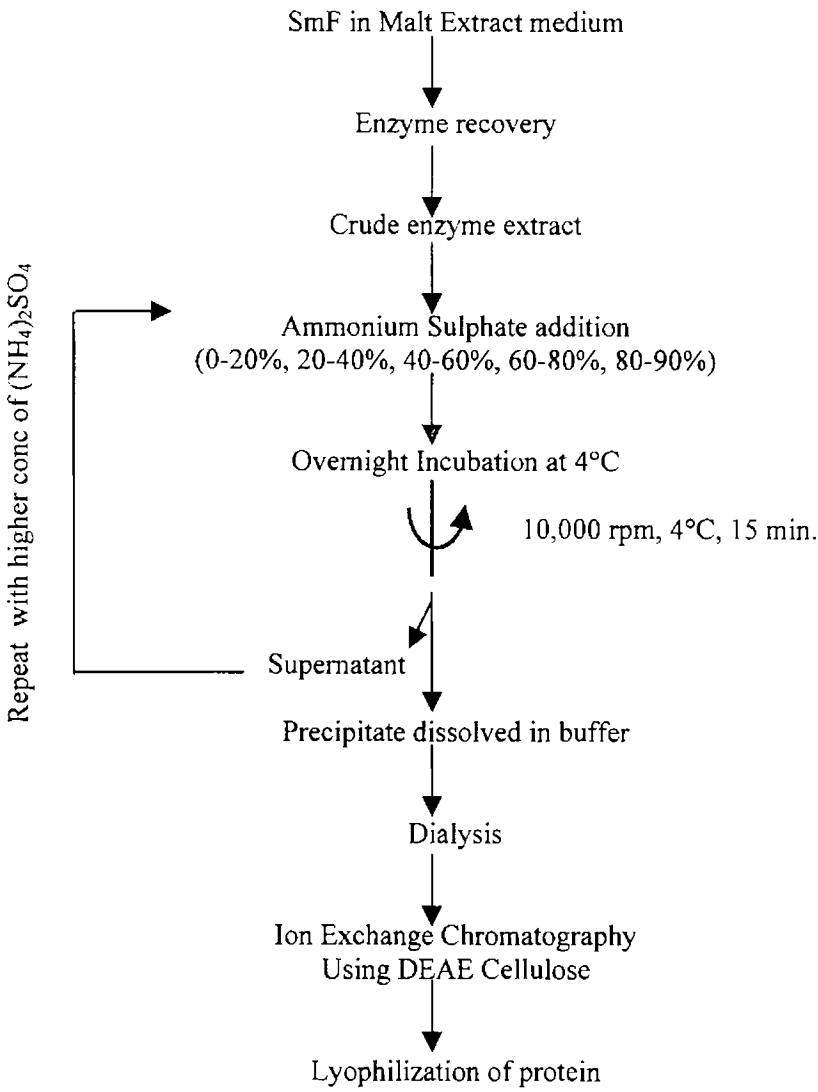
(ii) After complete dissolution of ammonium sulphate, the solution was kept at 4°C for overnight.

(iii) Protein precipitated was collected by centrifugation at 10,000 rpm for 15 min at 4°C.

(iv) The precipitate was resuspended in minimum quantity in 0.1 M phosphate buffer (pH 7.0).

(v) To the supernatant, ammonium sulphate required for next level of saturation was added and the procedure as mentioned above was repeated. This exercise was continued upto 90 % of ammonium sulphate saturation.

Fig. 3.1 ENZYME PURIFICATION PROTOCOL



3.4.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialysed against phosphate buffer in order to remove the ammonium sulphate from the precipitate. The precipitated protein, which was resuspended in minimum quantity of 0.1 M phosphate buffer (pH 7.0), was dialysed in the pretreated dialysis tube (section 3.4.2.1) (cut off value 12kDa) against 0.01 M solution of phosphate buffer of pH 7.0 for 24 h, at 4°C with 6 changes of buffer. The dialysate was assayed for lipase activity, protein content and specific activity as described in section 3.4.4. Yield and fold of purification were calculated as described in section 3.4.4.1.

3.4.2.1 Pretreatment of Dialysis Tube

Dialysis tube (Sigma-Aldrich) was treated to remove the humectants and protectants like glycerin and sulfur compounds present in it, and to make the pores of the tube more clear. The treated tube retain most of the proteins of molecular weight 12kDa or greater. The method followed for the treatment of the dialysis tube was as follows.

(a) Washed the tube in running water for 3-4 h.
(b) Dipped in 0.3 % (w/v) solution of sodium sulfide, at 80°C for 1 min.
(c) Washed with hot water (60°C) for 2 min.
(d) Acidified with 0.2 % (v/v) sulphuric acid.
(e) Rinsed with hot water (60°C).

3.4.3 Ion Exchange Chromatography (Rossomando, 1990).

Active fraction obtained after ammonium sulphate fractionation followed by dialysis was further purified by ion exchange chromatography using the anion exchanger DEAE cellulose as the column material. (solid stationary phase).

3.4.3.1 Standardization of binding pH of lipase to DEAE Cellulose

The pH at which the enzyme binds at its maximum to the anion exchanger was standardized by eluting the enzyme solution after incubating with DEAE cellulose equilibrated to each pH. DEAE Cellulose was activated by following the method described in section 3.4.3.2, suspended in distilled water and equilibrated to each pH using 0.01 M buffers of HCl-KCl buffer (pH 2.0), Glycine-HCl (pH 2.5), Citrate-Phosphate buffer (pH 3.0 to 6.5), Phosphate buffer (pH 7.0 & 7.5), Tris-HCl buffer (pH 8.0-9.0) and Carbonate-bicarbonate buffer (pH 9.5, 10.0 & 10.5). One milliliter of diluted sample of 40-90 % ammonium sulphate precipitated fraction was mixed with 2 ml slurry of DEAE cellulose equilibrated to each pH, incubated at 4°C for overnight, and the supernatant was collected by decanting without disturbing the suspension and assayed for lipase activity and protein as described in section 3.4.4. Added 2ml of 0.4 M NaCl and incubated for 2 h to elute the bound protein from the DEAE Cellulose. Supernatant collected was centrifuged at 10,000 rpm for 10 min to remove fine particles and assayed for lipase activity and protein content as described in section 3.4.4.

3.4.3.2 Activation of DEAE Cellulose

The following method was adopted for the activation of DEAE Cellulose

(a) Ten gram of DEAE Cellulose (Sisco Research Laboratories Pvt. Ltd., India) was soaked in Phosphate buffer (pH 7.5, 0.01 M) and fine particles were removed by decanting.

(b) It was then suspended in 1 M NaCl solution for overnight.

(c) Decanted sodium chloride solution and washed several times with distilled water in sintered glass funnel (G-1, Buchner Type, BOROSIL®, Mumbai) using vacuum filtration, until the pH of washings became neutral.

(d) It was equilibrated in Phosphate buffer of pH 7.5 (0.01 M) by repeated washing with the same.

3.4.3.3 Purification Using DEAE Cellulose column

DEAE Cellulose activated as described in section 3.4.3.2 was carefully packed in XK16/26 column (Amersham Biosciences) without trapping any air bubble. The column was equilibrated with Phosphate buffer of pH 7.5 (0.01 M) for overnight.

Dialysate (15 ml) prepared as in section 3.4.2 with protein content of 1.41 mg/ml was applied to the pre-equilibrated DEAE Cellulose column with height 20 cm. After the complete entry of sample in to the column the unbound proteins were washed with Phosphate buffer of pH 7.5 (0.01 M) until the OD_{280} reached near zero. Stepwise elution was done at a flow rate of 2 ml/min using 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl in the same buffer. Fractions (5 ml) were collected and protein content was estimated by measuring the absorbance at 280 nm. Peak fractions from the column were pooled and assayed for lipase activity, protein content and specific activity as described in section 3.4.4.

Yield and fold of purification was calculated as described in section 3.4.4.1.

3.4.4 Analytical Methods

Lipase activity, protein content and specific activity was determined as described earlier in sections 3.3.5.1, 3.3.5.2 and 3.3.5.3; expressed as U/ml, mg/ml and U/mg protein respectively. All the experimental data were statistically analysed using Microsoft Excel.

3.4.4.1 Calculation of Yield of Protein, Yield of Enzyme Activity and Fold of Purification

Yield of protein and enzyme activity of each fraction obtained during purification is the percentage activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract as the case may be.

$$\text{Yield of Protein} = \frac{\text{Total Protein content of the fraction x 100}}{\text{Total Protein content of the crude extract}}$$

$$\text{Yield of activity} = \frac{\text{Total activity of the fraction x 100}}{\text{Total activity of the crude extract}}$$

$$\text{Fold of Purification} = \frac{\text{Specific activity of the fraction}}{\text{Specific activity of the crude extract}}$$

3.5 CHARACTERIZATION OF PURIFIED ENZYME

Lipase purified by ammonium sulphate fractionation, dialysis, and ion exchange chromatography was further characterized for its biophysical and biochemical properties such as molecular mass determination, zymogram profile, glycoprotein staining, optimal pH and temperature, pH and temperature stability, effect of oxidizing agents, reducing agents, organic solvents, metal ions, enzyme kinetics, hydrolytic activity, and positional specificity as described in the following sections.

3.5.1 Electrophoretic Methods

Ammonium sulphate precipitated sample and active fractions collected after ion exchange chromatography were electrophoresed by Native-PAGE and SDS-PAGE in a 10 % polyacrylamide gel according to the method of Laemmlli (1970). SDS-PAGE of purified enzyme was carried out under reductive and non-reductive conditions, i.e., with and without β-mercaptoethanol respectively.

3.5.1.1 Reagents for Polyacrylamide Gel Electrophoresis

1) *Stock acrylamide solution* **(30:0.8)**
   Acrylamide (30 %) - 60.0 g
   Bis-acrylamide (0.8 %) - 1.6 g
   Distilled water (DW) - 200.0 ml
   Stored at 4°C in amber coloured bottle

2) *Stacking gel buffer stock* **(0.5 M Tris-HCl, pH 6.8)**
   Tris buffer - 6 g in 40 ml DW
   Titrated to pH 6.8 with 1M HCl (~48 ml) and made up to 100ml with DW
   Filtered with Whatman No:1 filter paper and stored at 4°C

3) *Resolving Gel buffer stock* **(3M Tris-HCl, pH 8.8)**
   Tris buffer - 36.3 g
   Titrated to pH 8.8 with 1M HCl (~48 ml) and made up to 100ml with DW
   Filtered with Whatman No:1 filter paper and stored at 4°C

4) ***Reservoir buffer for Native-PAGE* (pH 8.3)**

Tris buffer	-	3.0 g
Glycine	-	14.4 g

Dissolved and made up to 1L with DW
Prepared in 10X concentration and stored at 4°C

5) ***Reservoir buffer for SDS-PAGE* (pH 8.3)**

Tris buffer	-	3.0 g
Glycine	-	14.4 g
SDS	-	1.0 g

Dissolved and made up to 1L with DW
Prepared in 10X concentration and stored at 4°C

6) ***Sample buffer for Native-PAGE***

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C

7) ***Sample buffer for Reductive SDS-PAGE***

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Dithiothreitol	-	0.1M
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C

8) ***Sample buffer for Non-Reductive SDS PAGE***

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Bromophenol blue	-	0.01%

Prepared in 2X concentration and stored at 4°C

9) ***SDS* (10 %)** - 1g in 10 ml DW

10) ***Sucrose* (50 %)** - 5g in 10 ml DW (autoclaved at 121°C for 10 min and stored at 4°C)

11) ***Protein Staining solution***

Coomassie brilliant blue (0.1%)	-	100mg
Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

12) *Destaining Solution*

Methanol (40 %)	-	40 ml
Glacial acetic acid (10 %)	-	10 ml
DW	-	50 ml

13) *Protein Marker for SDS-PAGE*

Low molecular weight marker mix of Amersham Pharmacia was used. Lyophilized marker mix was reconstituted in 1X sample buffer for reductive SDS-PAGE, boiled for 5 min, and 5 µl of marker was loaded on to the gel. The composition of the marker mix is as given below.

Components		MW(M_r)
Phosphorylase b	-	97,000
Bovine Serum Albumin	-	66,000
Ovalbumin	-	45,000
Carbonic anhydrase	-	30,000
Trypsin inhibitor	-	20,100
α-Lactalbumin	-	14,400

3.5.1.2 Native- Polyacrylamide Gel Electrophoresis (Native-PAGE)

3.5.1.2.1 Gel Preparation

Resolving gel (10 %)

Acrylamide : bis-acrylamide (30: 0.8)-		10.0 ml
Resolving gel buffer stock	-	3.75ml
Ammonium persulphate (APS)	-	0.15ml
Water	-	16.25ml
TEMED	-	15.0µl

Stacking gel (2.5 %)

Acrylamide : bis- acrylamide (30: 0.8)-		2.5 ml
Stacking gel buffer stock	-	5.0 ml
Ammonium persulphate (APS)	-	0.15ml
Water	-	12.5 ml
TEMED	-	15.0 µl

Sample buffer (1X)

Native-PAGE sample buffer (2X)	-	1.0 ml
50 % Sucrose	-	0.4 ml
DW	-	0.6 ml

3.5.1.2.2 Sample preparation

Added 100 µl of 1X sample buffer to lyophilized sample or 20 µl of 2X sample buffer and 10 µl of 50 % sucrose to 30 µl liquid sample, mixed well and 30 µl sample and 5 µl marker mix was loaded on to the gel.

3.5.1.2.3 Procedure

(a) The gel plates were cleaned using absolute ethanol and acetone, and assembled.

(b) **Resolving gel** - All the components except APS were added into a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and poured a layer of butanol over the gel and allowed to solidify at least for 1h.

(c) **Stacking gel**- The components of stacking gel except APS was added into a beaker, mixed gently and finally added APS. Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed it to solidify at least for 30 min.

(d) Gel was placed in the electrophoresis apparatus, and upper and lower reservoir was filled with reservoir buffer for Native-PAGE.

(e) The gel was pre run for 1 h at 80V.

(f) Loaded the gel with the protein sample.

(g) The gel was run at 80V till the sample entered the resolving gel.

(h) When the dye front entered the resolving gel, increased the current to 100 V.

(i) The current was stopped when the dye front reached 1 cm above the lower end of the glass plate.

(j) Removed the gel from the cast and stained for at least 1h in the staining solution.

(k) Destained till the bands became clear and observed under a transilluminator.

3.5.1.3 Sodium Dodecyl Sulphate- Polyacrylamide Gel electrophoresis (SDS-PAGE)

Purified protein was subjected to reductive or non-reductive SDS-PAGE i.e., with or without β-mercaptoethanol. Low molecular weight markers of Amersham Pharmacia was used as standard and molecular weight of lipase was determined using Quantity One@ Software of Biorad.

3.5.1.3.1 Reductive SDS-PAGE

3.5.1.3.1.1 Gel Preparation

Resolving gel (10%)

Acrylamide : bis-acrylamide (30: 0.8)-		10.0 ml
Resolving gel buffer stock	-	3.75ml
10% SDS	-	0.3 ml
Ammonium persulphate (APS)	-	0.15 ml
Water	-	15.95 ml
TEMED	-	15.00 µl

Stacking gel (2.5%)

Acrylamide : bis-acrylamide (30: 0.8)-		2.5 ml
Stacking gel buffer stock	-	5.0 ml
10% SDS	-	0.2 ml
Ammonium persulphate (APS)	-	0.15 ml
Water	-	12.3 ml
TEMED	-	15.0 µl

Sample buffer (1x)

SDS-PAGE sample buffer (2x)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

3.5.1.3.1.2 Sample preparation

Added 100 µl of 1x sample buffer to purified and lyophilized sample, mixed well, boiled for 5 min in a water bath, cooled to room temperature, and 30 µl sample and 5 µl low molecular weight markers were loaded on to the gel.

3.5.1.3.1.3 Procedure

Procedure followed for electrophoresis and staining was essentially same as described in section 3.5.1.2.3, with the exception that the reservoir buffer used was that of SDS-PAGE.

3.5.1.3.2 Non-reductive SDS- PAGE

3.5.1.3.2.1 Gel preparation

Resolving and Stacking gel was prepared as described in section 3.5.1.3.1.1.

Sample buffer (1X)

Sample buffer for Non-reductive SDS-PAGE (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

3.5.1.3.2.2 Sample preparation

Added 100 µl of 1X sample buffer to lyophilized sample or 20 µl of 2X sample buffer and 10 µl of 50 % sucrose to 30 µl liquid sample, mixed well, and 30 µl sample and 5 µl low molecular weight markers were loaded on to the gel.

3.5.1.3.2.3 Procedure

Procedure followed for electrophoresis and staining was essentially the same as described in section 3.5.1.2.3 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.5.1.4 Zymogram

Lipase activity of enzyme protein band was confirmed by zymogram analysis (Diaz *et al.*, 1999) using the fluorogenic substrate 4-methylumbelliferyl butyrate (MUF-butyrate) (Sigma, St. Louis, USA). Ammonium sulphate precipitated (40-90 %) fraction and active fractions pooled from ion exchange chromatography were lyophilized in 1 ml aliquots, resuspended in 0.1 ml of sample buffer under non-reducing condition (0.0625 M Tris-HCl, 2 % SDS, 10 % sucrose, 0.01% bromophenol blue, pH 6.8), and subjected to electrophoresis in a 10 % polyacrylamide gel at 4°C as described in section 3.5.1.3.2. After electrophoresis, SDS was removed from the gel by soaking the gel for 30 min in 2.5 % (v/v) Triton X-100 at room temperature. The gels were then briefly washed in 50 mM phosphate buffer, pH 7.0, and covered by a solution of 100 µM methylumbelliferyl butyrate prepared by diluting in 50 mM phosphate buffer from a stock of 25 mM MUF-butyrate in Methyl cellosolve (Sisco Research Laboratories Pvt. Ltd., India). Activity bands became visible in a short time (1-15 min) under UV illumination. Following zymogram analysis, SDS-PAGE gel was stained with Coomassie brilliant blue and protein bands were visualized.

3.5.2 Molecular Weight determination by Gel Filtration Chromatography

Gel filtration chromatography was performed for the precipitate obtained after ammonium sulphate fractionation (40-90 %) using Sephadex G-100 (Sigma-Aldrich) in order to determine the molecular weight of lipase.

3.5.2.1 Preparation of Column

(a) 25 g of Sephadex G-100 (Sigma-Aldrich) was suspended in distilled water and allowed to hydrate for 3 h at 100°C in a water bath, and fine particles were removed by decantation.

(b) Hydrated gel suspension was degassed under vacuum to remove the air bubbles.

(c) Filled the column (Amersham Biosciences XK26/70 column) with eluent without air bubble. Gel suspension was carefully poured into the column without trapping air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column.

(d) Column was stabilized by allowing two times the bed volumes of eluent (0.1 M Phosphate buffer, pH 7.5) to pass through the column bed in descending eluent flow.

3.5.2.2 Sample preparation and application

Dialysed sample (2 ml) prepared as in section 3.4.2 with a protein content of 1.41 mg/ml was applied to the column. Care was taken to make sure that the sample was completely free of undissolved substances. After the complete entry of sample in to the column the proteins were eluted using 0.1 M Phosphate buffer of pH 7.5, at a flow rate of 1 ml/minute. One ml fractions were collected and protein content was estimated by measuring the absorbance at 280 nm in a

UV-Visible Spectrophotometer (Shimadzu, Japan). Peak fractions from the column were pooled and assayed for lipase activity and protein content as described in sections 3.5.17.

3.5.2.3 Calculation of molecular weight

The molecular weight of the eluted protein was calculated by calibrating the column with low molecular weight gel filtration protein markers from Amersham Pharmacia.

3.5.2.4 Column calibration

The gel filtration column prepared using Sephadex G-100 was calibrated with low molecular weight gel filtration protein markers from Amersham Pharmacia.

i) Prepared a fresh solution of Blue Dextran 2000 (0.1mg/ml) (Sigma-Aldrich) in the eluent buffer (0.1 M phosphate buffer, pH 7.5)

ii) Blue Dextran (1-2% of the total gel bed volume) was applied to the column to determine the void volume (V_0)

iii) Dissolved the proper combination of the calibration kit proteins in the eluent buffer. The concentration of each protein was between 5-20 mg/ml.

The calibration markers used included the following:

Components		MW(M_r)
Ribonuclease A	-	13,700
Bovine Serum Albumin	-	67,000
Ovalbumin	-	45,000
Chymotrypsinogen A	-	25,000

iv) The calibration kit proteins were applied to the column. The volume of calibration solution was 1 % of the total gel bed volume.

v) The elution volume (V_e) for each kit protein was determined by measuring the volume of the eluent from the point of application to the center of the elution peak.

vi) The K_{av} value (Partition coefficient) for each protein was calculated and a calibration curve of K_{av} versus log molecular weight was prepared

K_{av} for each protein was calculated by the formula,

$$K_{av} = V_e / V_0$$

where V_e is the elution volume of each protein and V_0 is the void volume of the column, which was calculated by running the column with Blue Dextran 2000.

3.5.2.5 Determination of molecular weight of the lipase enzyme

Molecular weight of lipase enzyme was calculated from the calibration curve prepared using the calibration kit proteins and by plotting the semilogarithmic graph for the K_{av} v/s molecular weight of the standard proteins.

3.5.3 Glycoprotein staining-Thymol-H_2SO_4 Method (Gander, 1984)

3.5.3.1 Principle:

This procedure detects glycoproteins containing at least 50 ng of carbohydrate. Glycoproteins bearing hexosyl, hexuronosyl, or pentosyl residues react with H_2SO_4 to form furfural derivatives, which, in turn, react with thymol to form a chromogen. The chromogen is stable for only a few hours at ambient temperature. Furfural derivatives are not formed when 2-deoxy- or 2-acetamido-2-deoxyhexosaminyl residues are allowed to react with H_2SO_4. Gels must be washed free of low molecular weight contaminants before they are treated with the acid.

3.5.3.2 Procedure:

1. SDS-PAGE was performed as described in section 3.5.1.3.2, except that in sample buffer, sucrose was replaced with glycerol.
2. After electrophoresis, the gel was washed twice for 2 h in glass tray with isopropanol-acetic acid-distilled water (25:10:65) to fix the proteins and remove low molecular weight substances. Glass tray was kept in gel rocker (Genei, Bangalore) for shaking.
3. Additional washes were performed to remove large concentration of sucrose or other soluble carbohydrates from the protein samples.
4. A final wash for 2 h in the same solvent containing 0.2 % (w/v) thymol (loba Chemie) was done which resulted in the formation of a stable gel.
5. After washing with thymol, the liquid was decanted and the gels were allowed to drain.
6. A solution of concentrated sulphuric acid-absolute ethanol (80:20) at ambient temperature was added to the gel. Atleast 10 ml of reagent per ml of gel was used.
7. The gel was shaken gently at room temperature for 2.5 h or until the opalescent appearance of gel disappeared.
8. Zones containing glycoprotein stained red, while the background was yellow. Other proteins did not form visible zones when treated in this manner.

3.5.4 Determination of carbohydrate content of purified enzyme

The carbohydrate content of purified lipase was determined by phenol-sulphuric acid method using glucose as standard (Dubios *et al.*, 1951).

3.5.5 Optimal pH for lipase activity

Optimum pH for maximal activity of the purified enzyme was determined by conducting enzyme assay at various levels of pH in the range of 2-13. The enzyme assay was essentially the same as described in section 3.3.5.1 with the following modification. The enzyme solution used was 0.2 ml of diluted sample and the substrate pNP Palmitate (pNP Caprylate was not used since it was unstable at pH above 8.0), was prepared in the respective buffer of each pH. The buffer systems used included, HCl-KCl buffer (pH 2.0), Citrate-Phosphate buffer (pH 3 to 6), Phosphate buffer (pH 7.0), Tris-HCl buffer (pH 8.0 & 9.0), Carbonate-bicarbonate buffer (pH 10.0), Titrisol@ pH buffer (pH 11.0, 12.0 & 13.0; Merck, Germany). Enzyme activity and relative enzyme activity were calculated as described in section 3.5.17 and 3.5.17.2 respectively.

3.5.6 Stability of lipase at different pH

Stability of the purified enzyme over a range of pH was determined by measuring the residual activity at pH 10.0 after incubating the enzyme in different buffer systems of pH 2-13 for 24 h, at 4°C. Purified enzyme as 0.2 ml aliquot was incubated in 3.8 ml of different buffer systems, which included, HCl-KCl buffer (pH 2.0), Citrate–Phosphate buffer (pH 3.0, 4.0, 5.0 and 6.0), Phosphate buffer (pH 7.0), Tris-HCl buffer (pH 8.0 and 9.0), Carbonate-bicarbonate buffer (pH 10.0), and Titrisol@ pH buffer (pH 11.0, 12.0 and 13.0; Merck, Germany). After incubation 20 µl sample was assayed for residual lipase activity as described in section 3.5.17.1. Enzyme activity was expressed as U/ml.

3.5.7 Optimal temperature for lipase activity

Temperature optimum for enzyme activity was determined by incubating 0.2ml of purified enzyme essentially following the method described in section 3.3.5.1 (not in microtitre plate) at the temperature range from 5 to 100°C. OD was measured at 415 nm using spectrophotometer (Shimadzu).

3.5.8 Enzyme stability at different temperatures

Temperature stability of purified enzyme was determined by incubating the enzyme sample at various temperatures ranging from 20-50°C and the enzyme assay was conducted after 30 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 12 h, 24 h and 48 h of incubation as described in section 3.3.5.1. Enzyme activity of the sample kept at 4°C was taken as control. OD was measured at 415 nm in Shimadzu spectrophotometer. Enzyme activity obtained was considered as residual activity and expressed as U/ml.

3.5.9 Kinetic studies

Purified enzyme was subjected to kinetic studies towards determining the K_m and V_{max}. K_m, the substrate concentration at which the reaction velocity is half maximum and V_{max}, the velocity maximum of the enzyme reaction were determined by incubating 20 µl of the purified enzyme at different conc of pNP Caprylate (72.0 µM to 650.0 µM), pH 7.0, for 30 min, at 37°C. Enzyme assay was essentially the same as described in section 3.3.5.1.

The initial velocity data was plotted as the function of the concentration of substrate by the linear transformation of the Michaelis-Menten equation for the calculation of K_m and V_{max} of the reaction.

3.5.10 Effect of oxidizing agent on enzyme activity

Activity and stability of the enzyme in the presence of oxidizing agent, hydrogen peroxide, was studied by measuring the residual activity after 30 min of incubation of the enzyme in different conc of H_2O_2 viz., 1, 2, 3, 4, 5, 8, 10 and 20 % (v/v). The enzyme activity and residual activity was assayed as described in section 3.3.5.1 and 3.5.17.1 respectively.

3.5.11 Effect of reducing agents on enzyme activity

Activity and stability of the enzyme in the presence of reducing agents were studied by incubating enzyme solution with 0.2, 0.4, 0.6, 0.8, 1 and 5 % (v/v) of ß-mercaptoethanol and sodium thioglycolate for 30 min and measuring the enzyme and residual activity as described in section 3.3.5.1 and 3.5.17.1 respectively.

3.5.12 Effect of organic solvents on lipase activity

Impact of various organic solvents on enzyme activity was evaluated by incubating the enzyme with each organic solvent for 24 h and assaying the residual activity after 30 min and 24 h as described in section 3.3.5.1 and 3.5.17.1. Organic solvents studied included 10, 30 and 60 % (v/v) of methanol, ethanol, acetone, dimethyl sulfoxide, hexane, benzene, butanol, diethyl ether and isopropanol.

3.5.13 Effect of various metal ions on enzyme activity

Effect of various metal ions on enzyme activity, was evaluated by incubating the enzyme along with different concentrations of various metal ions in the enzyme reaction mixture for 30 min followed by measuring the residual

enzyme activity (section 3.3.5.1 and 3.5.17.1). The metals studied included 1, 5, 10, 15 and 20 mM final concentrations of sodium chloride, calcium chloride, magnesium sulphate, zinc sulphate, potassium chloride, cupric sulphate, ferric chloride, manganese chloride, nickel sulphate, cobalt chloride, mercury chloride, barium chloride, cadmium sulphate, lithium chloride, sodium molybdate, aluminium sulphate and chromium oxide which contribute the metal ions, Na^{+}, Ca^{2+}, Mg^{2-}, Zn^{2+}, K^{+}, Cu^{2-}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Co^{2-}, Hg^{2+}, Ba^{2+}, Cd^{2+}, Li^{+}, Mo^{6+}, Pb^{2+}, Al^{3-} and Cr^{3+} respectively.

3.5.14 Substrate specificity of the purified lipase

Lipase activity on different nitrophenyl esters (pNPAcetate-C2, Butyrate-C4, Caprylate-C8, Laurate-C12 and Palmitate–C16) was studied by the method of Prim *et al.*, (2003). Enzyme activity was calculated as described in section 3.3.5.1.

3.5.15 Positional specificity of the purified lipase

The positional specificity of the lipase was examined by thin-layer chromatography of the reaction product obtained by using pure triolein (C18:1) as a substrate (Rashid *et al.*, 2001; Saxena *et al.*, 2003a). The reaction mixture composed of 1 ml of triolein (Sisco Research Laboratories, Mumbai), 4 ml of 50 mM phosphate buffer (pH 7.0), and 1 ml of enzyme (300 U) was incubated at 37°C for 30 min with constant stirring at 200 rpm. After incubation, the reaction product was extracted with 20 ml of diethyl ether. Aliquots of ether layer were applied on to a silica gel G-60 plate (Merck, Germany) and developed with a solvent mixture containing petroleum ether, diethyl ether and acetic acid in the ratio 80:30:1. Spots were visualized using a saturated iodine chamber.

3.5.16 Lipase hydrolytic activity of purified lipase

Hydrolytic activity of the lipase was determined according to the method of Ota and Yamada (1966) as described in section 3.3.5.6. The liberated free fatty acids were titrated against 0.05 M NaOH using phenolphthalein as indicator. One unit of hydrolytic activity of lipase was defined as the amount of enzyme which catalyses the release of one μmol of free fatty acid per min under the assay conditions.

3.5.17 Analytical Methods

Lipase activity, protein content and specific activity were determined as described earlier in sections 3.3.5.1, 3.3.5.2 and 3.3.5.3 and were expressed as U/ml, mg/ml and U/mg protein respectively. All the experimental data were statistically analysed using Microsoft Excel.

3.5.17.1 Residual Activity

Residual activity is the enzyme activity of the sample with respect to the original enzyme activity of the control sample, which is expressed in percentage.

$$\text{Residual activity} = \frac{\text{Activity of sample (U/ml) x 100}}{\text{Activity of the Control (U/ml)}}$$

3.5.17.2 Relative Activity

Relative activity is the percent enzyme activity of the sample with respect to the sample for which maximum activity was obtained.

$$\text{Relative activity} = \frac{\text{Activity of sample (U/ml) x 100}}{\text{Activity of the maximal enzyme activity obtained sample (U/ml)}}$$

3.6 APPLICATION STUDIES

The industrial applications of lipases have grown rapidly in recent years and are likely to markedly expand further in the coming years. Lipases are the most versatile biocatalyst and bring about a range of bioconversion reactions such as hydrolysis, transesterification, esterification, alcoholysis, acidolysis and aminolysis.

In the present study, as part of application studies an attempt was made to evaluate the use of lipase in biodiesel production, sugar fatty acid synthesis and in bioremediation towards its industrial applications.

3.6.1 Biodiesel production

Transesterification reaction using lipase for biodiesel production was conducted according to the method described by Shah *et al.*, (2004).

Rice bran oil and methanol were taken in the ratio of 1:4 (mol/mol) in a screw-capped vial. To this mixture, 50 mg of enzyme preparation (lyophilized) was added and incubated at 40°C with constant shaking at 200 rpm for 24 h. The reaction products were appropriately diluted (with hexane), and passed through anhydrous sodium sulphate to remove any trace of water before analysis by gas chromatography and thin-layer chromatography.

3.6.1.1 Gas-Chromatography analysis

The formation of methyl esters of rice bran oil was analyzed on a Nucon-5700 gas chromatograph with a flame-ionization detector. The capillary column used was made up of 70 % phenyl polysilphenylenesiloxane, had a length of 30 m with an internal diameter of 0.25 mm. Nitrogen was used as the carrier gas at a

constant flow rate of 4 kg/cm^2. The column oven temperature was programmed from 150 to 250°C, at the rate of 10°C per min with injector and detector temperatures at 240 and 250°C, respectively.

3.6.1.2 Thin-Layer Chromatography

The formation of methyl esters of rice bran oil in the reaction mixture was also analyzed by thin-layer chromatography with silica gel G-60 F_{254} (E. Merck, Mumbai, India). The solvent system consisted of hexane, ethyl acetate and acetic acid in the ratio of 90:10:1 (v/v). The spots were detected in the iodine chamber.

3.6.2 Fatty acid sugar ester synthesis

Lipase-catalyzed synthesis of fatty acid sugar esters was performed in a mainly solid-phase system consisting of insoluble sugar, fatty acid and product in a small amount of organic solvent (Gulati *et al.*, 2001; Sabeder *et al.*, 2006).

The sugars used were fructose and glucose; fatty acids were myristic acid and palmitic acid; and the organic solvent was hexane. In order to increase the reaction yield, the water generated during esterification was continuously removed from the reaction mixture by evacuation in vacuum.

For a typical reaction, 100 mmol/L of the fatty acid was mixed with 50 mmol/L of the sugar in 4 ml n-hexane in 15 ml screw capped vials. The reaction mixture was equilibrated for 24 h in an evacuated desiccator saturated with $MgCl_2.6H_2O$, which was found to be the best salt hydrate for the ester synthesis reaction. Lyophilized lipase (50 mg) was then added to the reaction mixture. The vials were incubated at 37°C for 48 h at 150 rpm. After 48 h of incubation samples were analyzed by thin layer chromatography (TLC) and by volumetric method. A control without lipase was also considered.

Product formation was qualitatively analysed by TLC using a solvent system comprising petroleum ether, diethyl ether and acetic acid in a ratio of 80:30:1. The plates (G60 F_{254}-Merck, Germany) were developed by spraying with 50% sulphuric acid and heating at 110°C for 5 min to develop ash coloured spots of sugar and sugar ester (Cao *et al.*, 1997).

The ester content was quantified by calculating the residual fatty acid amount in the reaction mixture, which was determined by volumetric method. 0.1 g of sample of the reaction mixture was diluted in 20 mL of 0.1 wt % phenolphthalein solution in absolute ethanol and then titrated with standardized sodium hydroxide solution of 0.1 mol/L in water (Habulin and Knez, 1990). The results were expressed in terms of percent conversion of fatty acid to ester.

3.6.3 Treatment of Effluent containing oils using *A. awamori* lipase

Potential of lipase for treatment of oil containing effluent was evaluated by treating a simulated oil effluent, blended with 10% each of 'used restaurant oil', 'used ayurvedic oil', 'used fish fry oil', palm oil, coconut oil, and dalda with lipase. The prepared effluent was treated with 1 % (v/v) enzyme (300 U/mL of lipase activity) for 24 h at 35°C, without agitation. Wastewater with 1 % heat inactivated enzyme was used as control. After 24 h of incubation, oil and grease content were estimated in both control and wastewater according to the AOAC manual and the percentage reduction in oil and grease was calculated and expressed.

3.7 ISOLATION OF LIPASE ENCODING GENE FROM ASPERGILLUS AWAMORI NAGAZAWA

3.7.1 Fungal and bacterial strains, and plasmid vector used

Fungus *Aspergillus awamori* Nagazawa, *E. coli* strain DH5α and the plasmid pGEM®-T Easy Vector (Promega) were used in the present study.

3.7.2 Enzymes and chemicals

The enzymes used included the following: Taq DNA Polymerase (Bangalore Genei) and T4 DNA Ligase (Promega).

All chemicals were of analytical or molecular biology grade. The chemicals used were sodium chloride (NaCl), Sodium dodecyl sulfate (SDS), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), and ethylenediamine tetraacetic acid (EDTA). The agarose (Qbiogene) used for the electrophoresis of DNA was of molecular biology grade. Ethidium bromide and ampicillin were obtained from Bangalore Genei; X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (Isopropyl-β-D-thiogalacto pyranoside) were procured from Sigma-Aldrich.

3.7.3 Cultivation of fungus and bacterial strains

The fungal strain, *Aspergillus awamori* Nagazawa was cultivated in ME broth. Medium preparation was done as described in section 3.3.1, without the addition of oil, in 100 ml in a 250 ml Erlenmeyer flask and cultivated at 28±2°C on a rotary shaker at 150 rpm for 5-7 days. Inoculation and incubation, was performed as detailed under section 3.3.3.

Escherichia coli DH5α was grown on LB media containing tryptone (10 g/L), NaCl (5 g/L), and yeast extract (5 g/L).

3.7.4 Isolation of Genomic DNA -Miniprep Method (Lee and Taylor, 1990)

Genomic DNA was isolated from *A. awamori.* After growth in ME medium for 7 days the culture was harvested by means of filtration through Whatman®No. 3 filter paper. The harvested mycelia was washed with distilled water and again filtered to remove residual media. The mycelia were then ground with liquid nitrogen in a -20°C pre-cooled mortar and pestle to a fine powder. Ground mycelia was transferred to 1.5 ml eppendorf tube, added 600 µl lysis buffer (200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA and 0.5 % SDS), mixed gently by inversion and incubated at 65°C for 1 h. A volume of 650 µl Phenol-chloroform (1:1) was added and carefully inverted a few times. After centrifugation at 12,000 rpm for 10 minutes in a centrifuge (Sigma-laboratory Centrifuge, Germany), the aqueous phase was transferred to a fresh tube and added with equal amount of (~500 µl) chloroform-isoamyl alcohol (25:1) and mixed several times by inversion. Centrifuged at 12,000 rpm for 10 minutes and the aqueous phase was transferred to a fresh tube and added 20 µl 3 M sodium acetate, followed by 0.6 volumes of isopropanol. The suspension was carefully mixed by inversion a few times and centrifuged at 12,000 rpm for 10 minutes. To the pellet 50-100 µl of 70 % ethanol was added and washed twice. The pellet was allowed to air dry. 30-50 µl TE buffer was added to the pellet and kept at 60°C for 10-20 min to dissolve, stored at -20°C.

3.7.4.1 Nucleic acid quantification

The quantitative analysis of DNA was done spectrophotometrically by measuring the optical density at 260 (A_{260}) and 280 nm (A_{280}). The reading at

260 nm allows the calculation of the concentration of the nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA. The ratio between the readings at 260 nm and 280 nm (A_{260} /A_{280}) provides an estimate of the purity of the nucleic acid. Pure DNA, free of protein contamination will have the A_{260} /A_{280} ratio close to 1.8. If there is protein contamination, the ratio A_{260} /A_{280} will be significantly less than 1.8. The quantification of DNA was done by using the DNA/Protein pack® software of Schimadzu UV-VIS spectrophotometer.

3.7.4.2 DNA agarose gel electrophoresis (Sambrook *et al.*, 2000)

The agarose gel electrophoresis was carried out in order to check the quality of the DNA obtained. Agarose gel (0.8 %) was prepared with electrophoresis grade agarose and known volume of TAE-buffer (0.1 M Tris, 0.05 M Na_2EDTA (pH 8.0) and 0.1 mM glacial acetic acid). The contents were mixed and melted in a microwave oven. The molten agarose at 55°C was poured without air bubbles into the gel-casting tray and allowed to solidify at room temperature. 10 µl of the DNA (in loading buffer) was loaded on to the gel and electrophoresis was carried out at 80 V for 1 h or until the tracking dye (Bromophenol blue) had traversed two-thirds distance of the gel. λ DNA/*EcoR*I *Hind* III double digest (Bangalore Genei) was used as the marker. The gel was stained in a freshly prepared 0.5 mg/ml ethidium bromide solution for 20 min, and viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Bio Rad).

3.7.5 Primer designing

Degenerate primers were designed and used for PCR based experiments. All the primers used in the study were synthesized by Integrated DNA Technologies, Inc. USA. Primers were designed based on the conserved domain

deduced from the already reported nucleotide sequence encoding lipase of several fungi. The nucleotide sequences coding for lipase were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) and aligned by CLUSTALW (http://align.genome.jp). The bases at the most conserved regions of alignment were considered for the primer designing. From this sequence two upstream and two downstream primers were designed for the amplification of the lipase gene from *A. awamori* (Table 3.5).

Table 3.5: Designed forward and reverse primers for the amplification of the lipase gene from *A. awamori*

Primer Name	Primer sequence	Use
ASP.F1	5'-GTCGAAATGGCCACTATCTCC-3'	1st PCR-forward
ASP.F2	5'-AACGGATGGATTCTCCGCGAC-3'	2nd PCR-forward
ASP.R1	5'-AACGCTCCAGTACTCTACACC-3'	1st PCR-reverse
ASP.R2	5'-CTCACAGCACTGCACTTCATC-3'	2nd PCR-reverse

3.7.6 Polymerase chain reaction (PCR)

A PCR was performed in the thermal cycler (Eppendorf, Germany) using the isolated genomic DNA from *A. awamori* to amplify the lipase gene. The PCR was performed with the primer pairs ASP.F1-ASP.R1, ASP.F1-ASP.R2, ASP.F2-ASP.R1 and ASP.F2-ASP.R2.

The PCR mixture contained 1 µl of isolated genomic DNA (360 ng) from *A. awamori*, 2 µl of each forward and reverse primer (100 pmol/µl), 2 µl 10 mM deoxy- ribonucleosidetriphoshate, 2.5 µl 10X PCR buffer containing $MgCl_2$, 2 µl of 3 U/µl *Taq* DNA Polymerase and deionsized water 13.5 µl to make up the total volume to 25 µl. The PCR reaction was conducted with the initial denaturation of the template DNA at 94°C for 2 minutes followed by denaturation at 94°C for

45 seconds, annealing at 56°C for 1 minute and elongation at 72°C for 2 minutes. This cycle was then followed by 29 cycles of denaturation, annealing and elongation, followed by an extended final elongation step at 72°C for 10 minutes.

All PCR products and fragments were electrophoresed in a 1% (w/v) agarose gel which was prepared and electrophoresed in TAE-buffer. The gel electrophoresis was conducted for 1 h at 80 V. DNA bands in the gel were observed on a UV transilluminator (BioRad) after staining with ethidium bromide solution.

3.7.7 Cloning of the lipase gene fragment from *A. awamori*

The amplicon obtained after PCR was cloned into the pGEM®-T Easy vector system II (Promega) according to the manufacturer's specifications. The ligated plasmids were transformed into $CaCl_2$ competent cells (*E. coli* DH5α) as described in section 3.7.7.1 and 3.7.7.2.

3.7.7.1 Ligation

The amplicon obtained was ligated to pGEM®-T vector (pGEM®-T Easy vector system II-Promega as per manufacturer's specifications). Ligation mix was prepared with 5 µl of 1X ligation buffer, 3 µl of insert, 1 µl of pGEMT vector and 1 µl of T4 ligase enzyme. All the components were incubated at 4°C overnight.

3.7.7.2 Preparation of competent cells (Sambrook *et al.*, 2000)

A single colony of *E. coli* host cell (DH5α) was inoculated in 5ml of Luria-Bertani (LB) broth and grown overnight with constant shaking at 150 rpm at 37°C. 500 µl of overnight culture was inoculated to 50 ml of LB broth and incubated at 37°C in an environmental shaker at 150 rpm for 2 h. The cells were harvested by

centrifuging at 10,000 rpm for 10 minutes at 4°C. The pellet was suspended in 20 ml of ice cold 0.1 M $CaCl_2$ and incubated in ice for 30 min. Then the cells were harvested again by centrifugation at 7,000 rpm for 5 min at 4°C. The pellet was resuspended in 2 ml of 0.1M $CaCl_2$. This was aliquoted as 80 µl fractions and added with chilled glycerol (~20 µl) and stored at -80°C until use.

3.7.7.3 Transformation

Ten µl of ligation mix was added to 100 µl of the prepared competent cells and incubated in ice for 30 minutes. Later the cells were given a heat shock at 42°C for 2 minutes in dry bath (Bangalore Genei) followed by quick chilling in ice for 5 minutes. A volume of 250 µl LB was added to the transformed cells in the tube and incubated at 37°C for 1h. The grown cells (50-100 µl) were plated on LB agar plates containing 50 µg/ml of ampicillin, 100 µg/ml IPTG and 40 µg/ml X-gal employing spread plate technique and incubated at 37°C. The plates were checked for transformants after overnight incubation.

3.7.7.4 Plasmid isolation from transformed colonies

Reagents:

Solution I

Glucose	-	50 mM
Tris-HCl (pH 8.0)	-	25 mM
EDTA (pH 8.0)	-	10 mM

Solution II*

NaOH	-	0.2 N
SDS	-	1 % (w/v)

* Freshly prepared

Solution III

Potassium acetate (pH 7.5)-5M	-	60.0 ml
Glacial acetic acid	-	11.5 ml
Deionized water	-	28.5 ml
pH	-	5.4

Plasmid DNA from the transformed colonies was isolated by a modified version of alkali lysis method (Sambrook *et al.*, 2000). Transferred a single white colony into 4 ml LB containing the appropriate antibiotic and incubated the culture overnight at 37°C at 150 rpm. The culture was centrifuged at 12,000 g for 2 min at 4°C. The pellet was suspended in 100 µl of ice cold solution I for 5 min. Freshly prepared solution II (200 µl), was added, mixed well by inversion and incubated in ice for 10 min. This was followed by the addition 150 µl of ice cold solution III, mixed by gentle inversion and then incubated in ice for 15 min. Subsequently, bacterial cell debris was separated by centrifugation of the sample at 12,000 g for 15 minutes at 4°C. The supernatant was transferred to a new centrifuge tube to which 0.6 volume of isopropanol was added, mixed by vortexing and incubated at room temperature for 10 minutes. The plasmid DNA was pelleted by centrifugation at 12,000 g for 15 minutes at 4°C, and was washed twice with 70 % ethanol, air dried and dissolved in 20 µl TE buffer (pH 8.0). The DNA sample was stored at -20°C.

The prepared plasmid DNA was subjected to agarose gel electrophoresis using a 1% agarose gel (section 3.7.4.2). The presence of the insert DNA was confirmed by restriction analysis using *EcoR*I. Reamplification of the plasmid DNA was done using the specific degenerate primers as described in section 3.7.5.

3.7.8 DNA sequencing

Nucleotide sequences of the PCR amplicon obtained from genomic DNA was determined by the ABI Prism 310 genetic analyzer, using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. Using BlastX programme the relationship of the amplicon nucleotide sequence with other reported lipase gene was determined towards confirmation of lipase gene of *A. awamori*.

Chapter 4

RESULTS

4.1 SCREENING, SELECTION, AND IDENTIFICATION OF POTENTIAL STRAIN FOR LIPASE PRODUCTION

4.1.1 Screening and Selection of potential strain

From the first phase of screening of fungal isolates, using rhodamine B-olive oil plate assay for lipase production, 14 cultures were recognized as potential lipase producers. The details of the cultures, which showed orange-red fluorescent halos upon UV irradiation in rhodamine B-olive oil plates, are presented below in Table 4.1. All the cultures are available as stock culture in the Microbial Technology Laboratory, DBT, CUSAT, Kerala.

Results obtained for the second phase of screening, which was based on the maximum lipase production using different oils as substrate and conducting enzyme assay, are presented in Fig. 4.1. From the results it is inferred that the fungal strain BTMFW 032 produces maximal lipase compared to other strains. Hence, this strain was selected as potential strain for lipase production.

Table 4.1: Details of lipase positive fungi selected after phase I screening

Culture code No.	Original Source of culture
BTMFW 010	Seawater/Kovalam beach, Trivandrum
BTMFW 015	Seawater/Shangumugham beach, Trivandrum
BTMFW 017	Seawater/Shangumugham beach, Trivandrum
BTMFS 020	Sediment/Ponnani azhimukam, Kozhikode
BTMFS 021	Sediment/Ponnani azhimukam, Kozhikode
BTMFW 028	Seawater/Nattika beach, Thrissur
BTMFW 032	Seawater/Munambam harbour, Kochi
BTMFW 035	Seawater/Ponnani beach, Kozhikode
BTMFW 042	Seawater/Ponnani beach, Kozhikode
BTMFW 044	Seawater/Nattika beach, Thrissur
BTMFW 046	Seawater/Chettuva beach, Thrissur
BTMFS 125	Sediment/Marine drive, Kochi
BTMFS 157	Sediment/Mangalavanam, Kochi
BTMFS 159	Sediment/Mangalavanam, Kochi

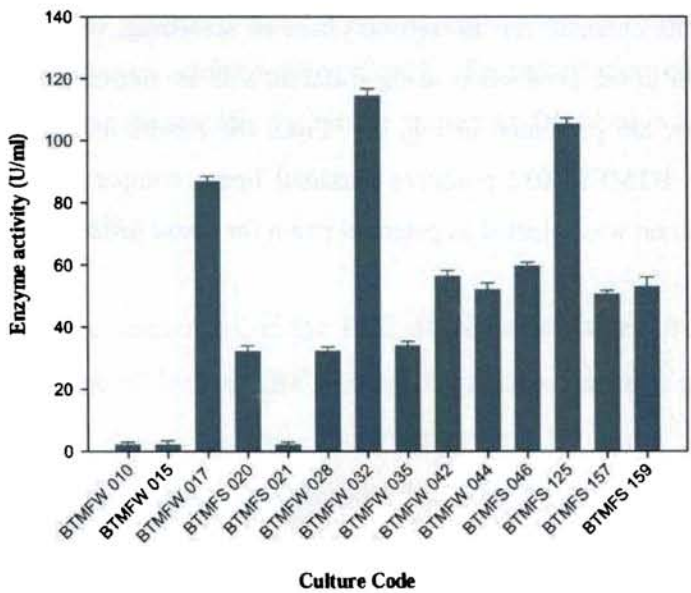


Fig 4.1 Enzyme activity of lipase positive fungi selected after phase I screening

4.1.2 Identification of the selected strain

The selected fungal strain, BTMFW 032 was identified as *Aspergillus awamori* Nagazawa at MTCC, IMTECH, Chandigarh. The lipase activity of *A. awamori* on rhodamine B-olive oil plate is presented in Fig. 4.2. The reddish brown conidial heads, fast growing colonies with biseriate sterigmata, and around 4.0 μm diameter conidia differentiate *A. awamori* from the closely related species *A. niger* (Fig. 4.3). The taxonomic classification of *A. awamori* Nagazawa is as given in Table 4.2.

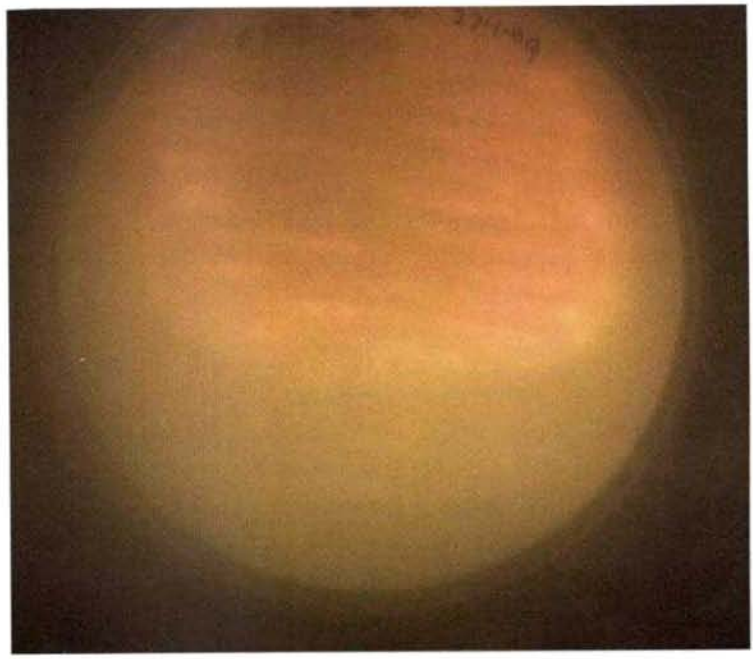


Fig 4.2 *A. awamori* Nagazawa in rhodamine B-olive oil agar plate

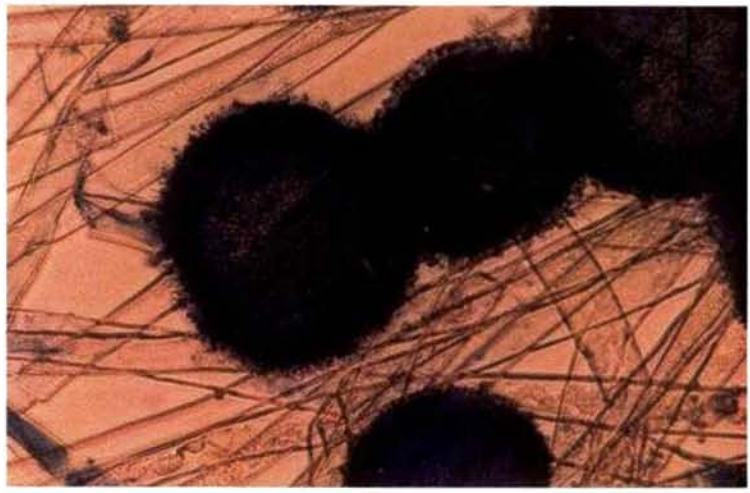


Fig 4.3 Photomicrograph (Nikon Phase Contrast Microscope, Japan) of conidial head (X 40x) of *A. awamori* Nakaz. (Slide culture-lactophenol cotton blue stain)

Table 4.2: *Aspergillus awamori* Nagazawa-Taxonomy
(GBIF Biodiversity Data Portal, Copenhagen, Denmark-http://www.gbif.org/)

Rank	**Name**
Kingdom	Fungi
Phylum	Ascomycota
Class	Ascomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Species	*awamori*

4.1.2.1 Ribotyping using 28S rRNA gene & Phylogenetic tree construction

The identity of the selected fungus was confirmed by carrying out ribotyping using partial sequence of 28S rRNA gene. The sequence of the amplified 28S rRNA gene (Fig.4.4) was compared with GenBank entries, using

BLAST programme and a phylogram was constructed using CLUSTALW N-J programme (Fig. 4.5).

AAAAACACGGATCGCCTACCCCACCGNAGGTTANAGGTAAGAGCCTTANCACTCTATGA
ACCTGCNAGGCTGTCACTTCCGGTNAGTACCGACTATGTGCTTCCGGCGTTGCGGGACCC
GCCGCANAAGTAGTCGCTGGGGAATCAACGAGGCGGGCATGAGGTAGGGGTGTAGAGA
GACACTACCGCGAATCATAGGTGGGCGGAGTCGTCCCAGTCGGCCCTGTGGTAGATAGC
CTTCCTATACGAACGAGTCGGAGTATGTATTGGGAATGCAGCTCTAAATGGGTGGTAAA
TATTCATACTAAAGAGCTAAATATTGGCNNGAGACCGATAGCGNACAAGTAGAGTGATN
CGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGAAGGTTGAAATGGTGGAAAG
GGAAAGNNGCTTTNGAAGGAACCANGAATTCGCTCCNGCGGGGCTNTCACGGGCGGTC
GNTANCGGNTTAGCCCGGAGTNATAATNTTTTCCCCGCGGAGTGGGGCNGTGACTNGAG
GTAGGTACGAGGAAATGGGTACGGGACCGTGTTCGCAGAATGGTCTTCAATANGGAATT
TCGTTATGGTGNCGCCTTNCACGTTGCTAGGCCTTGTAAATATGGACCGGGAGGGCGGTC
GCAAGTTGGCGCGCTTCAATGGGCGCCTGGGGGACGCCTAGCGAAACCCTTCCCCTCTTC
ACGAGCATNGAAAATTTGCTTGGGATTTAAAATTGGTCTTAAAAACCCAAACCGTG

Fig. 4.4. The sequence obtained from ribotyping using partial 28S rRNA gene of *A. awamori* Nagazawa.

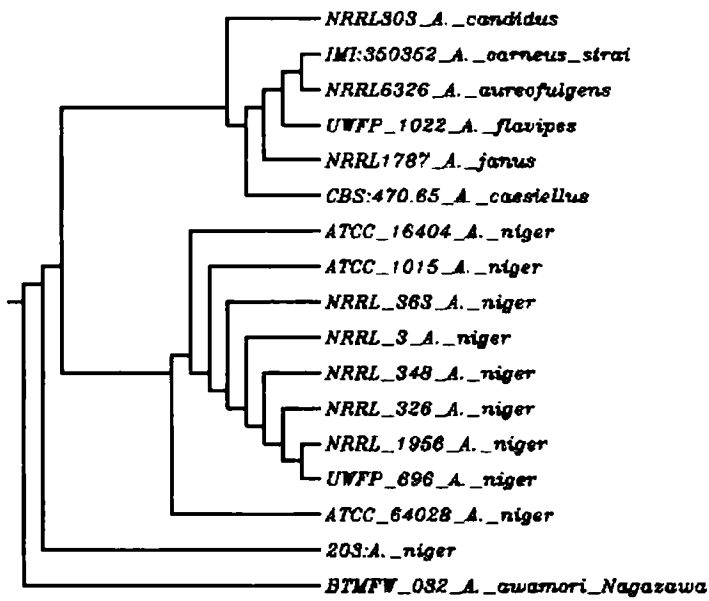


Fig. 4.5 Phylogram of *A. awamori* Nagazawa (BTMFW 032)

From the phylogram, it is inferred that *A. awamori* Nagazawa has close similarity to *A. niger* with 91% similarity compared to other species of *Aspergillus.*

Partial sequence of the 28S rRNA gene (764 bp) was submitted to GenBank (Accession number EF524198) through BankIt programme, at NCBI site.

4.2 SUBMERGED FERMENTATION FOR PRODUCTION OF LIPASE BY ASPERGILLUS AWAMORI NAGAZAWA

4.2.1 Optimization of Bioprocess variables for lipase production by *Aspergillus awamori* Nagazawa

4.2.1.1 Optimization of incubation time

Results documented in Fig 4.6 testify that this fungus produced maximum enzyme (64 U/ml) after 96 h (4 days) of incubation. Lipase production was observed to commence after 12 h of incubation and the activity declined after 96 h of incubation. Hence, 4 days of incubation was considered as optimum during the later optimization studies.

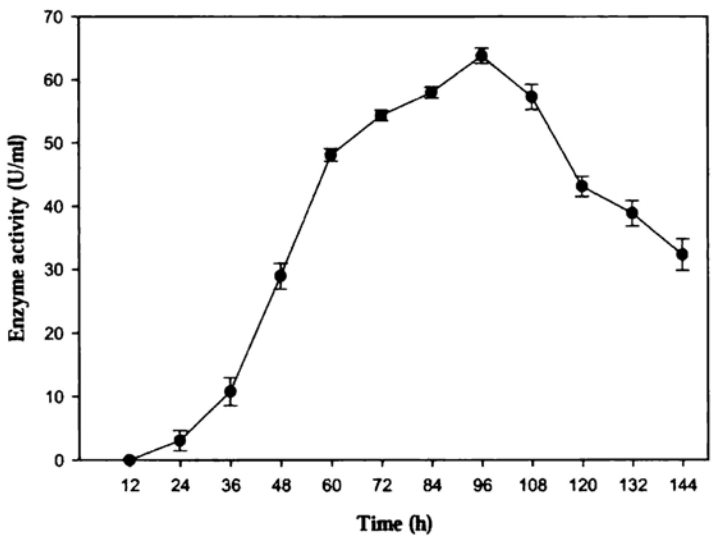


Fig 4.6 Optimization of incubation time for lipase production by *A. awamori* under SmF

4.2.1.2 Selection of substrate

Data obtained for the studies conducted with different oils as substrate suggest that almost all the oils used in the present study supported lipase production (Fig. 4.7). However, among the 12 different oil substrates studied, maximum enzyme production was recorded with rice bran oil (202.16 U/ml) followed by mustard oil (113.5 U/ml) and groundnut oil (107.78 U/ml). Rice bran oil (2 %) was selected as the suitable substrate.

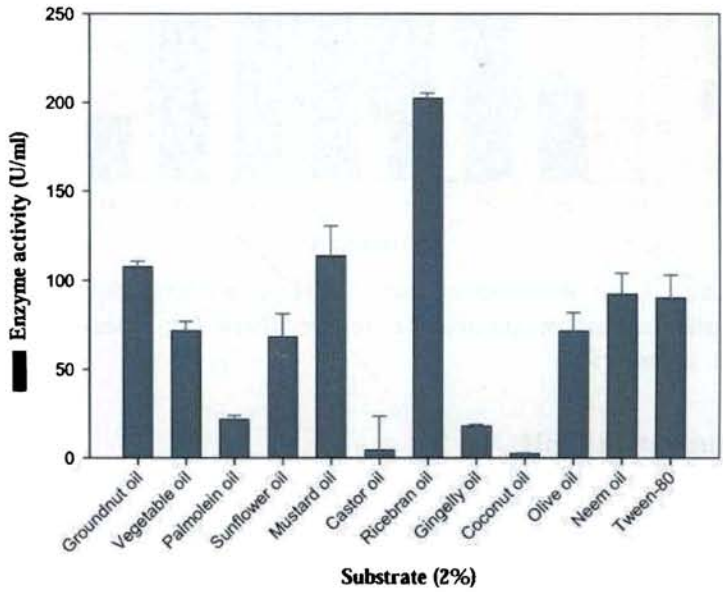


Fig 4.7 Effect of different oil substrates on lipase production by *A. awamori*

4.2.1.3 Optimization of incubation temperature

Results presented in Fig 4.8 clearly evidence that the optimal incubation temperature for maximal enzyme production is 40°C. Nevertheless, there was

considerable level of enzyme production at all the lower temperatures studied. However, incubation at temperatures 45°C and above did not support lipase production.

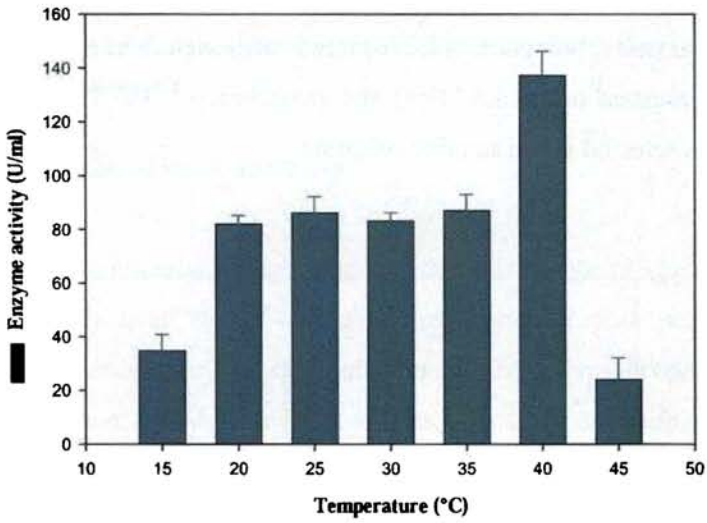


Fig 4.8 Optimization of incubation temperature for lipase production by *A. awamori*

4.2.1.4 Optimization of pH

Studies conducted for optimization of pH indicate that the fungus is capable of producing lipase over a broad range of initial pH of the medium from pH 3.0 to pH 12.0 (Fig. 4.9), although the enzyme activity varied considerably from 16.0 U/ml to 122.9 U/ml. Apparently, it seems that, this fungus has two pH optima, one at pH 3.0 (122.9 U/ml) and another at pH 10.0 (119.0 U/ml) for maximal lipase production. It was also noted that the enzyme activity declined along with increase in pH from 3.0 to 8.0. However, there was a sudden increase in enzyme activity at pH 9.0 followed by a gradual increase at pH 10.0. At high alkaline pH (10.0 to 13.0), there was rapid decrease in enzyme activity. Interestingly, irrespective of the initial pH of the medium that supported maximal

lipase production by the fungus, the pH of the fermented medium after 4 days of incubation was considerably acidic (pH 3.0-5.0).

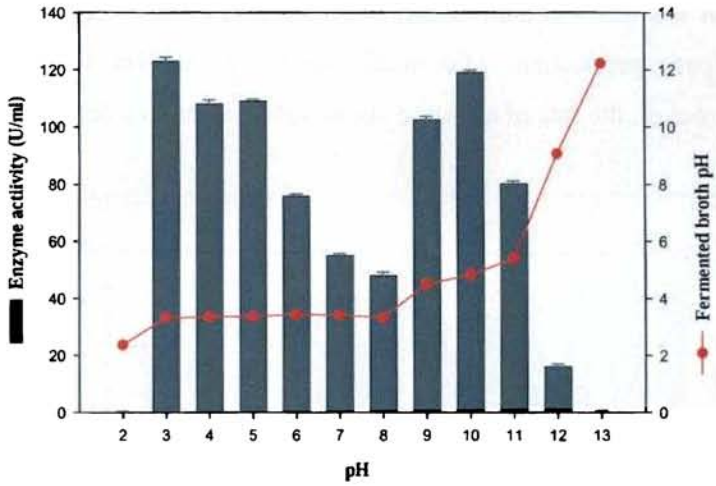


Fig 4.9 Optimization of pH for lipase production by *A. awamori*

4.2.1.5 Effect of agitation

It was inferred from the results presented in Fig 4.10, that agitation is required for this fungus for lipase production since there was no enzyme production at stationary condition, and agitation at 100 rpm and 150 rpm led to enhanced lipase production. Maximum enzyme production was recorded at 150 rpm. However, the rate of agitation above 150 rpm led to a decrease in enzyme production.

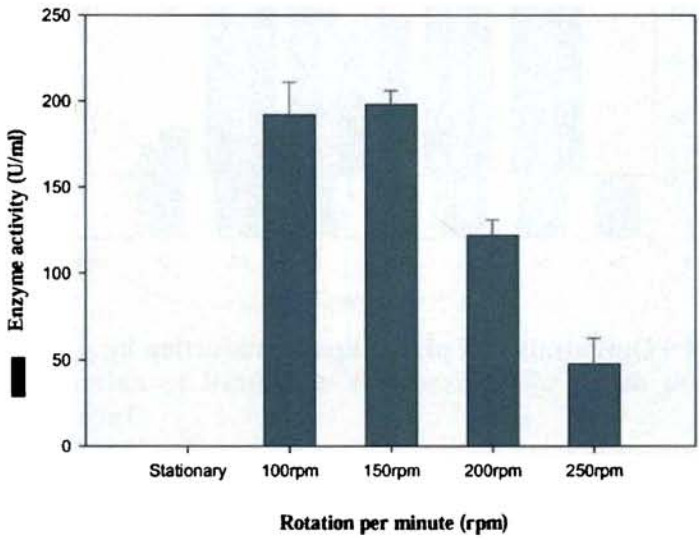


Fig 4.10 Effect of agitation on lipase production by *A. awamori*

4.2.1.6 Inoculum Concentration

Studies conducted for the optimization of inoculum concentration indicated that variation in the level of inoculum conc. from 1 % to 15 % did not influence the rate of enzyme production (Fig 4.11). The enzyme activity varied from 103.8 U/ml (1%) to 128.5 U/ml (15%).

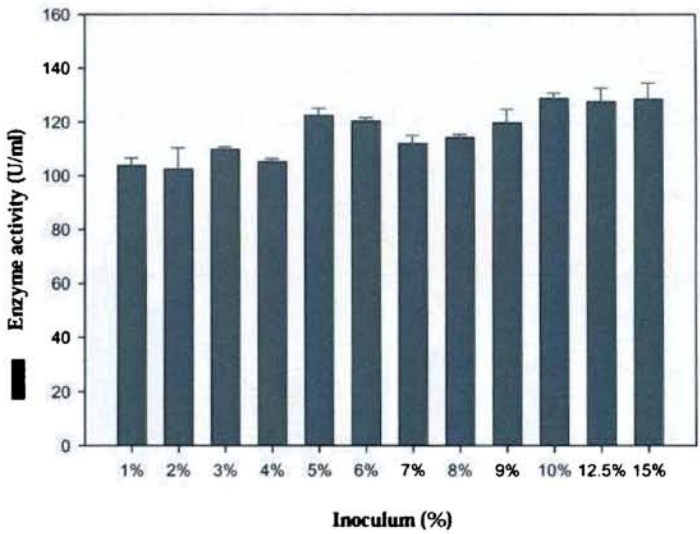


Fig. 4.11 Effect of inoculum on lipase production by *A. awamori*

4.2.1.7 Effect of additional carbon source

Data obtained for the studies conducted on the effect of additional carbon source evidence that none of the sugars supplied as additional carbon source enhanced lipase production compared to control (ME medium)(Fig. 4.12). Maltose, lactose and sucrose inhibited lipase production. Whereas, all the other sugars tested led to a reduced level of enzyme production when compared with that of the control.

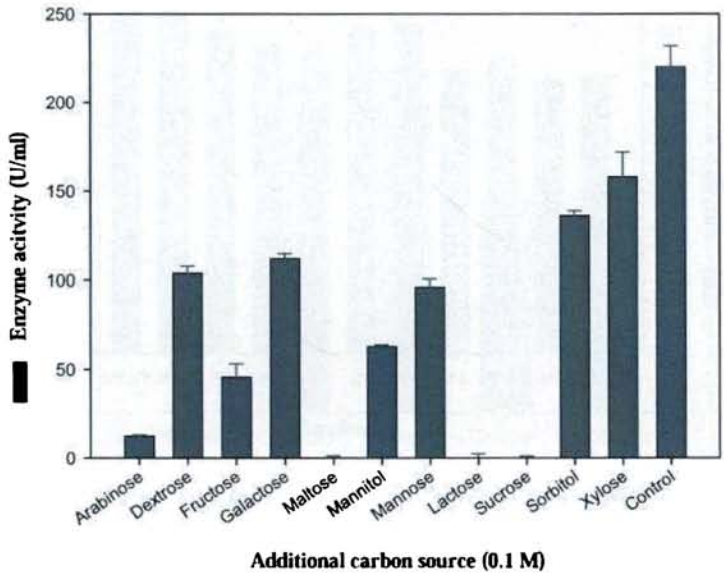


Fig. 4.12 Effect of sugars as additional carbon source for lipase production by *A. awamori*

4.2.1.8 Effect of Nitrogen sources

4.2.1.8.1 Additional amino acids

Results presented in Fig. 4.13 indicate that the amino acids tested as additional nitrogen source influenced lipase production. Among the 19 different amino acids tested, methionine (220.0 U/ml) followed by lysine (198.7 U/ml) and proline (182.3 U/ml) supported enhanced enzyme production compared to the control medium (162.0 U/ml). Methionine could effect about 37 % more enzyme production compared to control. On the other hand, cysteine, histidine, leucine, isoleucine, tryptophan and arginine caused a considerable reduction in enzyme production.

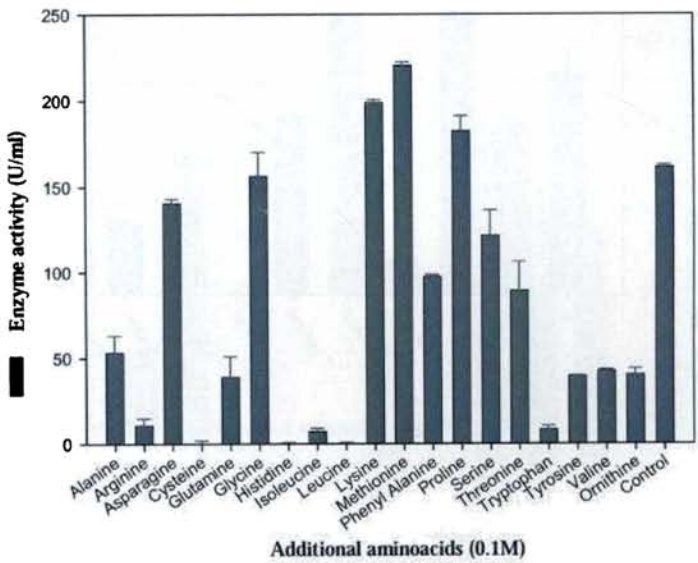


Fig. 4.13 Effect of additional aminoacids on lipase production by *A. awamori*

4.2.1.8.2 Organic nitrogen sources

Among the various organic nitrogen sources tested, except urea, all the other nitrogen sources supported enhanced lipase production (Fig.4.14). The control medium, which was devoid of any nitrogen source, did not support production of lipase. Beef extract (219.3 U/ml) and soyabean meal (221.0 U/ml) supported maximal level of enzyme production followed by peptone, tryptone, yeast extract, casein, gelatin and malt extract.

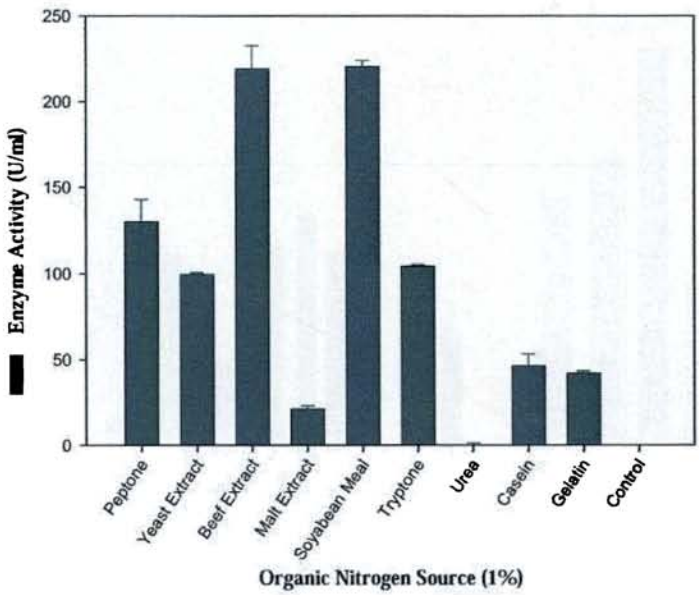


Fig 4.14 Effect of organic nitrogen source on lipase production by *A. awamori*

4.2.1.8.3 Inorganic Nitrogen sources

It is evident from the results presented in Fig. 4.15 that among the inorganic nitrogen sources tested, ammonium nitrate, ammonium chloride, ammonium dihydrogen orthophosphate, ammonium sulphate, sodium nitrate and potassium nitrate had a positive effect on enzyme production. The control medium, which was devoid of any nitrogen source, did not support production of lipase. Presence of ammonium hydrogen carbonate, ammonium acetate, ammonium oxalate, and ammonium iron sulphate in the medium inhibited enzyme production. Maximum enzyme production was supported by ammonium sulphate (224.3 U/ml) and ammonium chloride (213.9 U/ml) followed by sodium nitrate, potassium nitrate, ammonium dihydrogen orthophosphate and ammonium nitrate.

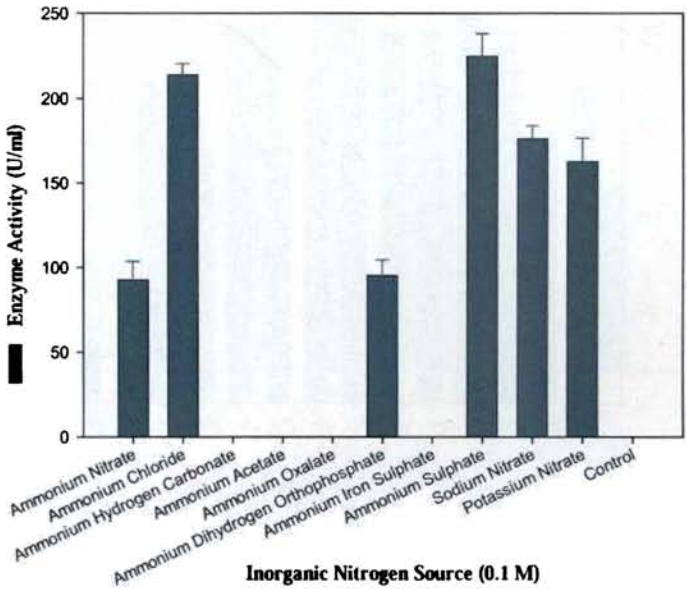


Fig. 4.15 Effect of inorganic nitrogen source on lipase production by *A. awamori*

4.2.1.9 Sodium chloride concentration

From the studies conducted on the impact of sodium chloride on lipase production it is evident that the presence of sodium chloride in the medium significantly affects lipase production (Fig. 4.16). In fact the fungus could produce lipase in a medium prepared without sodium chloride in distilled water (168.6 U/ml). Addition of sodium chloride at concentrations 1-3.5 % (w/v) enhanced lipase production, recording a maximum activity with 1.5 % (206.6 U/ml). Sodium chloride concentrations above 3.5 % led to a gradual decline in lipase production compared to control (without NaCl). Results also suggest that the fungus is halotolerant in nature since it is capable of tolerating upto 12.5 % NaCl for production of lipase, although at reduced level.

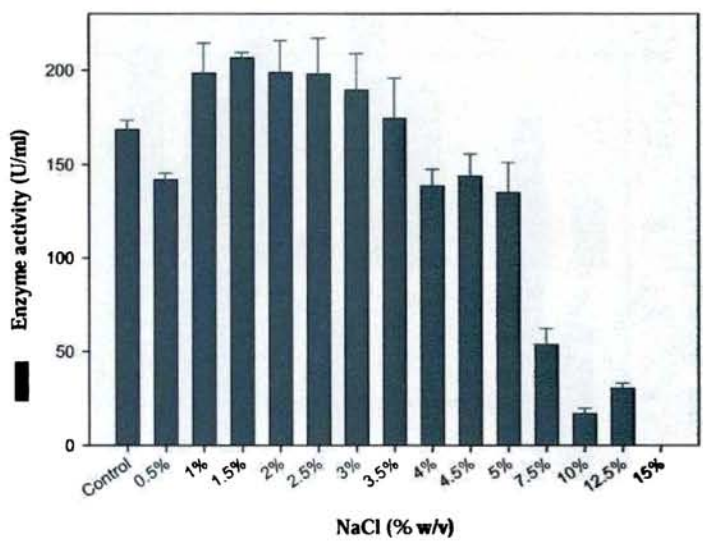


Fig. 4.16 Effect of sodium chloride on lipase production by *A. awamori*

4.2.1.10 Seawater concentration

An attempt was made to study the impact of seawater concentration on lipase production by preparing the medium with seawater and distilled water at various ratio of combinations. Results presented in Fig. 4.17 suggest that although this fungus can produce lipase in the presence of seawater, addition of seawater more than 25 % led to decline in enzyme production. Only at a combination of 25 % seawater plus 75 % distilled water there was 29 % enhanced lipase production (217.5 U/ml) compared to control medium without seawater and NaCl. Further, the level of enhancement in enzyme production with 25 % seawater was only 9 % compared to that of the control medium containing 1 % NaCl.

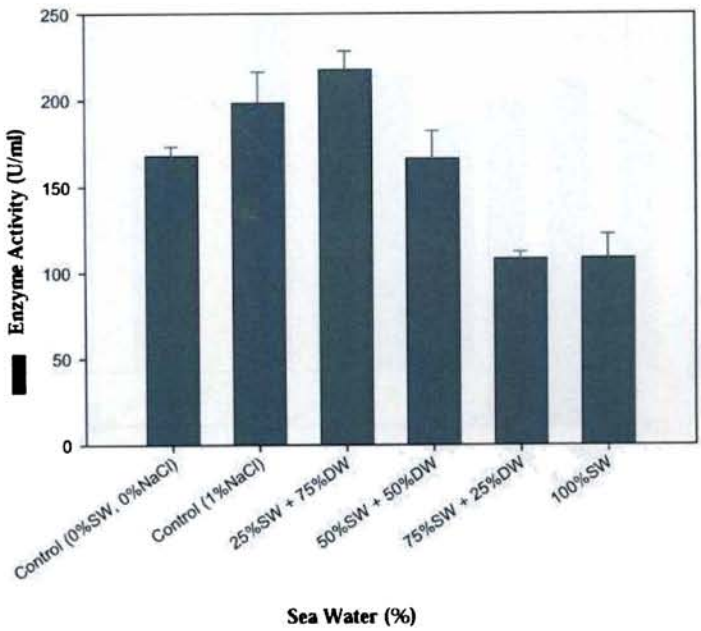


Fig. 4.17 Effect of seawater on lipase production by *A. awamori*

4.2.1.11 Inorganic salts

Data obtained for the studies conducted to evaluate the impact of inorganic salts in the medium on lipase production (Fig. 4.18) very clearly demonstrate the influence of inorganic salts. Among the various inorganic salts tested only calcium chloride (80.16 U/ml) and magnesium sulphate (75.2 U/ml) supported considerable enhancement in lipase production. The control medium, which was devoid of any inorganic salts, supported lipase production (5.25 U/ml). Whereas, all the other inorganic salts including zinc sulphate, potassium chloride, copper sulphate, ferrous sulphate, manganese chloride, and cobalt chloride inhibited lipase production.

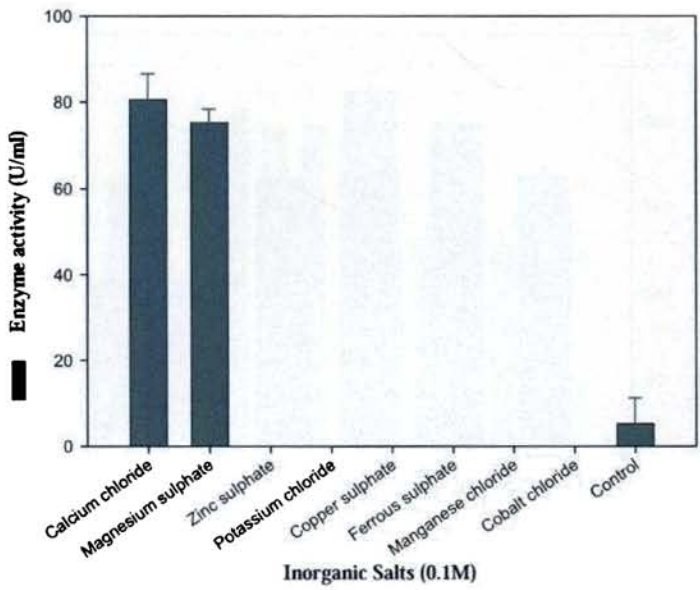


Fig. 4.18 Effect of inorganic salts on lipase production by *A. awamori*

4.2.1.12 Effect of surfactants

Among the different surfactants studied for their impact on lipase production by fungi, only PEG 6000 (133.8 U/ml) and Tween 20 (110.3 U/ml) supported enhanced lipase production in the fermented medium compared to control medium (Fig. 4.19). There was about 38 % enhancement in lipase production with PEG 6000 compared to control. Sodium dodecyl sulphate (SDS) and sodium deoxycholic acid inhibited lipase production. All the other surfactants had a marginal impact such that the lipase activity decreased compared to control medium.

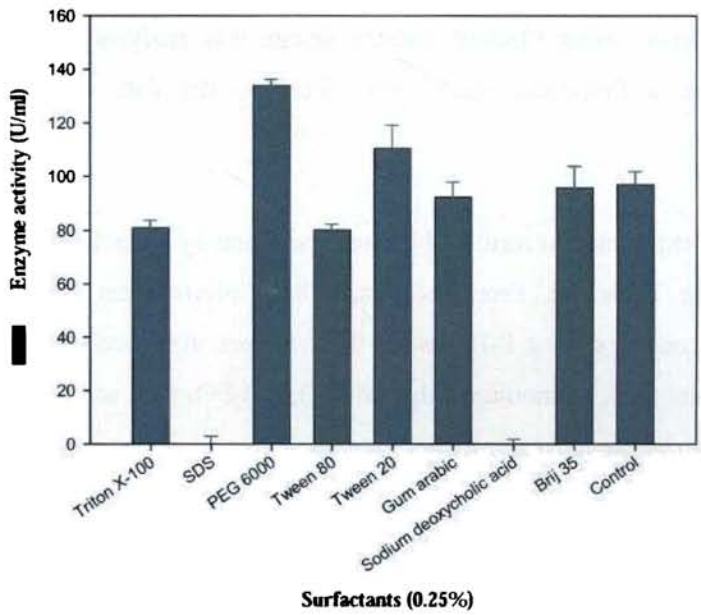


Fig. 4.19 Effect of surfactants on Lipase production by *A. awamori*

4.2.2 Optimization of bioprocess variables for lipase production by Statistical approach

Medium that could support maximal lipase production by *A. awamori* was optimized employing statistical approach. Initially process variables were optimized using Plackett-Burman design and in the second stage Response Surface Methodology was adopted towards selection of optimal variables and understanding the interrelationship among significant variables.

4.2.2.1 Plackett-Burman Design (P-B design)

Data obtained for the studies conducted on optimization of medium for lipase production using Plackett-Burman design was analyzed by Design expert software and a first-order model was fitted to the data obtained from the experiment.

The experimental results of lipase production by a Plackett–Burman design are shown in Table 4.3. From the results it is inferred that among the eleven variables screened during P-B design, five factors viz. incubation period (X_1), soyabean meal (X_3), ammonium sulphate (X_4), KH_2PO_4 (X_8) and temperature (X_{11}) were found to be the most significant variables.

First-order model equation:

Enzyme activity Y (U/ml) = $40.69 - 11.28\ X_1 + 16.61\ X_3 + 4.69\ X_4 - 25.0\ X_8 - 9.75\ X_{11}$

The statistical significance of the model equation was evaluated by the *F*-test analysis of variance (ANOVA), which revealed that this regression is statistically significant (Table 4.4). The Model F-value of 71.55 implied that the model is significant. Values of "Prob>F" less than 0.05 indicate that the model terms are significant. This fit of the model was checked with the coefficient of determination R^2, which was calculated to be 0.9835. In fact 99.9 % of the

variability in the response could be explained by the model ($P < 0.1$) at 90% of confidence level.

The effect of individual parameters studied in P-B design is presented as Pareto chart in Fig. 4.20. The data evidence that while soyabean meal and ammonium sulphate had a positive effect in enhancing enzyme production along with increase in their concentration, KH_2PO_4 followed by incubation period and temperature had a negative effect on enzyme production along with increase in the variable.

Table 4.3: The matrix of the Plackett-Burman design experiment, together with the observed experimental data for lipase production by *A. awamori*

Run	Incubation period (Days) (X_1)	pH (X_2)	Soyabean meal (%) (X_3)	Ammonium Sulphate (M) (X_4)	NaCl (%) (X_5)	Ricebran oil (%) (X_6)	$CaCl_2$ (M) (X_7)	KH_2PO_4 (M) (X_8)	PEG 6000 (%) (X_9)	Inoculum (%) (X_{10})	Temperature (°C) (X_{11})	Lipase activity (U/ml) (Y)
1	3	5	0.5	0.1	2	2	0.1	0.1	0.05	1	40	0
2	5	5	0.5	0.1	1	4	0.05	0.05	0.05	4	40	39.52
3	3	5	1	0.1	1	4	0.1	0.05	0.1	1	30	103.6
4	5	5	1	0.05	1	2	0.05	0.1	0.1	1	40	0
5	3	3	0.5	0.05	1	2	0.05	0.05	0.05	1	30	66.45
6	3	3	1	0.1	2	2	0.05	0.05	0.1	4	40	88.4
7	5	3	1	0.05	2	4	0.1	0.05	0.05	1	40	57.7
8	5	5	0.5	0.05	2	2	0.1	0.05	0.1	4	30	38.466
9	3	3	0.5	0.05	1	4	0.1	0.1	0.1	4	40	0
10	5	3	0.5	0.1	2	4	0.05	0.1	0.1	1	30	0
11	3	5	1	0.05	2	4	0.05	0.1	0.05	4	30	53.37
12	5	3	1	0.1	1	2	0.1	0.1	0.05	4	30	40.75

Table 4.4: ANOVA for the experiments with Plackett-Burman design for lipase production by *A. awamori*

Term	Lipase yield
F-value	71.55
Prob > F	<0.0001
Mean	40.69
R-squared	0.9835
Adjusted R-squared	0.9698
Coefficient of variance	15.24
Adequate precision	25.578

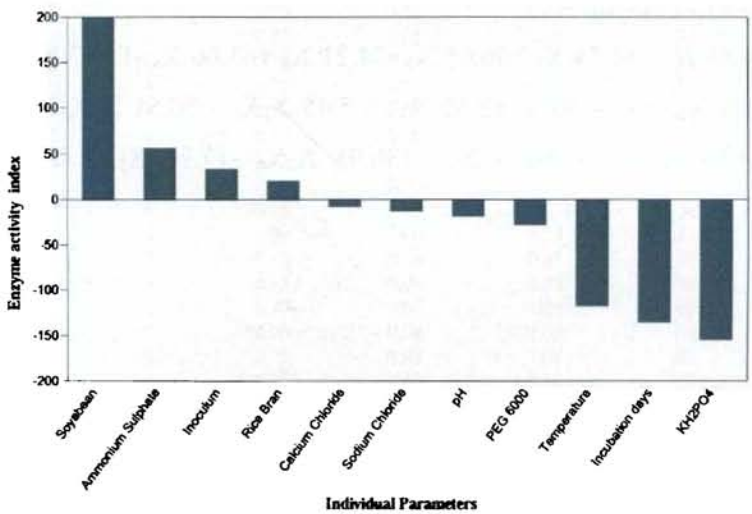


Fig. 4.20 Pareto Chart showing effect of individual factors on lipase production by *A. awamori*

4.2.2.2 Response surface methodology

Response surface methodology (using Box-Behnken design experiment) was adopted towards selection of optimal level of the significant variable (incubation period (X_1), soyabean meal (X_2), ammonium sulphate (X_3), KH_2PO_4 (X_4) and temperature (X_5) identified based on the P-B design experiment. The design matrix (Box-Behnken design) and the corresponding experimental data obtained are shown in Table 4.5.

The results obtained for the Box-Behnken design experiment were analyzed by ANOVA, which yielded the following regression equation for the level of lipase production (Y):

Lipase activity, Y (U/ml) =

$$316.88 + 54.85 X_1 + 34.74 X_2 + 36.05 X_3 - 74.21 X_4 + 63.66 X_5 - 15.67 X_1^2 - 5.03 X_2^2 - 2.05 X_3^2 + 9.94 X_4^2 - 80.65 X_5^2 + 7.45 X_1X_2 - 50.51 X_1X_3 - 26.02 X_1X_4 + 14.09 X_1X_5 - 5.80 X_2X_3 + 30.93 X_2X_4 - 17.70 X_2X_5 - 31.22 X_3 X_4 - 16.93 X_3X_5 + 8.84 X_4X_5.$$

Table 4.5: Effect of individual variable on lipase production by *A. awamori* studied using Box-Behnken design experiment

BLOCK	RUN	Incubation period X_1 (Days)	Soyabean meal X_2 (%w/v)	Ammonium Sulphate X_3 (M)	KH_2PO_4 X_4 (M)	Temperature X_5 (°C)	*Lipase activity Y (U/ml)
1	1	5	0.50	0.08	0.08	35	324.75
1	2	4	1.00	0.08	0.08	30	193.90
1	3	4	0.75	0.05	0.05	35	253.96
1	4	3	0.50	0.08	0.08	35	130.00
1	5	5	0.75	0.10	0.08	35	272.95
1	6	4	1.00	0.08	0.08	40	404.20
1	7	4	0.75	0.08	0.08	35	169.34
1	8	3	0.75	0.05	0.08	35	229.50
1	9	4	0.50	0.08	0.08	40	338.00
1	10	4	0.75	0.08	0.10	40	180.18
1	11	5	0.75	0.05	0.08	35	278.70
1	12	3	1.00	0.08	0.08	35	237.50
1	13	4	0.75	0.08	0.08	35	423.70
1	14	4	0.75	0.08	0.05	40	403.20
1	15	4	0.50	0.08	0.08	30	56.90
1	16	5	1.00	0.08	0.08	35	462.03
1	17	3	0.75	0.10	0.08	35	425.78
1	18	4	0.75	0.05	0.10	35	283.14
1	19	4	0.75	0.08	0.05	30	322.39
1	20	4	0.75	0.08	0.08	35	269.73
1	21	4	0.75	0.08	0.10	30	64.02
1	22	4	0.75	0.10	0.10	35	298.69
1	23	4	0.75	0.10	0.05	35	394.40
2	1	4	0.50	0.05	0.08	35	287.00
2	2	5	0.75	0.08	0.05	35	478.20
2	3	4	1.00	0.08	0.10	35	271.12
2	4	3	0.75	0.08	0.08	40	56.73
2	5	3	0.75	0.08	0.05	35	330.75
2	6	4	0.75	0.05	0.08	40	338.40
2	7	5	0.75	0.08	0.08	30	302.87
2	8	5	0.75	0.08	0.08	40	270.48
2	9	5	0.75	0.08	0.10	35	302.87
2	10	4	0.50	0.08	0.05	35	412.80
2	11	3	0.75	0.08	0.10	35	259.50
2	12	4	0.75	0.08	0.08	35	266.40
2	13	4	0.75	0.08	0.08	35	394.20
2	14	4	0.75	0.05	0.08	30	78.85
2	15	4	1.00	0.05	0.08	35	297.80
2	16	4	0.50	0.08	0.10	35	154.50
2	17	3	0.75	0.08	0.08	30	145.50
2	18	4	0.75	0.10	0.08	30	190.47
2	19	4	0.50	0.10	0.08	35	336.00
2	20	4	0.75	0.08	0.08	35	377.90
2	21	4	0.75	0.10	0.08	40	382.30
2	22	4	1.00	0.10	0.08	35	323.60
2	23	4	1.00	0.08	0.05	35	405.70

* Lipase production is considered as the Response.

The ANOVA analysis of lipase production (Table 4.6) showed that Prob > F value was less than 0.05, which indicate that the model is significant. Three linear coefficient, incubation time (X_1), KH_2PO_4 (X_4) and temperature (X_5); and one quadratic term (X_5^2) were significant model terms for the response.

Table 4.6 ANOVA for the response surface experiments conducted using Box-Behnken design for lipase production by *A. awamori*

Term	Lipase yield
F-value	2.07
Prob > F	0.0457
Mean	284.37
R-squared	0.6325
Adjusted R-squared	0.3262
Coefficient of variance	31.65
Adequate precision	6.065

The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination (R^2) of 0.6325. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 99 % of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different numbers of independent variables, was 0.3262. All the selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on lipase production. The coefficient of variance was 31.65. The adequate precision that measures the "signal to noise ratio" was 6.065. A ratio greater than 4 is desirable as it indicates an adequate signal. Thus, this model could be used to navigate the design space.

4.2.2.2.1 Analysis of factors influencing lipase production

Three-dimensional response surface curves were plotted to study the interaction among various physicochemical factors, and to determine the optimum concentration of each individual factor for maximum lipase production. The model predicted maximum lipase production up to 482.85 U/ml that could be achieved using 0.8 % w/v soyabean, 0.1 M ammonium sulphate and 0.05 M KH_2PO_4 at 35°C for 5 days incubation. An overall 2.8-fold increase in lipase production was achieved after validation of RSM in shake flasks.

4.2.2.2.2 Interactions between factors

The pairwise interaction among the factors in terms of lipase production in the optimized set were assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two.

4.2.2.2.2.1 Interaction between incubation time and concentration of soyabean meal

When the concentrations of ammonium sulphate and KH_2PO_4, and the incubation temperature were held at their optimum levels (0.09 M, 0.05 M and 38°C, respectively), the lipase production increased along with increase in incubation time (Fig. 4.21). Whereas, enzyme production remained unchanged in response to increase or decrease in the concentration of soyabean meal suggesting a neutral interaction between soyabean meal and incubation time.

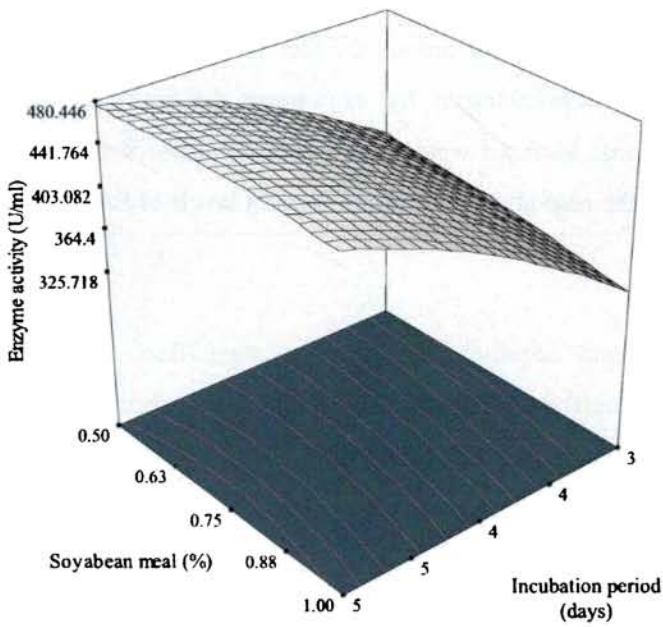


Fig. 4.21 Effect of incubation days and conc. of soyabean meal on lipase production by *A. awamori*.

4.2.2.2.2.2 Interaction between incubation days and ammonium sulphate concentration

There was an increase in lipase production along with an increase in incubation period and increase in the concentration of ammonium sulphate (Fig. 4.22). Lower concentrations of ammonium sulphate significantly reduced enzyme production when the concentrations of soyabean meal and KH_2PO_4, and the incubation temperature were held at their optimum levels (0.8 %, 0.05 M and 38°C, respectively).

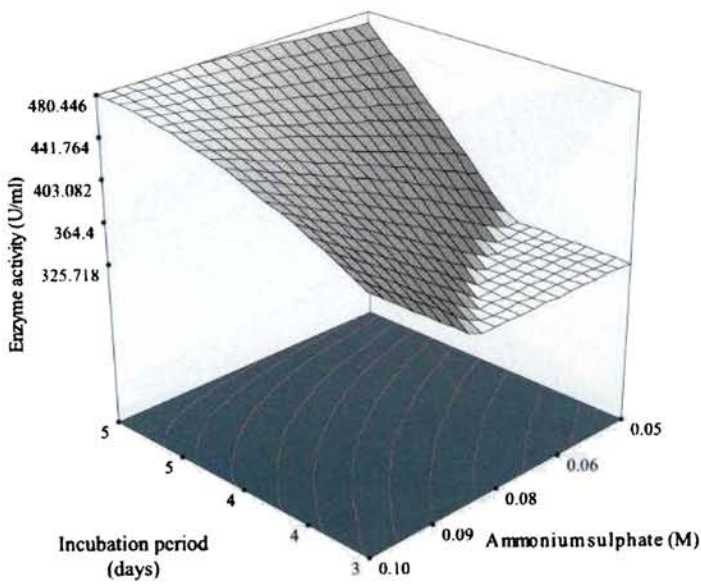


Fig. 4.22 Effect of incubation period and conc. of ammonium sulphate on lipase production by *A. awamori.*

4.2.2.2.2.3 Interaction between incubation days and KH_2PO_4 concentration

From the data presented in Fig. 4.23 it is evident that at lower concentrations of KH_2PO_4 and extended period of incubation there was enhanced lipase production, while the concentrations of soyabean meal, ammonium sulphate, and the incubation temperature were held at their optimum levels (0.8 %, 0.09 M and 38°C, respectively). There was a steep decrease in activity along with increase in the concentration of KH_2PO_4.

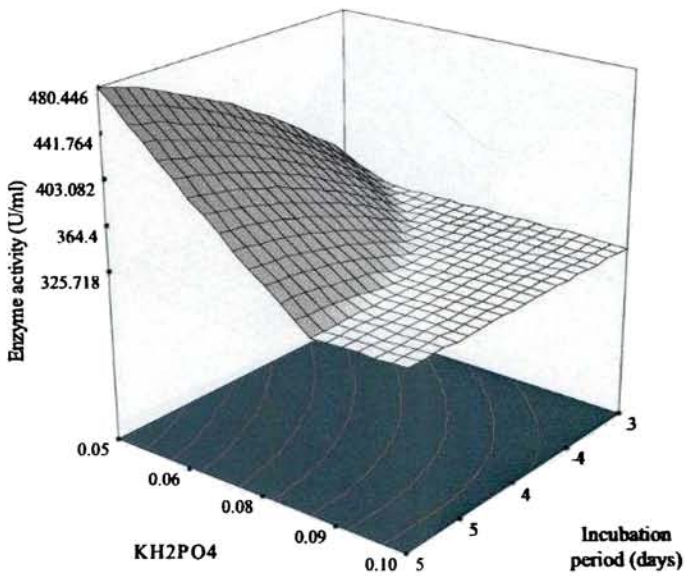


Fig. 4.23 Effect of incubation period and conc. of KH_2PO_4 on lipase production by *A. awamori*

4.2.2.2.2.4 Interaction between incubation period and temperature

The lipase production showed a parabolic trend in response to variation in incubation temperature from 35 to 38°C, recording a maximal activity at 37°C and 5 days of incubation, under the conditions when the concentrations of soyabean meal, ammonium sulphate and KH_2PO_4 were at their optimum levels (Fig. 4.24). The enzyme production decreased at other incubation temperatures, both at higher and lower levels other than 37°C.

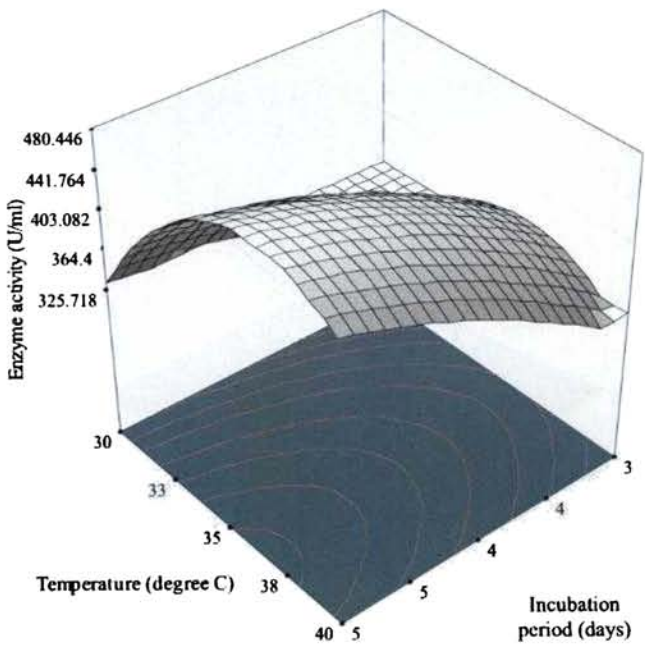


Fig. 4.24 Effect of temperature and incubation period on lipase production by *A. awamori*

4.2.2.2.2.5 Interaction between concentrations of soyabean meal and ammonium sulphate

From the results presented in Fig. 4.25, it is clearly evident that there was no prominent interaction between concentrations of soyabean meal and ammonium sulphate during lipase production when the incubation period was 5 days. The concentration of KH_2PO_4 and incubation temperature was at their optimum level.

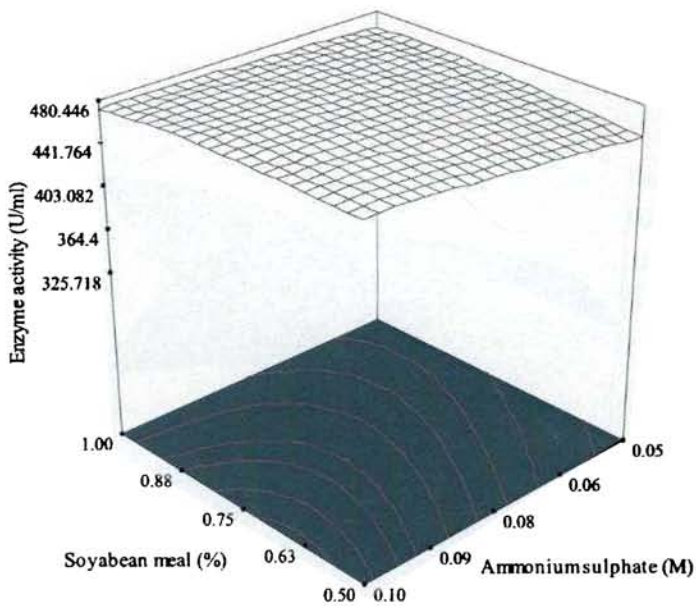


Fig. 4.25 Effect of concentrations of soyabean meal and ammonium sulphate on lipase production by *A. awamori*

4.2.2.2.2.6 Interaction between concentrations of soyabean meal and KH_2PO_4

There was no significant interaction between conc. of soyabean meal and KH_2PO_4 during lipase production, since enzyme yields were higher at lower concentrations of KH_2PO_4, irrespective of soyabean meal concentration (Fig. 4.26). The maximal enzyme yields were obtained at the lower KH_2PO_4 concentrations of 0.05-0.06 M. The concentration of ammonium sulphate, incubation days and incubation temperature were at their optimum levels, 0.09 M, 5 days and 38°C respectively.

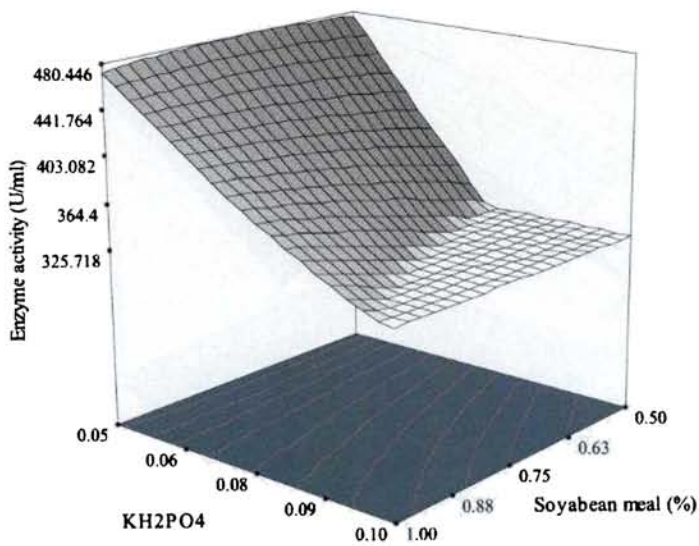


Fig. 4.26 Effect of concentrations of soyabean meal and KH_2PO_4 on lipase production by *A. awamori*

4.2.2.2.2.7 Interaction between concentration of soyabean meal and incubation temperature

There was a parabolic change in the lipase production pattern with respect to incubation temperature and maximum enzyme yield was recorded over 36-38°C. Soyabean meal concentration showed maximum response over the range of 0.75-0.88 % concentration (Fig. 4.27). The concentrations of ammonium sulphate and KH_2PO_4; and incubation days were at their optimum levels, 0.09 M, 0.05 M and 5 days respectively.

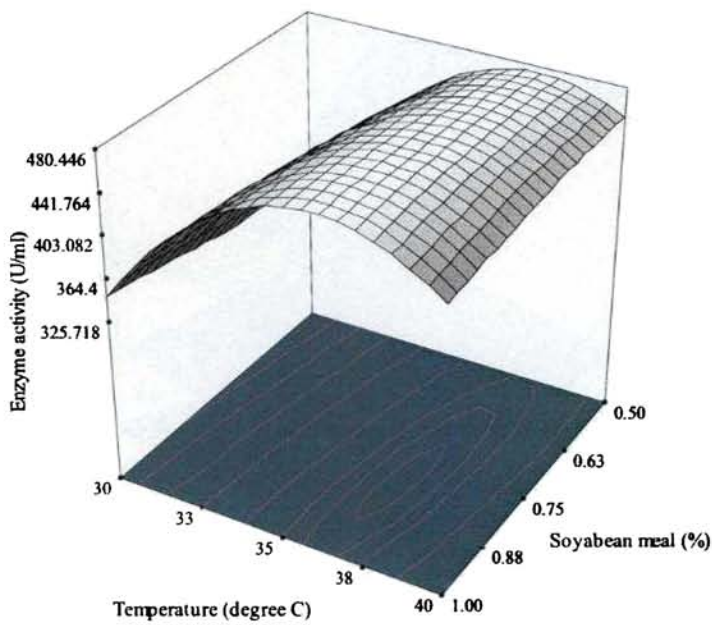


Fig. 4.27 Effect of concentration of soyabean meal and incubation temperature on lipase production by *A. awamori*

4.2.2.2.2.8 Interaction between concentrations of KH_2PO_4 and ammonium sulphate

Enzyme yields were higher at lower KH_2PO_4 concentrations irrespective of ammonium sulphate concentration (Fig. 4.28) indicating a neutral interaction. The maximal enzyme yield was obtained at 0.05 M concentration of KH_2PO_4. The concentration of soyabean meal, incubation days and incubation temperature were at their optimum levels, 0.8 %, 5 days and 38°C respectively.

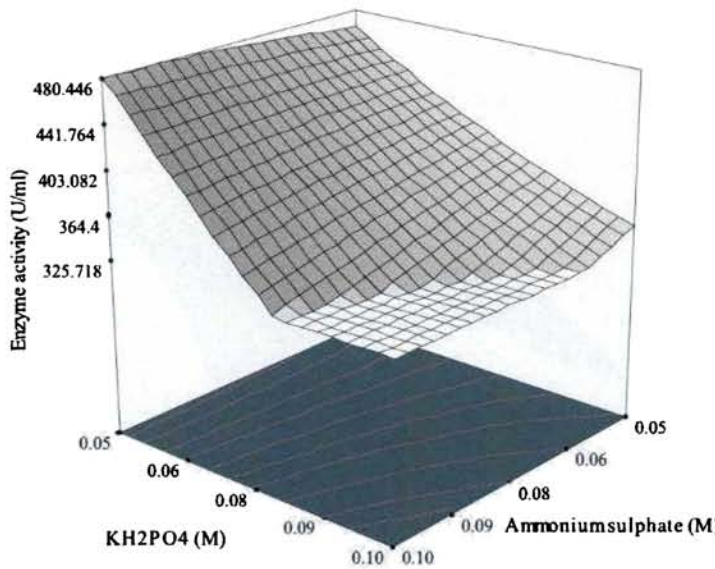


Fig. 4.28 Effect of concentrations of KH_2PO_4 and ammonium sulphate on lipase production by *A. awamori*

4.2.2.2.2.9 Interaction between incubation temperature and ammonium sulphate concentration

The maximal yields were obtained at temperature range between 36-38°C and in the concentration range between 0.09-0.10 M ammonium sulphate. There was a positive interaction between incubation temperature and the conc. of ammonium sulphate as is evidenced by the data presented in the Fig. 4.29. The enzyme yield was reduced at temperature below 35°C and above 38°C. The concentrations of soyabean meal and KH_2PO_4, and incubation days were at their optimum levels, 0.8 %, 0.05 M and 5 days respectively.

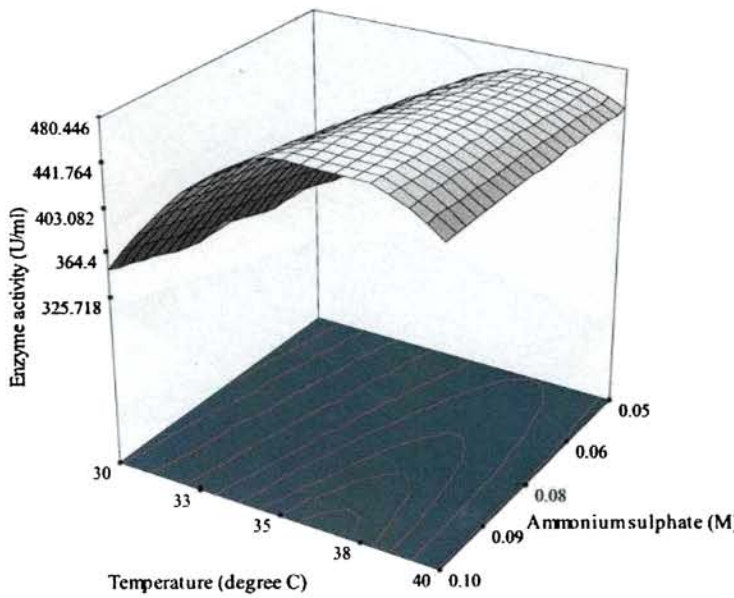


Fig. 4.29 Effect of incubation temperature and ammonium sulphate concentration on lipase production by *A. awamori*

4.2.2.2.2.10 Interaction between incubation temperature and KH_2PO_4 concentration on lipase production by *A. awamori*

Results presented in Fig. 4.30 show a very clear interaction between incubation temperature and KH_2PO_4 conc. in their cumulative effect on lipase production. Maximum enzyme yield was obtained in the temperature range 35-39°C and 0.05-0.06 M KH_2PO_4 concentration. It is also evident from the data that concentrations higher than 0.06 M of KH_2PO_4 led to reduction in the enzyme yield. The concentrations of soyabean meal and ammonium sulphate, and incubation days were at their optimum levels, 0.8 %, 0.05 M and 5 days respectively.

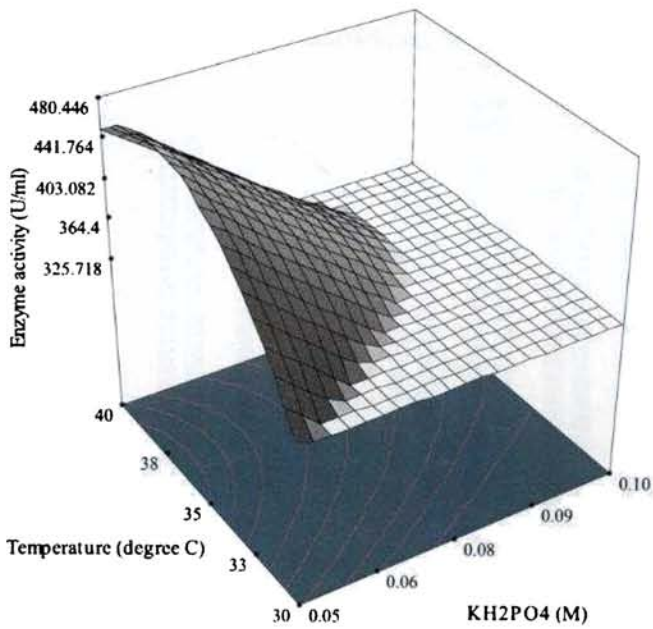


Fig. 4.30 Effect of incubation temperature and KH_2PO_4 concentration on lipase production by *A. awamori*

4.2.2.3 Validation of the response surface model

Validation of the deduced response surface model based on the previous experiments was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated (Table 4.7 & Fig. 4.31). The temperature range that supported maximal enzyme yield was 35-38°C, and the KH_2PO_4 conc. was 0.05 M.

The optimized conditions for lipase production were as follows:

Soyabean meal-0.77 % (w/v); ammonium sulphate-0.1 M; KH_2PO_4-0.05 M; Rice bran oil-2 % (v/v); $CaCl_2$-0.05 M; PEG 6000-0.05 % (w/v); NaCl-1 % (w/v); inoculum-1 % (v/v); pH 3; incubation temperature-35°C and incubation period-5 days.

Table 4.7: Predicted and experimental values of lipase production obtained for validation of the RSM model

S.No.	Incubation (days)	Soyabean meal (%)	Ammonium sulphate (M)	KH_2PO_4 (M)	Temperature (°C)	Enzyme activity (U/ml)	
						Predicted	Experimental
1	5	0.77	0.10	0.05	35	482.857	484
2	5	1.00	0.07	0.05	37	478.212	468
3	5	0.93	0.08	0.05	37	479.931	472
4	5	0.82	0.07	0.05	37	480.123	472
5	4	0.56	0.10	0.05	37	479.017	468
6	5	0.95	0.05	0.05	37	475.098	478
7	5	0.55	0.07	0.05	37	474.701	477

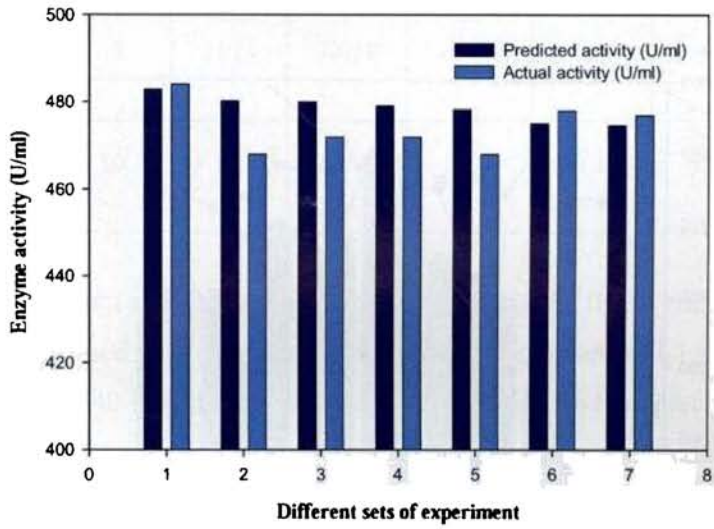


Fig. 4.31 Predicted and experimental values of lipase production by *A. awamori* obtained for validation of the RSM model

4.2.3 Time course study under optimal conditions

Data obtained from the time course experiment conducted over a period of 192 h (8 days) under optimised condition (Fig. 4.32) testify that enzyme production commenced after 36 h of incubation and reached a peak after 96 h (495.0 U/ml). While maximal specific activity was recorded at 108 h (1164.63 U/mg protein). Further incubation beyond 108 h did not favor enhanced enzyme activity and instead resulted in a decline. Maximum biomass (29.5 mg/ml) was attained within 48 h of incubation and was maintained almost stable during the entire study period.

Protease assay was conducted during the entire period of study and no protease was found even after 192 h of incubation. It is inferred that the lipase produced by *A. awamori* is a 'protease free lipase'.

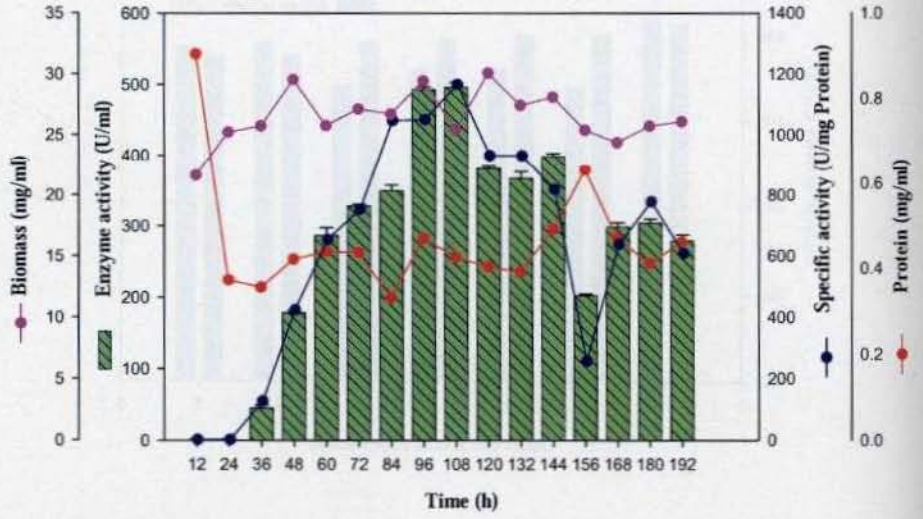


Fig. 4.32 Time course study under optimized condition for lipase production by *A. awamori*

4.3 ENZYME PURIFICATION

Lipase was purified employing standard protein purification procedures, which included ammonium sulphate fractionation followed by dialysis and ion exchange chromatography. Results obtained for purification of crude enzyme is summarised in Table 4.8.

Table 4.8: Yield and fold of purification of lipase produced by *A. awamori*

Sample	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude extract	1300	643.5	79612	123.7	100	100	1
Ammonium sulphate fractionation 40-90%	15	21.24	32712	1540.1	3.3	41	12.5
Ion Exchange chromatography (DEAE)	80	7.2	26880	3733	1.1	33.7	30.2

Ammonium sulphate required for precipitating the lipase enzyme from the supernatant obtained after growth of *A. awamori* was standardized. Lipase could be precipitated with 40-90% ammonium sulphate saturation and the precipitate, which showed a 12.5 fold increase in specific activity, compared to the crude sample, was used for further purification employing Ion exchange chromatography (Table 4.8). A 30.2 fold purified enzyme was obtained after ion exchange chromatography.

4.3.1 Standardization of binding pH of lipase to DEAE-Cellulose

The binding affinity which slowly increased from pH 3.5, showed a maximum at pH 7.5. The enzyme eluted from the DEAE cellulose and equilibrated to pH 7.5 showed maximal activity. Hence, the binding pH to DEAE cellulose was maintained as pH 7.5 (Fig. 4.32).

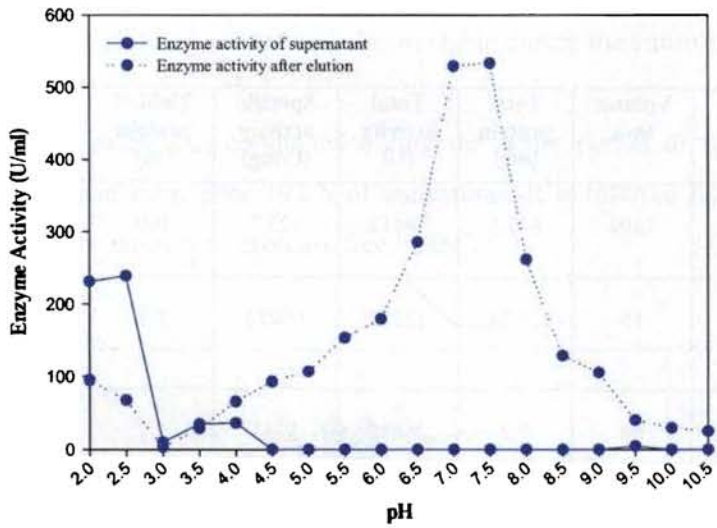


Fig. 4.32 Optimization of binding pH for binding of lipase to the anion exchange resin DEAE-Cellulose

Elution profile of the enzyme fractions obtained from the DEAE-Cellulose column is presented in Fig. 4.33. The single peak obtained indicate that enzyme with activity could be eluted with buffer containing 0.2 M NaCl. This step resulted in 33.7 % of recovery of lipase (30.2 fold of purification) with a specific activity of 3733.0 U/mg protein.

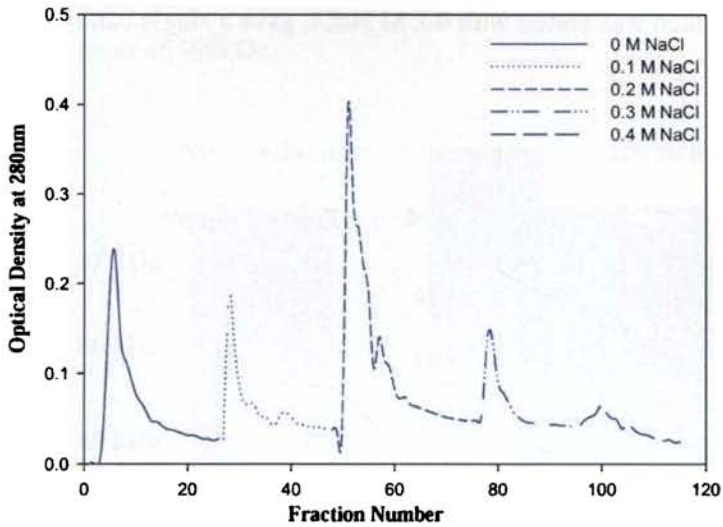


Fig. 4.33 Ion exchange chromatography profile for *A. awamori* lipase

4.4 CHARACTERIZATION OF PURIFIED ENZYME

4.4.1 Native-Polyacrylamide Gel Electrophoresis (Native-PAGE)

All the fractions, which had significant lipase activity, obtained after ion exchange chromatography, were pooled and lyophilized. They were subjected to Native polyacrylamide gel electrophoresis and confirmed their homogenity. Enzyme protein, which was eluted with 0.2 M NaCl, gave a single band in Native-PAGE (Fig. 4.34).

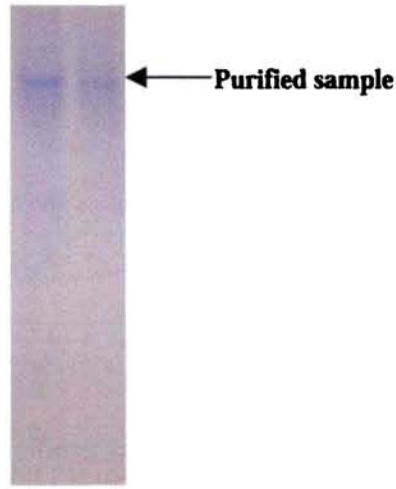
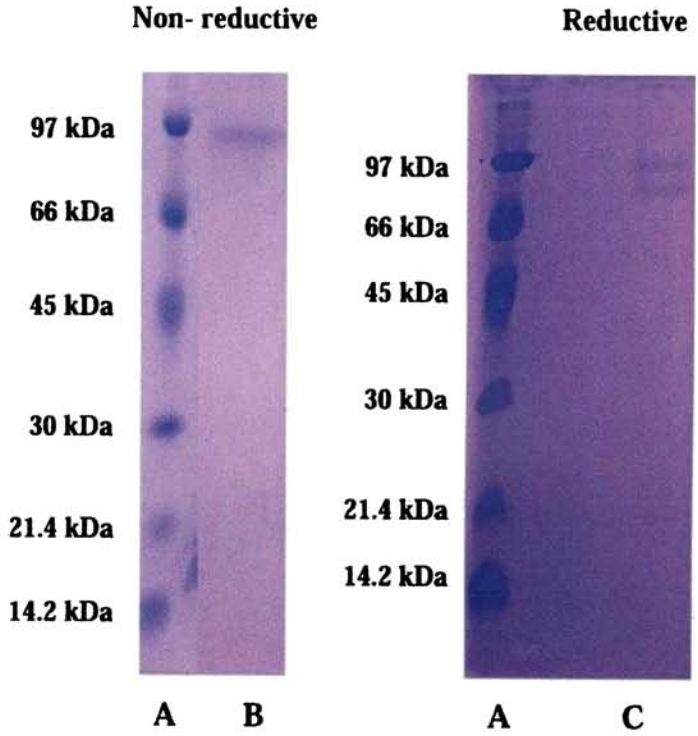


Fig. 4.34 Native PAGE of purified sample

4.4.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of the purified enzyme, performed under non-reducing and reducing conditions, yielded a single band and double band respectively, testifying the dipeptide nature of the enzyme (Fig. 4.35). The molecular mass of lipase, estimated by comparison with the electrophoretic mobility of marker proteins indicate that the *A. awamori* lipase has an apparent molecular mass of ~90kDa.

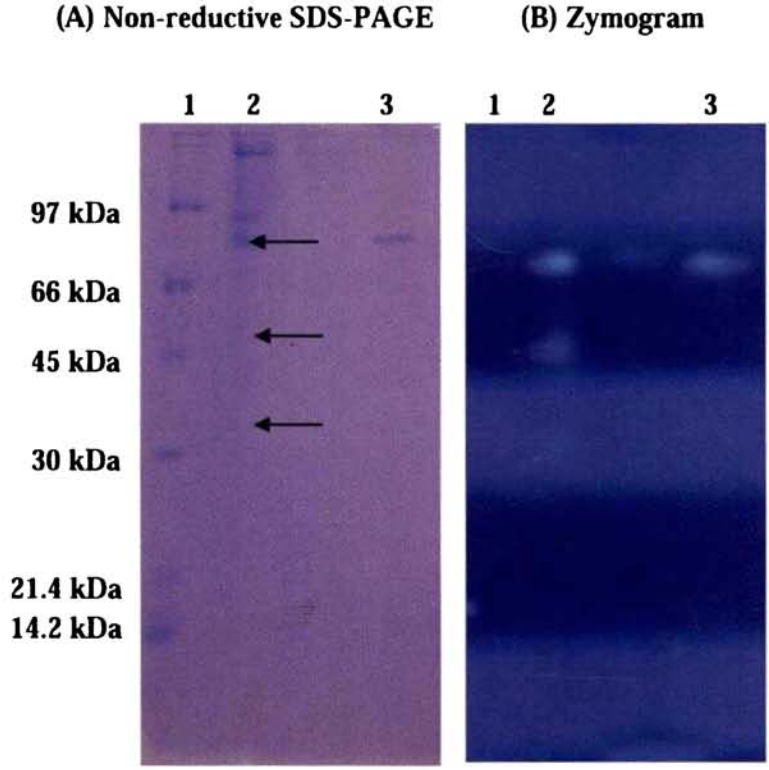


Lane A. Marker
Lane B. Purified sample (non-reductive condition)
Lane C. Purified sample (reductive condition)

Fig. 4.35 SDS-PAGE of purified sample

4.4.3 Zymogram

The lipolytic activity of the purified enzyme protein was further confirmed by zymogram analysis as described in section 3.5.1.4. The zymogram showed three fluorescent bands in the case of ammonium sulphate precipitated sample, compared to a single fluorescent band obtained with ion exchange purified sample (Fig. 4.36 A & B). Results confirm the purity and activity of the purified lipase obtained from *A. awamori*.



Lane 1. Marker
Lane 2. Sample after ammonium sulphate fractionation
Lane 3. Sample after ion exchange chromatography

Fig. 4.36 Zymographic analysis of lipase

4.4.4 Gel filtration chromatography for estimation of molecular weight of lipase enzyme

Gel filtration chromatography of the partially purified enzyme protein using Sephadex G100, yielded three peaks with lipase activity (Fig. 4.37). The molecular weight of the proteins was calculated from the standard graph plotted for K_{av} versus log molecular weight of the standard proteins. From the K_{av} value, the molecular weight of the enzyme for the three fractions (I, II & III) were found to be 95 kDa, 65 kDa and 38 kDa respectively.

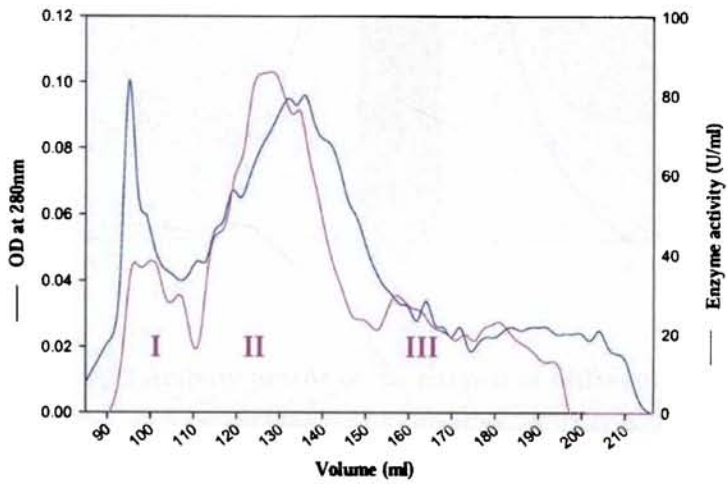


Fig. 4.37 Elution profile of lipase on Sephadex G100

4.4.5 Glycoprotein staining

The purified lipase was stained with Thymol-H_2SO_4 reagent, in order to assess whether the lipase is a glycoprotein. A purple band obtained after staining suggest that *A. awamori* lipase is a glycoprotein (Fig.4.38).

Fig. 4.38 Glycoprotein staining using purified lipase

4.4.6 Carbohydrate content of purified enzyme

Carbohydrate content of purified enzyme was tested by phenol-sulphuric acid assay. It was found that *A. awamori* lipase was glycosylated to the extent of 3.6 %.

4.4.7 Determination of Optimal pH for lipase activity

From the results depicted in Fig. 4.39 it is inferred that the lipase has an optimum pH between 7.0 and 8.0 for maximal activity. pH 7.0 is considered as

optimal pH for maximal activity. In general, the lipase was active over a pH range of 5.0-9.0 and an increase in pH from 5.0 to 7.0 led to proportionate increase in enzyme activity. It was also noted that the enzyme was totally inactive at pH below 4.0 and above 10.0. Moreover more than 60 % of maximal enzyme activity was recorded at pH in the range between 6.0-8.0 (Table 4.9).

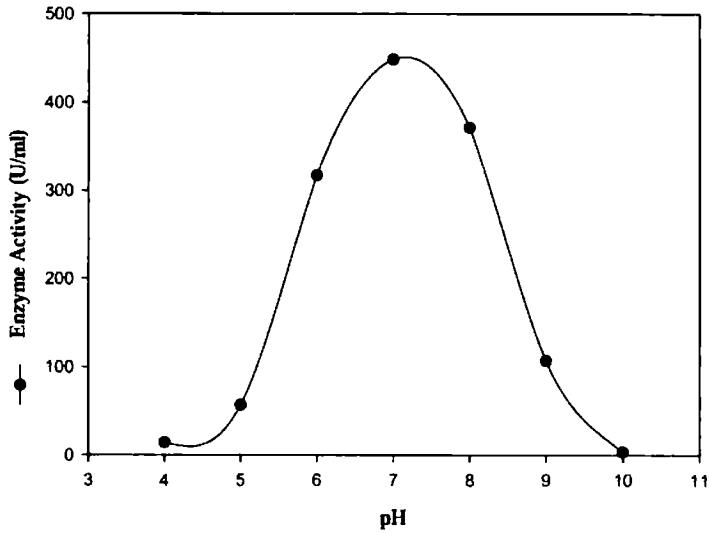


Fig. 4.39 Activity profile of the enzyme at different pH

Table 4.9: Relative activity of lipase enzyme at different pH

pH	Relative Activity (%)
2	0
3	0
4	2.55
5	10.12
6	62.78
7	**100.00**
8	86.16
9	27.72
10	0.458
11	0
12	0

4.4.8 Determination of pH stability of lipase enzyme

The data obtained for the pH stability studies of the lipase (Fig. 4.40) testify that the enzyme is stable over a wide range of pH from 2.0-12.0. However, maximal residual enzyme activity was recorded only with the sample incubated in the buffer having pH 7.0. Nevertheless, when compared to other levels of pH tested from 2.0-8.0, the difference in enzyme activity was only marginal.

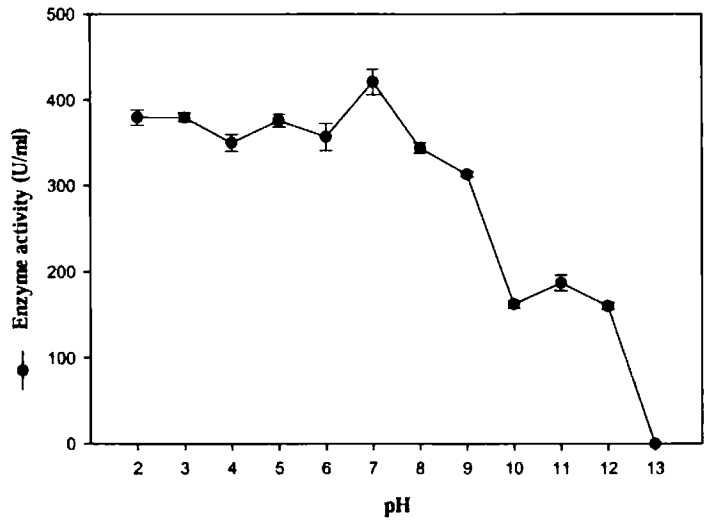


Fig. 4.40 Stability profile of enzyme in different pH

4.4.9 Determination of optimal temperature for lipase activity

Results presented in Fig. 4.41 indicated that the enzyme was active over a broad range of incubation temperature while recording maximal activity at 40°C. Temperatures above 45°C led to a sharp decline in enzyme activity. In fact, the lipase activity showed a linear increase along with increase in temperature, and the increase was rapid over a range of temperature varying from 20°C to 40°C. Nevertheless, the enzyme showed activity even at 5°C with 31.85 % relative

activity. Data documented in Table 4.10 clearly indicated the preference for a higher temperature of 35°C-45°C for maximal enzyme activity.

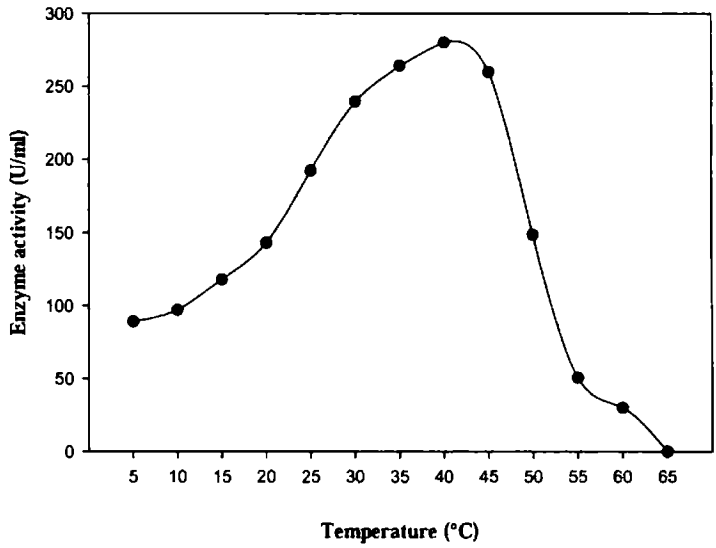


Fig. 4.41 Effect of temperature on lipase activity

Table 4.10: Relative activity of lipase enzyme at different temperatures

Temperature (°C)	Relative Activity (%)
5	31.85
10	34.68
15	42.13
20	51.05
25	68.71
30	85.50
35	**94.28**
40	**100**
45	**92.85**
50	53.04
55	18.05
60	10.70
65	0

4.4.10 Determination of temperature stability of lipase enzyme

It is evident from the results presented in Fig. 4.42, for the temperature stability studies conducted using lipase, that the enzyme was stable over 20-30°C and could retain 100 % residual activity even after 48 h. Whereas, at 40°C, the optimal temperature for maximal activity, the enzyme could retain only 38 % of activity after 12 h of incubation. Results suggest that at high temperatures above 50°C the enzyme lost activity within 30 minutes of incubation. Hence, it is inferred that this lipase is not a thermo stable enzyme.

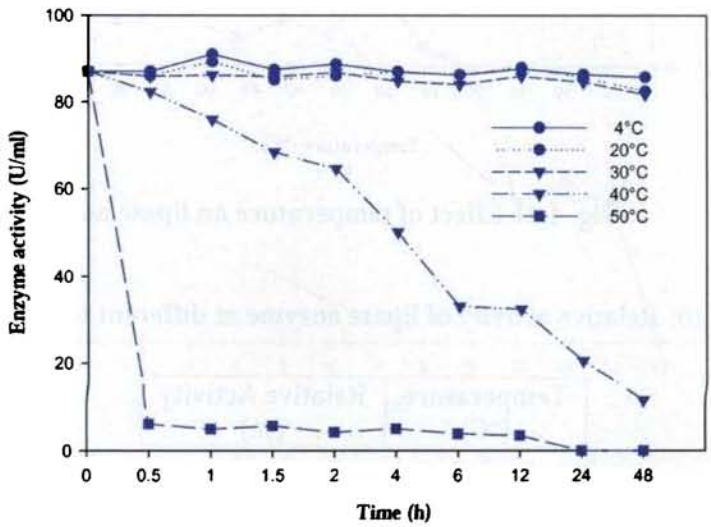


Fig. 4.42 Thermostability of lipase at different temperatures

4.4.11 Kinetic studies

Kinetic studies were performed for lipase using pNP caprylate as the substrate and the data obtained is presented in Fig. 4.43. K_m and V_{max} were estimated by plotting the initial velocity data as the function of the concentration of substrate, linear transformation of the Michaelis-Menten equation using Graphpad Prism software. K_m and V_{max} were recorded as 73.67 µmol and 344.7 U/ml respectively.

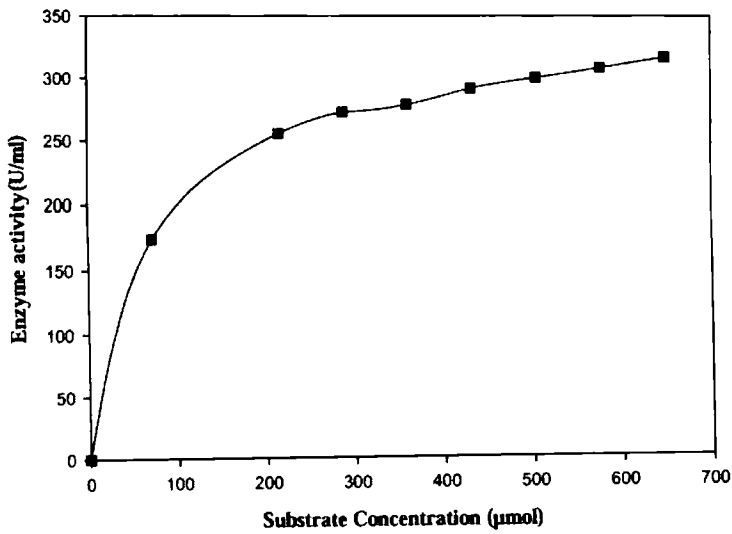


Fig. 4.43 Kinetic studies using pNP caprylate as substrate

4.4.12 Effect of oxidizing agent on enzyme activity

Effect of oxidizing agent on enzyme activity was studied using H_2O_2 and the results obtained are documented in Fig. 4.44 and Table 4.11. From the data, it is evident that 99.9 % of enzyme activity was retained in the presence of 1 % H_2O_2 and 50 % of enzyme activity was retained even upto 5 % conc. studied. The enzyme was inactive at conc. of H_2O_2 above10 %. Thus it is concluded that the enzyme stability declined along with increase in conc. of H_2O_2.

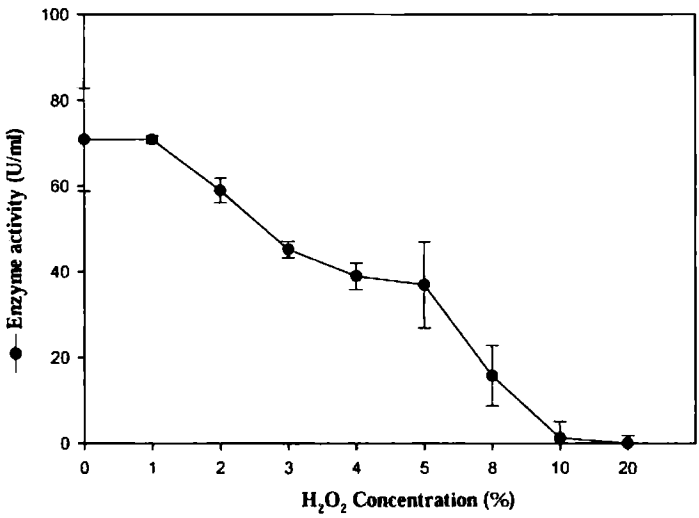


Fig. 4.44 Effect of oxidizing agent on enzyme activity

Table 4.11: Effect of oxidizing agent on enzyme activity

Concentration of Hydrogen peroxide (%)	Residual activity (%)
1	**99.90**
2	83.18
3	63.78
4	54.93
5	52.12
8	22.29
10	1.80
20	0

4.4.13 Effect of reducing agents on enzyme activity

Effect of reducing agents on lipase activity was evaluated using β-mercaptoethanol and sodium thioglycolate. Results documented in Table 4.12, indicate that the reducing agents have a positive effect on enzyme activity except at highest concentration tried. β-mercaptoethanol led to an enhancement in enzyme activity along with increase in conc. up to 1 % concentration. In the case of sodium thioglycolate increase in conc. above 0.2 % led to gradual decline in residual enzyme activity along with increase in concentration. Both β-mercaptoethanol and sodium thioglycolate, at their highest concentration (5 %) studied, caused reduction in residual enzyme activity to 90.05 % and 57.92 % respectively.

Table 4.12: Relative activity of lipase enzyme with different reducing agents

Reducing agent	Concentration (%)	Residual activity (%)
β-Mercaptoethanol	0.2	109.3
	0.4	108.6
	0.6	103.6
	0.8	103.2
	1	101.3
	5	90.05
Sodium thioglycolate	0.2	100.70
	0.4	82.40
	0.6	86.30
	0.8	84.08
	1	62.64
	5	57.92

4.4.14 Effect of organic solvents on lipase activity

Effect of organic solvents on lipase activity was tested using 9 different organic solvents up to 24 h. Results obtained for the study (Fig. 4.45, Table 4.13 A & B) indicate that all the organic solvents, except butanol, at 10 % concentration

level enhanced enzyme activity more than 100 %, even after 24 h. Butanol was found to be a strong inhibitor of lipase even at lower concentration. From the results it is clearly evident that at both 30 % & 60 % concentrations of hexane, benzene and diethyl ether the enzyme was stable even after 24 h with residual activities 106.25, 112.32 and 133.40 % respectively. Among other solvents tested, only methanol at 30 % conc. showed slight enhancement of residual lipase activity (109.10 %) after 30 min, compared to others. However, on further incubation up to 24 h there was loss in enzyme stability.

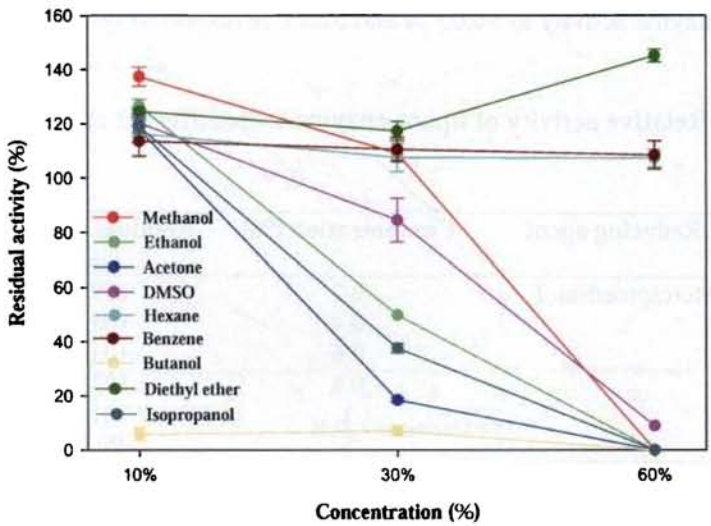


Fig. 4.45 Effect of different organic solvents on lipase activity at different concentrations after 30 minutes of incubation

Table 4.13: (A) Effect of different organic solvents on lipase activity at different concentrations after 30 minutes of incubation

Organic solvents	Residual activity (%) at different concentrations (after 30min)		
	10%	30%	60%
Methanol	**137.40**	**109.10**	0
Ethanol	**126.59**	49.86	0
Acetone	**117.90**	18.50	0
DMSO	**120.38**	84.57	9
Hexane	**116.42**	**107.60**	**107.00**
Benzene	**113.65**	**110.60**	**108.48**
Butanol	5.80	7.0	0
Diethyl ether	**124.60**	**117.39**	**145.20**
Isopropanol	**119.07**	37.62	0

Table 4.13: (B) Effect of different organic solvents on lipase activity at different concentrations after 24 h of incubation

Organic solvents	Residual activity (%) at different concentrations (after 24 h)		
	10%	30%	60%
Methanol	130.70	67.90	0
Ethanol	123.87	11.96	0
Acetone	114.58	11.86	0
DMSO	113.60	45.48	0
Hexane	114.40	102.36	106.25
Benzene	118.90	115.65	112.32
Butanol	0	0	0
Diethyl ether	118.19	90.11	133.40
Isopropanol	101.97	10.2	0

4.4.15 Effect of various metal ions on enzyme activity

Of the various metals evaluated for their effect on lipase activity, only Co^{2+} enhanced lipase activity, at all the conc. tested upto 20 mM concentrations (Table 4.14). All the other metals tested had a negative effect on lipase and led to a decline in residual activity at all the concentrations tested, except ferric chloride, which supported a marginal raise in enzyme activity at 1 mM conc. In general, the increase in conc. of metal ions led to proportionate decrease in enzyme activity. Even at 1mM concentration lipase activity was considerably decreased in the presence of Hg^{2+} and Cd^{2+}. At 20 mM concentration there was no activity in the presence of $Al^{3+}$and the activity got reduced considerably in the presence of Zn^{2+}, Cd^{2+} and Hg^{2+}.

Table 4.14: Effect of various metal ions on lipase activity (Residual activity in %)

Metal ions	Concentration (mM)				
	1	5	10	15	20
Aluminium sulphate (Al^{3+})	90.4	77.3	56.09	23.80	0
Barium chloride (Ba^{2+})	90.36	74.7	73.87	62.20	61.20
Cadmium sulphate (Cd^{2+})	35.36	33.10	23.90	20.03	16.60
Calcium chloride (Ca^{2+})	82.70	74.02	70.59	62.90	53.30
Chromium oxide (Cr^{3+})	86.30	85.20	83.70	82.40	65
Cobalt chloride (Co^{2+})	122.60	124.30	126.19	118.40	116.60
Cupric sulphate (Cu^{2+})	92.50	85.30	75.90	64	50.78
Ferric chloride (Fe^{3+})	103	95.66	89.40	82.97	55.20
Lithium chloride (Li^{+})	91.70	85.50	82.37	81.04	78.36
Magnesium sulphate (Mg^{2+})	95.95	94.17	91.70	85.80	73.97
Manganese chloride (Mn^{2+})	89.85	76.39	59.75	60.75	58.12
Mercury chloride (Hg^{2+})	33.80	33.50	33.20	27.27	11.30
Nickel sulphate (Ni^{2+})	71.46	73.40	71.46	65.70	65.20
Potassium chloride (K^{+})	90.46	86.40	84.79	82.80	83
Sodium chloride (Na^{+})	91.40	87.20	86.80	79.40	75.20
Sodium molybdate (Mo^{6+})	84.98	84.60	79.50	76.80	71.40
Zinc sulphate (Zn^{2+})	75.86	48.09	42.90	37.80	32.86

4.4.16 Substrate specificity

All the substrates tested, for specificity, with *A. awamori* lipase showed high level of affinity. Maximal affinity was shown by pNP caprylate, followed by pNP butyrate, pNP laurate and pNP palmitate (Fig. 4.46). These results clearly indicate the affinity of *A. awamori* lipase towards C-8 fatty acids.

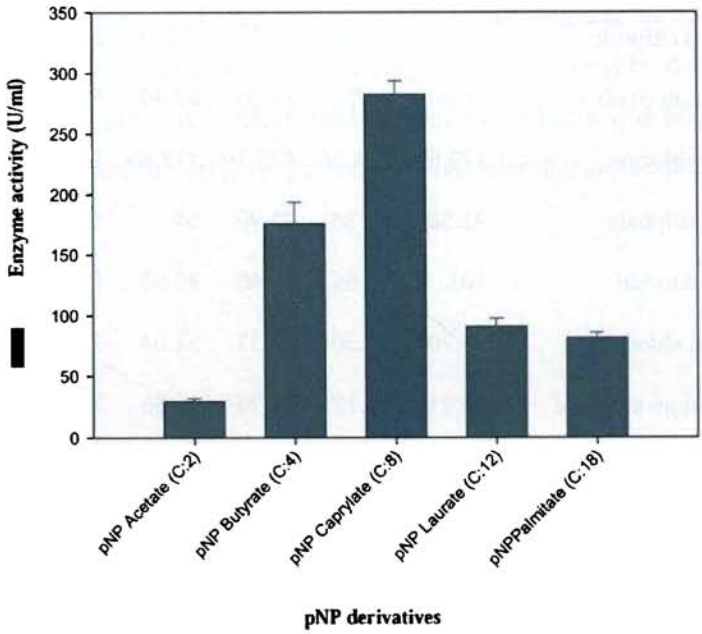
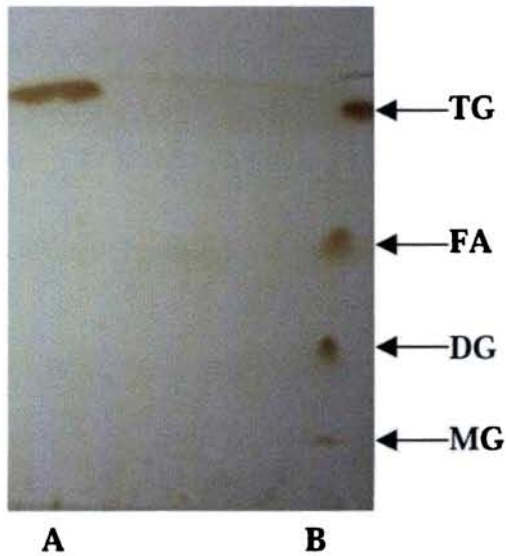


Fig 4.46 Affinity of *A. awamori* lipase towards different p-nitrophenyl esters

4.4.17 Positional specificity

The hydrolytic product of the lipase activity with triolein (C18:1) as a substrate was examined by thin-layer chromatography. Triolein was hydrolysed into diolein, mono-olein and oleic acid, evidencing the 1,3-regiospecific nature of *A. awamori* lipase (Fig.4.47).



Lane A- Standard triolein
Lane B- Enzymic reaction products
Abbreviations: TG, triolein; FA, oleic acid; DG, diolein; MG, mono-olein

Fig. 4.47 Thin-layer chromatogram showing the 1,3-regiospecific nature of *A. awamori* lipase

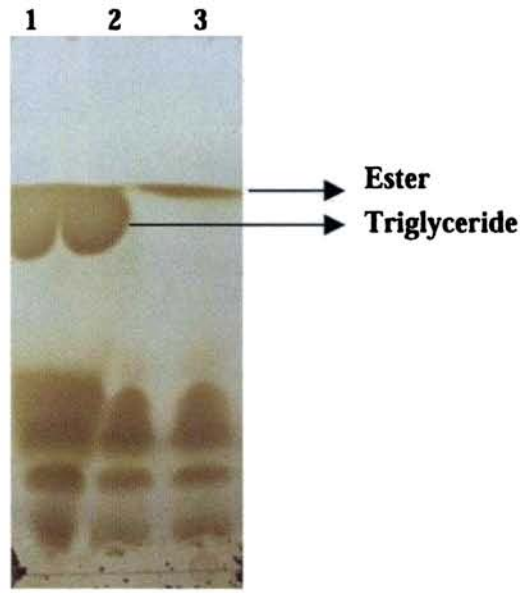
4.4.18 Hydrolytic activity of *A. awamori* lipase

Hydrolytic activity of *A. awamori* lipase with olive oil as substrate was 4.0 U/ml.

4.5 APPLICATION STUDIES

4.5.1 Biodiesel production

Transesterification of rice bran oil and methanol with lipase was tested towards biodiesel production. Data analysed by Gas chromatography did not yield any positive result. Whereas, the thin-layer chromatogram clearly showed that almost all the triglyceride form of rice bran oil was converted to its hydrolysis product (Fig. 4.48), indicating scope for biodiesel production subject to further standardisation of the reactants molar concentration.



Lane 1. RB Oil alone
Lane 2. RB Oil + Methanol
Lane 3. RB Oil + Methanol + Enzyme

Fig. 4.48 Thin-layer chromatogram showing the action of A. *awamori* lipase towards biodiesel production

4.5.2 Fatty acid sugar ester synthesis

Use of lipase for fatty acid sugar ester synthesis was evaluated. Qualitative analysis of the fatty acid sugar ester was unsuccessful with TLC because spraying of 50 % H_2SO_4 got absorbed to the silica and the coating got cracked, and detached from the alumina. Whereas, quantitative analysis of fatty acid sugar ester yielded 16 % fructose myristate, 21.4 % glucose myristate, 8.3 % glucose palmitate, and formation of fructose palmitate was nil. The lower percentage of fatty acid sugar ester formation might be due to reverse reaction that could have occurred due to the water generated during esterification reaction.

4.5.3 Treatment of effluent containing oils using *A. awamori* lipase

Potential of lipase for treatment of oil containing effluent was evaluated by treating a simulated oil effluent, blended with10 % each of 'used restaurant oil', 'used ayurvedic oil', 'used fish fry oil', palm oil, coconut oil and dalda with lipase. From the results obtained (Fig. 4.49) it is inferred that there was 91.4 % reduction in oil and grease content after treatment of ayurvedic oil with *A. awamori* lipase, followed by palm oil, fish fry oil and coconut oil with 80.16 %, 33.3 % and 30 % reduction in oil and grease respectively.

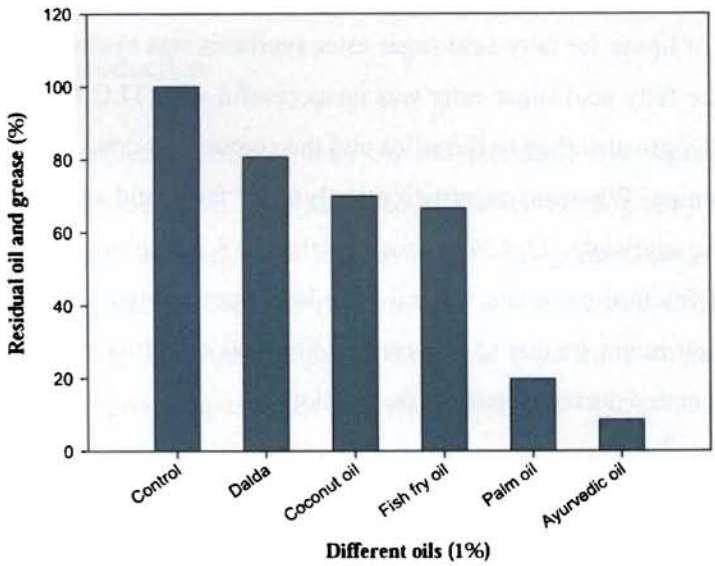


Fig. 4.49 Effect of *A. awamori* lipase in reducing oil and grease content in effluent

4.6 ISOLATION OF GENE ENCODING LIPASE

4.6.1. Isolation of genomic DNA

Genomic DNA was isolated from *A. awamori*. Approximately 120 µg of DNA obtained from 2.5 gram of fresh mycelia. From the Fig. 4.50 it may be noted that the isolated genomic DNA was almost pure.

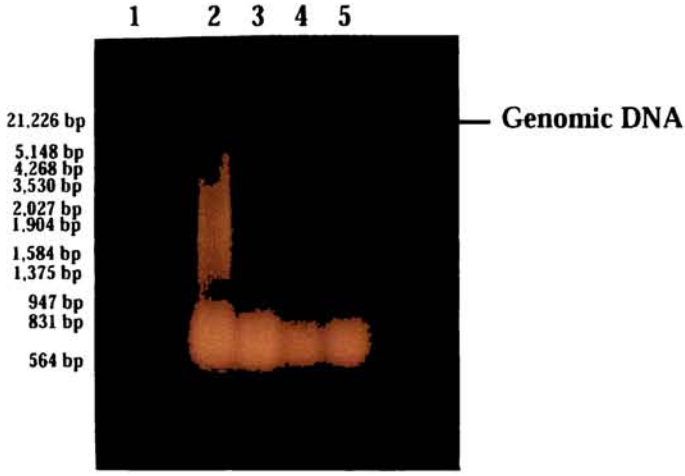


Fig. 4.50 An ethidium bromide stained 1 % agarose gel showing the isolated fungal genomic DNA. Lane 1, Lamda DNA/*EcoR1-Hind* III Double Digest marker. Lane 2-5, genomic DNA isolated from *A. awamori*

4.6.2 PCR amplification of the lipase gene from *A. awamori*

The degenerate primers were designed from the complementary DNA (cDNA) sequence available for lipase from *Aspergillus* sp deposited at GenBank as described in section 3.7.1.5. Amplification of the genomic DNA with degenerate primers forward ASP.F1 and reverse ASP.R1 yielded an amplicon (LIP.ASP1) of approximately 800 bp at an annealing temperature of 56°C (Fig. 4.51)

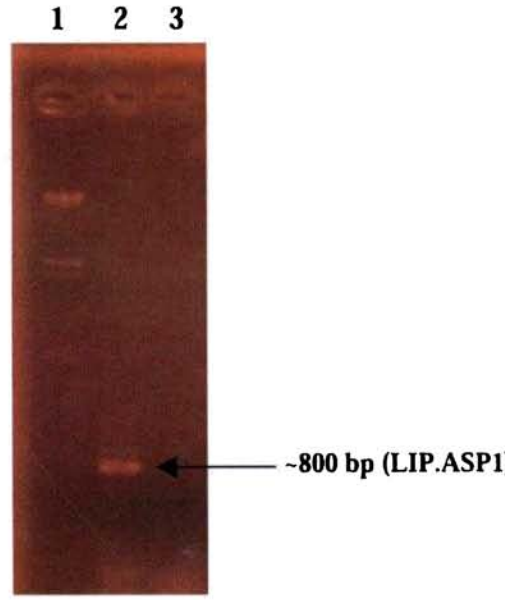


Fig. 4.51 An ethidium bromide stained 1 % agarose gel showing the 800 bp PCR product. Lane 1, Lamda DNA/*EcoR1-Hind* III Double Digest marker. Lane 2, represents the putative amplified lipase gene from the genome of *A. awamori*

The 800 bp PCR product was cloned into the pGEMT plasmid vector, transformed in *E.coli* DH5α and plated. White colonies were selected and checked for recombinant plasmids.

Recombinant plasmid with the insert was reconfirmed by amplification of the insert with specific primer ASP.F1-ASP.R1 (Fig. 4.52).

Fig. 4.52 An ethidium bromide stained 1% agarose gel showing the insert amplified with specific primer. Lane 1, Lamda DNA/*EcoR1-Hind* III Double Digest marker. Lane 2, amplicon. Lane 3, -ve control (blue colony). Lane 4, amplified plasmid.

The 800 bp amplicon was cloned into the pGEMT plasmid vector, and later subjected to sequence analysis using the primer T7 in order to confirm the lipase gene sequence. A 463 bp sequence was obtained for LIP.ASP1 (Fig. 4.53). Blastx was conducted and the relationship of the *A. awamori* lipase sequence with other reported lipase was checked.

GCGGCCGCGGGAATTCGATTCGATTCTCACAGCACTGCACTTCATC
CTCATCCGCCCAGCGGAACGCACAATGCTCCTCCGGATTCAACTTC
AATGAATTAGTCTCCTCAACCTCCACCAAAAACCCCCACTTGATCA
CCTTCTTCTCTCCCTGACCCCCCGGAAGTGCCTTCCTCCACTCATC
AACAGCAACCAAATCCACAACCTTAGTTACCCGTAGCCCGGTCTCC
TCGACTGTCTCGCGACATAGTGCCGAGAGGAGGGTGTCGTCGAGA
GAGGGGTCTGCTCCTCCTCCCGTGGATTCCCAGTGCAGGGGGTAG
CTGTCTGTTGGGGAGCGGTGAAGGAGGAGAAGGAGTGGCTTCGAT
GTTGGATTAAGATTGTTTTGCTGGAGTTGGGATTTGGTGGTGAGAG
ATGTAGGCGCCCACGCCAAAGTCCGTGTAGGTGGGGTAGTGGGTG
GTGGGGA

Fig. 4.53. The sequence obtained for LIP-ASP1 gene of *A. awamori* Nagazawa.

The lineage report for the LIP-ASP1 gene is as follows:

```
Lineage Report
cellular organisms
. Eukaryota          [eukaryotes]
.. Fungi/Metazoa group [eukaryotes]
... Fungi            [fungi]
.... Pezizomycotina  [ascomycetes]
..... Trichocomaceae [ascomycetes]
...... Aspergillus   [ascomycetes]
....... Aspergillus niger ---------- 244 2 hits [ascomycetes]  unnamed protein product [Aspergillus niger]
....... Aspergillus clavatus NRRL 1 ..... 87 2 hits [ascomycetes]  NUDIX domain protein [Aspergillus clavatus NRRL 1] >gi|1193
....... Aspergillus oryzae ..... .... 83 4 hits [ascomycetes]  unnamed protein product [Aspergillus oryzae]
....... Aspergillus fumigatus Af293 ..... 83 1 hit [ascomycetes]  NUDIX domain protein [Aspergillus fumigatus Af293] >gi|6684
....... Aspergillus terreus NIH2624 .....40 1 hit [ascomycetes]  predicted protein [Aspergillus terreus NIH2624] >gi|1141920
...... Neosartorya fischeri NRRL 181 -----87 1 hit [ascomycetes]  NUDIX domain protein [Neosartorya fischeri NRRL 181] >gi|11
...... Aspergillus nidulans FGSC A4 ..... 63 2 hits [ascomycetes]  hypothetical protein AN0503.2 [Aspergillus nidulans FGSC A4
..... Neurospora crassa OR74A ----------70 2 hits [ascomycetes]  hypothetical protein [Neurospora crassa OR74A] >gi|28921747
..... Chaetomium globosum CBS 148.51 . 63 2 hits [ascomycetes]  hypothetical protein CHGG_09486 [Chaetomium globosum CBS 14
..... Gibberella zeae PH-1 ..... 59 2 hits [ascomycetes]  hypothetical protein FG02574.1 [Gibberella zeae PH-1]
..... Magnaporthe grisea 70-15 ..... 56 2 hits [ascomycetes]  hypothetical protein MG08699.4 [Magnaporthe grisea 70-15] >
..... Phaeosphaeria nodorum SN15 ..... 48 1 hit [ascomycetes]  hypothetical protein SNOG_12764 [Phaeosphaeria nodorum SN15]
```

Distribution of blast hits on the query sequence

Sequences producing significant alignments:	Score (Bits)	E Value
ref|XP_001268247.1| NUDIX domain protein [Aspergillus clavatu...	92.0	8e-18
ref|XP_001258266.1| NUDIX domain protein [Neosartorya fischer...	91.7	1e-17
dbj|BAE58976.1| unnamed protein product [Aspergillus oryzae]	88.2	1e-16
ref|XP_751032.1| hypothetical protein Afu6g11490 [Aspergillus...	88.2	1e-16
ref|XP_960262.1| hypothetical protein [Neurospora crassa OR74...	74.3	2e-1
ref|XP_001227413.1| hypothetical protein CHGG_09486 [Chaetomi...	67.4	2e-10
ref|XP_658107.1| hypothetical protein AN0503.2 [Aspergillus n...	67.4	2e-10
dbj|BAE61688.1| unnamed protein product [Aspergillus oryzae]	66.6	3e-10
dbj|BAE64502.1| unnamed protein product [Aspergillus oryzae]	63.5	3e-09
ref|XP_382918.1| hypothetical protein FG02742.1 [Gibberella z...	63.5	3e-09
ref|XP_382750.1| hypothetical protein FG02574.1 [Gibberella z...	62.8	5e-09
ref|XP_363115.1| hypothetical protein MG08699.4 [Magnaporthe gri	60.5	3e-08
gb|EAU83380.1| predicted protein [Coprinopsis cinerea okayama7#1	59.7	4e-08
ref|XP_958246.1| hypothetical protein [Neurospora crassa OR74...	59.3	6e-08
ref|XP_368408.1| hypothetical protein MG00836.4 [Magnaporthe gri	57.0	3e-07
ref|XP_001221573.1| hypothetical protein CHGG_05478 [Chaetomi...	55.1	1e-06
ref|YP_121862.1| putative MutT family protein [Nocardia farci...	51.2	2e-05
gb|EAT80062.1| hypothetical protein SNOG_12764 [Phaeosphaeria no	50.8	2e-05
ref|NP_927607.1| hypothetical protein plu0244 [Photorhabdus l...	48.1	1e-04
ref|XP_424973.1| PREDICTED: similar to Nudix (nucleoside diph...	47.4	2e-04
ref|NP_822648.1| ATP/GTP-binding protein [Streptomyces avermi...	47.0	3e-04

Based on this Blastx analysis it is concluded that the DNA fraction obtained through PCR amplification contain partial gene sequence that encodes lipase in *A. awamori*. The sequences were submitted to GenBank with the accession no. **EF524197** as *Aspergillus awamori* Nagazawa partial lipase gene related NUDIX protein.

Chapter 5

DISCUSSION

5.1 SCREENING, SELECTION, AND IDENTIFICATION OF POTENTIAL STRAIN FOR LIPASE PRODUCTION

Marine environments, in general, are unique by virtue of their salinity, wide range of mineral content and well knitted ecosystem when compared to terrestrial environments which is constantly disturbed by human activities. Results obtained in the present study during screening of fungal isolates for lipase very clearly indicate that marine environments, both water and sediment, are potential sources of lipase. In fact lipase producing fungi were more abundant in marine sediments than in seawater. Probably fungi in the sediments participate in the secondary productivity of the sea, particularly in the process of cycling of the organic carbon and consequently in the maintenance of fertility of sea (Zobell, 1946).

Fungi are one of the most important lipase source for industrial application, for they are usually excreted extracellularly, facilitating easy extraction from the fermentation media. A large number of filamentous fungi have been studied for lipase production (Elibol and Ozer, 2002; Mahadik *et al.*, 2002). In the present study, primary screening was done in Rhodamine B-olive oil agar plate, which gave satisfactory results. Many reports are available for screening lipase activity using Rhodamine B-olive oil agar plate because this method enables the screening of lipase activity in large number of microorganisms (Wang *et al.*, 1995).

Among the different genera of fungi, capable of producing lipase, *Aspergillus* is known as the dominating group for industrial production of enzymes, particularly lipase. In the present study, the strain selected as potential lipase producer, isolated from sea water was identified as *Aspergillus awamori* Nagazawa, is a closely related species of *A. niger*, which is well known for production of several industrial enzymes.

A. awamori is known well for the industrial production of glucoamylase, α-amylase, protease and tannase. Most reports made earlier on *A. awamori* were focused on strain improvement for a specific product (Gouka *et al.*, 1996; Smith and Wood, 1991; Ward, 1996). Whereas, so far there are no reports on lipase production by *A. awamori*, irrespective of the source of isolation, whether it is from marine or terrestrial.

Molecular techniques utilizing amplification of target DNA provide alternate methods for diagnosis and identification (Kurtzman and Robnett, 1997). PCR-based detection of fungal DNA sequences is rapid, sensitive, and specific (Makimura *et al.*, 1994). Coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes evolve slowly, relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationships (White *et al.*, 1990). The identity of the selected fungus was confirmed by carrying out ribotyping using partial sequence of 28S rRNA gene. From the phylogram, it is inferred that the selected lipase producing fungi, *A. awamori* Nagazawa has close similarity to *A. niger* with 91 % similarity compared to other species of *Aspergillus*. This observation on phylogram strongly suggests that *A. awamori*, a close relative of *A. niger* could have migrated from nearby terrestrial environment into marine environment through surface run-off and got settled down in sediments and adapted to local environment.

Lipase assay was conducted by spectrophotometric method in microtitre plate using pNP caprylate as the substrate. This assay gave reliable results in the screening of potential strains as well in all other critical experiments. The caprylate (pNPC8) is a good compromise between maximum activity of esterase, usually obtained with fatty acids containing 2-4 carbon atoms, and that of lipases which are more active on esters containing 16-18 carbon atoms. In addition, pNPC8 is soluble under the conditions used which greatly facilitated the spectrophotometric assay and detection of activity was highly sensitive (Kademi *et al.*, 1999).

Triton X-100 and gum arabic were used in the assay mixture, both acts as emulsifiers. The addition of Triton X-100 in the assay mixture, dispenses with the tedious and time-consuming step of fatty acid precipitation, making the handling of a large number of samples possible. Also, the current trends in enzyme research have distinctly reduced the reaction volumes to micro or nanotitre plates (Gupta *et al.*, 2002).

5.2 SUBMERGED FERMENTATION FOR PRODUCTION OF LIPASE BY ASPERGILLUS AWAMORI NAGAZAWA

Microbial lipases are produced mostly employing submerged fermentation system (Ito *et al.*, 2001), although solid state fermentation equally holds potential for industrial production of enzymes (Chisti, 1999). Lipase production by *A.awamori* under submerged fermentation was optimized for various physicochemical parameters that influence lipase production. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration (Elibol and Ozer, 2001). Lipid as carbon sources seem to be generally essential for obtaining a high lipase yield.

The incubation time for enzyme production is governed by the characteristics of the culture and is based on growth rate. In the present study, lipase production was observed to commence after 12 h of incubation and the activity declined after 96 h of incubation, indicating that incubation for 96 h is optimal requirement for maximal enzyme production. Similar results were reported for *Aspergillus,* which showed highest lipase activity on day 4 of incubation (Cihangir and Sarakaya, 2004). and Maximum lipase production was observed at 96 h for *Penicillium roquefortii* (Petrovic *et al.*, 1990) and *Aspergillus niger* (Ellaiah *et al.*, 2004) respectively.

Oils were good carbon sources for biomass and lipase production by *Aspergillus* sp (Cihangir and Sarakaya, 2004) and *Candida rugosa* (Valerio *et al.*, 1991). *Geotrichum candidum* released lipase into the culture broth when cultivated in a medium supplemented with triglyceride such as olive oil (Sugihara *et al.*, 1990). Almost all the oils used in the present study supported lipase production, although maximum enzyme production was obtained with rice bran oil (2 %) as the suitable substrate. The variation in preferential utilization of specific oil as substrate for maximal enzyme may be attributed to the diversity in nutritional requirement of each species of fungi.

Temperature is a critical parameter that has to be controlled and it varies from organism to organism. Temperature influences secretion of extra cellular enzymes by changing the physical properties of the cell membrane. The optimal incubation temperature for maximal enzyme production by *A. awamori* was recorded as 40°C. Nevertheless, there was considerable level of enzyme production at all the lower temperatures studied. 45°C was reported as the best temperature for lipase production by *Talaromyces emersonii* (Oso, 1978). Whereas 22-35°C was observed to be optimum for maximum lipase production for *A. wentii* (Chander

et al., 1980b), *R. nigricans* (Chander *et al.*, 1981), *M. heimalis* (Akhtar *et al.*, 1980), and *R. oligosporus* (Nahas, 1988).

The initial pH of the growth medium influences the rate of lipase production. In the present study, it was noted that the fungus is capable of producing lipase over a broad range of initial pH of the medium varying from pH 3.0 to pH 12.0. Apparently, it seems that, this fungus has two pH optima, one at pH 3.0 (122.9 U/ml) and another at pH 10.0 (119.0 U/ml) for maximal lipase production.This dual optimum for growth is characteristic of most marine fungi (Suresh and Chandrasekaran, 1998). At high alkaline pH (10.0 to 13.0), there was rapid decrease in enzyme activity. Interestingly, irrespective of the initial pH of the medium that supported maximal lipase production by the fungus, the pH of the fermented medium after 4 days of incubation was considerably acidic (pH 3.0-5.0), probably fatty acid formed by the action of lipase enzyme, would have led to the decline in pH of the medium.

From the results obtained in the present study, it is inferred that agitation is required for lipase production, since there was no enzyme activity when incubated at stationary condition. Maximum enzyme production was recorded at 150 rpm. Further, the rate of agitation above 150 rpm led to a decrease in enzyme production. It was reported earlier that growth of *Aspergillus* sp. was significantly enhanced in shaking cultures (Cihangir and Sarakaya, 2004). The increase in lipase production could be attributed by increased oxygen transfer rate, increased surface area of contact with the media components and better dispersability of the oil substrate during fermentation under agitated condition. However, at higher agitation rates, there was a reduction in growth as well as lipase production probably due to shearing stress on the organism, and fragmentation of the mycelium (Gulati *et al.*, 2000).

Variation in the level of inoculum conc. from 1 % to 15 % did not influence the rate of enzyme production. The data clearly showed that there was no direct relation between inoculum concentration and enzyme activity. Of course, it is desirable to produce maximum enzyme activity using lower concentration of spore inoculum, when industrial production of any product is the primary concern of an industry.

In the present study none of the sugars supplied as additional carbon source enhanced lipase production by *A. awamori* compared to control. Maltose, lactose and sucrose inhibited lipase production. Whereas, all the other sugars tested led to a reduced level of enzyme production when compared with that of the control. Lipase activity was not detected in media with glucose as sole carbon source with *A. nidulans*, in which biosynthesis appears to be repressed by glucose, and it requires oil as inducer (Mayordomo *et al.*, 2000). Similar results has been previously reported for lipase of different origin- *Aspergillus oryzae* and *A. niger* (Ohnishi *et al.*, 1994a; Pokorny *et al.*, 1994). In the present study also repression of lipase production by glucose was noted. Whereas, lipase production was stimulated by the addition of glucose to the production medium for *Mucor hiemalis* (Akhtar *et al.*, 1980) and *Aspergillus wentii* (Chander *et al.*, 1980).

The amino acids tested as additional nitrogen source influenced lipase production. Methionine could affect about 37 % more enzyme production compared to control. On the other hand, cysteine, histidine, leucine, isoleucine, tryptophan and arginine caused a considerable reduction in enzyme production. A mixture of arginine, lysine and glutamic acid in medium was observed to be effective for lipase production by *P. fragii* (Alford and Pierce, 1963).

Among the various organic nitrogen sources tested, except urea, all the other nitrogen sources supported enhanced lipase production. Beef extract

(219.3 U/ml) and soyabean meal (221.0 U/ml) supported maximal level of enzyme production followed by peptone, tryptone, yeast extract, casein, gelatin and malt extract. Presence of ammonium hydrogen carbonate, ammonium acetate, ammonium oxalate, and ammonium iron sulphate in the medium inhibited enzyme production. Maximum enzyme production was supported by ammonium sulphate (224.3 U/ml) and ammonium chloride (213.9 U/ml) followed by sodium nitrate, potassium nitrate, ammonium dihydrogen orthophosphate and ammonium nitrate. Similar reports were made earlier where soyabean meal extract supported good growth and lipase production in *Rhizopus oligosporus* (Nahas, 1988).

Poor yield of lipase and biomass in medium containing urea was reported earlier for *Aspergillus* sp. (Cihangir and Sarakaya, 2004). In the present study also supplementation of urea and nitrate salts did not increase lipase yields. The requirement of the type of nitrogen varies among microorganisms, some prefer inorganic form while others prefer organic nitrogen (Nahas, 1988). Higher production of lipase was observed in the presence of di-ammonium hydrogen orthophosphate with *Burkholeria cepacia* (Rathi *et al.*, 2001). Whereas, *P. fragi* utilized ammonium sulphate for maximum lipase production (Alford and Pierce, 1963).

The presence of sodium chloride in the medium significantly affected lipase production. In fact the fungus could produce lipase in a medium prepared without sodium chloride in distilled water (168.6 U/ml). Addition of sodium chloride at concentrations 1-3.5 % (w/v) enhanced lipase production, recording a maximum activity with 1.5 % (206.6 U/ml). Sodium chloride concentrations above 3.5 % led to a gradual decline in lipase production compared to control (without NaCl). Results also suggest that the fungus is halotolerant in nature since it is capable of tolerating upto 12.5 % NaCl for production of lipase, although at reduced level. The fungus can produce lipase in the presence of seawater, addition

of sea water more than 25 % led to decline in enzyme production. Only at a combination of 25 % seawater plus 75 % distilled water there was 29 % enhanced lipase production compared to control medium without seawater and NaCl. In fact the phylogram analysis performed during the present study very clearly indicated that this fungus is a close relative of terrestrial *Aspergillus* sp. Hence, the results obtained with NaCl requirement testify that *A. awamori* could have adopted to marine environment and thus could show a halotolerant phenomenon.

Among the various inorganic salts tested, only calcium chloride (80.16 U/ml) and magnesium sulphate (75.2 U/ml) supported considerable enhancement in lipase production. Whereas, all the other inorganic salts including zinc sulphate, potassium chloride, copper sulphate, ferrous sulphate, manganese chloride, and cobalt chloride inhibited lipase production. Magnesium chloride in presence of calcium chloride was reported to tremendously increase lipase production in *Burkholderia cepacia* (Rathi *et al.*, 2001).

It is well documented that various compounds, such as surfactants can increase cell permeability, facilitating the export of several molecules across the cell membrane. These compounds may alter the cell permeability increasing protein secretion, or by facilitating the contact between enzyme and substrate. The effects of surfactants improving lipase secretion have been studied in several microorganisms with different results (Corzo and Revah, 1999). In *M. anisopliae*, SDS and Tween 80 were found to be the best surfactants (Orlando Beys Silva *et al.*, 2005). A two-fold increased recovery of *Aspergillus niger* lipase was recorded when Triton X-100 was used as surfactant (Mahadik *et al.*, 2002). In this study impact of Triton X-100, SDS, PEG 6000, Tween 80, Tween 20, Gum Arabic, Sodium deoxycholic acid, and brij 35 on lipase production was evaluated. From the results it was inferred that about 38 % enhancement in lipase production with PEG 6000 compared to control. Sodium dodecyl sulphate (SDS) and sodium

deoxycholic acid inhibited lipase production. All the other surfactants had a marginal impact such that the lipase activity decreased compared to control medium.

5.2.1 Optimization of bioprocess variables for lipase production by Statistical approach

In fermentation processes, where the operational variables interact and influence each other's effect on response, it is essential that the optimization method account for these interactions, so that a set of optimal experimental conditions can be determined (Sen and Swaminathan, 1997). Optimization through factorial design and response surface analysis particularly fulfils this requirement (Elibol and Ozer, 2002). In recent years, use of statistical approach involving Plackett Burman (P-B) designing and response surface methodology (RSM) has gained lot of impetus for medium optimization and for understanding the interactions among various physico-chemical parameters using a minimum number of experiments (Gupta *et al.*, 2004; Rathi *et al.*, 2002). Medium that could support maximal lipase production by *A. awamori* was optimized employing statistical approach. Initially process variables were optimized using Plackett-Burman design and in the second stage Response Surface Methodology was adopted towards selection of optimal variables and understanding the interrelationship among significant variables.

From the results of Plackett–Burman design it is inferred that among the eleven variables screened during P-B design, five factors viz. incubation period, soyabean meal, ammonium sulphate, KH_2PO_4 and temperature were found to be the most significant variables. The statistical significance of the model equation was evaluated by the *F*-test analysis of variance (ANOVA), which revealed that this regression is statistically significant. The Model F-value of 71.55 implied that the model is significant. Values of "Prob>F" less than 0.05 indicate that the model

terms are significant. This fit of the model was checked with the coefficient of determination R^2, which was calculated to be 0.9835. In fact 99.9 % of the variability in the response could be explained by the model ($P < 0.1$) at 90 % of confidence level. The effect of individual parameters studied in P-B design, evidence that while soyabean meal and ammonium sulphate had a positive effect in enhancing enzyme production along with increase in their concentration, KH_2PO_4 followed by incubation period and temperature had a negative effect on enzyme production along with increase in the variable.

Among the five variables tested, incubation time, KH_2PO_4 and temperature were found to be the major significant factor influencing both the production of lipase. All other factors and interactions had very little or a negligible effect. However, rice bran oil concentration also influenced the activity of the enzyme. Lipases are generally induced by lipid substrates and are thus produced with high specific activity in the presence of oil as a carbon source (Ghosh *et al.*, 1996; Shirazi *et al.*, 1998).

Response surface methodology (using Box-Behnken design experiment) was adopted towards selection of optimal level of the significant variable. The results obtained for the Box-Behnken design experiment were analyzed by ANOVA showing that Prob > F value was less than 0.05, which indicates that the model is significant. Three linear coefficient, incubation time, KH_2PO_4 and temperature; and one quadratic term temperature were significant model terms for the response. The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination (R^2) of 0.6325. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 99 % of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different numbers of independent

variables, was 0.3262. All the selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on lipase production. The coefficient of variance was 31.65. The adequate precision that measures the "signal to noise ratio" was 6.065. A ratio greater than 4 is desirable as it indicates an adequate signal. Thus, this model could be used to navigate the design space.

Three-dimensional response surface curves were plotted to study the interaction among various physicochemical factors, and to determine the optimum concentration of each individual factor for maximum lipase production. The model predicted maximum lipase production up to 482.85 U/ml that could be achieved using 0.8 % w/v soyabean, 1 M ammonium sulphate and 0.05 M KH_2PO_4 at 35°C for 5 days incubation. An overall 2.8-fold increase in lipase production was achieved after validation of RSM in shake flasks.

Validation of the deduced response surface model based on the previous experiments was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. The temperature range that supported maximal enzyme yield was 35-38°C, and the KH_2PO_4 conc. was 0.05 M.

The statistical approach employed in process optimization led to the identification of the variables - Soyabean meal-0.77 % (w/v); ammonium sulphate-0.1 M; KH_2PO_4-0.05 M; Rice bran oil-2 % (v/v); $CaCl_2$-0.05 M; PEG 6000-0.05 % (w/v); NaCl-1 % (w/v); inoculum-1 % (v/v); pH 3.0; incubation temperature 35°C and incubation period-5 days as optimal conditions for obtaining maximal lipase production by *A. awamori*.

Response surface methodology used for medium optimization for maximum lipase production from *B. cepacia* indicated the importance of various physicochemical factors at different levels. A high similarity was also observed between the predicted and experimental results, which reflected the accuracy and applicability of the central composite design for process optimization. An overall 2.5-fold increase in lipase production and a 1.8-fold increase in specific activity was achieved after validation of RSM in shake flasks using *Burkholderia cepacia* (Rathi *et al.*, 2002). Similarly, up to a ninefold increase in lipase production was observed for *Penicillium cyclopium* using response surface methodology (Vanot *et al.*, 2001).

The use of RSM in the present study showed that the total time required for lipase production was 96 h of incubation. Extended incubation periods up to 48 and 72 h for lipase production have been reported for *B. stearothermophilus* SB-1 (Bradoo *et al.*, 2002) and *Rhizopus oligosporus* (Nahas, 1988). It is concluded that production of lipase in *A. awamori* could be improved by controlling various physical and nutritional factors, and a statistical approach aids rapid identification of the signal parameters and their optimal concentrations.

Data obtained for the time course experiment conducted over a period of 192 h (8 days) under optimized condition after statistical modeling testify that enzyme production commenced after 36 h of incubation and reached a peak after 96 h (495.0 U/ml), while maximal specific activity was recorded at 108 h (1164.63 U/mg protein). Further incubation beyond 108 h did not favor enhanced enzyme activity and instead resulted in a decline. Maximum biomass (29.5 mg/ml) was attained within 48 h of incubation and was maintained almost stable during the entire study period. Protease assay was conducted during the entire period of study and no protease was found even after 192 h of incubation. It is inferred that the

lipase produced by *A. awamori* is a 'protease free lipase'. In fact protease free lipase has been reported in literature for *A. carneus* (Saxena *et al.*, 2003a).

5.3 ENZYME PURIFICATION AND CHARACTERIZATION

Lipases were reported to be extensively purified and characterized in terms of their activity and stability profiles relative to pH, temperature, and effects of metal ions, oxidizing agents, reducing agents and organic solvents. In many cases, lipases have been purified to homogeneity and crystallized (Sharma *et al.*, 2001). Lipases from *Aspergillus niger* and *A. oryzae* have been purified by conventional purification and chromatography (Brush *et al.*, 1999; Sughihara *et al.*, 1988; Toida *et al.*, 1995). In the present study, lipase could be purified by employing conventional methods and a 30.2 fold purified enzyme was obtained after ion exchange chromatography. Further 33.7 % of recovery of lipase with a specific activity of 3733.0 U/mg protein, could be achieved.

SDS-PAGE analysis of the purified enzyme, performed under non-reducing and reducing conditions, yielded a single band and double band respectively, testifying the dipeptide nature of the enzyme. The molecular mass of lipase, estimated by comparison with the electrophoretic mobility of marker proteins indicate that the *A. awamori* lipase has an apparent molecular mass of ~90kDa.

The molecular mass of lipase in the range between 11 and 67 kDa was reported (Saxena *et al.*, 2003b). Most of the lipase purification schemes in literature focused on purifying small amounts of the enzyme to homogeneity towards characterization of the same. Whereas there is hardly any information published on large scale processes for commercial purification of lipase. May be the reason is that most commercial applications of lipase do not require highly pure

enzyme and excessive purification is expensive and reduces overall recovery of the enzyme (Chisti, 1998).

The zymogram showed three fluorescent bands in the case of ammonium sulphate precipitated sample, compared to a single fluorescent band obtained with ion exchange purified sample. Results confirm the purity and activity of the purified lipase obtained from *A. awamori*. In contrast to other zymographic systems, the sensitivity range of MUF-butyrate technique is extremely high, allowing detection of 1.5 X 10^{-7} units of Pancrealipase® in less than 15 min. The short time required for activity detection on gels greatly contributes to prevent protein diffusion, thus allowing a most accurate determination of the protein molecular weight. An important advantage of the zymographic technique is that after activity detection, the same gels can subsequently be stained with a conventional dye in order to determine the molecular mass of the active proteins (Prim *et al.*, 2003).

Gel filtration chromatography of the partially purified enzyme protein using Sephadex G100, yielded three peaks with lipase activity. The molecular weight of the proteins was calculated from the standard graph plotted for K_{av} versus log molecular weight of the standard proteins. From the K_{av} value, the molecular weight of the enzyme for the three fractions (I, II & III) were found to be 95 kDa, 65 kDa and 38kDa respectively. A similar observation was reported earlier where three different peaks with different molecular weight were obtained, during SDS-PAGE, indicating that different lipases are isoforms (Benjamin and Pandey, 2000).

Most lipases are reported to be acidic glycoprotein, glycosylated to the extent of 2-15% with mannose (Saxena *et al.*, 2003a). In the present study the purified lipase was stained with Thymol-H_2SO_4 reagent, and a purple band was

obtained. The results suggest that the lipase is a glycoprotein, which was glycosylated to the extent of 3.6 %.

The lipase has an optimum pH between 7.0 and 8.0 for maximal activity. pH 7.0 is considered as optimal pH for maximal activity. It was also noted that the enzyme was totally inactive at pH below 4.0 and above 10.0. More than 60 % of maximal enzyme activity was recorded at pH in the range between 6.0-8.0. In general, lipases from *Aspergillus* strains was reported to be active between pH 4.0 and 7.0 and at temperatures between 40 and 50°C (Pabai *et al.*, 1995a).

The data obtained for the pH stability studies of the lipase testify that the enzyme is stable over a wide range of pH from 2.0-12.0. However, maximal residual enzyme activity was recorded only with the sample incubated in the buffer having pH 7.0. Nevertheless, when compared to other levels of pH tested from 2.0-8.0, the difference in enzyme activity was only marginal.

A. awamori enzyme was active over a broad range of incubation temperature while recording maximal activity at 40°C. Temperatures above 45°C led to a sharp decline in enzyme activity. In fact, the lipase activity showed a linear increase along with increase in temperature, and the increase was rapid over a range of temperature varying from 20°C to 40°C. Interstingly, the enzyme showed activity even at 5°C with 31.85 % relative activity. A synergistic effect was observed when the reaction mixture was incubated at 20°C - 40°C, similar to lipases of *Fusarium solani* (Camargo-de-Morais *et al.*, 2003), *P. citrinum* (Pimentel *et al.*, 1994), *C. rugosa* (Camargo-de-Morais *et al.*, 1998), *F. solani* (Maia *et al.*, 2001).

Temperature stability studies conducted using lipase, showed that the enzyme was stable over 20-30°C and could retain 100 % residual activity even

after 48 h. Whereas, at 40°C, the optimal temperature for maximal activity, the enzyme could retain only 38 % of activity after 12 h of incubation. Results suggest that at high temperatures above 50°C the enzyme lost activity within 30 minutes of incubation. Hence, it is inferred that this lipase is not a thermo stable enzyme. Velocity of enzymatic reaction is enhanced by a raise in temperature, but at temperatures close to that supporting maximal activity, the enzyme suffers denaturation and thus inactivation (Dixon and Webb, 1979).

In many cases, lipases appear to obey Michaelis–Menten kinetics (Guit *et al.*, 1991; Malcata *et al.*, 1992). Michaelis–Menten kinetics are characterized by two parameters, K_m and V_{max}. The latter is the maximum rate of reaction and K_m is a measure of the affinity of an enzyme for a particular substrate. A low K_m value represents a high affinity. The K_m values of the enzyme range widely, but for most industrially relevant enzymes, K_m ranges between 10^{-1} and 10^{-5} M (Fullbrook, 1996). Michaelis–Menten parameters K_m and V_{max} of a purified lipase of *P. fragi* CRDA 323. The K_m and v_{max} values were 0.7 mg/mL and 0.97 x 10^{-3} U/min, respectively were reported (Pabai *et al.*, 1995b). Kinetic studies were performed for *A. awamori* lipase using pNP caprylate as the substrate and the linear transformation of the Michaelis-Menten equation recorded a K_m and V_{max} as 73.67 µmol and 344.7 U/ml respectively.

Effect of oxidizing agent on enzyme activity was studied using H_2O_2. It is evident from the data that 99.9 % of enzyme activity was retained in the presence of 1 % H_2O_2 and 50 % of enzyme activity was retained even upto 5 % conc. studied. The enzyme was inactive at conc. of H_2O_2 above10 %. Thus it is concluded that the enzyme stability declined along with increase in conc. of H_2O_2. Lipolase® marketed by Novo Nordisk, Denmark exhibited only 43 % residual activity after 1 h of treatment, whereas *Burkholderia cepacia* exhibited better resistance (100 % residual activity) towards strong oxidizing agents especially hypochlorite (Rathi

et al., 2001). The current thrust for novel enzymes that tolerate oxidative stress makes the present lipase of high commercial value.

Effect of reducing agents on lipase activity was evaluated using β-mercaptoethanol and sodium thioglycolate. Results clearly indicate that the reducing agents have a positive effect on enzyme activity except at the highest concentration tried. β-mercaptoethanol led to an enhancement in enzyme activity along with increase in conc. up to 1 % concentration. In the case of sodium thioglycolate increase in conc. above 0.2 % led to gradual decline in residual enzyme activity along with increase in concentration. Both β-mercaptoethanol and sodium thioglycolate, at their highest concentration (5 %) studied, caused reduction in residual enzyme activity to 90.05 % and 57.92 % respectively.

Lipases are known for their ability to work in aqueous as well as in organic solvents. Effect of organic solvents on lipase activity was tested using 9 different organic solvents up to 24 h. Results obtained for the study indicate that the all the organic solvents, except butanol, at 10 % concentration level enhanced enzyme activity more than 100 %, even after 24 h. Butanol was found to be a strong inhibitor of lipase even at lower concentration. From the results it is clearly evident that at up to 60 % concentrations of hexane, benzene and diethyl ether the enzyme was stable even after 24 h. The stability of *A. awamori* lipase in hexane, benzene and diethyl ether makes it an ideal candidate for use in biotransformations in organic solvent systems.

Of the various metals evaluated for their effect on lipase activity, only Co^{2+} enhanced lipase activity, at all the conc. tested upto 20 mM concentrations. All the other metals tested had a negative effect on lipase and led to a decline in residual activity at all the concentrations tested, except ferric chloride, which supported a marginal raise in enzyme activity at 1 mM conc. In general, the increase in conc. of

metal ions led to proportionate decrease in enzyme activity. Even at 1mM concentration lipase activity was considerably decreased in the presence of Hg^{2+} and Cd^{2+}. At 20 mM concentration there was no activity in the presence of Al^{3+} and the activity got reduced considerably in the presence of Zn^{2+}, Cd^{2+} and Hg^{2+}. The result strongly indicates the absence of the requirement for a co-factor for lipase activity, which is in agreement with previous studies (Gilbert *et al.*, 1991; Yadav *et al.*, 1998a). Salts of heavy metals (Fe^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}) strongly inhibited the lipase, suggesting that they were able to alter the enzyme conformation (Sharon *et al.*, 1998). The lipase (L3) of *A. oryzae* was inactivated by Ag^{+}, Hg^{2+}, Cu^{2+}, Al^{3+} and Zn^{2+} (Toida *et al.*, 1998). Ferric ion inhibited lipase activity of *A. oryzae* (Toida *et al.*, 1995), *P. roqueforti*, *A. niger* (Sughihara *et al.*, 1988), *R. javanicus* (Aisaka and Terada, 1980).

All the substrates tested, for substrate specificity, with *A. awamori* lipase showed high level of affinity. Maximal affinity was shown pNP caprylate, followed by pNP butyrate, pNP laurate and pNP palmitate. These results clearly indicate the affinity of *A. awamori* lipase towards C-8 fatty acids.

The hydrolytic product of the lipase activity with triolein (C18:1) as a substrate was examined by thin-layer chromatography. Triolein was hydrolysed into diolein, mono-olein and oleic acid, evidencing the 1,3-regiospecific nature of *A. awamori* lipase. The *A. awamori* showed pronounced regio-specificity, hydrolyzing triolein into its intermediate products diolein and monoolein. This lipase therefore seemed to have a strong specificity towards the outer chains of triglycerides. The pattern of hydrolysis of A. awamori was similar to that of earlier reported *A. carneus* (Saxena *et al.*, 2003a), *A. niger* and *Rhizopus delemar* (Okumura *et al.*, 1976).

The hydrolytic reaction catalysed by lipases generally takes place at the oil-water interface. The hydrolytic activity is the basic characteristic of lipases; it is on this basis that lipases are generally sold and guaranteed by the producer (Wu *et al.*, 1996). Hydrolytic activity of *A. awamori* lipase with olive oil as substrate was 4.0 U/ml.

The characterization of purified lipase produced by *A. awamori* showed stability in organic solvents, oxidizing agent and reducing agents, 1,3-regiospecificity and hydrolytic activity. These properties make this lipase an ideal candidate for biocatalysis in organic media for the production of novel compounds.

5.4 APPLICATION STUDIES

The industrial applications of lipases have grown rapidly in recent years and are likely to markedly expand further in the coming years. Lipases are the most versatile biocatalyst and bring about a range of bioconversion reactions such as hydrolysis, transesterification, esterification, alcoholysis, acidolysis and aminolysis.

5.4.1 Biodiesel production

Biodiesel production is a very modern and technological area for researchers due to the relevance that it is winning everyday because of the increase in the petroleum price and the environmental advantages. Methyl and ethyl esters of fatty acids, better known as biodiesel, are nontoxic, biodegradable, and an excellent replacement for petroleum diesel. Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional physiochemical process. Transesterification of rice bran oil and methanol with

A. awamori lipase was tested towards biodiesel production. Data analysed by Gas chromatography did not yield any positive result. Whereas, the thin-layer chromatogram clearly showed that almost all the triglyceride form of rice bran oil was converted to its hydrolysis product, indicating scope for biodiesel production subject to further standardisation of the reactants molar concentration. The molar ratio of the reactants, oil and methanol, is an important factor that determines the production of methyl esters of fatty acids (biodiesel). It also depends on the hydrolytic activity of lipase. Further standardisation is necessary for biodiesel production. Zullaikah *et al.*, (2005) have developed a two-step acid-catalyzed process for the production of biodiesel from rice bran oil. Shah *et al.*, (2004) has reported biodiesel production by lipase catalysed transesterification of Jatropha oil.

5.4.2 Fatty acid sugar ester synthesis

Fatty acid sugar esters are non-ionic surfactants with high emulsifying, stabilizing and detergency effect, which are widely used in the food, cosmetic, detergent and pharmaceutical industry. Use of *A. awamori* lipase for fatty acid sugar ester synthesis was evaluated. Qualitative analysis of the fatty acid sugar ester was unsuccessful with TLC because spraying of 50 % H_2SO_4 got absorbed to the silica and the coating got cracked, and detached from the alumina. Whereas, quantitative analysis of fatty acid sugar ester yielded 16 % fructose myristate, 21.4 % glucose myristate, 8.3 % glucose palmitate, and formation of fructose palmitate was nil. The lower percentage of fatty acid sugar ester formation might be due to reverse reaction that could have occurred due to the water generated during esterification reaction (Sabeder *et al.*, 2006).

5.4.3 Treatment of effluent containing oils using *A. awamori* lipase

Bioremediation refers to the use of biological systems, such as bacteria, fungi, and enzymes, to degrade environmental pollutants. Fats, oil and grease are contributed to the effluent wastewater through dairy industry, slaughter houses, restaurants, ayurvedic massage centre etc. It is necessary to reduce the concentration of fat, oils and proteins or to eliminate these materials altogether, in order to enable the biological treatment to proceed without any inhibition of the biological reduction of organic matter in wastewater. A large number of pretreatment systems (grease-trap, tilted plate separators, dissolved air flotation systems and physical–chemical treatment) are employed to remove oil and grease (O&G) from these wastewaters prior to the main treatment process itself, which is generally of a biological nature (Cammarota *et al.*, 2000; Wakelin and Forster, 1997). The use of extracellular enzymes is a standard practice in many industries for many years. Whereas, only recently they are being studied as a means for enhancing bioremediation. Potential of lipase for treatment of oil containing effluent was evaluated by treating a simulated oil effluent, blended with10 % each of 'used restaurant oil', 'used ayurvedic oil', 'used fish fry oil', palm oil, coconut oil and dalda with lipase. From the results obtained it is inferred that there was 91.4 % reduction in oil and grease content after treatment of ayurvedic oil, followed by palm oil, fish fry oil and coconut oil with 80.16 %, 33.3 % and 30 % reduction in oil and grease respectively.

5.5 ISOLATION OF GENE ENCODING LIPASE

In the present study, amplification of the genomic DNA with degenerate primers (forward ASP.F1 and reverse ASP.R1) yielded an amplicon (LIP.ASP1) of approximately 800 bp at an annealing temperature of 56°C. The 800 bp amplicon cloned into the pGEMT plasmid vector, was later subjected to sequence analysis

using the primer T7 in order to confirm the lipase gene sequence. A 463 bp sequence was obtained for LIP.ASP1 and Blastx was conducted. Based on the Blastx analysis it is concluded that the DNA fraction obtained through PCR amplification contain partial gene sequence that encodes lipase in *A. awamori*. The sequences were submitted to GenBank with the accession no. EF524197 as *Aspergillus awamori* Nagazawa partial Lipase gene related NUDIX protein.

'Nudix' hydrolases are able to cleave substrates containing an X-linked nucleoside diphosphate (X = phosphate, sugar, nucleoside mono/diphosphate, etc.). Their metabolic role has been related to the control of the cellular concentration of toxic compounds and physiological metabolites whose accumulation could be harmful for the cell (Bessman *et al.*, 1996). It is demonstrated that this protein is a bifunctional enzyme, endowed with NMN adenylyltransferase and 'Nudix' hydrolase activities. It is notable that a ferulic acid esterase from *A. niger* (deVries *et al.*, 1997) possesses a similar pattern of conserved residues as EDS1, raising the possibility that EDS1 hydrolyzes a nonlipid substrate. The *Aspergillus niger* protein faeA contains the lipase consensus motifs but has been shown to be a ferulic acid esterase (deVries *et al.*, 1997). Thus, it is inferred that available partial gene sequence encodes the precursor protein for lipase. The partial sequence available can be used as probe for pulling the full gene for lipase from *A. awamori*.

Chapter 6

SUMMARY AND CONCLUSION

A potential lipase producing marine fungus was selected among 14 lipase producers isolated from seawater and sediments of South Indian coastal environments which was identified as *Aspergillus awamori* Nagazawa. The selected fungus was subjected to ribotyping using partial 28S rRNA gene, for confirming its identity, which could be amplified and sequenced. Partial sequence of the 28S rRNA gene was submitted to GenBank (accession number EF524198) through BankIt programme, at NCBI site. The identity of the *A. awamori* could be compared with the sequences available in the GenBank, by BLAST programme. From the phylogram, it is inferred that *A. awamori* Nagazawa has close similarity to *A. niger* with 91% similarity.

Various bioprocess parameters affecting lipase production by fungus under submerged fermentation were optimized towards maximal enzyme production using Malt Extract medium. Strategy adopted for the optimization was to evaluate individually the effect of different parameters ('one-variable-at-a-time' method) on lipase production under SmF, conduct statistical optimization, and perform a time course experiment under optimized condition.

Optimization of incubation period on lipase production showed that enzyme production was observed to commence after 12 h of incubation and the activity declined after 96 h of incubation. Hence, 4 days of incubation was considered as optimum during the later optimization studies.

Rice bran oil induced enhanced lipase production (202.16 U/ml) by *A. awamori.* 40°C was recorded as optimal incubation temperature for maximal lipase production. The enzyme production was recorded almost at all the lower temperatures and incubation at temperatures above 45°C did not support lipase production under SmF.

Lipase production was observed over a broad pH range from pH 3.0 to pH 12.0, eventhough the enzyme activity varied considerably in the range of 16.0 U/ml to 122.9 U/ml. Apparently, it seems that, this fungus has two pH optima, one at pH 3.0 (122.9 U/ml) and another at pH 10.0 (119.0 U/ml) for maximal lipase production.

Maximum enzyme production was recorded at 150 rpm and increase in rpm led to a decrease in enzyme production. There was no enzyme production at stationary condition indicating requirement for agitation. Increase in inoculum concentration did not enhance enzyme activity in *A. awamori.*

None of the sugars supplied as additional carbon source enhanced lipase production compared to ME medium (control). Maltose, lactose & sucrose inhibited lipase production. Whereas in the presence of xylose and sorbitol there was enzyme production, was lesser than in the control medium.

Among the 19 different amino acids tested, only methionine and lysine showed an enzyme activity of 220.0 U/ml and 198.7 U/ml compared to control medium, which showed an enzyme activity of 162.0 U/ml. On the other hand, cysteine, histidine, leucine, isoleucine, tryptophan and arginine caused a considerable reduction in enzyme production.

Among the inorganic nitrogen sources tested ammonium nitrate, ammonium chloride, ammonium dihydrogen orthophosphate, ammonium sulphate, sodium nitrate and potassium nitrate had a positive effect on enzyme production while, ammonium hydrogen carbonate, ammonium acetate, ammonium oxalate, and ammonium iron sulphate inhibited enzyme production. Maximum enzyme production was obtained using ammonium sulphate and ammonium chloride with an activity of 224.3 U/ml and 213.9 U/ml respectively.

Sodium chloride significantly affected lipase production by *A. awamori* and at concentrations 1-3.5 % (w/v), it enhanced lipase production with maximum activity at 1.5 % (206.6 U/ml). The fungus was capable of growth upto 12.5 % NaCl and produced low levels of lipase.

Among the different inorganic salts tested, only calcium chloride and magnesium sulphate enhanced lipase production by *A. awamori*. The activity was 80.6 U/ml with calcium chloride and 75.2 U/ml with magnesium sulphate. The other inorganic salts zinc sulphate, potassium chloride, copper sulphate, ferrous sulphate, manganese chloride, and cobalt chloride inhibited lipase production.

Among the different surfactants studied PEG 6000 and Tween 20 enhanced lipase production compared to control. PEG 6000 showed maximum enzyme activity of 133.8 U/ml. Sodium dodecyl sulphate (SDS) and sodium deoxycholic acid inhibited lipase production by *A. awamori*.

Incubation days, concentration of soyabean, ammonium sulphate, KH_2PO_4, and incubation temperature greatly influenced lipase production as it is evident from the P-B design. Further analysis using RSM, showed that incubation time, KH_2PO_4 and temperature; were significant model terms for the response. Three-dimensional response surface curves were plotted to study the interaction among

various physicochemical factors, and to determine the optimum concentration of each individual factor for maximum lipase production. The model predicted maximum lipase production (up to 482.85 U/ml) that could be achieved using 0.8 % w/v soyabean, 0.1 M ammonium sulphate and 0.05 M KH_2PO_4 at 35°C for 5 days incubation. An overall 2.8-fold increase in lipase production was achieved after validation of RSM in shake flasks. Validation of the deduced response surface model was carried out in shake flasks under conditions predicted by the model and the experimental values were found to be very close to the predicted values. Hence, the model was successfully validated. The optimized conditions for lipase production were as follows: Soyabean meal-0.77 % (w/v); ammonium sulphate-0.1 M; KH_2PO_4-0.05 M; Rice bran oil-2 % (v/v); $CaCl_2$-0.05 M; PEG 6000-0.05 % (w/v); NaCl-1 % (w/v); inoculum-1 % (v/v); pH 3.0; incubation temperature 35°C and incubation period-5 days.

The time course experiment conducted over a period of 192 h (8 days) under optimized condition testify that enzyme production commenced after 36 h of incubation and reached a peak after 96 h (495.0 U/ml). While maximal specific activity was recorded at 108 h (1164.63 U/mg protein). Further incubation beyond 108 h did not favor enhanced enzyme activity and instead resulted in a decline. Maximum biomass (29.5 mg/ml) was attained within 48 h of incubation and was maintained almost stable during the entire study period. Protease assay was conducted during the entire period of study and no protease was found even after 192 h of incubation. It is inferred that the lipase produced by *A. awamori* is a 'protease free lipase'.

Lipase was purified employing standard protein purification procedures, which included ammonium sulphate fractionation followed by dialysis and ion exchange chromatography. Lipase could be precipitated with 40-90 % ammonium sulphate saturation and showed a 12.5 fold increase in specific activity, compared

to the crude sample. The lipase was purified upto 30.2 fold after ion exchange chromatography. Elution profile from the DEAE-Cellulose column furnished a single peak with lipase activity, which could be eluted with buffer containing 0.2 M NaCl. This step resulted in 33.7 % lipase recovery (30.2 fold of purification) with a specific activity of 3733.0 U/mg protein.

Native and SDS-PAGE were performed to analyse the nature of the protein. From the SDS-PAGE analysis, the enzyme was found to have a dipeptide nature. The molecular mass was found to be ~90kDa.

The zymogram showed three fluorescent bands in the case of ammonium sulphate precipitated sample, whereas a single fluorescent band was seen with ion exchange purified sample.

Gel filtration chromatography of the partially purified enzyme protein using Sephadex G100 yielded three peaks with lipase activity and the molecular weight of the enzyme for the three fractions (I, II & III) were found to be approximately 95 kDa, 65 kDa and 38 kDa respectively.

The purified lipase when stained with Thymol-H_2SO_4 reagent, gave a purple band indicating that the lipase is a glycoprotein. Phenol-sulphuric acid assay using purified lipase revealed that the lipase is glycosylated to the extent of 3.6%.

The pH optima was found to be 7.0 and the enzyme was found to be stable from pH 2.0-9.0. The optimal temperature for maximal activity was 40°C. Results suggest that at high temperatures, above 50°C, the enzyme had denatured and lost activity within 30 minutes.

Lipase kinetic studies were conducted using pNP caprylate as the substrate and the data obtained and from this, the K_m and V_{max} were recorded as 73.67 µmol and 344.7 U/ml respectively.

The lipase was stable upto 5 % H_2O_2 as oxidizing agent and presence of the reducing agents, β-mercaptoethanol and sodium thioglycolate had a positive effect on enzyme activity except at highest concentration tried. All the organic solvents except butanol at 10 % concentration enhanced more than 100 % enzyme activity even after 24 h. Butanol is found to be a strong inhibitor of lipase even at lower concentration. At both 30 % & 60 % concentrations of hexane, benzene and diethyl ether the enzyme was stable even after 24 h with residual activities 106.25, 112.32 and 133.40 % respectively.

Of the various metals evaluated for their effect on lipase activity, only Co^{2+} enhanced lipase activity even upto 20 mM concentrations. Even at 1 mM concentration lipase activity was considerably decreased in the presence of Hg^{2+} and Cd^{2+}. At 20 mM concentration the activity was nil in the presence of $Al^{3+}$and activity was lowered considerably in the presence of Zn^{2+}, Cd^{2+} and Hg^{2+}.

The lipase showed high level of affinity towards pNP caprylate, followed by pNP butyrate, pNP laurate and pNP palmitate indicating the affinity of the lipase towards C-8 fatty acids.

The lipase shows 1,3- regiospecificity. Hydrolytic activity of lipase was studied using olive oil as substrate and the activity was found to be 4.0 U/ml.

The importance of lipase in biodiesel production, fatty acid sugar ester synthesis and effluent treatment were assessed. More than 90 % reduction in oil and grease was observed with ayurvedic oil.

Fungal genomic DNA and lipase gene fragment isolation was conducted. The partial lipase gene sequence was submitted to Genbank with the accession no. EF524197 as *Aspergillus awamori* Nagazawa partial lipase gene related NUDIX protein.

Conclusion

The results obtained from the present study indicate the scope for the utilization of the marine fungus *Aspergillus awamori* Nagazawa BTMFW 032 for extracellular lipase production employing submerged fermentation. To the best of our knowledge this is the first report on lipase production by a marine fungus employing statistical modeling towards industrial production. The characterization of purified lipase produced by *A. awamori* showed stability in organic solvents, oxidizing agent and reducing agents, 1,3-regiospecificity and hydrolytic activity. These properties make this lipase an ideal candidate for biocatalysis in organic media for the production of novel compounds such as biodiesel and sugar fatty esters. 91.4 % reduction in oil and grease content in ayurvedic oil by the treatment of *A. awamori* lipase indicates that there is a scope for this enzyme in the treatment of oil effluents and bioremediation. There is ample scope for further research on the biochemistry of the enzyme, structure elucidation and enzyme engineering towards a wide range of further applications, besides enriching scientific knowledge on marine enzymes.

Chapter 7

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Table 3.5: Ammonium Sulphate solubility chart (Jayaraman J, 1981)

Initial concentration of ammonium sulphate (Percentage saturation)	Final Concentration of Ammonium sulphate (percentage saturation)																
	10	**20**	**25**	**30**	**33**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**90**	**100**
	Grams solid ammonium sulphate to be added to 1 litre of solution																
0	56	114	144	176	196	209	243	277	313	351	390	430	472	516	561	662	767
10		57	86	118	137	150	183	216	251	288	326	365	406	449	494	592	694
20			29	59	78	91	123	155	189	225	262	300	340	382	424	520	619
25				30	49	61	93	125	158	193	230	267	307	348	390	485	583
30					19	30	62	94	127	162	198	235	273	314	356	449	546
33						12	43	74	107	142	177	214	252	292	333	426	522
35							31	63	94	129	164	200	238	278	319	411	506
40								31	63	97	132	168	205	245	285	375	469
45									31	65	99	134	171	210	250	339	431
50										33	66	101	137	176	214	302	392
55											33	67	103	141	179	264	353
60												34	69	105	143	227	314
65													34	70	107	190	275
70														35	72	153	237
75															36	115	198
80																77	157
90																	79